MICROVESICLES AND THEIR ROLES IN EARLY DEVELOPMENT

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MICROVESICLES AND THEIR ROLES IN EARLY DEVELOPMENT

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Mouse embryonic stem (ES) cells generate and shed small, vesicular structures from their plasma membranes. These microvesicles (MVs) have emerged as a novel form of intercellular communication with important roles in a variety of biological and pathological processes. Here, I show that ES cells, which are derived from the inner cell mass (ICM) of a blastocyst, communicate with the surrounding layer of trophoblasts using MVs. Two extracellular matrix proteins, fibronectin and laminin, were found to be major protein cargo in these MVs and were shown to interact with integrins expressed on the surfaces of trophoblasts. This resulted in the activation of the signaling proteins FAK and JNK and increased the ability of the trophoblasts to migrate, a critical step during embryo implantation. In fact, injection of MVs generated by ES cells into blastocysts was sufficient to increase their implantation efficiency, highlighting a novel role for MVs in one of the earliest and most important events in pregnancy.

In addition to promoting implantation, trophoblasts are essential for placental formation. Interestingly, I determined that trophoblasts generate and shed MVs similar to ES cells, and that the trophoblast MVs are able to impact the behavior of endothelial cells, the cell type that gives rise to the placental vasculature. For example, when incubated with MVs derived from trophoblasts, endothelial cells undergo tubulation and exhibit enhanced survival under nutrient-limiting conditions. Proteomics performed on the trophoblast MVs revealed that they contained a large percentage of metabolic enzymes. Since placental formation involves the extensive

remodeling and formation of blood vessels, which requires a large amount of energy and biomass, these data suggest that the transfer of metabolic enzymes from the MVs to the endothelial cells is important for promoting this outcome.

BIOGRAPHICAL SKETCH

Laura Desrochers was born in Montreal, Canada and was raised in a small town in Quebec called Blainville until she was twelve years old. Her family then moved to Massachusetts where she completed her middle school and high school years. Her parents strengthened her early fascination with science and the natural world by filling their house with a large supply of science and nature books for children and by being fascinated by science themselves. Her mother worked for many years as a medical laboratory technician, while her father is fascinated by astronomy, and would occasionally wake the entire family up at four in the morning to watch meteor showers when the skies were darkest. When Laura applied to college, she decided to return to Montreal to obtain a Bachelor of Science in Biochemistry from McGill University. While at McGill, she first performed behavioral and addiction research with rats in Dr. Paul Clarke's laboratory. Next, she studied peroxisome proliferator-activated receptor-gamma coactivator (PGC)-1α and its role in mitochondrial biogenesis in the context of breast cancer in Dr. Julie St-Pierre's laboratory, where she discovered a love for cell biology. Upon graduation, she was accepted into the Biochemistry, Molecular and Cell Biology program at Cornell University and joined Dr. Richard Cerione's laboratory after her first year. There, she focused on determining the role of a novel form of intercellular communication, microvesicles, in the context of mouse embryonic stem cells and early development. After graduation, she plans to move to New York City where she has secured a post-doctoral research position in Dr. Neal Rosen's laboratory at the Memorial Sloan Kettering Cancer Center to study cancer cell signaling. Although she will miss the quiet, natural surroundings found in Ithaca, she is excited to live and perform research in a city, once again.

ACKNOWLEDGEMENTS

I would like to thank my parents for their constant love and support over the years, as well as my brother, a fellow nerd and occasional partner-in-crime.

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I would also like to thank the Cerione lab as a whole, and especially my office mates, for providing such a warm, friendly and welcoming environment. I could not imagine completing my PhD in any other place.

The Department of Molecular Medicine has been a great help to me throughout my time here. I would like to give a special thanks to Debbie, Cindy, Val and Robin for their administrative support and constant cheerfulness.

Finally, I would like to thank my friends for being there for me over the past few years, especially my fellow MV researcher, Bridget, who has become one of my best friends, and Dan, who has helped me through this last stretch.

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LIST OF ABBREVIATIONS

2-DG: 2-deoxyglucose
ABs: apoptotic bodies
Arf6: ADP-ribosylation factor 6
ARRDC1: arrestin domain-containing protein 1
bFGF: basic fibroblast growth factor
CD: cluster of differentiation
Cdc42: Cell division control protein 42 homolog
CM: conditioned medium
CSCs: cardiac stem cells
DLS: dynamic light scattering
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
EGFRvIII: epidermal growth factor receptor variant III
ERK: extracellular signal-regulated kinase
ES cells: embryonic stem cells
ESCRT: endosomal sorting complex required for transport
EVs: extracellular vesicles
EVTs: extravillous trophoblasts
F-actin: filamentous actin
FAK: focal adhesion kinase
FBS: fetal bovine serum
GAC: glutaminase C
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GFP: green fluorescent protein
GLS1: glutaminase 1
GPI: glycosylphosphatidylinositol
hCG: human chorionic gonadotrophin
HIF1α: hypoxia inducible factor 1α
HUVECs: human umbilical vein endothelial cells
ICM: inner cell mass
IL: interleukin
ILVs: intraluminal vesicles
iPSCs: induced pluripotent stem cells
IUGR: intrauterine growth restriction
JAG1: protein jagged-1
JNK: c-Jun N-terminal kinase
KGA: kidney type glutaminase

LDHA: lactate dehydrogenase A
LIF: leukemia inhibitory factor
LimK: Lim kinase
MAPK: mitogen-activated protein kinase
MET: hepatocyte growth factor receptor
MI: myocardial infarction
MIF: macrophage inhibitory factor
miRNA: microRNA
MLC: myosin light chain
MLCK: myosin light chain-kinase
MMPs: matrix metalloproteinases
mRNA: messenger RNA
MSCs: mesenchymal stem (or stromal) cells
MVB: multivesicular body
MVEs: multivesicular endosomes
MVs: microvesicles
NOTCH3: Neurogenic locus notch homolog protein 3
NS: not significant
PBS: phosphate-buffered saline
PDAC: pancreatic ductal adenocarcinoma
PDGF: platelet-derived growth factor
PGC-1α: proliferator-activated receptor-gamma coactivator-1α
PKM2: pyruvate kinase M2
PLD: phospholipase D
PM-GFP: plasma membrane-targeted green fluorescent protein
PS: phosphatidylserine
Rab: Ras-related protein Rab
Rac1: Ras-related C3 botulinum toxin substrate 1
RhoA: Ras homolog gene family member A
ROCK: Rho-associated protein kinase
RVG: rabies virus glycoprotein
s.e.m.: standard error of the mean
siRNA: small interfering RNA
STAT1: Signal transducer and activator of transcription 1-alpha/beta
TEM: transmission electron microscopy
TGFβ: transforming growth factor β
TIM4: T cell/transmembrane, Ig, and mucin-4
TSG101: tumor susceptibility gene 101
tTG: tissue transglutaminase
UTR: untranslated region

VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor
WCL: whole cell lysate

CHAPTER 1

[*]Introduction

Intercellular communication is a process that is critical for the development and functioning of any multicellular organism and classically involves either the secretion of soluble factors or direct cell-to-cell contact.[1] However, more recently, extracellular vesicles (EVs), or small, membrane-enclosed packages of information, have emerged as a novel and exciting form of intercellular communication. [2-6]

EVs were initially discovered in the 1980s as part of an effort to understand the maturation of reticulocytes into red blood cells.[7] The differentiating reticulocytes were shown to shed EVs that contained the transferrin receptor, a protein whose expression needs to be rapidly down-regulated for this maturation process to occur. Thus, the generation of EVs in this case was thought to help the reticulocytes to rapidly eliminate unwanted material (e.g. the transferrin receptor). While additional studies in the 1990s focused on EVs in the context of immune function and showed that they might also be used to help generate an immune response,[1,8-10] the scientific community largely dismissed EVs as nothing more than cellular debris. However, a few key studies in the field of cancer cell biology many years later raised the exciting possibility that EVs were more than just "debris."[2-6,11,12] Rather, it appeared that in certain contexts, EVs could play an important role in promoting cancer progression, causing researchers to take a closer look at EVs and their potential functions.

It is now appreciated that most, if not all cancer cells have the ability to generate and shed EVs.[7,13,14] Cancer cell-derived EVs have been shown to be critical for mediating tumor growth,

[*] Select parts of this introduction are currently under review as a perspective for *Developmental Cell* by LM Desrochers, MA Antonyak and RA Cerione.

chemoresistance, metastasis, and even tumor vascularization.[11,15-21] EVs have also attracted a good deal of attention due to their ability to transport unusual cargo, such as messenger RNAs (mRNAs), microRNAs (miRNAs), and non-classically secreted proteins to other cells.[5,11,12,22,23] Due to their unique contents and packaging, EVs have wide-ranging effects in many biological contexts. Additionally, because EVs can be found in many bodily fluids, such as blood, urine, cerebrospinal fluid and saliva, translational researchers and pharmaceutical companies are aggressively pursuing EVs as a potential source of diagnostic information in the context of cancer, as well as other diseases.[14,24,25] Currently, research is beginning to emerge suggesting that it might be possible to take advantage of EVs as a potent and effective way to deliver therapeutics to specific cell types,[2-6,26] or even use EVs from specific cell types, such as mesenchymal stem cells (MSCs), to help the body recover from acute insults to tissues.[7,27,28] Given all the potential surrounding EVs, it is becoming clear that we need to learn much more about EV biogenesis and how they function as satellites of information transfer to impact a variety of physiological and pathological conditions.

Formation and shedding of EVs

There are considered to be three distinct forms of EVs: microvesicles (MVs), exosomes and apoptotic bodies (ABs).[6,8-10] Microvesicles and exosomes are generally distinguished by their size and are thought to be generated via distinct mechanisms by viable cells (Figure 1.1 and Table 1.1), whereas ABs are only released by cells that have undergone programmed cell death or apoptosis.

Figure 1.1. Microvesicles and exosomes are generated by distinct mechanisms.
Microvesicles (in purple, bottom) bud directly from a cell's plasma membrane, potentially from lipid raft domains, while exosomes (in red, smaller) are made through the re-routing of the endosomal pathway. Specifically, multivesicular endosomes (MVEs) containing intraluminal vesicles (ILVs) are directed to fuse with the cell's plasma membrane where they release their ILVs as exosomes into the extracellular space. Normally, MVEs fuse with the lysosome to degrade their contents, including ILVs.

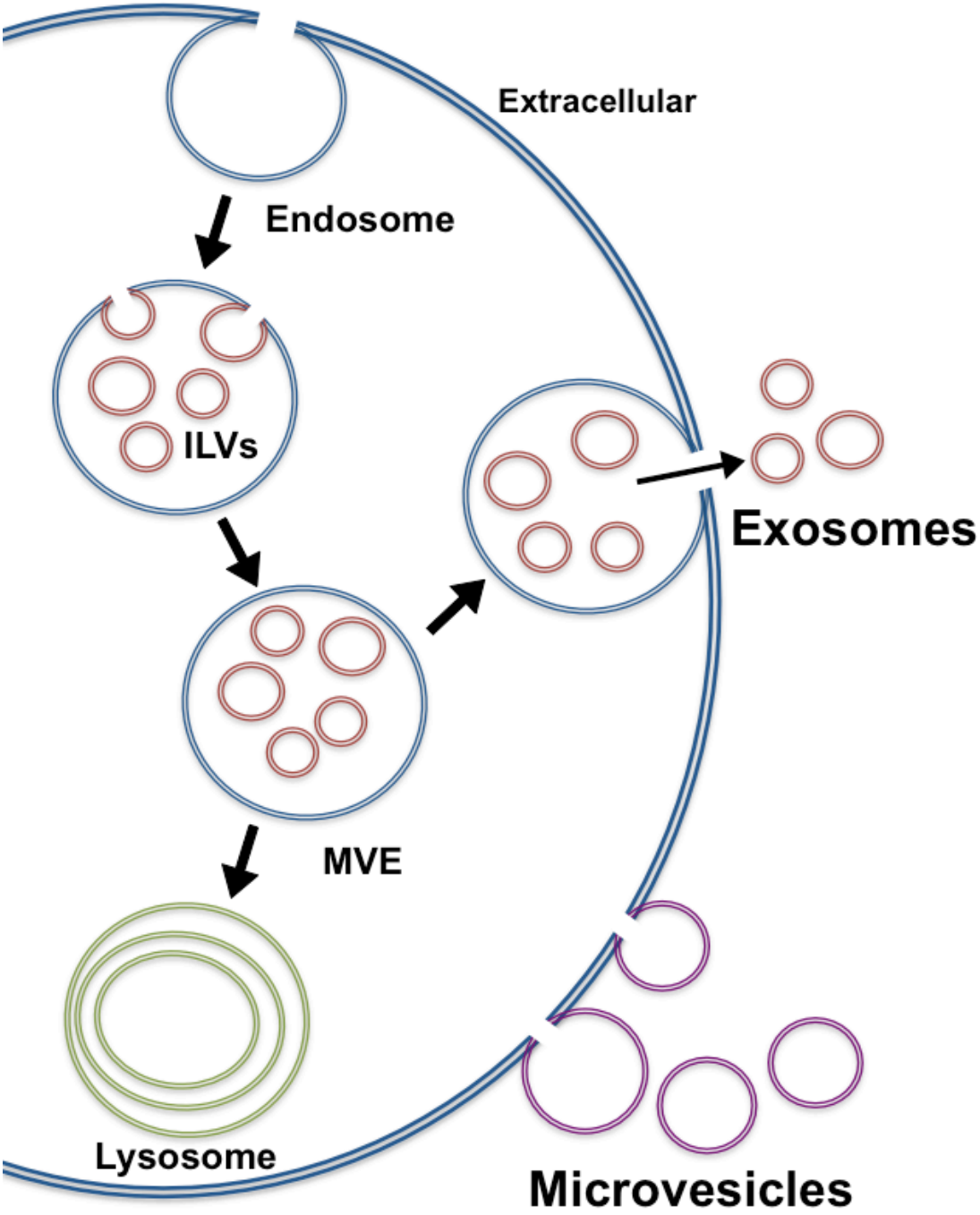

Extracellular
Endosome
ILVs
Exosomes
MVE
Lysosome
Microvesicles

Table 1.1. Sizes of different extracellular vesicles (EVs).
Apoptotic bodies are the largest type of EVs, followed by microvesicles and then exosomes.

Extracellular Vesicles	Size (µm)
Microvesicles (MVs)	0.2 - 3
Exosomes	0.03 - 0.1
Apoptotic Bodies (ABs)	2 - 10

MV biogenesis

MVs are vesicular structures that bud directly from cell plasma membranes and range in size from 0.2-2 μm in diameter (Figure 1.1 and Table 1.1).[6,11-13] They go by a variety of names such as shedding vesicles, microparticles, ectosomes, and when shed by cancer cells, they have also been referred to as oncosomes.[5,13,14] The exact mechanism underlying their formation is still an area of active investigation. However, in recent years, studies have linked the rearrangement of the actin cytoskeleton as well as specific lipid components of the plasma membrane to MV formation. For example, in the context of cancer cells, researchers have implicated both Ras homolog gene family member A (RhoA) and ADP-ribosylation factor 6 (Arf6) in the formation and release of MVs (Figure 1.2 and 1.3).[11,15-21,29,30] Both of these proteins are small GTPases, which function as molecular switches in cells. RhoA and Arf6 are commonly activated in response to extracellular cues such as growth factors and cellular stressors and can cause changes in the actin cytoskeleton that appear to underlie MV formation.

Our laboratory first noticed that the stimulation of HeLa cervical carcinoma cells with epidermal growth factor (EGF) resulted in an increase in both MV formation and shedding.[5,11,12,15,22,23] They also noticed that MV formation in these cancer cells appeared to involve the assembly of filamentous (F-) actin into ring-like structures.[15,29] Since the Rho family of small GTPases, including Cdc42, RhoA and Rac are known to function downstream of the epidermal growth factor receptor (EGFR)[31] and play important roles in regulating the actin cytoskeleton,[32] our laboratory became attracted to the idea that MV biogenesis might be regulated by small GTPases. Indeed, they went on to show that RhoA was necessary for MV formation and even localized to the neck of the outward budding sites of MVs.[29] More

Figure 1.2. RhoA-dependent signaling can induce MV budding.
Active, GTP-bound RhoA signals through ROCK to LimK, which then phosphorylates cofilin and inhibits its ability to sever filamentous actin. Consequently, actin polymerization occurs at sites of MV budding.

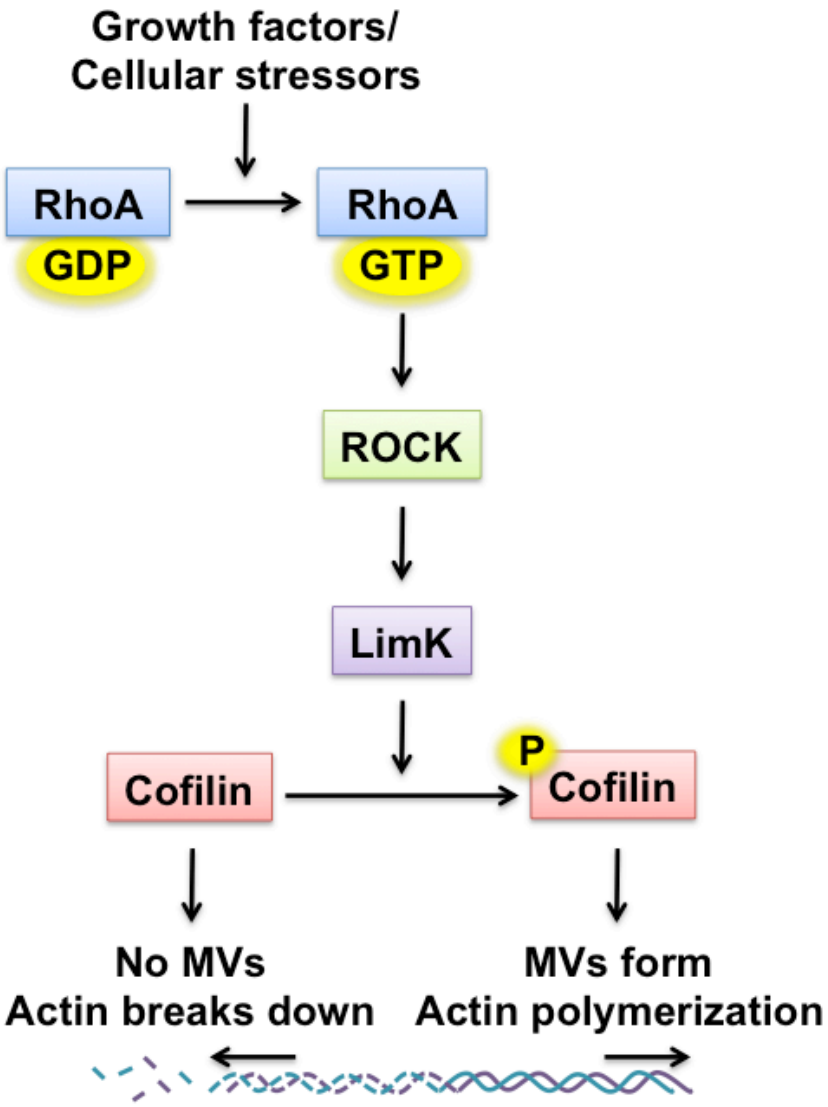
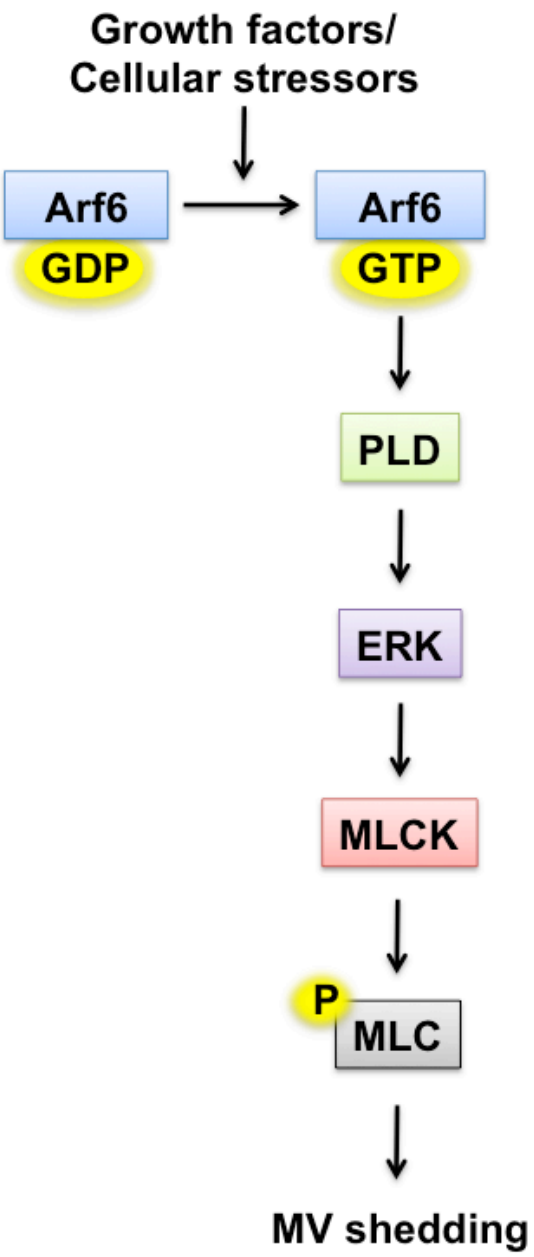

Growth factors/
Cellular stressors
RhoA
GDP
RhoA
GTP
ROCK
LimK
Cofilin
P
Cofilin
No MVs
Actin breaks down
MVs form
Actin polymerization

Figure 1.3. Arf6 regulates MV shedding.
Active, GTP-bound Arf6 signals through PLD and ERK to activate MLCK, which then phosphorylates myosin light chain (MLC). This functions to tighten the actin cables at the base of the MV, allowing it to be released from the cell surface.


Growth factors/
Cellular stressors
Arf6
GDP
Arf6
GTP
PLD
ERK
MLCK
P
MLC
MV shedding

specifically, as outlined in Figure 1.2, for cancer cells to generate MVs upon stimulation with EGF or other growth factors, RhoA is converted from its inactive, GDP-bound state to its active, GTP-bound state. Activated RhoA then releases Rho-associated protein kinase (ROCK) from its auto-inhibitory conformation, allowing it to bind to and phosphorylate Lim Kinase (LimK). The phosphorylated or activated form of LimK then phosphorylates cofilin on serine 3 and inhibits its actin-severing function.[33,34] This promotes actin polymerization at sites where MVs are budding from the surfaces of cancer cells and can explain why MVs forming on the surfaces of cancer cells can be visualized by staining for F-actin. Importantly, interfering with any step in this pathway using inhibitors or small interfering RNAs (siRNAs) targeting components of this pathway blocked MV formation.[29]

Another study by Muralidharan-Chari, et al. showed that Arf6 GTPase is involved in MV release or shedding in cancer cells (Figure 1.3).[30] Arf6 is another small GTPase family member and signals through phospholipase D (PLD) to activate extracellular signal-regulated kinase (ERK).[35] Activated ERK then causes myosin light chain-kinase (MLCK) to phosphorylate myosin light chain (MLC),[36-38] allowing for the contraction of actin cables and the eventual release (shedding) of MVs. Consistent with this pathway being involved in the final step of releasing MVs from the cell surface, inhibition of this pathway led to the accumulation of MVs along the surfaces of cells and fewer MVs shed into the medium.[30]

While both the RhoA- and Arf6-mediated signaling events play important roles in the ability of highly aggressive cancer cells to generate and release MVs, we now have preliminary data suggesting that non-transformed cell types use an alternative mechanism to generate and shed MVs. For example, the ability of mouse embryonic stem (ES) cells to shed MVs is not blocked by inhibiting RhoA or one of its downstream effectors, and ES cell MVs cannot be

detected by staining the cells with rhodamine-conjugated phalloidin (to detect F-actin). These data suggest that there must be other mechanisms that regulate MV biogenesis in non-cancerous cells. One interesting possibility comes from another study that suggested that, like viral budding from the plasma membrane, MVs can be generated through the exploitation of the endosomal sorting complex required for transport (ESCRT) protein tumor susceptibility gene 101 (TSG101).[39] This study showed that TSG101 interacted with arrestin domain-containing protein 1 (ARRDC1) and required the activity of VPS4 ATPase, another ESCRT protein, to form MVs. The knockdown of TSG101 or ARRDC1 in cells resulted in fewer MVs being released.

There are also indications that MVs are generated from specialized domains in a cell's plasma membrane called lipid rafts, which are enriched in specific proteins and lipids, such as cholesterol and sphingolipids.[40] Studies have shown that pharmacological depletion of cholesterol from cells inhibited MV formation,[41,42] and that proteins such as flotillin and integrins, which are present in lipid rafts,[43,44] can be detected in isolated MVs.[6] In fact, flotillin is widely used as a marker of MVs.[45] Similarly, a negatively charged lipid, phosphatidylserine (PS), is enriched on the outer surfaces of MVs, despite normally only being present on the inner leaflet of healthy (non-apoptotic) cells' plasma membranes.[46,47] It has been suggested that increasing PS on the outer surface of the cell helps to promote membrane curvature while the MV is budding from the cell surface.[47]

Exosome biogenesis

Exosomes are a better-studied form of EVs. They are significantly smaller in size than MVs, ranging from 30-100 nm in diameter and are formed via a distinct mechanism (Figure 1.1 and Table 1.1).[2,6] Specifically, exosomes are formed through the re-routing of multivesicular

endosomes (MVEs) (also known as multivesicular bodies (MVBs)) from the lysosome to the plasma membrane.[2] The MVEs then fuse with the plasma membrane, releasing their intraluminal vesicles (ILVs) as exosomes into the extracellular space. MVEs are derived from early endosomes when ESCRT machinery sequesters cargo and causes the inward budding of the early endosome's membrane to form ILVs.[48] Certain proteins are known to be specifically involved in generating ILVs that will be released as exosomes. These include: syndecan, syntenin and ALIX.[49] Syndecan is a heparan sulfate proteoglycan, which interacts with syntenin, an adaptor protein that links syndecan to ALIX. ALIX interacts with ESCRT machinery and is involved in the budding and abscission of ILVs. Syntenin and ALIX have been shown to be necessary for loading cargo into exosomes, as well as their release into the extracellular environment.

MVEs traditionally fuse with lysosomes to degrade their ILVs.[48] However, in order to generate exosomes, the MVEs need to be redirected to the plasma membrane where they fuse to release their contents (e.g. exosomes) into the extracellular space. While the signals that traffic MVEs to the cell surface are poorly understood, the signaling events that regulate the docking and tethering of MVEs to the plasma membrane require Ras-related protein Rab (Rab) 27a and 27b as well as Rab 35 and Rab 11.[50-53] Knockdown of any of these Rab GTPases by siRNAs caused an accumulation of MVEs in cells, as well as a reduction in the amount of exosomes being shed into the medium. Rab GTPases are already known to be involved in most vesicle trafficking steps,[54] so it is not necessarily surprising to find that they are also involved in trafficking MVEs to the plasma membrane.

Similar to MVs, exosomes are also derived from lipid rafts since they contain lipids and proteins enriched in these specialized plasma membrane domains.[47] Ceramide, a sphingolipid enriched in exosomes, was shown to be necessary for the formation of ILVs destined to be

released as exosomes.[55] Inhibition of neutral sphingomyelinases to deplete ceramide in cells reduced the amount of exosomes secreted by cells.

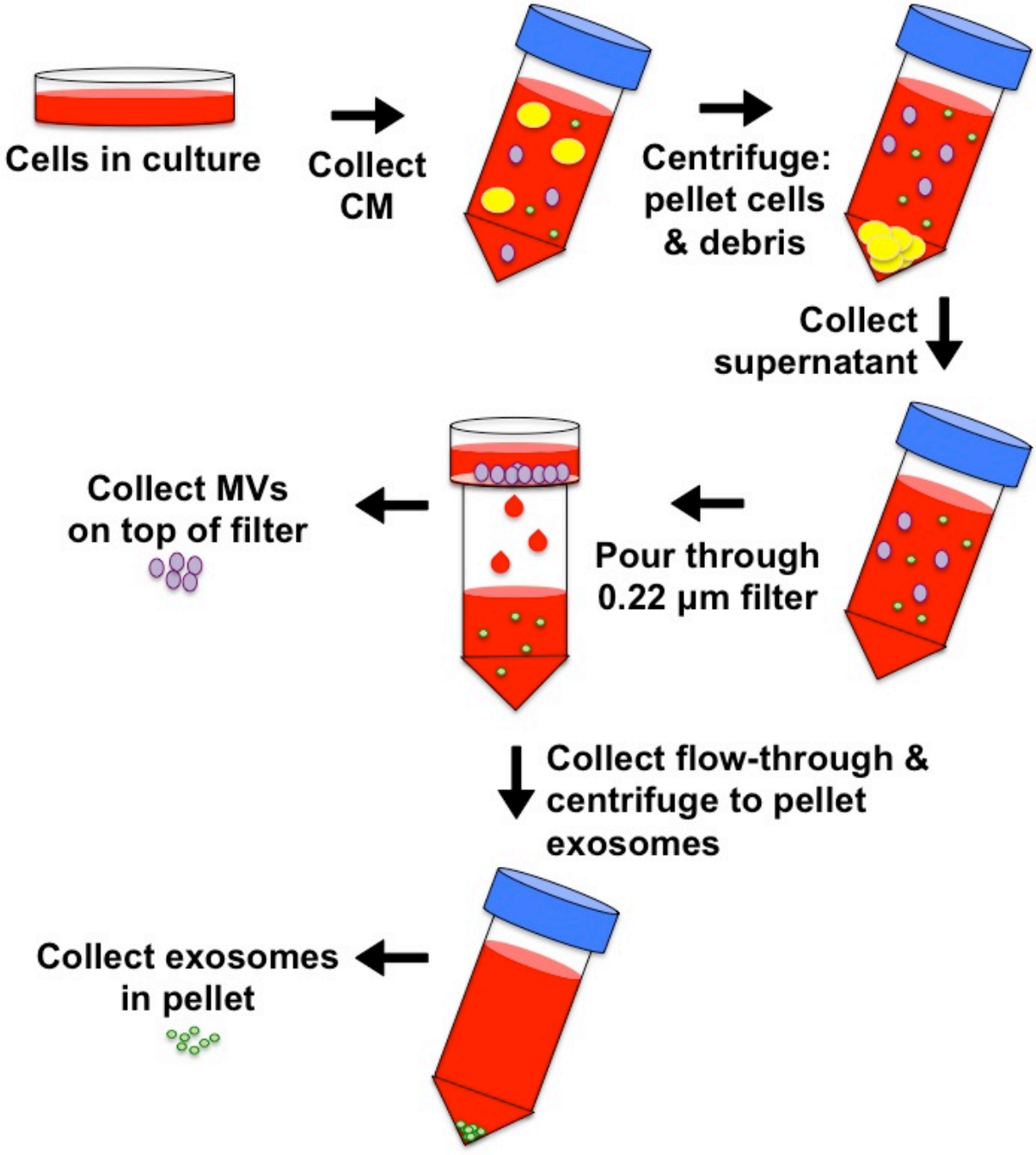
Apoptotic Bodies

The third class of EVs is ABs, which are formed when a cell is undergoing programmed death.[56] These vesicular structures are much larger than exosomes and MVs and can range in size from 2-10 μm in diameter (Table 1.1).[57] They can contain entire organelles, pieces of DNA, and large fragments of the apoptotic cell.[56] ABs are generally phagocytosed by neighboring cells and degraded. While ABs are categorized as a subtype of EV, the field's current focus is on MVs and exosomes, which are both produced by healthy, viable cells.

Isolating MVs and exosomes

Although it has been difficult to separate exosomes from MVs, we have made significant strides to do so. Most EV collection procedures rely on a series of centrifugations during which cells and cell debris are first removed from conditioned medium using low-speed centrifugations, and then the EVs are collected via sequential high-speed centrifugation steps (Figure 1.4).[57,58] Specifically, MVs are pelleted at ~10 000 x *g*, while exosomes are pelleted at ~100 000 x *g*. Alternatively, MVs can be isolated from exosomes by filtering the conditioned medium (cleared of cells and cell debris) with a 0.22 μm filter.[15] Since MVs are typically larger than 200 nm in size, they are retained by the filter and can be collected. This filtration step is also often performed prior to isolating exosomes by centrifugation to remove MVs from the sample.[59] Markers specific for MVs are still being elucidated, while protein markers considered to be specific for exosomes include CD63, CD81, CD9, ALIX and TSG101.[2,60,61]

Figure 1.4. Standard procedure to collect MVs and exosomes.
Conditioned medium (CM) is collected from cultured cells and then centrifuged to remove cells and large debris. The supernatant is collected and then poured over a 0.22 μm filter, trapping the MVs on top of the filter for collection. Exosomes pass through the filter, and can be collected from the flow-through by a final, high-speed centrifugation step.


Cells in culture
Collect CM
Centrifuge: pellet cells & debris
Collect supernatant
Pour through 0.22 µm filter
Collect MVs on top of filter
Collect flow-through & centrifuge to pellet exosomes
Collect exosomes in pellet

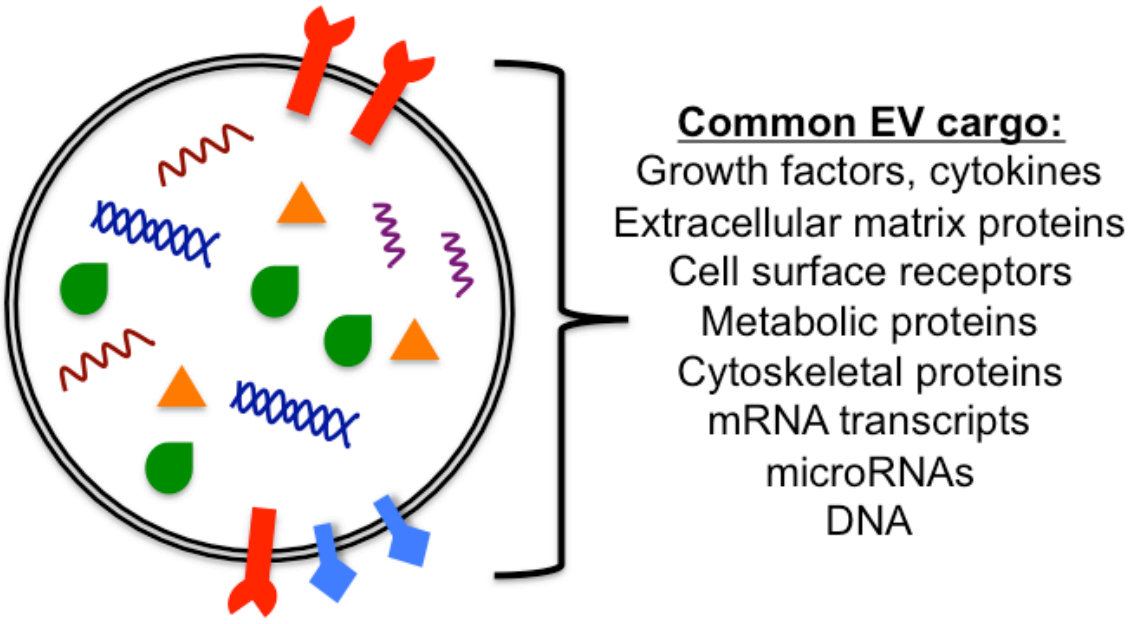
While most laboratories studying EVs use these or similar approaches to isolate MVs or exosomes, it is worth noting that they are difficult, time-consuming, and unless care is taken, the resulting preparations contain a mixture of exosomes and MVs. Thus, new, more selective, affinity-based methods are needed to obtain pure preparations. Additionally, while there are some differences in the cargo contained in exosomes and MVs, much of the cargo appears to be common to both types of EVs, making their physical separation and functional distinction even more challenging. Comparing studies between laboratories also becomes difficult with the realization that few researchers use exactly the same methods of purification. Currently, many in the field have decided to only use the term "EVs," citing the difficulties in separation and the uncertainty of whether the two EV populations perform separate functions.

Cargo in EVs

EVs have been shown to contain a wide variety of cargo including tyrosine kinase signaling receptors, extracellular matrix proteins, small GTPases, metabolic proteins, cytoskeletal proteins, miRNAs, mRNAs, and DNA (Figure 1.5).[6,11,12,23,62] EVs also generally contain markers from their cell of origin. Online, open-source databases, such as ExoCarta (exocarta.org) or Vesiclepedia (microvesicles.org), exist to catalogue the protein, lipid and nucleic acid contents of EVs. Both databases already contain entries from virtually hundreds of studies and several different species.

How cargo is transported into EVs is still relatively unknown, especially in regards to MVs. For exosomes, it is known that the syndecan-syntenin-ALIX pathway affects the loading of certain proteins into exosomes, as mentioned above.[49] Additionally, certain protein

Figure 1.5. Common cargo in extracellular vesicles.
This list represents some basic categories of cargo that are present in EVs.


Common EV cargo:
Growth factors, cytokines
Extracellular matrix proteins
Cell surface receptors
Metabolic proteins
Cytoskeletal proteins
mRNA transcripts
microRNAs
DNA

modifications that help localize proteins to the cell's plasma membrane, such as glycosylphosphatidylinositol (GPI) anchors, acyl, myristoyl or palmitoyl groups, help direct proteins into EVs.[63] For example, the GPI-anchored proteins CD55, CD58 and CD59 are trafficked into reticulocyte EVs, as is the lipid-modified Lyn tyrosine kinase. Interestingly, both lipid and GPI-anchor modifications are known to localize proteins to lipid rafts, from which both exosomes and MVs are thought to originate.

However, results from our laboratory and others have shown that many proteins without membrane-targeting modifications are major EV cargo. For instance, many metabolic proteins, such as glutaminase C or mitochondrial ATP synthase, are present in EVs derived from both cancerous and non-cancerous cell types.[64-66] Interestingly, both of these enzymes predominantly localize to mitochondria, making it unclear as to how these proteins are selectively included as EV cargo. Even more intriguing, I have found that ES cell MVs contain transcription factors, such as Oct3/4 and Nanog, which are expressed in the nucleus. How proteins that are localized to the mitochondria or the nucleus are trafficked into EVs is currently unknown, but there must be a fundamental sorting mechanism at play.

As for mRNAs, microarray comparisons of parental cancer cells and their EVs revealed that many mRNAs are exclusively present or significantly enriched in EVs, indicating that trafficking of mRNAs into EVs must also be a tightly regulated process.[12,62] In fact, when one group aligned mRNA transcripts derived from glioblastoma EVs, they discovered a 25-base pair "zipcode"-like sequence that was conserved in the 3' untranslated region (UTR) of several of the mRNAs.[67] When this "zipcode" sequence was deleted from certain mRNA transcripts, they were no longer targeted to the EVs, while its addition to the 3' UTR of other mRNA transcripts resulted in the enrichment of the altered mRNAs in the EVs released by cells.

Unfortunately, similar screens have not been done for miRNAs, DNA or proteins in EVs, and it is probable that there are other sequences present in transcripts, awaiting discovery, that direct loading into EVs.

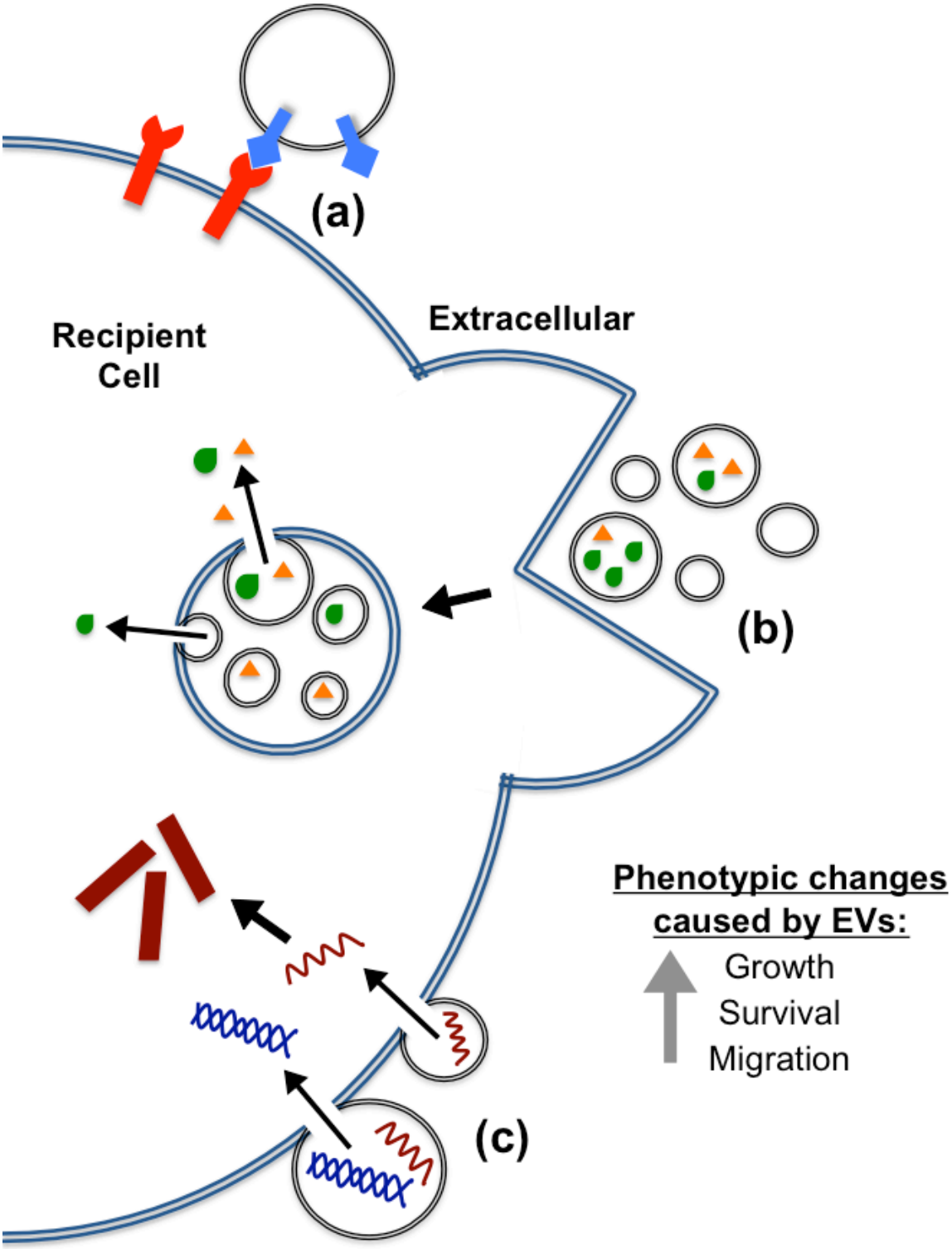
Interactions of EVs with recipient cells

There is no consensus as to how EVs are taken up by, or interact with, recipient cells, and studies have suggested that many different routes can potentially be used. Mechanisms that have been proposed to mediate EV uptake include: clathrin-, caveolin- or lipid raft-mediated endocytosis, macropinocytosis or phagocytosis, and direct membrane fusion (Figure 1.6),[68] but the majority of studies have focused on different methods of endocytosis. For instance, in multiple studies, inhibiting clathrin-mediated endocytosis with inhibitors that targeted the actin cytoskeleton or dynamin-2, a GTPase involved in the pinching off of endocytosed vesicles from the plasma membrane, resulted in reduced EV uptake.[69-78] Other studies depleted lipid raft components, such as cholesterol or sphingolipids, in recipient cells to block lipid raft-mediated endocytosis, and this similarly lead to reduced EV uptake by cells.[69,72,79,80]

Macropinocytosis is used by cells to surround or sample small parts of the extracellular environment and requires membrane ruffling, mediated by the small GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1).[81] Macropinocytosis has been shown to be important for EV uptake by microglia and ovarian cancer cells.[74,76] However, other studies using inhibitors of macropinocytosis showed that they had no effect on EV uptake, suggesting that only specific cell types may use macropinocytosis to internalize EVs.[73,82,83]

Phagocytosis, on the other hand, is more widely accepted as a mechanism for EV uptake. PS is known to be enriched on the outer membranes of EVs,[46] and cells commonly express its

Figure 1.6. Different mechanisms of EV uptake by recipient cells.
(**a**) Contact-dependent signaling can occur when signaling molecules present on the surface of the EV activate receptors present on the surface of the recipient cell. In this case, uptake of the EV contents is not necessary. (**b**) Endocytosis (e.g. clathrin-, caveolin-, or lipid raft-mediated), phagocytosis (i.e. cell eating) or macropinocytosis (i.e. cell drinking) can all be used to take up EVs. (**c**) The contents of EVs can be taken up by direct fusion of the EV membrane with the recipient cell's plasma membrane. Finally, all of these different mechanisms of uptake can lead to phenotypic changes within the recipient cell, including increased growth, survival and migration.


(a)
Recipient Cell
Extracellular
(b)
(c)
Phenotypic changes caused by EVs:
Growth
Survival
Migration

receptor, TIM4, on their surfaces.[84] When an EV with PS on its membrane comes in contact with a target cell, the PS binds to its receptor and stimulates phagocytosis. Blocking TIM4 with an antibody or blocking PS on EVs with Annexin V can both efficiently reduce EV uptake by recipient cells.[73,85,86]

Proteins including tetraspanins (i.e. CD63, CD81, CD9), integrins, immunoglobulins, and lectins present on the surfaces of EVs have also been shown to play roles in EV uptake.[71,75,77,87-92] Additionally, proteoglycans, or proteins with carbohydrate modifications, appear to be critical membrane components in order for the recipient cell to take up EVs.[82,93] For instance, cancer cells depleted of the heparan sulfate proteoglycans, glypican and syndecan, demonstrate a reduced ability to internalize EVs.[82]

The internalization of EVs is not necessarily required to mediate their effects. Rather, in some cases, the effects of EVs can be mediated by contact-dependent signaling alone (Figure 1.6).[13,25] For instance, our laboratory showed that the activation of surface integrins on non-transformed fibroblasts by cross-linked fibronectin present on the surfaces of cancer cell MVs was sufficient to activate signaling events in the fibroblasts that promoted their growth and survival.[15]

Because the methods of uptake are so diverse and the studies were performed in many different cell types, it is still unclear which of these different EV-transfer mechanisms are predominant or whether uptake of EVs is cell-type specific. This is an area of study that clearly needs to be expanded upon, especially since there is great therapeutic potential in being able to target EVs to a specific cell type. For instance, one study took advantage of the fact that the rabies virus glycoprotein (RVG) peptide is specifically taken up by cells of the central nervous system.[26] The authors loaded the RVG peptide onto EVs containing siRNA that targeted the

metabolic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). When these EVs were intravenously injected into mice, they delivered the siRNA specifically to neurons, oligodendrocytes and microglia, knocking down GAPDH expression in these cell types alone. They also demonstrated that they could knockdown a therapeutic target in Alzheimer's disease (e.g. BACE1). However, there have been very few studies as successful as this particular example. If researchers determine how to block or direct the uptake of EVs loaded with specific cargo to specific cell types, then this information could be potentially used to develop a number of therapeutic applications.

EVs in cancer progression

While many of the initial studies on the function of EVs were performed using immune cells,[8,9] the field of EVs came into greater prominence when several pivotal discoveries were made several years later in the context of cancer biology.[11,12] The first such study involved the transfer of EVs between cancer cells, specifically, high-grade brain tumor or glioblastoma cells.[11] The authors used human U373 glioma cells stably expressing epidermal growth factor receptor variant III (EGFRvIII), a truncated and highly oncogenic form of the EGFR often found in the most aggressive brain tumors. They showed that EGFRvIII expression enhanced the aberrant growth properties of the glioma cells, and, interestingly, also increased the number of EVs that the U373 cell line produced. When the EVs shed by the U373 glioma cells expressing EGFRvIII were isolated and added to cultures of parental U373 glioma cells, the cells began to grow more quickly. The authors went on to determine the cargo in the EVs responsible for mediating this effect and made the unexpected discovery that the mutant EGFR itself was present in the EVs and could be transferred to parental U373 cells. Once transferred, EGFRvIII activated

growth-promoting signaling events (e.g. mitogen-activated protein kinase (MAPK) and AKT), stimulated vascular endothelial growth factor (VEGF) production, and enhanced malignant transformation. These findings were particularly exciting, since cancer researchers were long puzzled by the fact that only a subset of the cancer cells that made up a brain tumor generally contained the genetic mutation encoding for EGFRvIII *in vivo*, despite the fact that a majority of the brain tumor cells expressed EGFRvIII at the protein level.[94] These results also highlighted how cancer-promoting proteins (e.g. EGFRvIII) could be transferred between cancer cells in EVs as a means to propagate oncogenic signaling.

In a second study also involving glioblastomas, it was discovered that these cancers shed EVs that contained a variety of mRNAs that encoded for growth-promoting proteins.[12] In fact, several of the RNA transcripts were notably enriched in EVs compared to the glioblastoma cells from which they were derived. The authors then went on to show that these mRNAs could be transferred to human brain microvascular endothelial cells, where they were then translated into functional protein. This resulted in the endothelial cells' ability to grow, survive, and undergo tubulation (a measure of angiogenesis, see below) better than the control cells that were not treated with EVs.

Interestingly, the authors discovered that the EVs shed by the glioblastoma cells also contained the mRNA encoding for EGFRvIII, raising the possibility that patient EVs could be analyzed for tumor-specific mRNA markers.[12] They went on to show that they could detect the EGFRvIII transcript in EVs isolated from serum from patients with EGFRvIII-positive tumors, but not in the serum of healthy individuals.

Other studies on EVs from cancer cells soon followed, and they confirmed that proteins, mRNAs, miRNAs and even DNA could be transferred between cancer cells via EVs to promote

tumor growth and cancer progression. [13,22,47,62,95-102] With the discovery that miRNAs and mRNAs encoding oncogenic markers, including EGFRvIII, were released from cancer cells in EVs and could even enter the bloodstream, this also opened up the possibility that EVs could be isolated from the blood of cancer patients and their contents used as diagnostic markers.[12,24,103-106] For tumors that are notoriously hard to biopsy (e.g. brain tumors), this novel approach to diagnose patients would be a remarkable advancement.

For example, a recent study used mass spectrometry analysis to identify a proteoglycan commonly overexpressed in breast and pancreatic cancer, glypican-1, that is uniquely present on the surfaces of breast and pancreatic cancer cell-derived EVs.[107] Using blood samples from healthy individuals or patients with breast or pancreatic (pancreatic ductal adenocarcinoma, or PDAC) cancer, the authors found that 75% of breast cancer patients had more glypican-1 positive EVs in their blood compared to healthy controls, while 100% of pancreatic cancer patients had higher levels of glypican-1 positive EVs. Pancreatic cancer has a high mortality rate due to the difficulty in diagnosing it in its early stages.[108-110] Due to the obvious benefits of finding a diagnostic marker for pancreatic cancer and the high correlation between PDAC and glypican-1 levels, the authors chose to focus on pancreatic cancer models. The authors also found that glypican-1 positive EVs predicted the development of pancreatic cancer in genetically engineered mouse models of pancreatic cancer and could potentially be used for prognosis. Other studies have identified potential cancer markers in EVs before,[5,12,24,103,104,106] and these studies will hopefully prove to be clinically useful and pave the way for less invasive diagnostic procedures.

Many studies have also found that there are higher numbers of EVs in the blood of cancer patients compared to healthy controls, making a simple count of the number of EVs in a patient's

blood a potential tool.[5,24,111] However, recent results from our laboratory even suggest that at least some transformed cells produce fewer EVs compared to non-transformed control cells. Given that it is now generally accepted that most normal cells are capable of generating EVs,[6,9,25,27] it appears unlikely that determining EV levels in a patient's blood will be an effective indicator for the presence of a tumor.

EVs and their effects on the tumor microenvironment

Besides transferring their contents to other cancer cells, cancer cell EVs can also impact normal cell types in their local environment, referred to as the "tumor microenvironment." [47,112,113] One study from our laboratory showed that breast cancer cell EVs can transfer their contents to both normal mammary epithelial cells and non-transformed fibroblasts, resulting in the normal cells acquiring some of the transformed properties of the cancer cells, including enhanced cell growth and survival, as well as the ability to form colonies under anchorage-independent conditions.[15] In the same study, the injection of mitotically arrested breast cancer cells that were still capable of generating EVs with non-transformed fibroblasts into immune-deficient mice resulted in tumors of fibroblast origin. Interestingly, in order for the non-transformed cells (i.e. fibroblasts or normal mammary epithelial cells) to maintain their growth and survival advantages, the EVs had to be reapplied at regular intervals (every 2 or 3 days). This strongly suggested that the effects of the cancer cell-derived EVs are transient in nature.

It is well documented that the stromal cells in the tumor microenvironment release secreted factors that promote tumor growth, survival and even resistance to chemotherapy.[112-114] Interestingly, in the context of breast cancer, stromal cells were shown to release EVs, which, in conjunction with a cell contact-dependent signaling event, helped mediate breast cancer

chemoresistance and tumor re-initiation.[16] Specifically, breast cancer cells took up mRNA from stromal cell EVs, resulting in the activation of interferon-stimulated genes, such as STAT1, in the breast cancer cells. These genes are normally activated as an antiviral response but are also often up-regulated in cancer cells to mediate radiation and chemotherapy resistance.[115] Stromal cells also express the transmembrane protein NOTCH3, which can activate JAG1 on neighboring breast cancer cells (i.e. activate juxtacrine signaling). The simultaneous activation of STAT1 and JAG1 in breast cancer cells increased the numbers of tumor-initiating cells, which are known to be highly resistant to most cancer therapies.[116-120] It is important to mention that the authors of the study also noted that stromal cells in the presence of breast cancer cells began to release more EVs through a Rab27b-dependent mechanism, suggesting that the breast cancer cells were inducing the stromal cells to produce more EVs. These findings highlight a complex process by which cancer cells and normal cells can communicate with each other via EVs to drive cancer progression.

EVs in angiogenesis

Besides their crosstalk with directly adjacent cells in the tumor microenvironment, tumor cell EVs have also been shown to impact angiogenesis or the recruitment of new blood vessels to the tumor.[3,13,21,99,101,121] Once a tumor reaches a certain size (1-3 mm in diameter), it needs to develop its own blood supply, or else its growth will be inhibited by the limited amount of oxygen and nutrients that can diffuse to it from the existing vasculature. In order to do this, the tumor must first clear paths in its microenvironment to generate the space needed for new blood vessel growth. Then, the tumor must stimulate the formation of blood vessels.[122] EVs have been implicated in both of these processes.

The stress of hypoxia and nutrient deprivation (i.e. serum starvation) has been shown to increase the release of EVs from cancer cells,[123,124] suggesting that an expanding tumor would begin to release more EVs as it reached a size that could no longer be supported by the available oxygen and nutrients. The EVs released by these cells often contain several different matrix metalloproteinases (MMPs),[125-128] which function by degrading extracellular matrix components. Potentially, tumor cells release MMP-loaded EVs so that they can diffuse further away from the tumor than freely secreted (soluble) forms of MMPs. The EVs would then rupture and release extracellular matrix-degrading MMPs into the microenvironment, creating the space around the tumor needed for new blood vessels to form.

Cancer cell EVs can also attract and stimulate endothelial cells. Cancer cells have been shown to release EVs containing activated EGFR, and when the EGFR is transferred to endothelial cells, signaling pathways are initiated that ultimately lead to the production of vascular endothelial growth factor (VEGF), which is known to stimulate angiogenesis.[21] Thus, cancer cell-derived EVs cause changes in the endothelial cells that recruit them to the tumor and promote blood vessel formation. It is worth mentioning that tumor-derived EVs can stimulate angiogenesis through the transfer of other cargo as well, including VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), interleukin-1 (IL-1) and IL-8.[129-134] Other studies have also implicated the mRNA and miRNA contents of tumor-derived EVs in the angiogenesis process. Clearly, there are a variety of contents in cancer cell EVs that can play a role in promoting angiogenesis.[99,101,135,136]

EVs in metastasis

One of the potentially more exciting roles that EVs have been found to play in tumor

progression is in metastasis. In fact, stromal cell EVs, besides promoting radiation and chemotherapy resistance as described above, can also promote changes that lead to increased metastasis.[13,25] Breast cancer cells treated with conditioned medium from tumor-associated fibroblasts demonstrated increased protrusive activity and motility.[17] When breast cancer cells were co-injected without or with fibroblasts into immune-compromised mice, the cancer cells injected together with fibroblasts were shown to metastasize better. This was shown to be mediated through the action of fibroblast-derived EVs, with CD81 being the critical cargo in the EVs responsible for stimulating cell migration and metastasis. Specifically, the CD81-containing EVs released by the fibroblasts were endocytosed by the breast cancer cells. The endocytosed EVs were then loaded with Wnt11 signaling factor and recycled back to the extracellular space, at which point the EV-tethered Wnt11 gave rise to an autocrine stimulation of the breast cancer cells, activating their core planar cell polarity components, which helped to stimulate directional cell motility. The complexity of this EV-mediated signaling process is astounding, but as more studies on EV-mediated cancer and stromal cell signaling paradigms emerge, these types of cell-cell interactions appear to be common.

While EVs can directly stimulate cancer cells to metastasize, several key studies have also shown that EVs can alter sites in the body distant from the primary tumor to be more hospitable to metastasizing cells. This is also known as "pre-metastatic niche" formation.[137,138] In a groundbreaking study, Peinado et al. showed that highly metastatic melanoma cells (B16-F10 cells) released EVs, which, when injected into mice, increased vascular leakiness and enhanced the metastasis of orthotopically injected melanoma cells.[18] On the other hand, EVs derived from poorly metastatic melanomas (B16-F1 cells) exhibited little ability to promote metastasis. The authors went on to discover the mechanism through which the EVs from the aggressive

melanoma cell line promoted metastasis. Specifically, they showed that the melanoma EVs transferred MET tyrosine kinase (also known as hepatocyte growth factor receptor) to bone marrow progenitor cells, which enhanced their mobilization into the bloodstream to help promote tumor vascularization and metastasis.

In a subsequent study performed by the same group, it was shown that pre-metastatic niche formation by pancreatic ductal carcinoma (PDAC) cells in the liver was mediated by EVs.[19] PDAC cells released EVs, which, when injected into the bloodstream of mice, increased the ability of PDAC cells to metastasize to the liver. Looking into the mechanism behind these results, the authors determined that Kupffer cells in the liver preferentially took up the circulating EVs, which contained macrophage migration inhibitory factor (MIF). In response to the MIF in the EVs, Kupffer cells then began to secrete transforming growth factor β (TGFβ). The increase in the local concentration of TGFβ in the liver stimulated hepatic stellate cells to secrete fibronectin, creating a fibrotic environment. Together, the increased TGFβ and fibronectin deposition recruited bone marrow-derived macrophages to the area, creating a fibrotic, pro-inflammatory site ideal for metastasis.

Finally, EVs have been implicated in explaining organotropic metastasis, or why certain cancers metastasize preferentially to certain organs, a mystery that has puzzled researchers for more than a century.[20] As described above, EVs from metastatic cancers form pre-metastatic niches in specific areas of the body, such as the lungs or liver. Hoshino, et al. showed that EVs from different metastatic cancer cell lines preferentially localize to whichever organs their cancer cells of origin normally metastasize. In fact, priming a mouse with EVs derived from a cancer cell line that normally metastasizes to the lung can redirect a bone-metastasizing cancer to the lung. Mass spectrometry analysis of liver-, lung- and brain-metastasizing cancer cell EVs

revealed that integrins were responsible for directing organotropism. Specifically, integrin α_6 was associated with lung metastasis, integrin $\alpha_V\beta_5$ with liver metastasis, and integrin β_3 with brain metastasis, and all metastatic EVs contained integrin $\alpha_2\beta_1$, a potential metastatic marker. EVs of different origins also preferentially interacted with specific cell types in the organ to which they localized, suggesting that the integrins on their surface directed the EVs to environments with specific extracellular matrix components. For instance, α_6, which pairs with β_1 or β_4 integrins, binds laminin, which is abundant in lung tissue. Once taken up by tissues, the EVs were found to activate pro-migratory and pro-inflammatory gene changes. These findings correlated well with metastatic tumors derived from patients, meaning that an analysis of the integrins present on tumor-derived EVs could help predict to which organ a tumor will metastasize.

ES cell and adult stem cell EVs

While great strides have been made in understanding the role of EVs in cancer progression, far less is known about how they contribute to physiological processes and outcomes. One field that is currently emerging is the role of EVs produced by stem cells. Many stem cell types, including adult mesenchymal stem cells (MSCs; also known as mesenchymal stromal cells) and ES cells have been shown to produce EVs, [28,66,139-143] but fewer studies have been directed at determining their function.

MSCs are defined as non-hematopoietic cells in adult tissues that are capable of adhering to plastic, undergoing self-renewal, as well as differentiating into multiple lineages *in vitro*, including osteoblasts, adipocytes and chondroblasts.[144] Additionally, to be considered MSCs, they must express CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules. While MSCs are present in most

adult tissues, they are most easily derived from bone marrow. Compared to ES cells, adult stem cells are readily available and their use does not come with the ethical quandaries that accompany ES cell research. Currently, researchers have been studying MSCs in the context of organ repair and tissue regeneration. [27,145] There are many diseases where an insult to a certain organ or tissue results in an injury that causes permanent, long-term damage. One common area of study in tissue injury is myocardial infarction (MI) and cardiovascular diseases[146] (although research has also been done in acute kidney and lung tissue injuries as well[147,148]). MI occurs when the heart is deprived of oxygen (ischemia) due to a blockage of the coronary arteries, causing portions of the heart muscle to die.[149] The damage caused by MI is permanent since heart tissue has little ability to regenerate on its own. Thus, for patients with heart failure, the only proven method to save their lives involves heart transplantation. However, if researchers could somehow stimulate any latent regenerative abilities in the remaining healthy heart tissue or minimize the damage to the heart caused by MI, then organ transplantation could possibly be avoided. This is why scientists have turned to MSCs.

MSCs have been injected into animal models of ischemia/reperfusion injury and patients with MI.[146,150] Studies have shown that the MSCs reduce the infarct (damaged tissue) size due to ischemia/reperfusion in rodent models.[151-156] The MSCs exerted these beneficial effects by promoting the survival and proliferation of resident cardiomyocytes, enhancing angiogenesis and suppressing inflammation.

While the beneficial effects of MSCs in the context of MI have been known for years, what causes these outcomes is less well defined. It has been postulated that MSCs can transdifferentiate into cardiomyocytes or fuse with resident cardiomyocytes, or even release paracrine factors to stimulate resident cardiac stem cells (CSCs).[156-159] However, studies have

shown that these outcomes are extremely inefficient and rare events that are unlikely to be the major cause of protection. Interestingly, injecting the conditioned medium (CM) from MSCs into animal models of MI promoted the same effects that were seen with injecting MSCs.[156,160-165] Consequently, this directed researchers to focus on the contents of the MSC secretome.

Studies have now shown that EVs shed by MSCs can also mediate cardioprotective outcomes. For instance, Lai, et al. provided some of the first evidence showing that EVs were the main component of MSC CM that were responsible for mediating protection against ischemia/reperfusion injury in a mouse model system.[166] Subsequent studies showed that when EVs were injected into mice after ischemia but before reperfusion, the EVs from the MSCs (but not the saline control) prevented the reduction in ATP and NADH levels in cardiac cells following ischemia.[139] It is interesting to note that these EVs contained several metabolic enzymes, which could have helped regenerate the ATP and NADH lost as a result of ischemia. The EVs also decreased oxidative stress and enhanced pro-survival signaling, such as Akt phosphorylation, in the damaged cardiomyocytes. As a result, the EVs reduced infarct sizes by 45% compared to controls.

Another study showed that CD34+ MSC EVs were responsible for mediating the pro-angiogenic effects of CD34+ MSCs, which have been shown to enhance the recovery of mouse models of MI when transplanted intramyocardially.[155] Additionally, studies have found that if MSCs or even cardiac cells *in vivo*, are subjected to anoxia followed by re-oxygenation (or ischemia/reperfusion *in vivo*), the EVs that are released by the cells subjected to ischemic injury or "pre-conditioning" offer greater protective effects in mouse models of MI.[167]

While EVs derived from MSCs are currently in the spotlight due to their potential therapeutic benefits for acute tissue injury, new information is quickly taking shape regarding the

roles played by EVs produced by ES cells and the embryos from which they are derived. ES cells are isolated from the inner cell mass of blastocyst stage embryos and can self-renew indefinitely as well as differentiate into any cell type in the body.[168] ES cells have been shown to make EVs, which can enhance the survival and expansion of hematopoietic progenitor cells.[142] In fact, studies have found that ES cell EVs could increase the intrinsic "stem-ness" properties of progenitor cells and have even shown that they can transfer cargo and alter gene expression in recipient fibroblasts, similar to cancer cell-derived MVs.[140,141,143] Of course, the hope would be that ES cell EVs could be used to induce pluripotency in differentiated cells (i.e. fibroblasts) or even enhance the efficiency of creating induced pluripotent stem cells (iPSCs), which remains an inefficient process.[169,170]

However, I have recently made the exciting discovery that EVs released from ES cells can also function in another important physiological process. This is the subject of Chapter 2 of my thesis. As a brief summary, I showed that ES cell-derived EVs can stimulate the migration of trophoblasts. Trophoblasts are derived from the trophectoderm layer of the blastocyst, and, in placental mammals, they are responsible for attaching the embryo to the endometrium, or uterine lining, and then migrating and invading through the endometrium to implant the embryo in the uterus.[171,172] My research showed that if EVs derived from mouse ES cells are injected into mouse blastocysts, and then those blastocysts are surgically placed into pseudo-pregnant mice, they can implant better compared to control blastocysts injected with vehicle alone. Not only does this suggest that EVs derived from the inner cell mass might be performing a normal physiological function during the implantation process, but that we might be able to clinically utilize EVs derived from ES cells to help treat problems with implantation, which are, for example, a major cause of the failure of *in vitro* fertilization treatments.[173]

However, it is becoming clear that other parts of the blastocyst, or the blastocyst as a whole, also generate and shed EVs.[174-178] In fact, researchers are beginning to look into how EVs shed by mammalian embryos might influence maternal cells in the uterus during the implantation process, or the establishment of a pregnancy.[178,179] Conversely, maternal endometrial cells also produce EVs, and could have an impact on early developmental processes.[179-183] Later in pregnancy, it is known that fetal EVs can be found in the maternal circulation, and most circulating EVs are derived from the placenta (i.e. trophoblasts).[179,184-188] Interestingly, studies have found that when there were problems with a pregnancy, such as preeclampsia, which is a serious and often deadly complication characterized by high maternal blood pressure and protein in the urine (proteinuria),[189] the numbers of placental EVs in the maternal circulation increased.[179,184,188,190] This presents the possibility that circulating fetal EVs could be used as a diagnostic marker for various pregnancy-related problems and could potentially provide new information about complications during pregnancy.[177,179,190]

Since these EVs have also been shown to contain embryonic DNA and mRNA, they could also potentially be used to determine sex and detect any genetic abnormalities in the embryo.[191-193] Additionally, EVs released by trophoblasts have been shown to play a role in modulation of maternal immune cells as well as other placental functions.[179,186,194]

In fact, in Chapter 3, I focus on the role of trophoblast MVs in the context of forming placental vasculature. Specifically, I showed that trophoblasts generated MVs, which promoted endothelial cell survival and tubulation, an *in vitro* measure of angiogenesis. Since trophoblasts interact extensively with endothelial cells during the first trimester of pregnancy to establish a blood supply to the growing embryo,[171,172] these data suggest that MVs shed by trophoblasts might play a role in shaping this critical developmental process.

Since EVs clearly play a role in the establishment of a pregnancy and embryonic developmental events, it is interesting to consider whether there are other aspects of development in which EVs might play a role. For instance, Wnt proteins are intercellular signaling molecules known to be important for many aspects of development, tissue homeostasis and stem cell maintenance,[195] and were shown to be secreted on EVs.[196] However, to this date, most examination of EV-bound Wnt activity has been done in the context of cancer.[17,197,198] There might be other developmental mediators that are carried on EVs that influence aspects of development. The more that we know about the physiological roles of EVs, the better we will be able to control their formation and contents, either to slow cancer progression, or to use them therapeutically for the treatment of disease.

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CHAPTER 2

[1]Microvesicles provide a novel mechanism for intercellular communication by embryonic stem cells during embryo implantation

Communication between the inner cell mass (ICM) of a blastocyst and its environment is known to occur, but its role in influencing essential developmental events is still poorly understood.[1,2] Here we describe how embryonic stem (ES) cells derived from the ICM mediate a critically important form of intercellular communication within the biological context of the interactions that take place between the mother and the developing embryo.[3,4] Specifically, we demonstrate that ES cells communicate with the surrounding layer of trophoblasts through the generation and shedding of a major class of extracellular vesicles called microvesicles (MVs). This vital form of intercellular communication takes advantage of the MV cargo proteins, fibronectin and laminin, to interact with integrins along the surfaces of trophoblasts, triggering the activation of two important signaling kinases, JNK and FAK, resulting in a marked stimulation in their ability to migrate. We further show that injecting MVs isolated from ES cells into blastocysts results in an increase in their implantation efficiency. Thus, these findings highlight a unique mechanism by which ES cells communicate with trophoblasts within the blastocyst-stage embryo, conferring them with the ability to migrate and invade into the uterus, and thereby promoting one of the earliest and most critically important steps during pregnancy.[5,6]

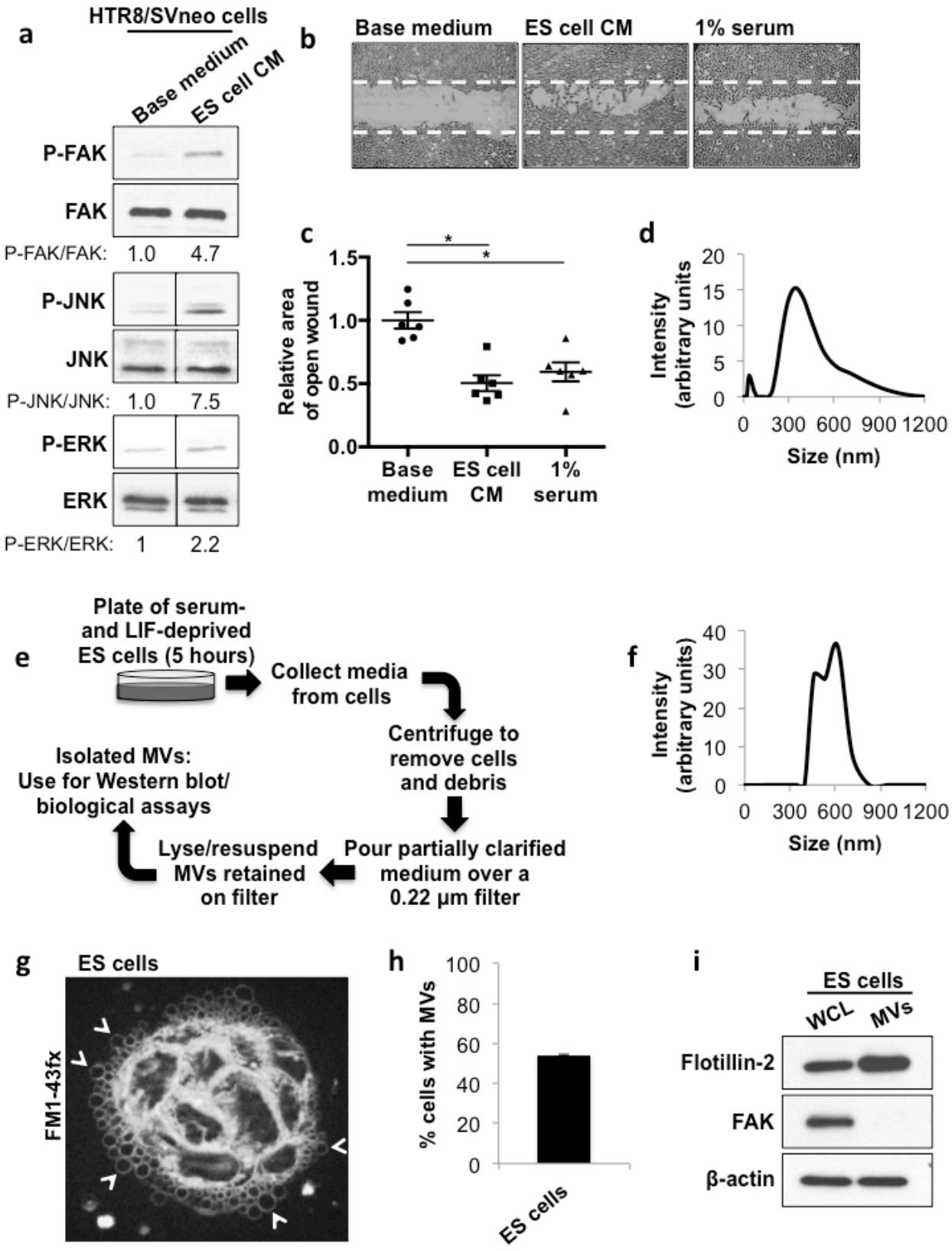
[1] Currently under review as a manuscript by LM Desrochers, F Bordeleau, CA Reinhart-King, RA Cerione and MA Antonyak at *Nature Communications*.

Results and Discussion

One of the major aspects of early embryogenesis that has been receiving a good deal of attention concerns to what extent the cells in the ICM of the blastocyst interact with their surroundings to shape fundamental physiological processes underlying normal development.[1,2] Here, we have examined how ES cells, which are derived from the ICM, engage in intercellular communication within the biological context of the blastocyst-stage embryo and its implantation into the uterus. Indeed, an important and as yet unanswered question is whether signaling between the cells that constitute the ICM and the surrounding layer of trophoblasts has a major influence on trophoblast function (i.e. migration and/or invasion). As a first step toward addressing this question, we treated the HTR8/SVneo trophoblast cell line[7] with either ES cell base medium lacking serum and leukemia inhibitory factor (LIF) or with conditioned medium (CM) from the pluripotent, feeder layer-independent E14tg2a.4 ES cell line[8] (Supplementary Figures 2.1a and 2.1b) that had been placed in the same base medium for 5 hours. The trophoblasts were then immunoblotted to detect the phosphorylated (activated) and total forms of signaling proteins frequently implicated in promoting cell migration, including focal adhesion kinase (FAK), c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase 1/2 (ERK1/2).[9-11] Figure 2.1a shows that the CM (lane labeled ES cell CM) up-regulated the phosphorylation levels of FAK (P-FAK) and JNK (P-JNK), compared to cells that were cultured in the ES cell base medium alone (lane labeled Base medium).

Wound closure (migration) assays were then performed to determine whether ES cell CM stimulates trophoblast migration. Wounds were struck through confluent monolayers of HTR8/SVneo trophoblasts placed in ES cell base medium (medium lacking serum and LIF), or in the same medium supplemented with either the CM collected from ES cells or serum. While

Figure 2.1. ES cells generate extracellular vesicles.
(**a**) Serum starved HTR8/SVneo trophoblasts treated with either ES cell base medium lacking serum and LIF (Base medium) or with the CM from E14tg2a.4 mouse ES cells cultured in the same medium (ES cell CM) were immunoblotted for phosphorylated FAK (P-FAK), JNK (P-JNK), and ERK (P-ERK). The blots were also probed for total FAK, JNK and ERK. The ratio of each phospho-protein/total protein pair examined was determined and included on the blots. (**b**) Images were taken of wound closure assays performed on HTR8/SVneo cells cultured in ES cell base medium (Base medium), the CM collected from ES cells cultured in the same base medium, or medium containing 1% serum. The dashed line indicates the width of the original wound. (**c**) The assays in **b** were quantified and plotted as relative area of open wound. (**d**) Dynamic light scattering plot of the E14tg2a.4 ES cell CM clarified of cells and debris. Note the detection of a ~30 nm exosome peak and a ~450 nm MV peak in the CM. **e**, Procedure for isolating MVs from CM. (**f**) Dynamic light scattering plot of the MV preparation from the E14tg2a.4 ES cell CM. Note the detection of a single peak of ~600 nm. (**g**) Fluorescence microscopy image of ES cells stained with the membrane dye FM1-43fx. Some of the MVs are denoted with arrowheads. (**h**) The percentage of cells in **g** with detectable levels of MVs on their surfaces was determined. (**i**) Lysates of ES cells (whole cell lysate, WCL) and the MVs generated by these cells were immunoblotted for the MV marker flotillin-2, the cytosolic-specific marker FAK, and β-actin as the loading control. All values shown are presented as mean ± s.e.m. ($n \geq 3$ independent experiments for each assay); *, $P<0.05$.


a
HTR8/SVneo cells
Base medium
ES cell CM
P-FAK
FAK
P-FAK/FAK: 1.0 4.7
P-JNK
JNK
P-JNK/JNK: 1.0 7.5
P-ERK
ERK
P-ERK/ERK: 1 2.2
b
Base medium
ES cell CM
1% serum
c
Relative area of open wound
1.5
1.0
0.5
0.0
*
*
Base medium
ES cell CM
1% serum
d
Intensity (arbitrary units)
20
15
10
5
0
0 300 600 900 1200
Size (nm)
e
Plate of serum- and LIF-deprived ES cells (5 hours)
Collect media from cells
Centrifuge to remove cells and debris
Pour partially clarified medium over a 0.22 µm filter
Lyse/resuspend MVs retained on filter
Isolated MVs: Use for Western blot/ biological assays
f
Intensity (arbitrary units)
40
30
20
10
0
0 300 600 900 1200
Size (nm)
g
ES cells
FM1-43fx
h
% cells with MVs
100
80
60
40
20
0
ES cells
i
ES cells
WCL
MVs
Flotillin-2
FAK
β-actin

trophoblasts maintained in only the base medium exhibited minimal migration after 12 hours, trophoblasts cultured in CM were capable of migrating into the wound to a degree comparable to that achieved upon serum-stimulation (Figures 2.1b and 2.1c). Inhibiting FAK and JNK activation blocked the ability of the ES cell CM to promote trophoblast migration, whereas inhibiting ERK activation had no effect (Supplementary Figures 2.2a-2.2f).

A mechanism by which cells communicate with their environment involves the shedding of extracellular vesicles (EVs). ES cells, like aggressive cancer cells,[12-14] generate distinct types of EVs called exosomes and microvesicles (MVs).[15,16] Exosomes range in size from 30-100 nm and are derived from the re-routing of multivesicular bodies to the cell surface where they fuse with the plasma membrane and are released.[17,18] MVs, which are considerably larger than exosomes (0.2-2 μm in diameter), bud and are shed from the plasma membrane.[19] EVs have been attracting considerable attention because of the diversity of proteins and nucleic acids that they contain as cargo, including cell surface receptors, cytosolic and nuclear signaling proteins, extracellular matrix proteins, RNA transcripts, micro-RNAs, and even DNA.[20] Moreover, EVs have the ability to transfer their contents to other cells and to stimulate signaling activities that lead to phenotypic and functional changes in the recipient cells.[12,15,18,21-24] Thus, we considered whether this unique form of cell-cell communication accounted for the ability of the CM from ES cells to affect the signaling events and functions of trophoblasts.

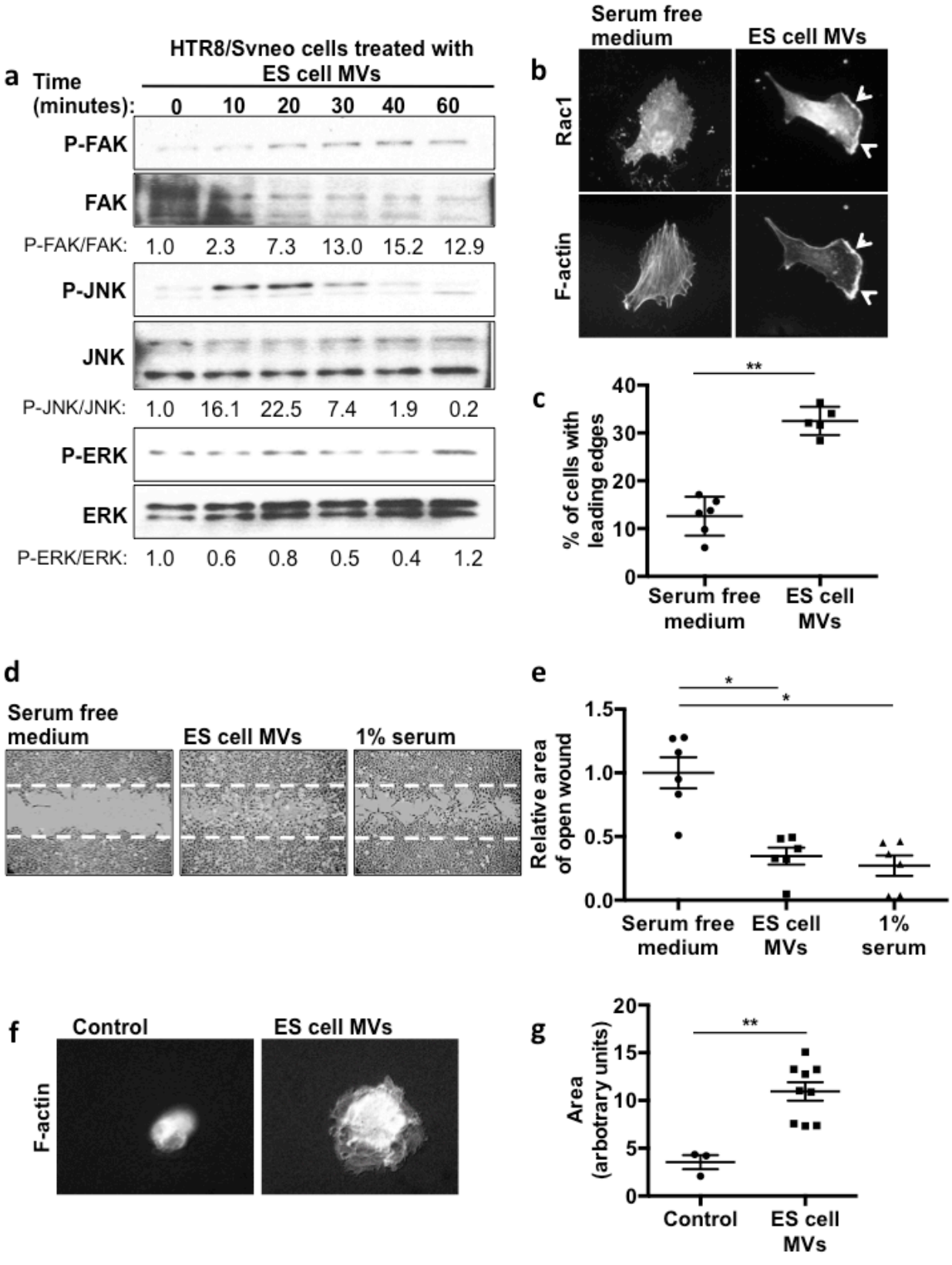
Following the removal of cells and debris by differential centrifugation, the CM was analyzed by dynamic light scattering (DLS) to determine the sizes of EVs present within these preparations. Figure 2.1d shows that ES cells produced EVs of two distinct sizes, averaging ~30 nm and ~450 nm. The size of the smaller vesicles corresponds to that of exosomes, while the larger vesicles represent MVs. Following an isolation procedure which involves an additional

filtration step of the partially clarified CM using a 0.22 μm filter (Figure 2.1e), a preparation of MVs, ranging from ~350-800 nm in diameter, was obtained (Figure 2.1f). Transmission electron microscopy (TEM) performed on the isolated MVs showed individual vesicles with sizes that were consistent with the DLS analysis (Supplementary Figure 2.3a). Likewise, microscopy experiments using the lipid-binding fluorescent dye FM1-43fx also revealed MVs of varying sizes decorating the surfaces of a significant percentage of the ES cell population (Figures 2.1g and 2.1h). The MV marker flotillin-2 (Figure 2.1i, top panel) was readily detected by immunoblot analysis of these MV preparations, while FAK (middle panel) was only found in the whole cell lysates (WCL). Moreover, control experiments carried out demonstrate that the MV isolation procedure efficiently removed soluble proteins from our MV preparations (Supplementary Figure 2.3b). Collectively, the findings indicate that the ES cell-derived MV preparations were largely homogeneous and devoid of exosomes, cytosolic contamination, as well as freely secreted proteins.

Because MVs from aggressive cancer cells activate signaling events in different types of recipient cells,[12,14,25,26] we examined whether MVs derived from ES cells could similarly affect trophoblasts, and in particular, if they were capable of activating the same protein kinases that were stimulated by the CM from ES cells. Indeed, we found that ES cell-shed MVs induced a time-dependent phosphorylation of FAK (P-FAK) and JNK (P-JNK), similar to when trophoblasts were incubated with the CM from ES cells (Figure 2.2a).

We then examined whether MVs generated by ES cells stimulated trophoblasts to form leading edges, a hallmark of polarized cell migration. Trophoblasts placed in serum free medium, either lacking or supplemented with ES cell-derived MVs for one hour, were stained for filamentous actin (F-actin) and Rac1, two proteins that localize at the leading edges of migrating

Figure 2.2. Trophoblasts treated with MVs from ES cells acquire a migratory phenotype. (**a**) Immunoblot of serum-starved HTR8/SVneo trophoblasts treated with serum free medium supplemented with MVs from ES cells for the indicated lengths of time. The ratio of each phospho-protein/total protein pair examined was determined and included on the blots. (**b**) HTR8/SVneo trophoblasts were incubated with serum free medium supplemented without (Serum free medium) or with MVs isolated from ES cells (ES cell MVs) for 1 hour, at which time the cells were stained with a Rac1 antibody and rhodamine-conjugated phalloidin to detect F-actin. Images of the cells maintained under each condition are shown. Arrowheads denote leading edges. (**c**) The percentages of cells in **b** with leading edges were determined. (**d**) Images were taken of wound closure assays performed on HTR8/SVneo cells cultured in serum free medium supplemented without (Serum free medium) or with either MVs from ES cells (ES cell MVs) or 1% serum. The dashed line indicates the width of the original wound. (**e**) The assays in **d** were quantified and plotted as the relative area of open wound. (**f**) E3.5 blastocysts were harvested and placed in dishes containing blastocyst culturing medium supplemented without (Control) or with ES cell MVs. Two days later, the blastocysts were stained with rhodamine-conjugated phalloidin to label F-actin and visualized by fluorescence microscopy. Images of the blastocysts maintained under each condition are shown. (**g**) The assays in **f** were quantified and graphed as relative area of blastocyst outgrowth. All values shown are presented as mean ± s.e.m. ($n \geq 3$ independent experiments for each assay); *, $P<0.05$, **, $P<0.01$.


a
HTR8/Svneo cells treated with ES cell MVs
Time (minutes):
0
10
20
30
40
60
P-FAK
FAK
P-FAK/FAK:
1.0
2.3
7.3
13.0
15.2
12.9
P-JNK
JNK
P-JNK/JNK:
1.0
16.1
22.5
7.4
1.9
0.2
P-ERK
ERK
P-ERK/ERK:
1.0
0.6
0.8
0.5
0.4
1.2
b
Serum free medium
ES cell MVs
Rac1
F-actin
c
% of cells with leading edges
**
Serum free medium
ES cell MVs
d
Serum free medium
ES cell MVs
1% serum
e
Relative area of open wound
*
*
Serum free medium
ES cell MVs
1% serum
f
Control
ES cell MVs
F-actin
g
Area (arbotrary units)
**
Control
ES cell MVs

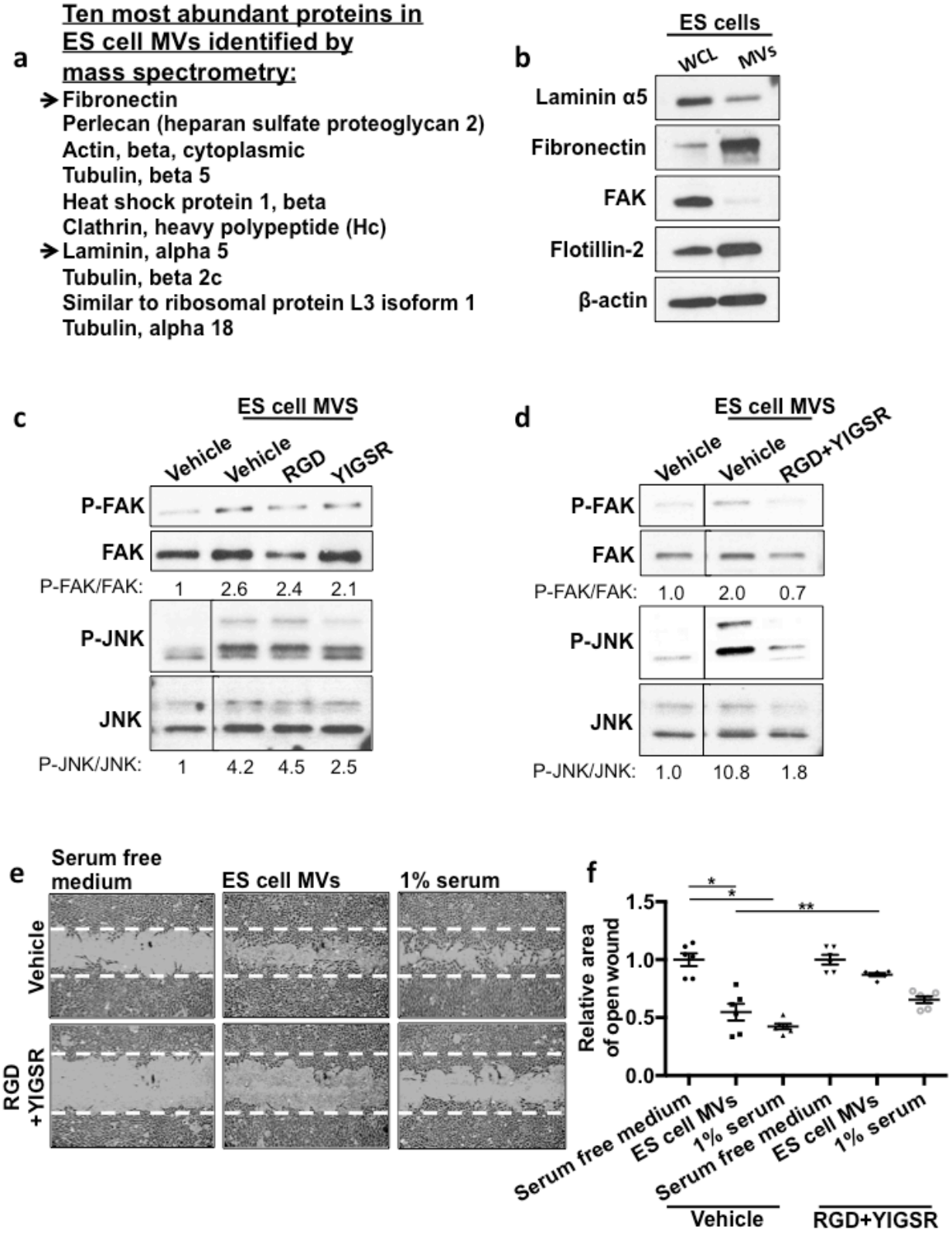
cells.[27,28] The resulting fluorescence microscopy images of the trophoblasts cultured in serum free medium showed that Rac1 was localized throughout the cell, with F-actin forming well-defined stress fibers (Figure 2.2b, left panels). However, the MV-treated trophoblasts exhibited a polarized morphology, with Rac1 being localized to discrete regions along their plasma membranes (Figure 2.2b, top right panel), and were largely devoid of detectable stress fibers. F-actin was aligned along the leading edges of the cells (Figure 2.2b, bottom right panel). Nearly 35% of the trophoblasts treated with ES cell-derived MVs showed well-defined leading edges, representing a greater than two-fold increase compared to the untreated control cells (Figure 2.2c).

The ability of ES cell MVs to induce trophoblasts to form leading edges was accompanied by an enhanced migration as measured in wound healing assays, which was comparable to the stimulation obtained with serum (i.e. Figures 2.2d and 2.2e). We then set out to determine whether MVs from ES cells promoted blastocyst outgrowth, which is thought to reflect the implantation process and the ability of the trophectoderm in the blastocyst-stage embryo to attach to a culture dish and migrate.[29] E3.5 blastocysts isolated from mice were randomly divided into two groups. One group was cultured in a dish containing blastocyst culture medium, while the second group was cultured in the same medium supplemented with MVs collected from ES cells. Figure 2.2f shows representative images of blastocysts that were cultured in medium lacking (Control, left) or containing ES cell MVs (right) for 48 hours, at which point they were stained with rhodamine-conjugated phalloidin to label F-actin and visualized by fluorescence microscopy. The trophoblasts in the blastocysts cultured with MVs showed enhanced migration, forming outgrowths that were at least three-fold greater in area than their control counterparts (Figure 2.2g).

We then set out to determine whether the MVs generated by ES cells were uniquely capable of influencing trophoblast migration. We discovered that the HTR8/SVneo trophoblast cell line, itself, generated MVs of similar sizes and amounts as ES cells, as read out by subjecting MVs isolated from this cell line to DLS analysis (Supplementary Figure 2.3c), and immunoblot analysis using the MV marker flotillin-2 (Supplementary Figure 2.3d). MVs could also be detected on the surfaces of trophoblasts stained with the FM1-43fx plasma membrane dye (Supplementary Figure 2.3e). However, the MVs generated by HTR8/SVneo trophoblasts, when collected and then added back to cultures of trophoblasts, were unable to promote their own migration (Supplementary Figure 2.3f and 2.3g).

Since MVs isolated from ES cells are capable of rapidly (within 1 hour) activating signaling events within recipient trophoblasts, as well as inducing leading edge formation, we suspected that proteins associated with the MVs were responsible for stimulating these outcomes, rather than transferred DNA or RNA transcripts, as phenotypic changes involving transcription and/or translation typically require several hours to occur. Therefore, a proteomic analysis was performed to identify the protein cargo of MVs isolated from the E14tg2a.4 ES cell line. A large number of proteins were detected, with the ten most abundant being listed in Figure 2.3a. The extracellular matrix proteins fibronectin and laminin α5 were especially noteworthy, given that fibronectin was previously shown to be essential for the growth- and survival-promoting activity of MVs from aggressive cancer cell lines,[12] whereas laminin α5 knock-out mice exhibited placental abnormalities.[30] These two extracellular matrix proteins were recently found to be expressed in the blastocyst at the time of implantation.[31] We confirmed the presence of both proteins in MVs derived from ES cells by immunoblot analysis (Figure 2.3b, top two panels). Fibronectin and laminin function as ligands that activate receptors

Figure 2.3. Fibronectin and laminin mediate MV-promoted trophoblast migration.
(a) Mass spectrometry was performed on MVs isolated from ES cells. The 10 most abundant proteins identified are listed. Arrows highlight proteins predicted to most likely mediate the migration-promoting effects of MVs. (**b**) Lysates of ES cells (WCL), and the MVs that they generated (MVs), were immunoblotted for fibronectin, laminin α5, the MV-marker flotillin-2, the cytosolic-specific protein FAK, and β-actin, as a loading control. (**c**) and (**d**) Serum starved HTR8/SVneo trophoblasts were treated with PBS (Vehicle) or with either **c**, the fibronectin inhibitory peptide RGD or the laminin inhibitory peptide YIGSR, or **d**, a combination of the two inhibitors. The cells were then stimulated with ES cells MVs for ~30 minutes before being lysed and immunoblotted for phosphorylated and total FAK and JNK. The ratio of each phospho-protein/total protein pair examined was determined and included on the blots. Note only the combination of RGD and YIGSR blocked integrin-mediating phosphorylation of FAK and JNK. (**e**) Images were taken of wound closure assays performed on HTR8/SVneo cells cultured in serum-free medium supplemented with the vehicle (top panels) or the RGD- and YIGSR-inhibitory peptides (bottom panels). Each culturing condition was further treated without (Serum free medium) or with either MVs from ES cells (ES cell MVs) or 1% serum. The dashed line indicates the width of the original wound. (**f**) The assays in **e** were quantified and plotted as the relative area of open wound. All values shown are presented as mean ± s.e.m. ($n \geq 3$ independent experiments for each assay); *, $P<0.05$, **, $P<0.01$.


a
Ten most abundant proteins in ES cell MVs identified by mass spectrometry:
Fibronectin
Perlecan (heparan sulfate proteoglycan 2)
Actin, beta, cytoplasmic
Tubulin, beta 5
Heat shock protein 1, beta
Clathrin, heavy polypeptide (Hc)
Laminin, alpha 5
Tubulin, beta 2c
Similar to ribosomal protein L3 isoform 1
Tubulin, alpha 18
b
ES cells
WCL
MVs
Laminin α5
Fibronectin
FAK
Flotillin-2
β-actin
c
ES cell MVS
Vehicle
Vehicle
RGD
YIGSR
P-FAK
FAK
P-FAK/FAK: 1 2.6 2.4 2.1
P-JNK
JNK
P-JNK/JNK: 1 4.2 4.5 2.5
d
ES cell MVS
Vehicle
Vehicle
RGD+YIGSR
P-FAK
FAK
P-FAK/FAK: 1.0 2.0 0.7
P-JNK
JNK
P-JNK/JNK: 1.0 10.8 1.8
e
Serum free medium
ES cell MVs
1% serum
Vehicle
RGD +YIGSR
f
Relative area of open wound
1.5
1.0
0.5
0.0
*
*
**
Serum free medium
ES cell MVs
1% serum
Serum free medium
ES cell MVs
1% serum
Vehicle
RGD+YIGSR

expressed by trophoblasts,[32] with the α5β1 integrin complex serving as the co-receptor for fibronectin,[33] while laminin binds to the 67 kDa laminin receptor, which acts as an integrin co-receptor for integrin α6.[34] Moreover, fibronectin- and laminin-induced integrins signal through FAK and JNK.[35,36] Both of these protein kinases were activated in trophoblasts treated with MVs from ES cells, and are required for trophoblast migration, i.e. trophoblasts incubated with either a JNK or FAK inhibitor failed to migrate in wound closure assays (Supplementary Figures 2.4a-2.4d). Consistent with these findings, treating isolated blastocysts with these same inhibitors also prevented their attachment and outgrowth onto a substrate (Supplementary Figure 2.4e).

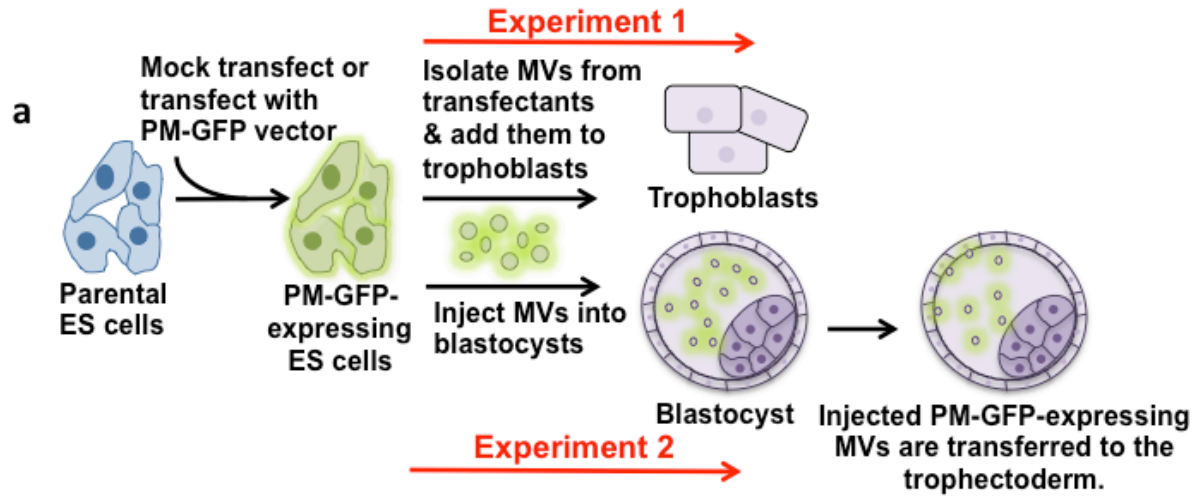
To determine whether MV-associated fibronectin and laminin were responsible for activating FAK and JNK, we examined the effects of blocking their ability to engage their respective receptors on the surfaces of trophoblasts. The interaction of fibronectin with α5β1 integrins was disrupted using the RGD peptide,[37] while the binding of laminin to its receptor was blocked using the YIGSR peptide.[38] Treating HTR8/SVneo trophoblasts with the RGD peptide alone, prior to incubating them with MVs isolated from ES cells, failed to prevent MV-induced FAK and JNK activation (Figure 2.3c, first and third panels). Similarly, treatment with the YIGSR peptide was not sufficient to block MV-stimulated FAK activation (Figure 2.3c, top panel) and caused only a modest reduction of JNK activity (Figure 2.3c, third panel). However, when the two inhibitors were used concurrently, they effectively blocked the ability of MVs to activate these protein kinases (Figure 2.3d, first and third panels).

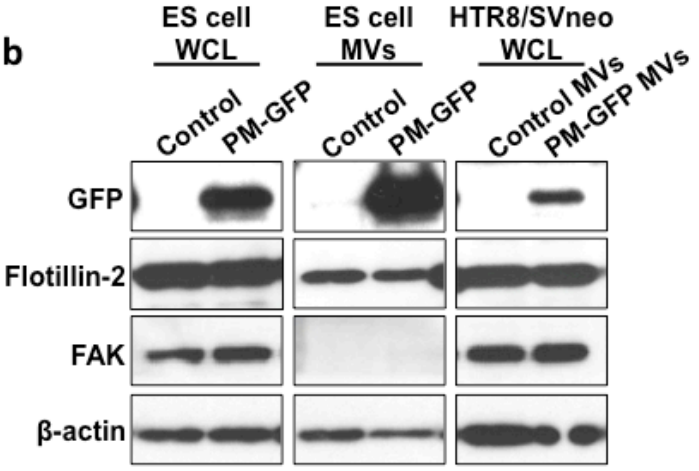
Confirmation that ES cell MVs require fibronectin and laminin to promote trophoblast migration was then obtained from wound closure assays in which the RGD and YIGSR peptides were added together to trophoblasts that were either cultured in serum free medium, or treated with medium containing ES cell MVs or serum. The addition of these peptides, in combination,

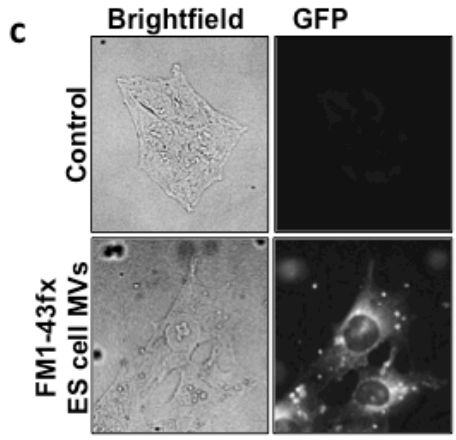
but not when added individually, efficiently inhibited MV-promoted trophoblast migration, reducing it to a level comparable to that observed in serum-starved conditions (Figures 2.3e and 2.3f; Supplementary Figures 2.5a-2.5d). In contrast, however, the exposure of trophoblasts to the combination of purified forms of fibronectin and laminin stimulated their migration to an extent similar to that obtained with full serum treatment (Supplementary Figures 2.5e and 2.5f). Taken together, these findings indicate that the extracellular matrix proteins fibronectin and laminin are essential for the ability of ES cell MVs to activate integrin-mediated signaling events necessary for promoting trophoblast migration.

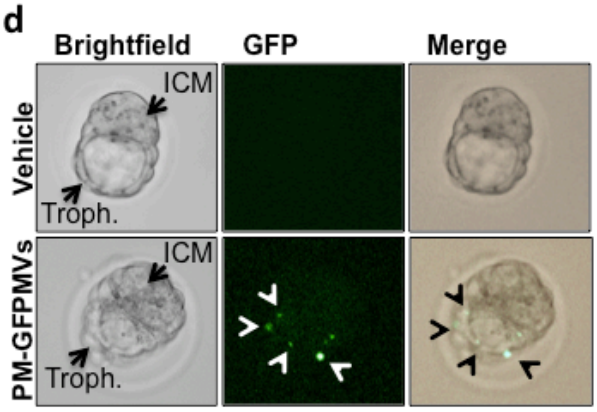
We next wanted to determine whether the MV-mediated intercellular communication between ES cells and trophoblasts occurs within blastocysts. To demonstrate that this is the case, we took advantage of earlier work by our laboratory showing that plasma membrane-targeted green fluorescent protein (PM-GFP) was capable of being incorporated into MVs generated by cancer cells and then transferred via MVs to recipient cells.[12] Duplicate sets of parental E14tg2a.4 ES cells were either mock transfected (Control) or transfected with PM-GFP (Figure 2.4a, Experiment 1). The MVs collected from one set of the transfected cells showed a significant incorporation of PM-GFP (Figure 2.4b, lanes labeled ES cell MVs, top panel), similar to what we had previously observed for MVs isolated from the highly aggressive MDA-MB231 breast cancer cell line expressing this fusion protein.[39] MVs isolated from either the second set of mock-transfected ES cells (Control), or from the ES cells ectopically expressing PM-GFP, were then added to cultures of trophoblasts for three hours, at which time the trophoblasts were extensively washed before being immunoblotted with a GFP antibody. PM-GFP was clearly detectable in the trophoblasts that had been incubated with the PM-GFP-labeled MVs (Figure 2.4b, lanes labeled HTR8/SVneo WCL, top panel).

Figure 2.4. MVs from ES cells are transferred to trophoblast lineage cells and promote blastocyst implantation.
(**a**) Description of experiments (Experiment 1 and 2) conducted to demonstrate that MVs transfer cargo from ES cells to trophoblasts. (**b**) Experiment 1; MVs generated by mock transfected ES cells (Control) or ES cells ectopically expressing a plasma membrane-targeted GFP (PM-GFP) construct were isolated and then either lysed or resuspended in medium. Lysates of the transfected ES cells (ES cell WCL, lanes 1 and 2), and the MVs they formed (ES cell MVs, lanes 3 and 4), were immunoblotted for GFP, the MV marker flotillin-2, the cytosolic-specific marker FAK, and β-actin, as a loading control. Note PM-GFP was incorporated into the MVs (lane 4). The MVs resuspended in medium were added to HTR8/SVneo trophoblasts for 3 hours, at which time the cells were extensively washed, lysed, and then immunoblotted as described above (HTR8/SVneo WCL, lanes 5 and 6). Note PM-GFP was detected in the trophoblasts treated with MVs from the ES cells expressing PM-GFP. (**c**) Basal medium (Control) or CM collected from ES cells clarified of cells and cell debris was treated with FM1-43fx plasma membrane dye for 5 minutes prior to being subjected to the MV isolation procedure. Each MV preparation was then added to cultures of trophoblasts for 3 hours, at which point they were fixed and visualized by brightfield and fluorescence (GFP) microscopy. Note that only the trophoblasts treated with MVs exhibited fluorescence. (**d**) Experiment 2; MVs derived from ES cells ectopically expressing PM-GFP were injected into E3.5 blastocysts. About 3 hours later, the blastocysts were visualized by microscopy. Shown are bright field (left panels), fluorescence (GFP, middle panels), and merged images (right panels) of two blastocysts. One was injected with vehicle (PBS) as a negative control (upper panels), while the other was injected with MVs expressing PM-GFP diluted in PBS (lower panels). Labeled on the blastocysts shown in the brightfield images are the inner cell mass (ICM) and the trophectoderm. On the lower panels, arrowheads are used to indicate GFP signal detected along thetrophectoderm layer of the blastocyst. (**e**) The ratio of blastocysts in **d** with detected levels of GFP signal in their trophectoderm. (**f**) E3.5 blastocysts were injected with either a PBS vehicle control or MVs from ES cells. The vehicle alone-injected blastocysts were surgically placed into the right uterine horn of a surrogate mouse, while the blastocysts injected with MVs were placed in the left uterine horn of the same mouse. Three days later (E6.5), the uteri were harvested and the rate of embryo implantation for each condition/mouse was determined and plotted as color-coded pairs. A total of 168 blastocysts (84 for each condition) and 12 surrogate mice were used in 4 independent experiments; *, $P \leq 0.05$.



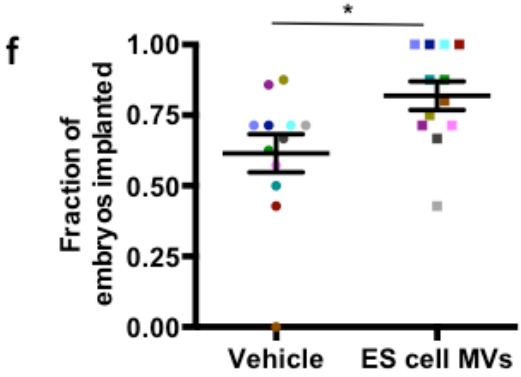






e

GFP-positive trophectoderm/vehicle-injected embryos	0/16
GFP-positive trophectoderm/PM-GFP MV-injected embryos	14/16



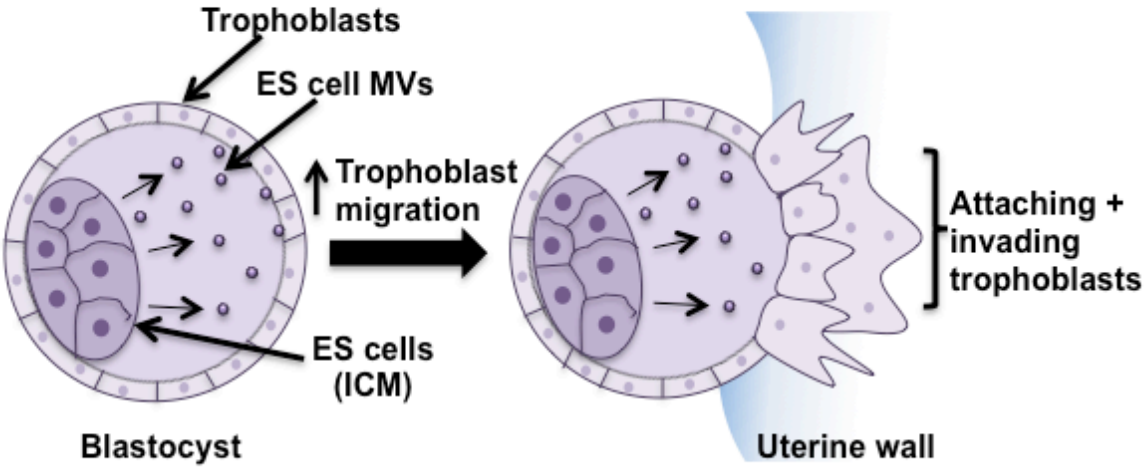
To further confirm the uptake of ES cell MVs by trophoblasts, basal medium (Control) or ES cell CM was treated with FM1-43fx plasma membrane dye prior to being subjected to the MV isolation procedure. The MV preparations were incubated with trophoblasts for three hours, at which time the cells were visualized by brightfield and fluorescence microscopy. While the control-treated trophoblasts failed to exhibit any detectable fluorescence (Figure 2.4c, panels labeled Control), nearly all of the trophoblasts treated with MVs labeled with FM-1-43fx did (Figure 2.4c, panels labeled FM1-43fx ES cell MVs).

We then examined whether MVs derived from ES cells can transfer their cargo to the layer of trophoblasts surrounding the ICM, called the trophectoderm (see Figure 2.4a, Experiment 2). MVs derived from ES cells ectopically expressing PM-GFP (Figure 2.4b, lanes labeled ES cell MVs, top panel) or vehicle alone were injected into the cavity of embryonic day 3.5 (E3.5) blastocysts, and then the blastocysts were visualized by microscopy ~3 hours later. Figure 2.4d shows bright field images (left panels), fluorescent images (middle panels), and merged images (right panels) of two blastocysts: one injected with vehicle (top panels), and another with MVs expressing PM-GFP (bottom panels). GFP signal was not detected in any of the blastocysts injected with the vehicle alone (Figure 2.4d, top panel and Figure 2.4e). In contrast, GFP signal could be clearly detected in discrete regions along the trophectoderm in 14/16 of the blastocysts injected with MVs (Figure 2.4d, bottom panels and Figure 2.4e), suggesting that MVs shed from ES cells were indeed capable of being transferred to the trophectoderm. In addition, the MV-mediated intercellular communication between ES cells and trophoblasts positively impacted the ability of the trophoblast to undergo implantation. Specifically, we found that injecting E3.5 blastocysts with MVs isolated from ES cells, prior to surgically placing them into the uteri of surrogate mice, gave rise to a statistically significant

enhancement in the likelihood that the blastocysts implanted when compared to blastocysts injected with vehicle alone (Figure 2.4f).

It is becoming increasingly appreciated that MVs provide an important mechanism for cell-cell-communication.[24,40] Thus far, this has been particularly well demonstrated within the context of tumorigenesis, where MVs shed by cancer cells can be transferred to (recipient) cells within the tumor microenvironment and alter their behavior in ways that drive cancer progression.[12,14,21,23,25,26] While it has been reported that ES cells also generate and shed MVs, as well as exosomes, we have barely scratched the surface in our understanding of the roles that these EVs play in different aspects of stem cell biology. We now show that ES cell-derived MVs play an important role in the ability of trophoblasts to migrate, invade and ultimately implant into the uterine wall (Figure 2.5). The early stages of pregnancy, especially implantation, are vital for successful pregnancies. Complications that arise during implantation are a major cause of infertility, but they can also lead to other serious conditions, such as preeclampsia.[3] Therefore, our findings shed new light on a novel form of ES cell function and intercellular communication in a critically important biological process.

Figure 2.5. Diagram showing how ES cells communicate with trophoblasts to promote embryo implantation.
In the blastocyst-stage embryo, the ES cells reside in the ICM and are surrounded by trophoblasts (left image). While it is known that the trophoblasts in the blastocyst respond to signals emanating from the mother to migrate and invade into the uterus (implantation), we have discovered that ES cells also contribute to this process by generating MVs (right image). The MVs are capable of activating signaling events in trophoblasts that enhance their ability to migrate.


Trophoblasts
ES cell MVs
Trophoblast migration
ES cells (ICM)
Blastocyst
Attaching + invading trophoblasts
Uterine wall

Materials and Methods

Antibodies and reagents

Antibodies that recognize the total or phosphorylated forms of JNK, FAK, ERK1/2, and flotillin-2 were from Cell Signaling Technology. The GFP and Oct3/4 antibodies were from Santa Cruz Biotechnology, the Ran antibody was from BD Transduction Laboratories, while the Nanog antibody was from Abcam. The fibronectin, β-actin and laminin α5 antibodies, as well as GRGDSP (RGD) peptide and YIGSR peptide were from Sigma-Aldrich. The 0.22 μm Steriflip filters, centrifugal filters, Rac1 antibody, FAK inhibitor III, PD98059, SP600125, embryonic stem (ES) cell-qualified fetal bovine serum (FBS), leukemia inhibitory factor (LIF) and M2 medium were from Millipore. The PM-GFP vector was a gift from Tobias Meyer (Addgene plasmid #21213). The rhodamine-conjugated phalloidin, Oregon green 488 goat anti-mouse secondary antibody, FM1-43FX plasma membrane dye, Lipofectamine, as well as all other tissue culture reagents were from Life Technologies.

Cell culture

E14tg2a.4 mouse ES cells were cultured on gelatin-coated dishes in Glasgow's Minimal Essential Medium supplemented with 1 mM sodium pyruvate, non-essential amino acids, 55 μM β-mercaptoethanol, 15% ES cell-qualified FBS, and 1000 U/ml LIF. HTR8/SVneo trophoblast cells, which were a gift from Charles Graham (Queen's University, Kingston, ON), were cultured in RPMI-1640 medium supplemented with 5% FBS.

Concentration of CM and MV isolation

One 150 mm dish of nearly confluent ES cells (~4.0 x 10^7 cells) was rinsed several times with phosphate-buffered saline (PBS) and incubated in serum- and LIF-free medium for 5 hours. The CM was removed from the cells, and first centrifuged at 300 x *g* for 10 minutes to pellet intact cells, and again at 3,000 x *g* for 20 minutes to remove cell debris. The partially clarified medium was concentrated to 1 ml using a centrifugal filter with a nominal molecular weight limit of 10 kDa. To isolate MVs, the CM from two nearly confluent 150 mm dishes of ES cells (~8.0 x 10^7 cells) was partially clarified as described above, filtered through a 0.22 μm Steriflip filter unit (Millipore), and then washed with 10 ml of PBS. If being used in biological assays, the MVs retained by the filter were resuspended in 1.5 ml of serum-free medium, treated as indicated, and added to ~2.0 x 10^5 trophoblasts. However, when used to generate lysates, the MVs were lysed with mammalian lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM β-glycerol phosphate, 1 μg/ml aprotinin, and 1 μg/ml leupeptin).

Signaling experiments in trophoblasts

Serum starved trophoblasts were trypsinized, counted, and ~2.0 x 10^5 cells were placed into two different tubes. The cells were centrifuged at 300 x *g* for 5 minutes, and the pelleted cells were then resuspended in serum-free medium supplemented without or with either CM or MVs isolated from ES cells. Following the indicated lengths of incubation, the cells were re-pelleted and lysed in mammalian lysis buffer. For the MV transfer experiments, MVs isolated from ES cells expressing the vector alone or PM-GFP or ES cell MVs treated with FM1-43fx were added to ~2.0 x 10^5 adherent trophoblasts for 3 hours, at which time the trophoblasts were lysed in mammalian lysis buffer or fixed with 3.7% formaldehyde in PBS for 20 minutes, as indicated.

Immunoblot analysis

Protein concentrations of MV and cell lysates were determined using the Bio-Rad DC protein assay. Equal amounts of each lysate were resolved by SDS-PAGE and then transferred to PVDF membranes. The membranes were incubated with the indicated primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20 (TBST). Primary antibodies were detected with HRP-conjugated secondary antibodies (Cell Signaling Technology), followed by exposure to ECL reagent. Where indicated, changes in protein phosphorylation were determined by normalization to total protein levels using ImageJ.

Mass spectrometry

Lysates of MVs isolated from E14tg2a.4 ES cells (30 μg) were resolved by SDS-PAGE and then stained with a Colloidal Blue Staining kit. The proteins were excised from the gel and trypsin-digested. Cornell's Proteomics Facility analyzed the resulting peptide fragments using a triple quadrupole linear ion trap (4000 Q Trap) on-line LC/MS/MS system (Applied Biosystems/MDS Sciex) or the Synapt HDMS system (Waters). Proteins were identified by performing peptide alignment searches using the NCBI mouse RefSeq protein database.

Fluorescence microscopy

To visualize MVs on the surfaces of ES cells and trophoblasts, cultures of cells were incubated with 5 μg/ml FM 1-43FX plasma membrane dye diluted in PBS for 1 minute, fixed in ice cold 3.7% formaldehyde, and then rinsed with PBS before being analyzed by fluorescence microscopy. To visualize leading edges on trophoblasts, cultures of cells that had been treated as indicated were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and then

blocked with 10% bovine serum albumin. The cells were incubated with a Rac1 antibody, washed, and then incubated with an Oregon Green 488-conjugated secondary antibody, rhodamine-conjugated phalloidin to stain F-actin, and DAPI to label nuclei. The cells were then visualized by fluorescence microscopy. All images of the cells were captured and processed using IPLABS software.

Dynamic Light Scattering (DLS)

To determine the sizes of EVs by DLS analysis, CM or MV preparations were loaded into microcuvettes and analyzed for particle size using a Zetasizer (Malvern Nano ZS; He-Ne laser; 173° backscattered light detection). At least three independent experiments were performed for each condition analyzed, and each result shown represents an average of 3 runs, with at least 5 measurements being taken per run.

Transmission electron microscopy (TEM)

Five µl of a MV preparation resuspended in PBS were added to a carbon-coated, 300-mesh copper grid and then stained with 1.75% uranyl acetate. Once dry, the samples were imaged using the FEI T12 Spirit 120 kV field emission TEM at Cornell's Center for Materials Research (CCMR), supported by NSF MRSEC award number: NSF DMR-1120296.

Wound closure (migration) assays

Wounds were struck through confluent monolayers of serum starved trophoblasts using a pipet tip. The cells were rinsed with PBS, placed in the indicated culturing conditions, and allowed to

migrate for 12 hours. The cells were then fixed in 3.7% formaldehyde. The extent of wound closure for each condition was imaged and plotted.

Blastocyst collection and outgrowth assays

Experiments were performed as described previously.[29] In brief, post-coital day 3.5 female BALB/c and B6-2J mice were sacrificed and their uteri removed. The uteri were flushed with M2 medium to remove the blastocysts, which were then cultured on gelatin-coated dishes in Dulbecco's Minimal Essential Medium supplemented with 10% FBS, non-essential amino acids, 55 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin and treated as indicated. Once the blastocysts attached to the dish and spread, they were fixed and stained with rhodamine-conjugated phalloidin. Images of the blastocysts were captured and processed using IPLABS software, and the areas of blastocyst outgrowth were determined using ImageJ.

Microinjection of blastocysts and determination of implantation rates

Isolated blastocysts were injected with MVs derived from ES cells ectopically expressing PM-GFP or vehicle (PBS) by Cornell's Induced Pluripotent Stem Cell Core Facility. The blastocysts were incubated in blastocyst culturing medium for ~3 hours, at which point they were visualized using light and fluorescence microscopy. For the *in vivo* studies, blastocysts injected with MVs derived from parental ES cells were surgically placed in the left uterine horn of a pseudo-pregnant recipient mouse, while an equal number of blastocysts injected with vehicle alone (PBS) were placed in the right uterine horn of the same mouse. Three days later the mouse was sacrificed, its uterus removed, and the percentages of embyros that implanted for each condition

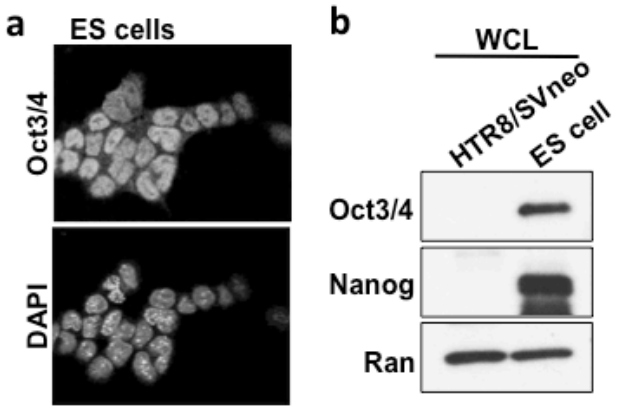
was determined. This experiment was carried out on four separate occasions, using a total of 168 blastocysts (84 for each condition) and 12 mice as embryo transfer recipients.

Statistical analysis

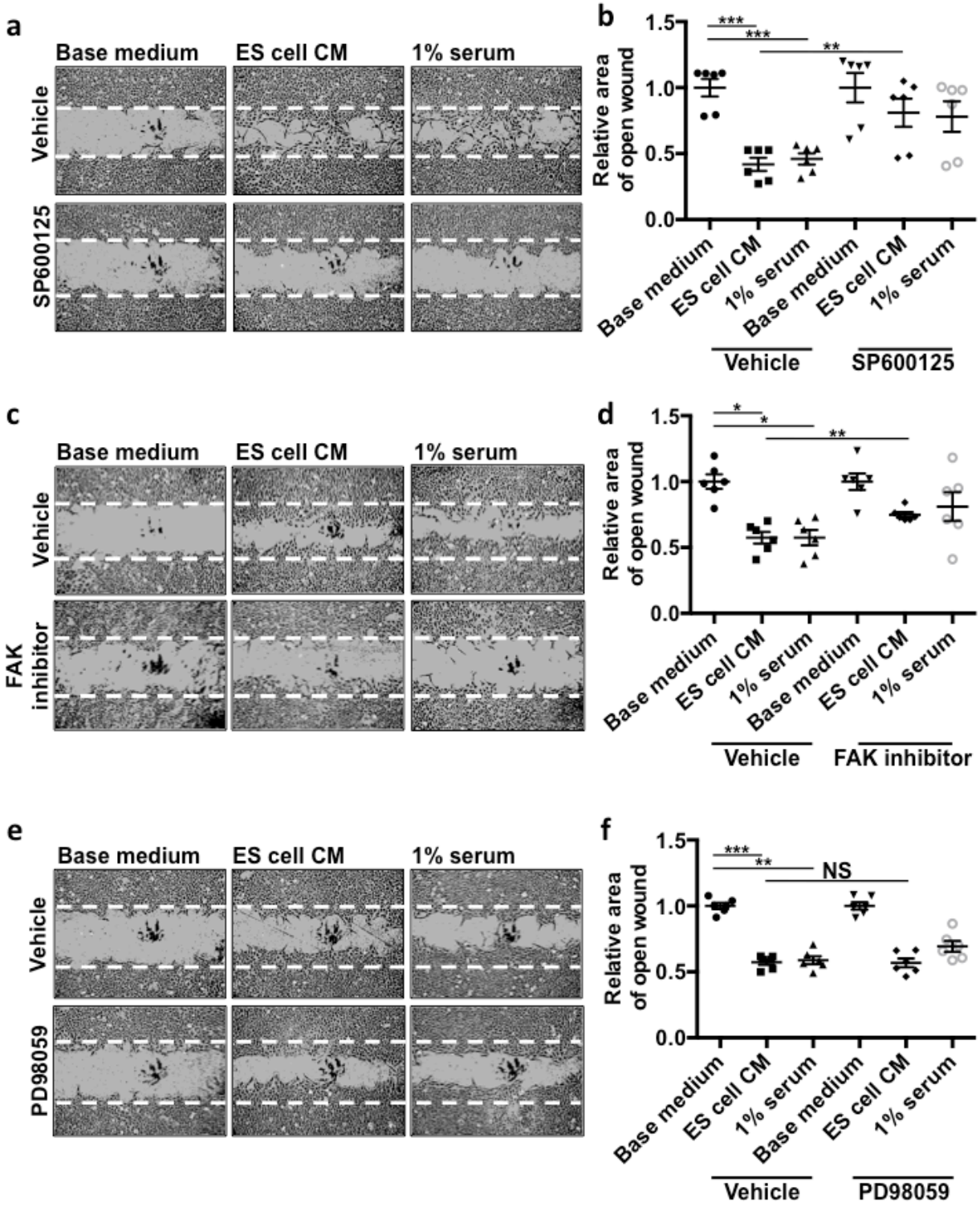
All experiments were performed a minimum of three independent times. Wound closure assays and Western blots were quantified using ImageJ. Many of the results were presented as scatter plots with mean and standard error of the mean (s.e.m.) plotted using GraphPad Prism 6. Student's t-tests were performed to assess statistical significance in all cases, except for the experiments involving the comparison of implantation rates, for which a Wilcoxon signed-rank test was used. P values ≤ 0.05 were considered significant and indicated with asterisks, as follows: *, $P \leq 0.05$, **, $P < 0.01$, ***, $P < 0.001$, NS, not significant.

Supplementary Information

Supplementary Figure 2.1. Feeder layer-independent E14tg2a.4 mouse embryonic stem (ES) cells are pluripotent.
(**a**) Immunofluorescence image of ES cells stained with an Oct3/4 antibody, a marker of pluripotency (top). The cells were also incubated with DAPI to label nuclei (bottom). Note that all of the cells in the field are Oct3/4-positive. (**b**) Whole cell lysates (WCL) of HTR8/SVneo trophoblasts and ES cells were immunoblotted for the pluripotency markers Oct3/4 and Nanog, and Ran GTPase as a loading control. Only the ES cells expressed Oct3/4 and Nanog, indicating that they are indeed pluripotent.

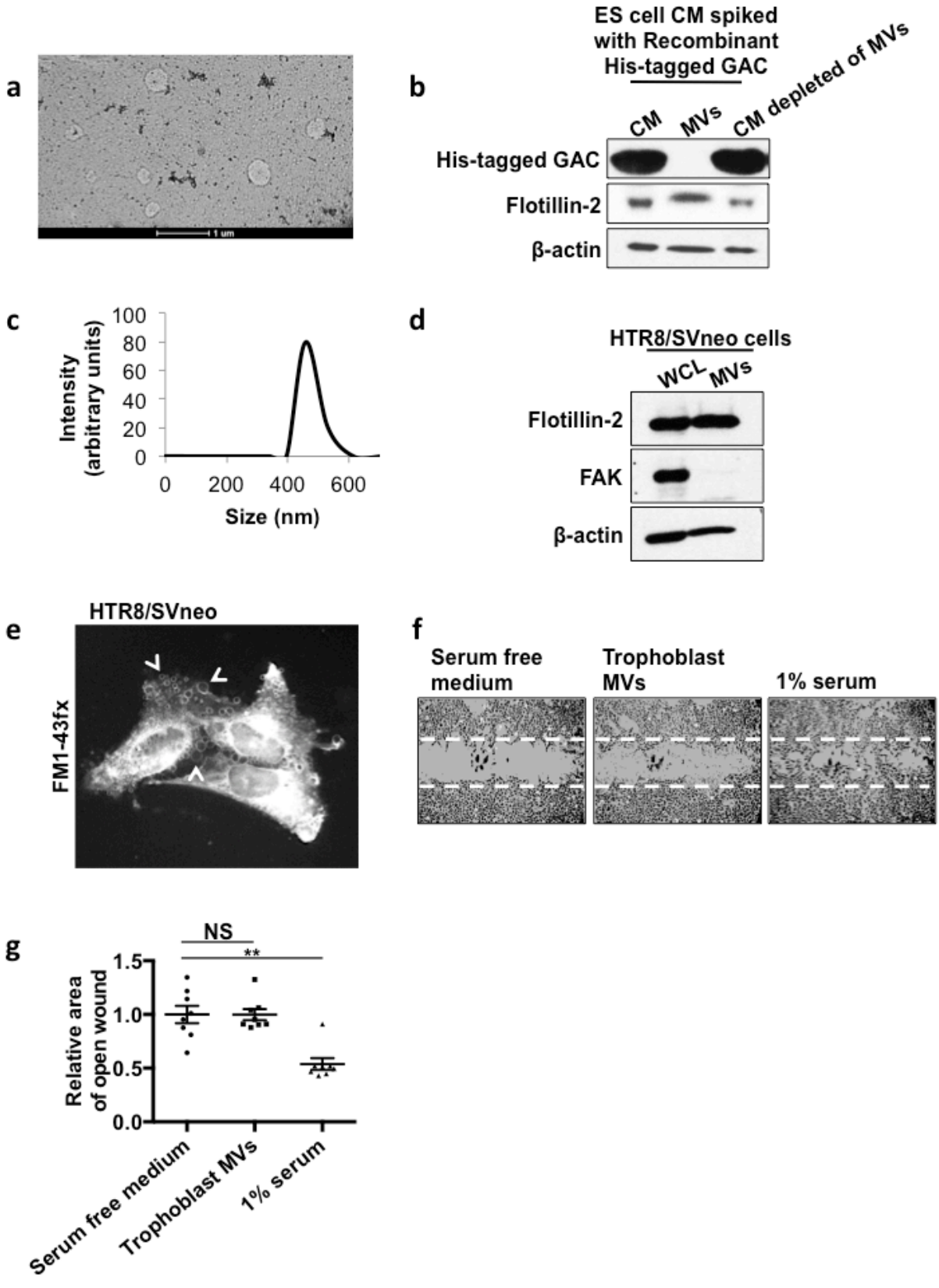

a
ES cells
Oct3/4
DAPI
b
WCL
HTR8/SVneo
ES cell
Oct3/4
Nanog
Ran

Supplementary Figure 2.2. FAK and JNK activation are important for HTR8/SVneo trophoblasts to migrate in response to treatment with ES cell CM.
(**a**-**f**) Wound closure assays were performed on HTR8/SVneo cells cultured in ES cell base medium lacking serum and LIF (Base medium), the CM collected from ES cells cultured in the same medium (ES cell CM), or medium containing 1% serum. Each culturing condition was treated further with either DMSO (Vehicle) or **a**, SP600125, **c**, FAK inhibitor III (FAK inhibitor) or **e**, PD98059. Images of the cells are shown. The dashed line indicates the width of the original wound. The assays in **a**, **c**, and **e** were quantified and plotted in **b**, **d**, and **f** as the relative area of open wound. All values shown are presented as mean ± s.e.m. ($n \geq 3$ independent experiments for each assay); *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$, NS, not significant.

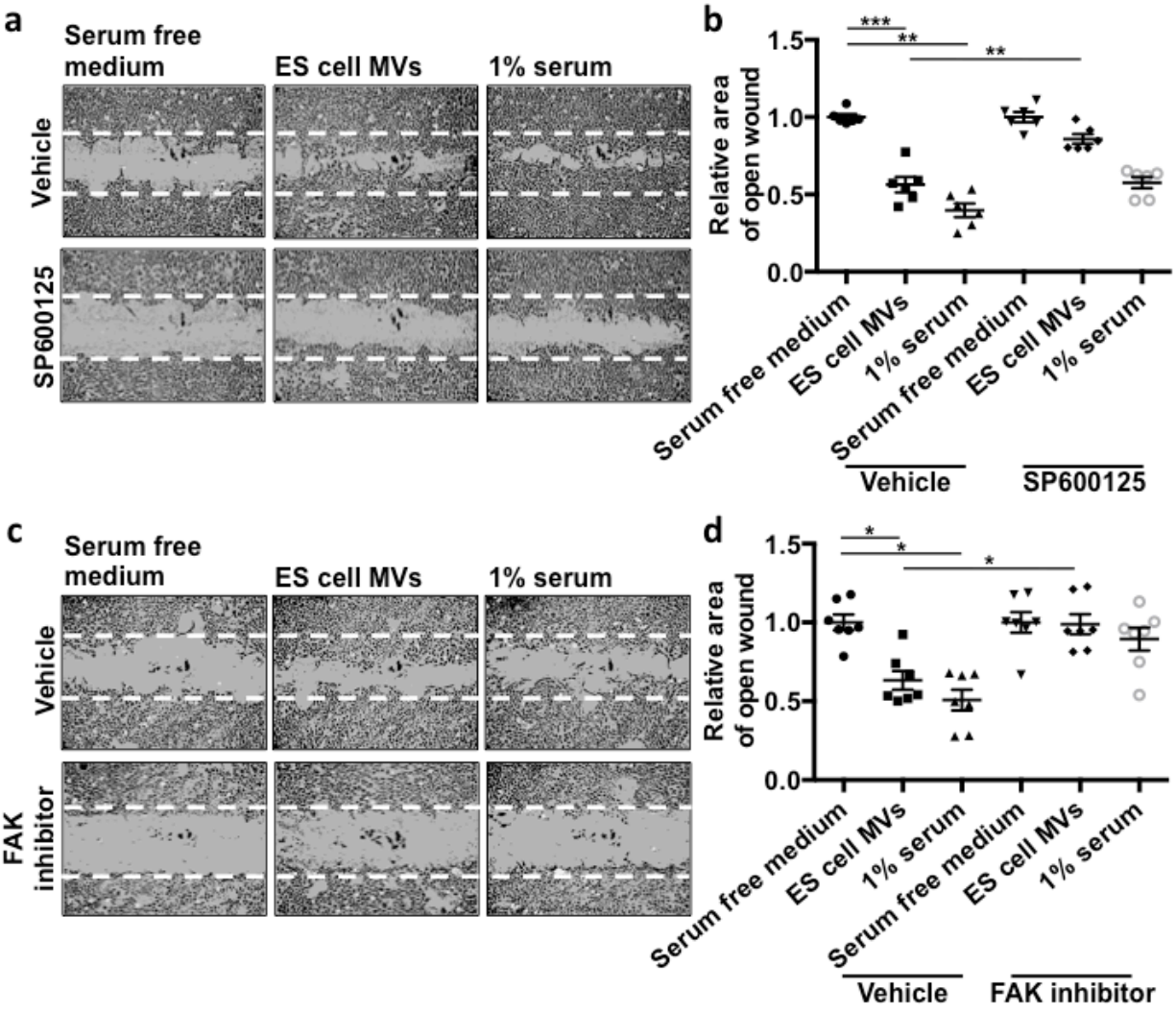

a
Base medium
ES cell CM
1% serum
Vehicle
SP600125
b
Relative area of open wound
1.5
1.0
0.5
0.0
***
***
**
Base medium
ES cell CM
1% serum
Base medium
ES cell CM
1% serum
Vehicle
SP600125
c
Base medium
ES cell CM
1% serum
Vehicle
FAK inhibitor
d
Relative area of open wound
1.5
1.0
0.5
0.0
*
*
**
Base medium
ES cell CM
1% serum
Base medium
ES cell CM
1% serum
Vehicle
FAK inhibitor
e
Base medium
ES cell CM
1% serum
Vehicle
PD98059
f
Relative area of open wound
1.5
1.0
0.5
0.0
***
**
NS
Base medium
ES cell CM
1% serum
Base medium
ES cell CM
1% serum
Vehicle
PD98059

Supplementary Figure 2.3. ES cells and trophoblasts generate MVs.
(**a**) Transmission electron microscopy (TEM) image of MVs isolated from ES cells. (**b**) To show that the MV preparations are free of soluble proteins, 40 ml of CM from ES cells was supplemented with 20 μg of a tagged recombinant form of Glutaminase C (His-tagged GAC) that has a molecular weight of ~65 kDa. The medium was clarified of intact cells and cell debris and divided into two equal parts. One part (20 ml) was directly concentrated to 400 μl using a centrifugal filter with a nominal molecular weight limit of 10 kDa. The rest was filtered with a 0.22 μm filter and then rinsed extensively with PBS. The MVs retained by the filter were lysed in 400 μl of mammalian lysis buffer, while the flow-through was concentrated to 400 μl as well. All of the samples were immunoblotted for His-tagged GAC, the MV marker flotillin-2, and β-actin as a loading control. Note that His-tagged GAC was detected in the concentrated CM (first lane) and the concentrated flow-through (third lane), but not in the MVs (second lane). These findings demonstrate that the MV isolation procedure efficiently removes soluble proteins. (**c**) DLS analysis performed on MVs isolated from trophoblasts show that they ranged in size from ~400-600 nm. (**d**) Lysates of HTR8/SVneo trophoblasts (WCL) and the MVs generated by these cells were immunoblotted for the MV marker flotillin-2, the cytosolic marker FAK, and β-actin as a loading control. (**e**) Fluorescence microscopy image of trophoblasts stained with the membrane dye FM1-43fx. Some of the MVs decorating the surfaces of the cells are denoted with arrowheads. (**f**) A wound closure assay was performed on HTR8/SVneo cells cultured in serum free medium supplemented without (Serum free medium), or with either trophoblast MVs or 1% serum. Images of the cells are shown. The dashed line indicates the width of the original wound. Trophoblasts failed to migrate in response to the HTR8/SVneo MVs. (**g**) The assay in **f** was quantified and plotted as the relative area of open wound, presented as mean ± s.e.m. (n=4 independent experiments); **, $P<0.01$, NS, not significant.

*The experiment described in (**b**) was performed by Marc A. Antonyak.


a
1 um
b
ES cell CM spiked with Recombinant His-tagged GAC
CM
MVs
CM depleted of MVs
His-tagged GAC
Flotillin-2
β-actin
c
Intensity (arbitrary units)
100
80
60
40
20
0
0
200
400
600
Size (nm)
d
HTR8/SVneo cells
WCL
MVs
Flotillin-2
FAK
β-actin
e
HTR8/SVneo
FM1-43fx
f
Serum free medium
Trophoblast MVs
1% serum
g
NS
**
Relative area of open wound
1.5
1.0
0.5
0.0
Serum free medium
Trophoblast MVs
1% serum

Supplementary Figure 2.4. FAK and JNK activation are important for HTR8/SVneo cells to migrate in response to treatment with ES cell MVs.
(**a**-**d**) Wound closure assays were performed on HTR8/SVneo cells cultured in serum free medium supplemented without (Serum free medium) or with either MVs from ES cells (ES cell MVs), or medium containing 1% serum. Each culturing condition was treated further with either DMSO (Vehicle) or **a**, SP600125 or **c**, FAK inhibitor III (FAK inhibitor). Images of the cells are shown. The dashed line indicates the width of the original wound. The assays in **a** and **c** were quantified and plotted in **b** and **d** as the relative area of open wound. (**e**) E3.5 blastocysts were harvested and placed in dishes containing blastocyst culturing medium supplemented without (Control) or with either FAK inhibitor III (FAK inhibitor) or SP600125 (JNK inhibitor). Two days later, the number of blastocysts that attached and formed outgrowths onto the dish was determined. All values shown are presented as mean ± s.e.m. ($n \geq 3$ independent experiments for each assay); *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$.



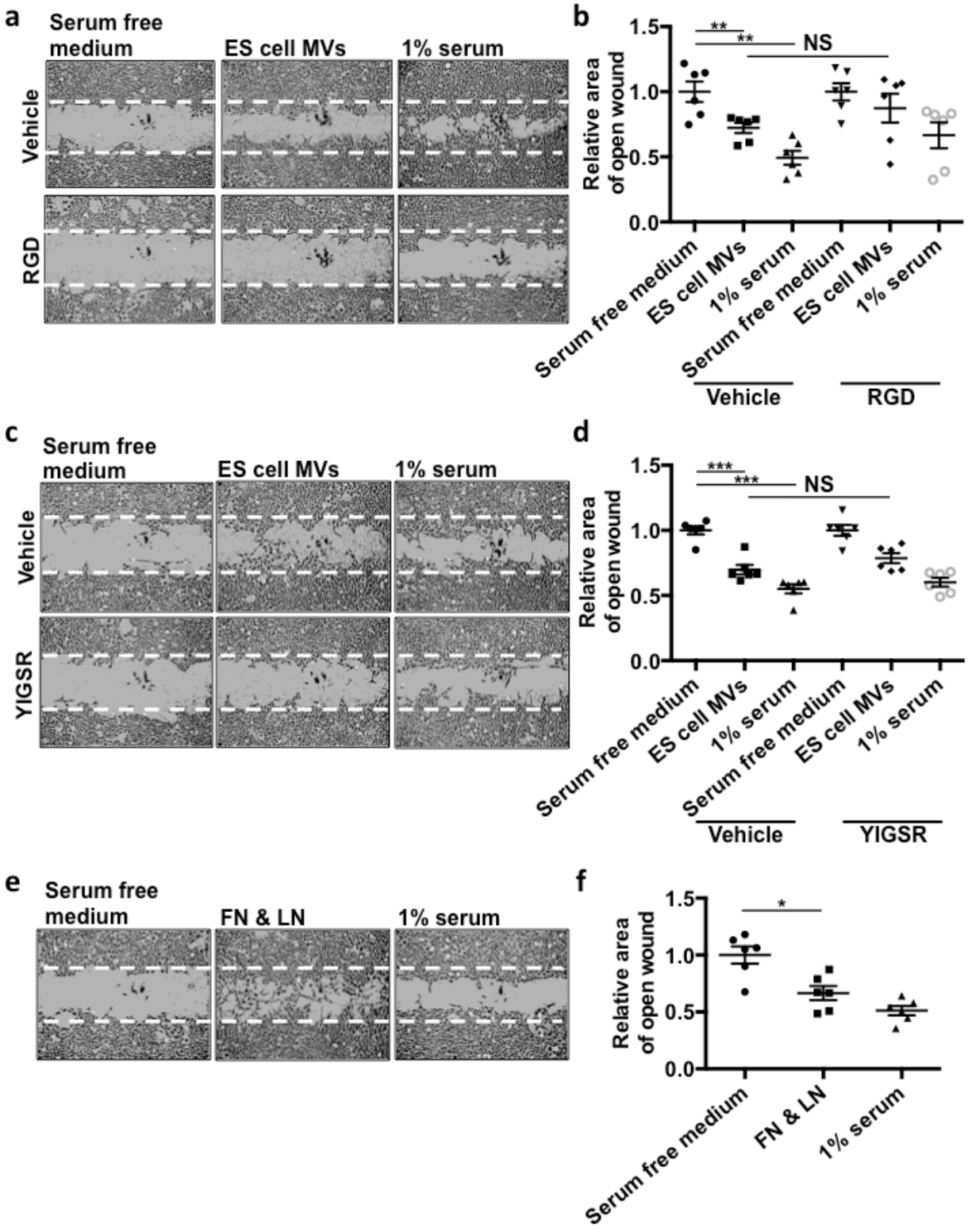
e

Blastocyst outgrowth assay

	Blastocyst attachment	Blastocyst outgrowth
Control (untreated)	9/9	9/9
FAK inhibitor	1/9	0/9
JNK inhibitor	0/8	0/8

Supplementary Figure 2.5. Inhibiting the MV-associated fibronectin or laminin from binding their corresponding receptors expressed on trophoblasts does not interfere with their ability to migrate.

(a) Wound closure assays were performed on HTR8/SVneo cells cultured in serum free medium supplemented without (Serum free medium) or with either MVs from ES cells (ES cell MVs) or 1% serum. Each culturing condition was treated further with vehicle (top panels) or the RGD inhibitory peptide (bottom panels). Images of the cells maintained under each condition are shown. The dashed line indicates the width of the original wound. (**b**) The assays in **a** were quantified and plotted as the relative area of open wound. (**c**) Wound closure assays were performed as in **a**, except that HTR8/SVneo cells were treated with the YIGSR inhibitory peptide (bottom panels). (**d**) The assays in **c** were quantified and plotted as the relative area of open wound. (**e**) Wound closure assays were performed on HTR8/SVneo cells cultured in serum free medium supplemented without (Serum free medium) or with either purified forms of fibronectin and laminin (FN & LN) or 1% serum. Images of the cells maintained under each condition are shown. The dashed line indicates the width of the original wound. (**f**) The assays in **e** were quantified and plotted as the relative area of open wound. All values shown are presented as mean ± s.e.m. ($n \geq 3$ independent experiments for each assay); *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$, NS, not significant.


a
Serum free medium
ES cell MVs
1% serum
Vehicle
RGD
b
Relative area of open wound
1.5
1.0
0.5
0.0
**
**
NS
Serum free medium
ES cell MVs
1% serum
Serum free medium
ES cell MVs
1% serum
Vehicle
RGD
c
Serum free medium
ES cell MVs
1% serum
Vehicle
YIGSR
d
Relative area of open wound
1.5
1.0
0.5
0.0
***
***
NS
Serum free medium
ES cell MVs
1% serum
Serum free medium
ES cell MVs
1% serum
Vehicle
YIGSR
e
Serum free medium
FN & LN
1% serum
f
Relative area of open wound
1.5
1.0
0.5
0.0
*
Serum free medium
FN & LN
1% serum

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CHAPTER 3

Microvesicles generated by trophoblasts promote endothelial cell tubulation

During the formation of the placenta, trophoblasts extensively remodel maternal spiral arteries to bring the necessary blood flow to the embryo.[1,2] Here we describe how trophoblasts use a novel form of intercellular communication to help promote this process. Specifically, trophoblasts shed a specific type of extracellular vesicle known as microvesicles (MVs),[3,4] which can interact with endothelial cells, promoting their ability to survive under nutrient-limiting conditions and their ability to tubulate (an *in vitro* readout of blood vessel formation). The MVs isolated from trophoblasts induce the activation of several important signaling proteins in endothelial cells, including JNK and ERK, which are required for endothelial cell migration and survival.[2,3,5-7] Moreover, proteomics performed on the trophoblast MVs revealed that they contained numerous metabolic proteins, which can potentially be transferred to and used by endothelial cells to boost their metabolism for tubulation. These findings highlight a unique form of intercellular communication that has potentially important roles in the early stages of placental formation.

Introduction

The blastocyst is composed of two distinct cell types: the trophectoderm, which forms the placenta, and the inner cell mass, which forms the embryo. To establish a pregnancy, the trophectoderm layer of the blastocyst attaches itself to the uterine lining and then migrates and invades past the uterine epithelium to implant the embryo into the uterus.[1,2] In chapter 2, I

demonstrated that MVs shed by ES cells derived from the inner cell mass promote trophoblast migration and influence the implantation process.

However, embryo implantation is just the first of many important roles played by trophoblasts during a successful pregnancy. During the first trimester of pregnancy, the trophoblasts begin to grow and differentiate into different types of trophoblasts.[1,2,8] The first type to form is the syncytiotrophoblast, which is formed from cytotrophoblast stem cells and creates a syncytial layer (i.e. a multinucleate layer of fused cells) surrounding the placenta. The syncytiotrophoblast exists to promote maternal-fetal nutrient, waste, and gas exchange, and also secretes hormones, such as human chorionic gonadotrophin (hCG), which can be found in the maternal circulation.[1-3,8]

The cytotrophoblast stem cells continue to proliferate beneath the syncytial layer. Some of these cells will differentiate into the syncytiotrophoblast to help maintain the syncytial layer, while others differentiate into cytotrophoblasts and form cell columns, or villi.[2,3,9] The cytotrophoblasts eventually break through the syncytiotrophoblast layer and migrate and invade into the uterine stroma as extravillous trophoblasts (EVTs). Eventually, these highly invasive and migratory cells reach the maternal spiral arteries (i.e. small, temporary arteries that supply blood to the endometrium during certain phases of the menstrual cycle). Interestingly, and somewhat counter-intuitively, this process culminates with the EVTs forming a barrier that completely blocks the blood flow in the maternal spiral arteries, creating a low oxygen or hypoxic environment.[2,10] The low oxygen condition actually induces EVTs to proliferate and rapidly expand their population. The EVTs then travel further into the maternal spiral arteries, replacing many of the endothelial cells lining the arteries and differentiating into endothelial-like cells to help generate new blood vasculature. Additionally, the EVTs cause the perivascular smooth

muscle cells lining the spiral arteries to migrate away, relaxing and opening the arteries to allow for lower resistance blood flow. Once these changes have occurred, usually by the end of the first trimester, the EVTs release their blockage of the maternal spiral arteries, allowing low resistance blood to flow to the growing embryo and exchange nutrients and gases with the syncytiotrophoblast.[2,8,11-13] If the trophoblasts fail to function properly early in this process, pregnancies are often terminated spontaneously (i.e. result in miscarriage). However, if problems occur later in this process, other serious complications such as preeclampsia and intrauterine growth restriction (IUGR) can arise.[1-3,8,14] Preeclampsia and IUGR result when EVTs fail to migrate and invade sufficiently into the maternal spiral arteries. Preeclampsia is characterized by high maternal blood pressure and protein in the urine (proteinuria), and is a serious complication that can lead to seizures and the death of both mother and child,[9,15-19] while IUGR is typically characterized by poor growth of the fetus.[10,20-23]

During the process of placentation, EVTs interact extensively with endothelial cells. For example, EVTs are known to secrete soluble forms of vascular endothelial growth factor (VEGF),[11-13,16,18,19] which can bind to and activate the VEGF receptor (VEGFR) expressed on endothelial cells, causing the cells to grow, migrate, and form new blood vessels.[14,24] More recently, trophoblast cells in the placenta have been shown to produce EVs, which can enter the maternal bloodstream.[15-19,25-28] The EVs shed by the trophoblasts, like those generated by cancer cells and ES cells, contain a wide variety of contents, including signaling and membrane proteins, mRNA, miRNA and even DNA.[20-23,29] These EVs come in two forms, exosomes and microvesicles (MVs). Although the function of EVs generated by trophoblasts is poorly understood, some recent work has suggested they could potentially be used to identify complications that occur during pregnancy.[16,18,19,30] For example, syncytiotrophoblast EVs are

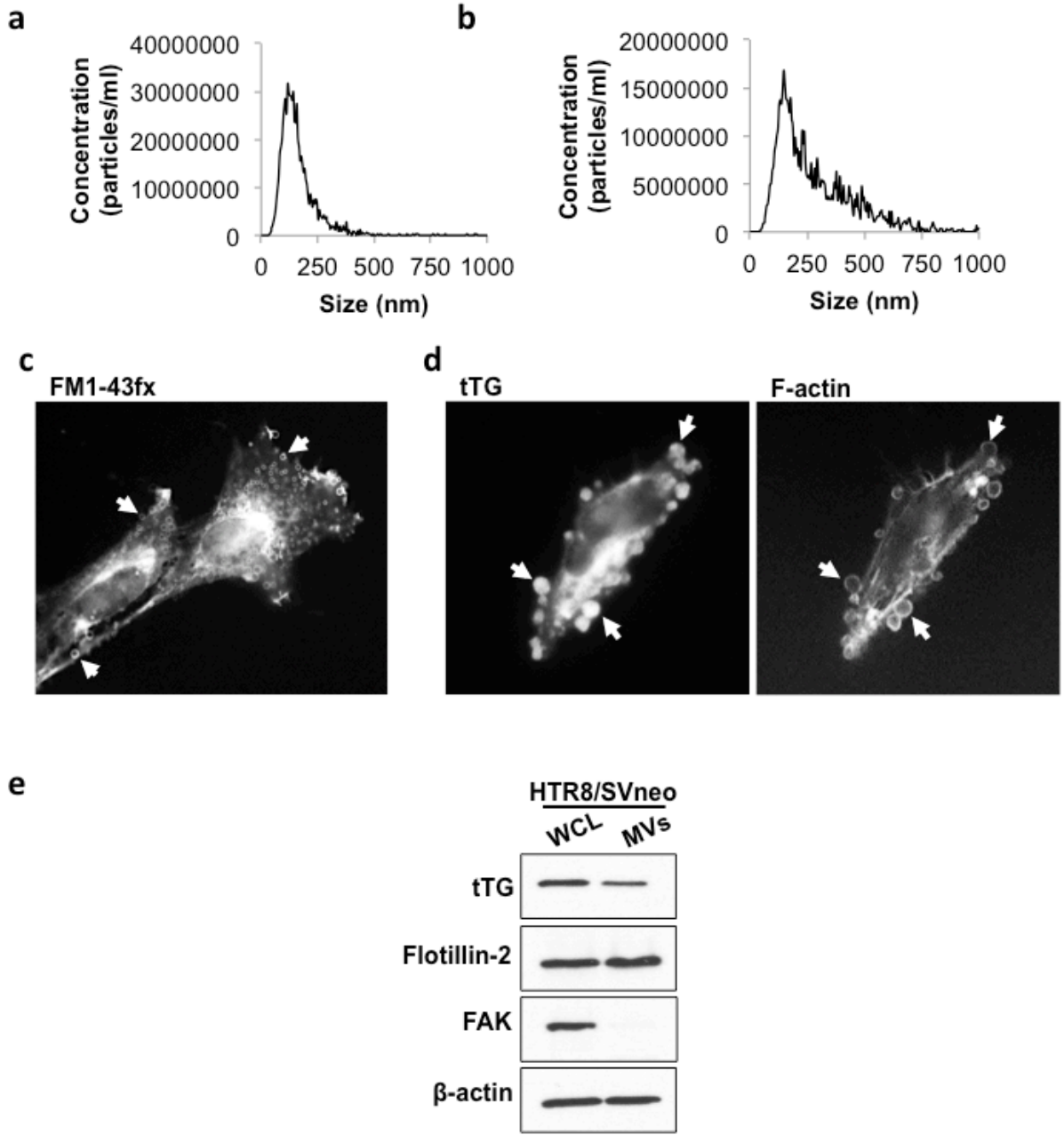
being studied for diagnostic purposes, since some studies have shown that preeclamptic women have higher levels of syncytiotrophoblast-derived EVs in their circulation.[24,30,31] Furthermore, other studies have shown that EVTs also produce EVs.[15,25-28,32] Thus, given the important roles played by EVTs in placental formation, we wanted to determine whether EVs shed by EVTs could potentially impact endothelial cell function and influence the process of maternal spiral artery remodeling.

Results

The HTR8/SVneo trophoblast cell line was derived from the developing placenta of a first trimester human pregnancy.[29,30] Since these trophoblasts had migrated away from the main villi of the placenta and invaded the decidualized uterus, they were termed EVTs. Because EVTs are known to contribute to placental vascularization, I set out to determine whether they formed and shed EVs that could influence the ability of endothelial cells to generate new blood vessels.

I first asked whether HTR8/SVneo cells generated EVs. The trophoblasts were incubated in serum free medium for ~5 hours, at which point the conditioned medium (CM) was collected and then cleared of cells and cell debris using consecutive low speed centrifugation steps, i.e. first at 300 x *g* for 5 minutes and then at 3,000 x *g* for 20 minutes.[30,33] The partially clarified CM was then subjected to Nanosight analysis to determine the sizes and amounts of EVs present in the CM. Figure 3.1a shows that the sample contained EVs that ranged in size from 50 nm to ~750 nm, indicating that both the smaller exosomes and the larger MVs are formed and shed by the HTR8/SVneo trophoblast cell line. The CM was then filtered using a 0.22 μm pore size filter to specifically isolate the MVs. The MV proteins, which were subjected to another round of Nanosight analysis, showed that the MV isolation procedure significantly enriched for EVs

Figure 3.1. HTR8/SVneo trophoblasts generate MVs.
(**a**) Representative plot of a Nanosight analysis performed on CM from HTR8/SVneo trophoblasts cleared of cells and cell debris. (**b**) Representative plot of a Nanosight analysis performed on MVs isolated from the CM of HTR8/SVneo trophoblasts. (**c**) Representative fluorescence microscopy image of trophoblasts stained with the plasma membrane dye FM1-43fx. (**d**) Immunofluorescence images of a trophoblast stained with a tTG antibody (left) as well as rhodamine-conjugated phalloidin to label F-actin (right). In both **c** and **d**, some of the MVs on the surface of the trophoblast are denoted with arrows. (**e**) MVs isolated from trophoblasts were lysed and then immunoblotted for tTG and flotillin-2 as markers of MVs, FAK as a cytosolic marker, and β-actin as a loading control. WCL, whole cell lysate.


a
Concentration (particles/ml)
40000000
30000000
20000000
10000000
0
0 250 500 750 1000
Size (nm)
b
Concentration (particles/ml)
20000000
15000000
10000000
5000000
0
0 250 500 750 1000
Size (nm)
c
FM1-43fx
d
tTG
F-actin
e
HTR8/SVneo
WCL
MVs
tTG
Flotillin-2
FAK
β-actin

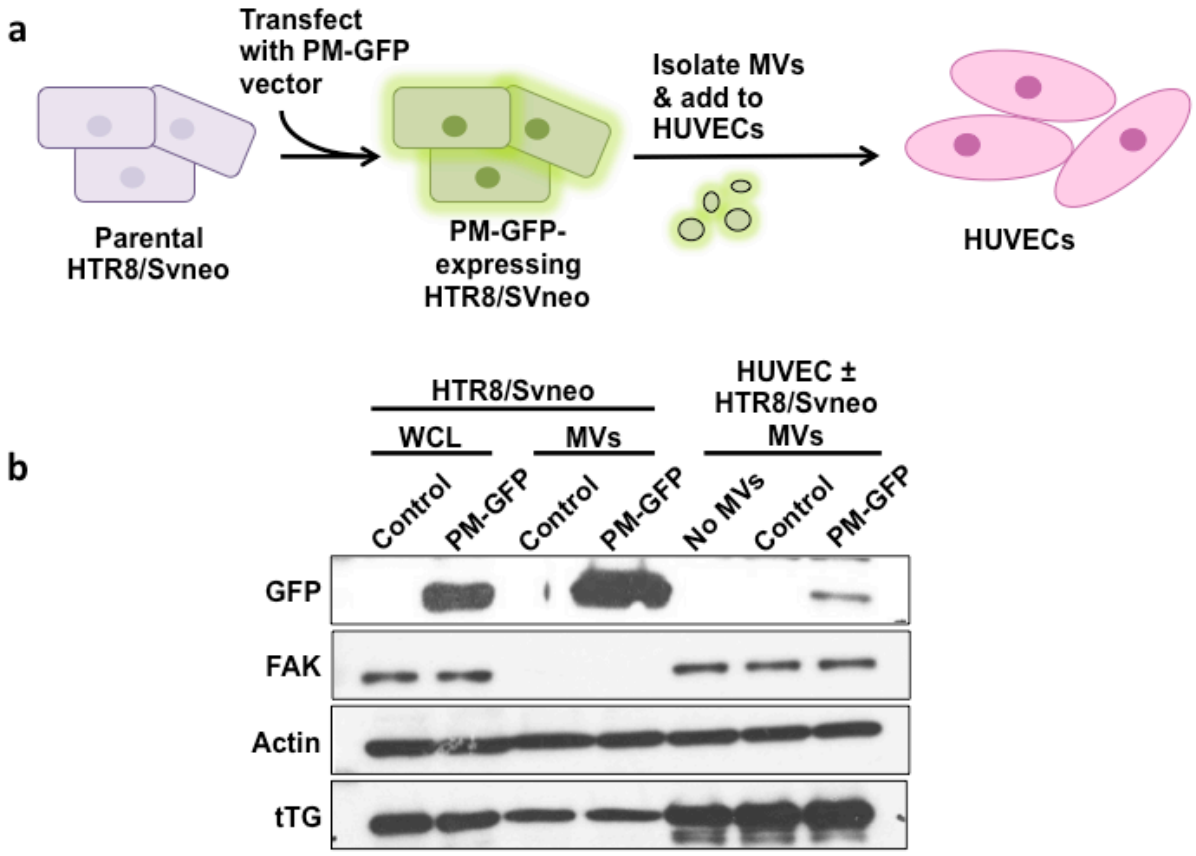
larger than 200 nm and effectively eliminated many of the smaller exosomes (Figure 3.1b).

To visualize EVs being generated by the HTR8/SVneo trophoblasts, cultures of cells were stained with FM1-43fx, a fluorescent plasma membrane dye. Fluorescence microscopy performed on the stained cells revealed that they were covered in small vesicular structures on their surfaces (Figure 3.1c). The trophoblasts were also immunostained for tissue transglutaminase (tTG) and filamentous (F-) actin, which are known to be present in MVs shed by aggressive cancer cells.[30,31,34-36] The resulting fluorescent images taken of these cells showed that both tTG and F-actin could label the MVs on the surfaces of the trophoblasts (Figure 3.1d), similar to what was seen previously on aggressive cancer cells.

Finally, the MVs isolated from the HTR8/SVneo trophoblasts were lysed and subjected to immunoblot analysis. Consistent with the immunofluorescence results, tTG and actin could be readily detected in the MV extracts (Figure 3.1e, first and last panels). They also contained flotillin-2, a marker of MVs (second panel from top), but lacked focal adhesion kinase (FAK; third panel from top), a cytosolic marker.

Once I had confirmed that trophoblasts generated and shed MVs, I next wanted to determine whether they could transfer their contents to endothelial cells. For this experiment, I used human umbilical vein endothelial cells (HUVECs), a primary cell culture frequently utilized to study angiogenesis.[32,37] As outlined in Figure 3.2a, parental HTR8/SVneo cells were either mock-transfected (Control) or transfected with a plasma membrane-targeted form of GFP (PM-GFP) that can efficiently be imported into the MVs shed by the cells[30,38] (Figure 3.2b, top panel, lanes labeled WCL and MVs). Medium supplemented with nothing (no MVs), MVs derived from the mock-transfected trophoblasts (Control), or MVs from the PM-GFP-expressing trophoblasts, were incubated with cultures of HUVECs for approximately one hour, at which

Figure 3.2. The contents of trophoblast-derived MVs can be transferred to HUVECs.
(**a**) Diagram of the experiment conducted in **b**. (**b**) MVs isolated from mock transfected trophoblasts (Control) or trophoblasts ectopically expressing PM-GFP were lysed or resuspended in serum free medium. Lysates of the transfected trophoblasts (lanes 1 and 2) and the MVs that they generated (lanes 3 and 4) were immunoblotted for GFP, the cytosolic marker FAK, β-actin as a loading control, and tTG, a known MV component. Note that PM-GFP was efficiently incorporated into the MVs (lane 4). Serum free medium supplemented without or with MVs from mock-transfected or PM-GFP-transfected trophoblasts was added to HUVECs for ~1 hour, at which time the cells were extensively washed, lysed and immunoblotted as described above. Note that PM-GFP was detected in HUVECs treated with MVs containing PM-GFP.


a
Transfect
with PM-GFP
vector
Isolate MVs
& add to
HUVECs
Parental
HTR8/Svneo
PM-GFP-
expressing
HTR8/SVneo
HUVECs
b
HTR8/Svneo
HUVEC ±
HTR8/Svneo
WCL
MVs
MVs
Control
PM-GFP
Control
PM-GFP
No MVs
Control
PM-GFP
GFP
FAK
Actin
tTG

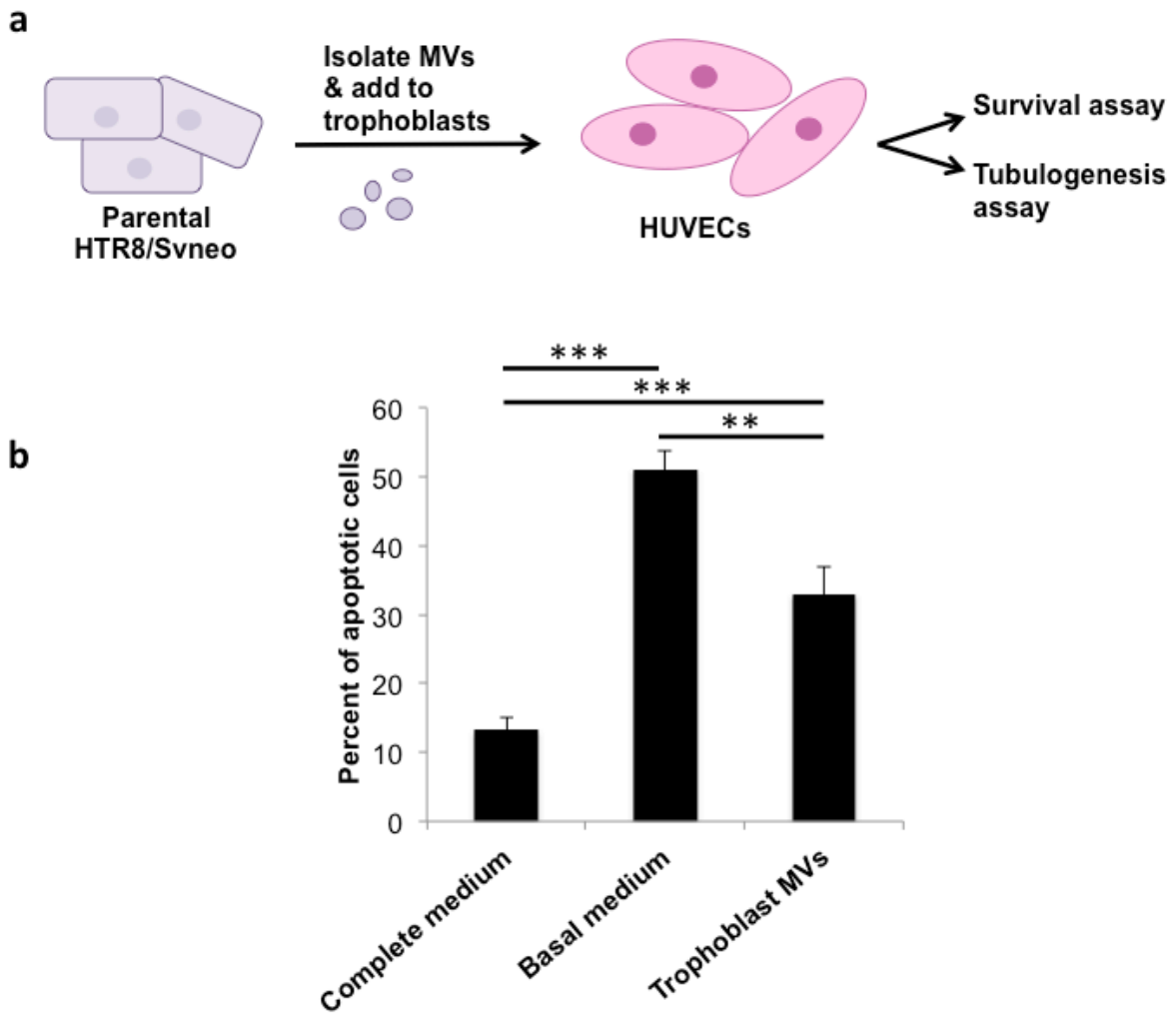
point the cells were rinsed extensively with PBS and lysed. Immunoblot analysis of the resulting HUVEC whole cell lysates (WCL) revealed that PM-GFP protein could only be detected in the HUVECs treated with MVs expressing PM-GFP, suggesting that a transfer of the fusion protein had occurred (Figure 3.2b, HUVEC ± HTR8/SVneo MVs, PM-GFP lane).

Next, I wanted to determine whether the trophoblast MVs had any effect on the ability of HUVECs to survive or tubulate (outlined in Figure 3.3a). For the survival assays, HUVECs were cultured in serum free medium supplemented without (Basal medium) or with trophoblast MVs. As a positive control, HUVECs were also cultured in complete medium. Approximately 40 hours later, the cells were collected, and the percentage of cells undergoing apoptosis in each condition was determined. While ~50% of the cells maintained in serum free medium were apoptotic, the HUVECs maintained in serum- or MV-containing medium showed less cell death, suggesting that trophoblast MVs can promote endothelial cell survival.

Under certain conditions, HUVECs can be stimulated to tubulate, which is thought to provide an *in vitro* read-out for the ability of endothelial cells to form blood vessels.[33,39] To examine whether trophoblast MVs could promote HUVEC tubulogenesis, the cells were plated on growth factor-depleted matrigel in serum free medium (Basal medium), medium containing growth factors and serum (Complete medium), or basal medium supplemented with trophoblast MVs for ~5 hours (HTR8/SVneo MVs; Figure 3.3c). As expected, very little tubulation occurred when the cells were cultured in the basal medium (Figure 3.3c, left), while complete medium stimulated tubulation (Figure 3.3c, middle). Interestingly, HUVECs treated with trophoblast MVs also exhibited tubulation that exceeded basal levels (Figure 3.3c, right), suggesting that MVs shed by trophoblasts might be capable of activating endothelial cells to generate blood vessels.

Figure 3.3. HTR8/SVneo trophoblast MVs promote both HUVEC survival and tubulogenesis.
(**a**) Diagram of experiments conducted in **b** and **c**. (**b**) Survival assays were performed on HUVECs that had been cultured for 40 hours in complete medium, or serum free medium supplemented without (Basal medium) or with MVs isolated from trophoblasts. The percentage of cells undergoing apoptosis was determined for each condition and the results were plotted. All values shown are presented as mean ± s.e.m. ($n \geq 3$ independent experiments for each assay); **, $P<0.01$, ***, $P<0.001$ (**c**) Tubulogenesis assays were performed on HUVECs that were cultured for 5 hours in serum free medium (Basal medium, left), medium containing serum, growth factors and VEGF (Complete medium, middle), and basal medium supplemented with trophoblast MVs (HTR8/SVneo MVs, right). All cells were treated with Calcein AM and visualized by fluorescence microscopy. Note that HUVECS that failed to undergo tubulogenesis with basal medium, but were able to do so either when treated with complete medium or with basal medium supplemented with HTR8/SVneo MVs.

*The experiment described in (**b**) was performed by Vinita Popat and Laura M. Desrochers.


a
Isolate MVs
& add to
trophoblasts
Parental
HTR8/Svneo
HUVECs
Survival assay
Tubulogenesis
assay
b
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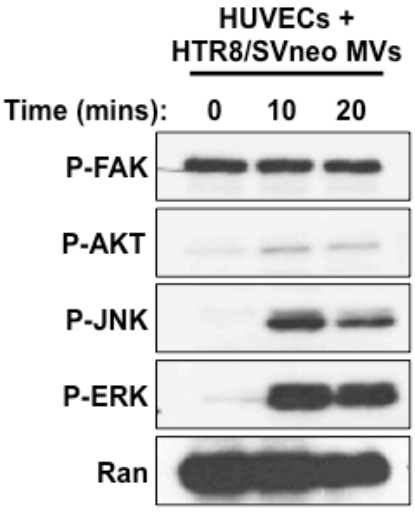
Percent of apoptotic cells
60
50
40
30
20
10
0
Complete medium
Basal medium
Trophoblast MVs
c
Basal medium
Complete medium
HTR8/SVneo MVs

To further determine how trophoblast MVs affect endothelial cells, I investigated whether trophoblast MVs influenced signaling events in HUVECs that are commonly linked to cell survival and migration. Serum starved cultures of HUVECs were treated without (Time = 0 min) or with trophoblast MVs for 10 or 20 minutes, and then cells were lysed and immunoblotted using antibodies that detect the activated (phosphorylated) forms of various signaling proteins. The phosphorylation levels of focal adhesion kinase (FAK) in endothelial cells were not changed by treatment with MVs, while AKT phosphorylation was increased slightly (Figure 3.4a, top two panels). In contrast, both c-Jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) were strongly activated by the MVs (Figure 3.4a, third and fourth panels from the top). JNK and ERK (and AKT) have been implicated in promoting cell survival,[34-36,40] while ERK activation is known to be required for HUVEC tubulogenesis.[37,41] Consistent with these findings, MV-mediated HUVEC tubulogenesis could be blocked under conditions where JNK activation (using SP600125)[38,42] or ERK activation (using PD98059)[39,43,44] was inhibited (Figure 3.4b).

Since tubulation occured over a period of hours, I next wanted to determine whether the ability of the trophoblast MVs to activate ERK and JNK activity in endothelial cells could be sustained over this time period. Therefore, HUVECs were treated with medium supplemented without (Basal medium) or with serum (Complete medium) or trophoblast MVs (HTR8/SVneo MVs) for either ten minutes or four hours. Once again, after ten minutes of treatment with MVs, both JNK and ERK were activated as read out by phosphorylation (Figure 3.4c, top two panels, third lane from left). After four hours, ERK and JNK phosphorylation levels were significantly diminished but still above baseline in HUVECs treated with trophoblast MVs (Figure 3.4c, top two panels, rightmost lanes).

Figure 3.4. HTR8/SVneo MVs activate signaling in HUVECs.
(**a**) Serum starved HUVECs treated with serum free medium, supplemented without (first lane, 0 mins) or with HTR8/SVneo MVs, for 10 or 20 minutes were immunoblotted for phosphorylated FAK (P-FAK), AKT (P-AKT), JNK (P-JNK), and ERK (P-ERK), as well as Ran as a loading control. (**b**) Tubulogenesis assays were performed on HUVECs that were cultured for 5 hours in serum free medium (Basal medium, top, left), medium containing serum, growth factors and VEGF (Complete medium, top, middle), and basal medium supplemented with trophoblast MVs (HTR8/SVneo MVs, top, right). In the bottom panels, tubulogenesis assays were performed with trophoblast MVs supplemented with either SP600125, the JNK inhibitor, or PD98059, the MEK inhibitor (to inhibit ERK activation). All cells were treated with Calcein AM and visualized by fluorescence microscopy. Note that all inhibitors (bottom) prevented trophoblast MV-mediated tubulation. (**c**) Serum starved HUVECs, treated with serum free medium supplemented without (Basal medium) or with serum (Complete medium), or with HTR8/SVneo MVs, for 10 mins or 4 hours were immunoblotted for P-JNK, P-ERK, VEGF, and HIF1α. Note that both JNK and ERK activation was above baseline in HUVECs even 4 hours after treatment with MVs. However, neither VEGF nor HIF1α expression increased.

a


HUVECs +
HTR8/SVneo MVs
Time (mins): 0 10 20
P-FAK
P-AKT
P-JNK
P-ERK
Ran

b

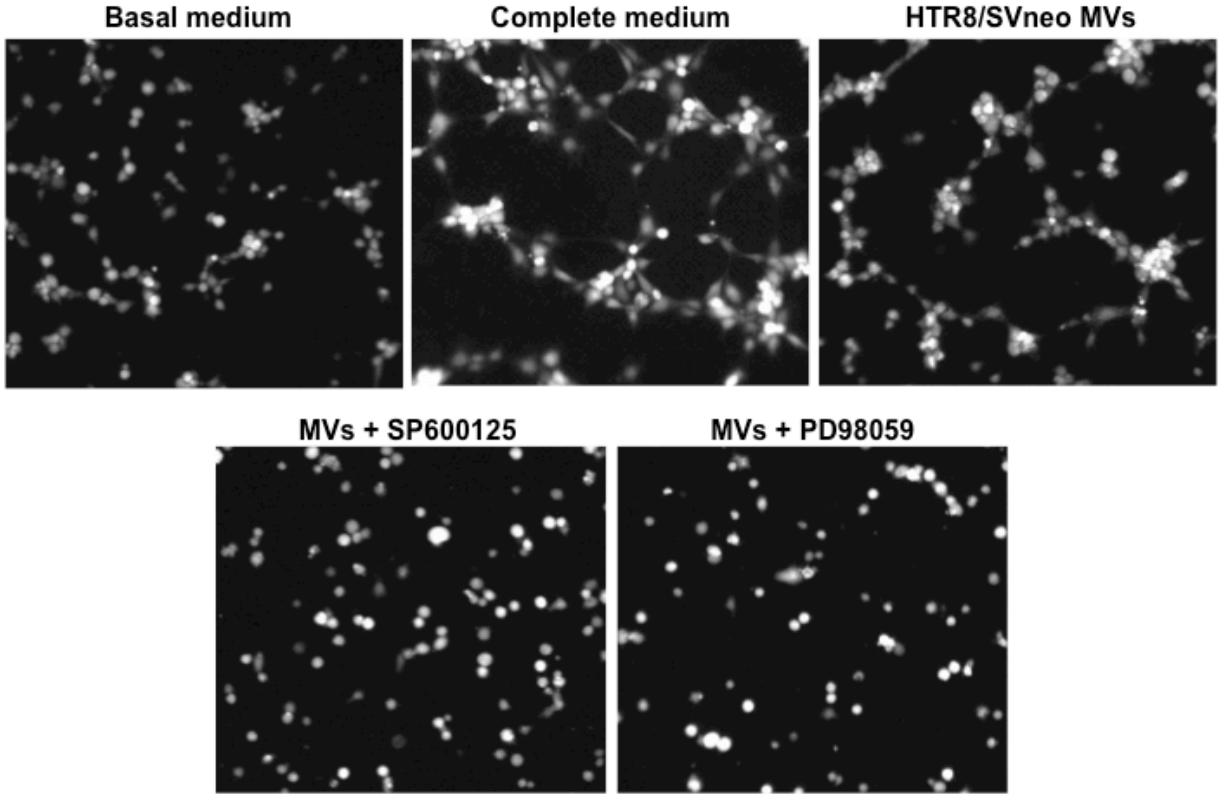
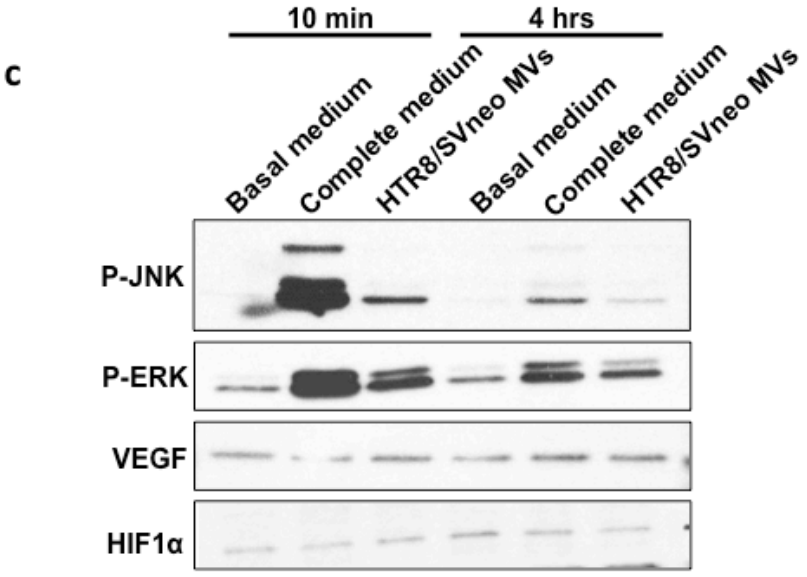

Basal medium
Complete medium
HTR8/SVneo MVs
MVs + SP600125
MVs + PD98059

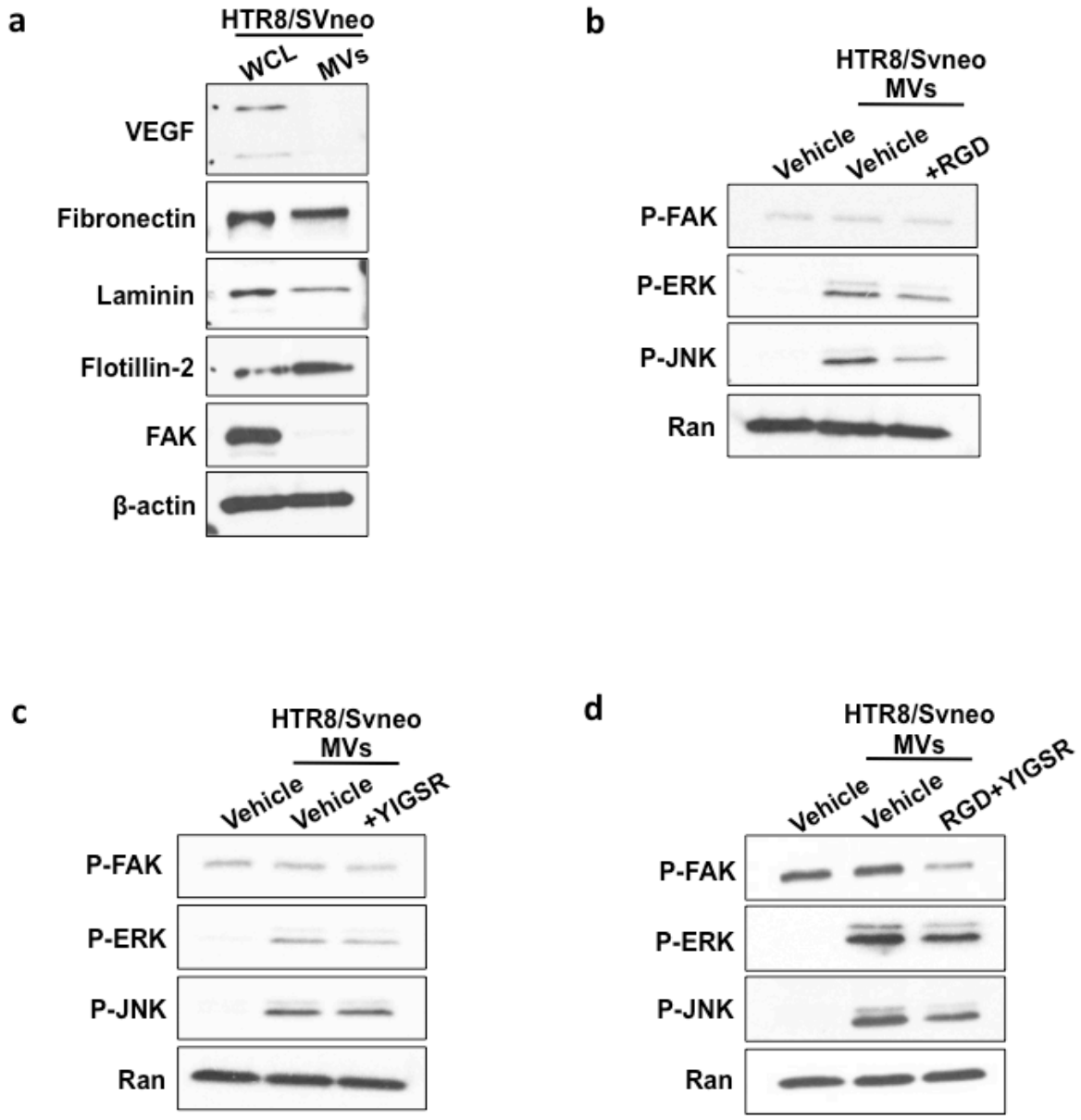
Figure 3.4 continued



We also determined whether the levels of VEGF and hypoxia inducible factor 1α (HIF1α) in HUVECs were altered by the trophoblast MVs. This is because it was recently shown that cancer cell-derived EVs cause endothelial cells to express VEGF and HIF1α.[40,45] However, the trophoblast MVs did not appear to increase the protein levels of VEGF or HIF1α expression (Figure 3.4c, bottom two panels). These findings suggest that the trophoblast MVs stimulate endothelial cell tubulation independent of the upregulation of VEGF or HIF1α expression.

Next, I became interested in determining which of the MV contents were responsible for stimulating HUVEC signaling events (i.e. activating JNK and ERK) and promoting tubulogenesis. Immunoblot analysis performed on lysates of MVs generated by trophoblasts revealed that they contained little, if any, detectable VEGF (Figure 3.5a, top panel), suggesting that this pro-angiogenic growth factor was not likely playing a role in the ability of the MVs to directly activate the VEGFR expressed on the surfaces of HUVECs and promote their tubulogenesis. However, the trophoblast MVs did contain both fibronectin and laminin (Figure 3.5a, second and third panels), two extracellular matrix proteins that I showed in Chapter 2 were important for the ability of ES cell MVs to stimulate trophoblast migration. The addition of the RGD peptide, which blocks fibronectin-integrin interactions,[41,46] caused a slight decrease in MV-induced ERK and JNK activation in HUVECs (Figure 3.5b), while the addition of the YIGSR peptide, which blocks laminin-laminin receptor interactions,[2,42] had no effect on trophoblast MV-stimulated signaling (Figure 3.5c). The combination of the two inhibitory peptides did not significantly alter the levels of ERK and JNK phosphorylation induced by the trophoblast MVs when compared to treating with RGD alone, suggesting that these extracellular matrix proteins were not important for the effects of the trophoblast MVs (Figure 3.5d).

Figure 3.5. VEGF, fibronectin and laminin are not responsible for the ability of trophoblast MVs to activate signaling events in HUVECs.
(**a**) MVs isolated from HTR8/SVneo cells were immunoblotted for VEGF, fibronectin and laminin. They were also immunoblotted for flotillin-2 as a marker of MVs, FAK as a cytosolic marker, and β-actin as a loading control. Note that while VEGF, fibronectin and laminin are present in the WCL, only fibronectin and laminin are detected in the MV lysates (MVs). (**b**), (**c**) and (**d**) Serum starved HUVECs were treated with PBS (Vehicle) or with either **b**, the fibronectin inhibitory peptide RGD, **c**, the laminin inhibitory peptide YIGSR, or **d**, a combination of the two inhibitors. The cells were then stimulated with ES cells MVs for ~30 minutes before being lysed and immunoblotted for phosphorylated FAK (P-FAK), ERK (P-ERK) and JNK (P-JNK).


a
HTR8/SVneo
WCL
MVs
VEGF
Fibronectin
Laminin
Flotillin-2
FAK
β-actin
b
HTR8/Svneo
MVs
Vehicle
Vehicle
+RGD
P-FAK
P-ERK
P-JNK
Ran
c
HTR8/Svneo
MVs
Vehicle
Vehicle
+YIGSR
P-FAK
P-ERK
P-JNK
Ran
d
HTR8/Svneo
MVs
Vehicle
Vehicle
RGD+YIGSR
P-FAK
P-ERK
P-JNK
Ran

In order to gain a better sense of what cargo in the HTR8/SVneo MVs accounted for their ability to promote survival and tubulogenesis, mass spectrometry analysis was performed on the trophoblast MVs. The analysis revealed that these MVs contained over 3000 different proteins. Interestingly, I noticed that many (~15%) of the most abundant proteins functioned in metabolic pathways (Figure 3.6a). Immunoblot analysis confirmed that several different metabolic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pyruvate kinase isoform 2 (PKM2) were indeed present in trophoblast MVs (Figure 3.6a, see No. 1 and No. 4, and Figure 3.6b). Additionally, other metabolic proteins which were not as highly abundant, such as lactate dehydrogenase A (LDHA) and glutaminase 1 (GLS1), including both of its isoforms (kidney type glutaminase (KGA) and glutaminase C (GAC)), were also detected in the MVs (Figure 3.6b).

Because endothelial cells must undergo dramatic metabolic alterations to promote the cell growth and migration required for angiogenesis,[33,43,44] we hypothesized that the transfer of several metabolic proteins in the MVs derived from trophoblast cells to HUVECs might help "jump-start" metabolism in endothelial cells, providing them with the essential energy and biomass that they need to grow and tubulate. Consistent with this idea, the ability of MVs to stimulate the tubulation of HUVECs could be completely blocked by using the glutaminase inhibitor, BPTES,[45,47] or an inhibitor of glycolysis, 2-deoxyglucose (2-DG)[14,46] (Figure 3.6c, bottom panels).

Figure 3.6. HTR8/SVneo trophoblast MVs contain several metabolic proteins.
(**a**) Mass spectrometry was performed on MVs isolated from the HTR8/SVneo cells, and of the top 100 most abundant proteins, 16 were metabolic proteins. Their relative abundance is indicated under "Rank." (**b**) MVs isolated from trophoblasts were immunoblotted for several metabolic proteins, included, GLS1 (the two isoforms, KGA and GAC are denoted with arrows), PKM2, LDHA and GAPDH, as well as FAK as a marker of cytosolic contamination. Note that all of these metabolic proteins were detected in the MV lysates. (**c**) In the top panels, tubulogenesis assays were performed on HUVECs cultured for 5 hours in serum free medium (Basal medium, left), medium containing serum, growth factors and VEGF (Complete medium, middle), and basal medium supplemented with trophoblast MVs (HTR8/SVneo MVs, right). In the bottom panels, tubulogenesis assays were performed with trophoblast MVs supplemented with either the glutaminase inhibitor BPTES, or the glycolytic inhibitor 2-DG. All cells were treated with Calcein AM and visualized by fluorescence miscroscopy. Note that all inhibitors (bottom panels) prevented trophoblast MV-mediated tubulation.

*The experiment described in (**b**) was performed by Vinita Popat and Laura M. Desrochers.

a

No.	Rank	Metabolic proteins in top 100 hits
1	3	glyceraldehyde-3-phosphate dehydrogenase
2	13	enolase 1
3	15	pyruvate kinase 3 isoform 1
4	22	pyruvate kinase 3 isoform 2
5	31	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor
6	32	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit precursor
7	59	fatty acid synthase
8	60	phosphoglycerate kinase 1
9	63	aldolase A
10	64	triosephosphate isomerase 1
11	71	enolase 3
12	72	enolase 2
13	78	mitochondrial trifunctional protein, alpha subunit precursor
14	82	transketolase
15	83	methylenetetrahydrofolate dehydrogenase 1
16	86	serine hydroxymethyltransferase 2 (mitochondrial)

b

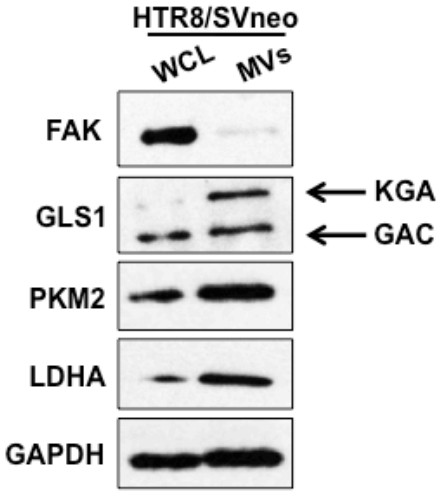
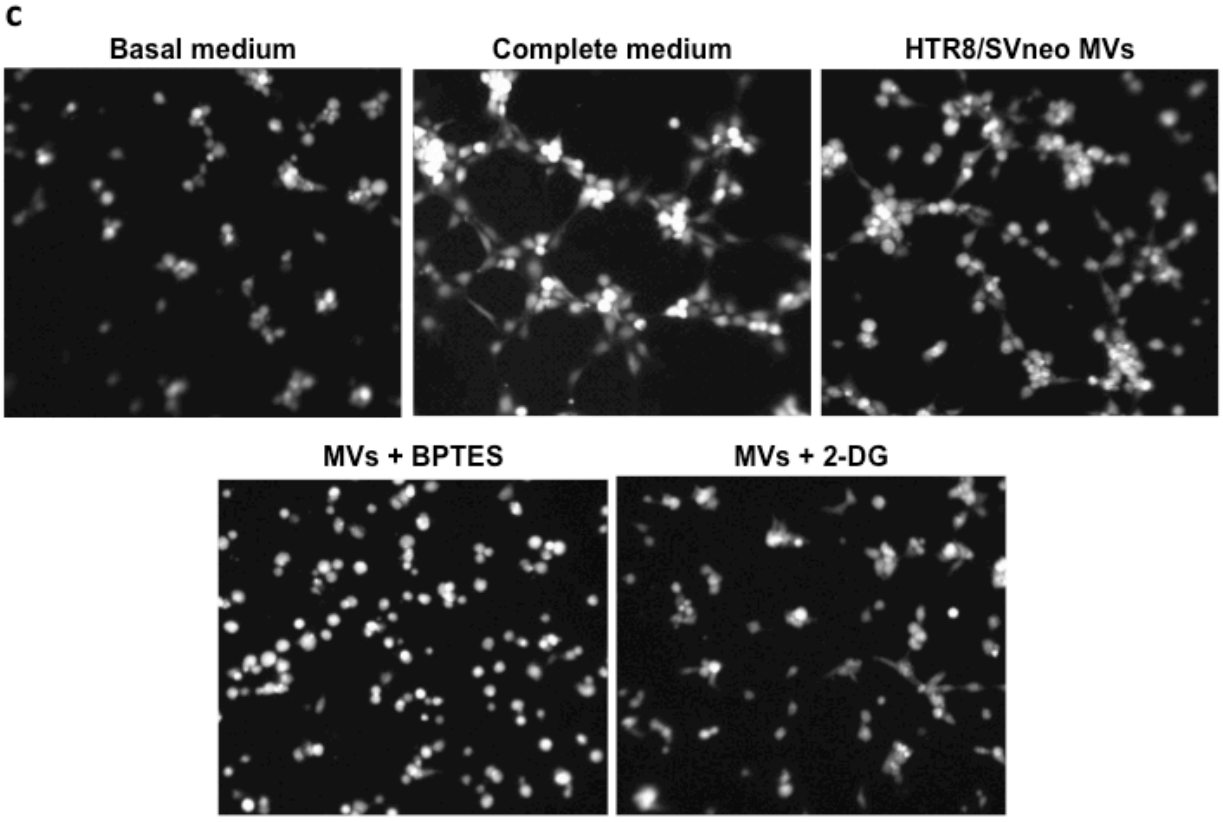


Figure 3.6 continued



Discussion

Because extravillous trophoblasts (EVTs) extensively remodel maternal spiral arteries through poorly understood mechanisms,[2,40] I wanted to know whether EVs played a role in this process. I first showed that HTR8/SVneo EVTs were indeed capable of producing EVs in a wide range of sizes, and that I could specifically enrich for the larger EVs, or MVs. Moreover, the MVs could be visualized along the surfaces of the trophoblasts by immunostaining for tTG and F-actin, revealing that HTR8/SVneo trophoblast MVs are more similar to MVs generated by cancer cells than other non-cancerous cell types (e.g. ES cells), which do not express tTG or exhibit F-actin rings.

HTR8/SVneo MVs were found to stimulate HUVEC survival as well as HUVEC tubulogenesis, an *in vitro* measurement of the ability of endothelial cells to form blood vessels.[33,43,44] These findings suggest that EVTs may play a vital role during maternal spiral artery remodeling by helping endothelial cells to survive better and to generate new placental vasculature. Another study has shown that under hypoxic conditions, placental mesenchymal stem cells (MSCs) produce EVs, which can stimulate placental microvascular endothelial cells to tubulate.[47] However, the study did not further examine the mechanisms behind tubulation, nor did it address whether first trimester EVTs produced EVs that could induce similar effects.

How do the trophoblast-derived MVs promote cell survival and tubulation? I showed that these MVs are capable of activating several signaling proteins, including ERK, AKT and JNK. ERK signaling has been shown to be necessary for HUVECs to undergo tubulogenesis, and I have shown that MV-mediated HUVEC tubulogenesis is also blocked when ERK activity is inhibited. Interestingly, I also found that JNK activity is important for this process, suggesting

that the trophoblast MVs activate multiple signaling events that are required to stimulate endothelial cell migration.

Additionally, I found that HTR8/SVneo trophoblast MVs do not contain VEGF, which can stimulate tubulogenesis in endothelial cells,[14] nor do they appear to stimulate endothelial cells to make VEGF. In the context of cancer, cancer cell MVs have been shown to stimulate the production of VEGF by endothelial cells, providing an autocrine substrate for the endothelial VEGFR and promoting angiogenesis.[40] Of course, the effects on tubulogenesis that we observed occurred over only ~5 hours, making it unlikely that transcriptional changes are ultimately responsible for promoting tubulogenesis.

To discover candidate proteins in trophoblast MVs that are potentially responsible for their effects, I carried out mass spectrometry analysis of the MVs, and found that they contained a variety of metabolic proteins. When endothelial cells are stimulated to undergo angiogenesis, they go through a wide variety of metabolic changes to transform themselves from quiescent cells to actively growing cells undergoing tubulogenesis.[43,44] The increase in metabolism is in some ways comparable to the metabolic changes that cancer cells undergo during malignant transformation.[1,2,44] Thus, it is possible that the trophoblast MVs can transfer their contents, i.e. metabolic proteins, to endothelial cells, helping to cause a boost in metabolism that helps endothelial cells undergo angiogenesis or even aids in their survival. Consistent with this idea, inhibiting glutaminase activity using BPTES or slowing glycolysis with 2-DG had a profound effect on HUVEC tubulation. To confirm these data, a knockdown of glutaminase or other key glycolytic proteins in trophoblast MVs with siRNA should be performed, and then control MVs as well as the MVs depleted of glutaminase (or glycolytic enzymes) should be added to HUVECs in a tubulation assay. If glutaminase plays an important role in promoting HUVEC

tubulation, the trophoblast MVs depleted of glutaminase should not be able to promote endothelial cell tubulation compared to the control MVs.

From these data, it is clear that EVTs release MVs, which can promote the survival and tubulation of endothelial cells. Given that EVTs interact extensively with endothelial cells during maternal spiral artery remodeling,[1,2,4] and that EVs derived from EVTs have already been shown to affect vascular smooth muscle migration during this critical step in placentation,[5-7,27] our results are consistent with the idea that EVs play an important role in this process. Serious complications later on in pregnancy, such as preeclampsia and intrauterine growth restriction, have been traced back to trophoblast migration and invasion into the maternal spiral arteries.[1,2,9,10] Therefore, it is important that we better understand this critical phase in pregnancy and the formation of the placenta. In the future, trophoblast-derived MVs could be a potential therapeutic for problems with maternal spiral artery remodeling.

Material and Methods

Antibodies and reagents

Antibodies that recognize the phosphorylated forms of JNK, FAK, ERK1/2, AKT (T308), EGFR as well as flotillin-2, PKM2, LDHA and GAPDH antibodies were from Cell Signaling Technology. The GFP and VEGF antibodies were from Santa Cruz Biotechnology, and the Ran antibody was from BD Transduction Laboratories. The tTG antibody was from Neomarkers, and the GLS1 antibody was from Abgent. The fibronectin, β-actin and laminin α5 antibodies, as well as GRGDSP (RGD) peptide and YIGSR peptide were from Sigma-Aldrich. The PM-GFP vector was a gift from Tobias Meyer (Addgene plasmid #21213). The rhodamine-conjugated phalloidin,

Oregon green 488 goat anti-mouse secondary antibody, FM1-43FX plasma membrane dye, Lipofectamine, as well as all other tissue culture reagents were from Life Technologies.

Cell Culture

HTR8/SVneo trophoblasts, which were a gift from Charles Graham (Queen's University, Kingston, ON), were cultured in RPMI-1640 medium supplemented with 5% fetal bovine serum (FBS). Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial basal medium-2 (EBM-2) supplemented with 10% FBS, hydrocortisone, human fibroblast growth factor (hFGF), VEGF, R3-insulin-like growth factor-1 (R3-IGF-1), ascorbic acid, human epidermal growth factor (hEGF), gentamycin and amphotericin (GA-1000), and heparin (packaged in EGM-2 BulletKit, Lonza).

Microvesicle isolation

Two 150 mm dishes of HTR8/SVneo cells were rinsed twice with 10 ml of phosphate-buffered saline (PBS) and incubated in serum free medium (RPMI-1460) for at least 5 hours. The conditioned medium was removed from the cells and then centrifuged at 300 x *g* for 10 minutes to pellet intact cells, and again at 3,000 x *g* for 20 minutes to remove cell debris. The partially clarified medium was then filtered through a 0.22 μm Steriflip filter unit (Millipore), and then rinsed with 10 ml of PBS. When being used for biological assays, MVs trapped on top of the filter were resuspended in ~1.5 ml of serum-free medium. However, if the MVs were used to generate lysates, they were lysed with mammalian lysis buffer (25 mM Tris, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM $NaVO_4$, 1 mM β-glycerol phosphate, 1 μg/ml aprotinin, and 1 μg/ml leupeptin).

Nanonoparticle tracking analysis

MVs isolated in 1.5 ml of serum free medium were assayed for size and concentration by nanoparticle tracking analysis with a Nanosight (Malvern NS300). A 405 nm laser was used, and five videos of 60 seconds in duration were taken for each sample. The videos were analyzed using NTA software v3.0, with settings kept constant between samples.

Fluorescence microscopy

To visualize MVs on the surface of trophoblasts, cells were incubated with 5 μg/ml FM1-43fx dye in cold PBS for 1 minute. The trophoblasts were fixed in ice cold 3.7% formaldehyde for 20 minutes and then rinsed with PBS. Alternatively, cultures of trophoblasts were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, and then blocked with 10% bovine serum albumin. The cells were incubated with a tTG antibody, washed, and then incubated with an Oregon Green 488-conjugated secondary antibody, rhodamine-conjugated phalloidin to stain F-actin, and DAPI to label nuclei. After both staining procedures, cells were visualized by fluorescence microscopy. All images of the cells were captured and processed using IPLABS software.

Immunoblot analysis

Protein concentrations of MVs and cell lysates were determined using the Bio-Rad Bradford protein assay. Equal amounts of each lysate were resolved by SDS-PAGE and then transferred to PVDF membranes. The membranes were incubated with the indicated primary antibodies diluted in 20 mM Tris, 135 mM NaCl, and 0.02% Tween 20 (TBST). Primary antibodies were

detected with HRP-conjugated secondary antibodies (Cell Signaling Technology), followed by exposure to ECL reagent.

Microvesicle transfer and signaling experiments

For the transfer experiments, HUVECs were plated in 6-well plates, and then incubated with serum free medium supplemented without or with MVs derived from HTR8/SVneo cells for one hour. The MVs were derived from either parental trophoblasts or trophoblasts ectopically expressing PM-GFP. For the signaling experiments, serum starved HUVECs in 6-well plates were treated with serum free medium supplemented without or with MVs derived from HTR8/SVneo cells, or serum for the indicated lengths of time. For both experiments, HUVECs were lysed in mammalian lysis buffer.

Survival assays

HUVECs were plated in 6-well dishes and then cultured in complete medium or serum free medium supplemented without or with HTR8/SVneo MVs. Approximately two days later, the HUVECs were collected, stained with DAPI and viewed by fluorescence microscopy. The number of cells undergoing apoptosis was determined based on nuclear condensation and fragmentation and then divided by the total number of cells to determine the percent of cell death. The experiment was performed at least three times and graphed.

Tubulogenesis assays

After 15 mins of treatment with 1 μM Calcein AM, 3 x 10^4 HUVECs were plated on reduced growth factor BME (matrigel) in a 96-well plate in 100 μl of complete medium or basal medium

supplemented without or with HTR8/SVneo MVs. The HUVECs treated with MVs were also supplemented without or with 15 μM BPTES, 25 mM 2-DG, 10 μM PD98059 or 10 μM SP600125. After ~5 hours, the cells were visualized by fluorescence microscopy.

Mass spectrometry

MV lysates collected from HTR8/SVneo trophoblast (30 μg) were resolved by SDS-PAGE and then stained with a Colloidal Blue Staining kit. The proteins were excised from the gel and trypsin-digested. Cornell's Proteomics Facility analyzed the resulting peptide fragments using a triple quadrupole linear ion trap (4000 Q Trap) on-line LC/MS/MS system (Applied Biosystems/MDS Sciex) or the Synapt HDMS system (Waters). Proteins were identified by performing peptide alignment searches using the NCBI human RefSeq protein database.

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CHAPTER 4

Conclusions

The study of extracellular vesicles (EVs) is still a young field, and much of the current work focuses on the role of EVs in cancer progression and their potential uses for diagnostics.[1-3] However, there is a growing appreciation that non-cancerous cell types also generate and shed EVs,[4,5] but their functions have been far less defined. Thus, for my thesis, I set out to expand our knowledge of the function of microvesicles (MVs), one type of EV, in the context of developmental biology. In particular, I investigated the role of MVs generated by embryonic stem (ES) cells and trophoblasts in the context of pregnancy.

In chapter 2, I first showed a novel mechanism by which MVs generated by ES cells influenced the implantation process. We demonstrated that conditioned medium (CM) from mouse ES cells (cell line E14tg2a.4) could activate signaling events in trophoblasts and promote their migration. These findings were particularly exciting since trophoblasts surround the inner cell mass, from which ES cells are derived, and are responsible for implanting an embryo into the uterus as well as establishing the placenta to nourish the embryo as it grows.[6,7] These steps are critical for initiating and maintaining a successful pregnancy. It is generally believed that signals generated by the mother are responsible for directing the implantation of the embryo and the formation of the placenta.[6,7] However, the findings reported here are, to the best of my knowledge, the first demonstration that the embryo also sends signals that promote trophoblast migration and direct the implantation process, making these findings particularly intriguing.

ES cells generated and shed both exosomes and MVs into the medium, and the MVs could be specifically isolated from the conditioned medium (CM) as well as visualized on the

surfaces of ES cells by taking advantage of the fluorescent membrane dye FM1-43fx. Furthermore, I showed that the MVs isolated from ES cells could recapitulate the effects of the CM, including activating signaling proteins (e.g. FAK and JNK) in trophoblasts and enhancing their ability to migrate. We also showed that ES cell MVs could enhance blastocyst outgrowth, an *in vitro* measure of a blastocyst's ability to implant.[8,9] These data indicated that MVs in the ES cell CM were sufficient to influence trophoblast function, specifically trophoblast migration.

It is worth mentioning that the HTR8/SVneo trophoblasts also make MVs, but that these MVs had no effect on trophoblast migration. Thus, the ES cell MVs appear to be uniquely capable of mediating trophoblast migration. Our laboratory has obtained similar findings in the past. For example, we have shown that while MVs from aggressive cancer cell lines can promote the growth and survival of other cancerous and non-cancerous cell types, such as fibroblasts,[10] they had little effect on the donor cells that generated the MVs (i.e. the aggressive cancer cells). These data suggest that perhaps a cell is somehow unable to efficiently take up the EVs that it generates. In support of this idea, a recent study showed that integrins on the surface of cancer cell-derived EVs direct the EVs to specific organs within the body, where they then adapt the local microenvironment, making it more hospitable to metastasizing cancer cells.[11] This suggests that only certain cell types may be able to efficiently take up specific EVs, depending on the proteins on the surface of the EVs. However, another possibility is that the contents of the MVs are already present in the cells that shed the MVs and are therefore unable to alter cellular function significantly.

Next, we determined that fibronectin and laminin, two components of the ES cell MVs, were necessary for the ES cell MVs to activate signaling events in trophoblasts that were important for promoting their ability to migrate. Both of these proteins were among the ten most

abundant identified by mass spectrometry in ES cell MVs. Our laboratory has previously identified fibronectin as a major protein component of MVs derived from the highly aggressive breast cancer MDAMB231 cell line and U87 glioblastoma cell line.[10] These MVs also contain tissue transglutaminase (tTG), a dual functioning GTPase and cross-linking enzyme that has been extensively linked to cancer progression.[12] tTG was shown to cross-link fibronectin on the surface of cancer cell MVs, enhancing fibronectin's ability to activate integrin-mediated signaling on recipient fibroblasts, thereby enhancing the MVs' growth- and survival-promoting capabilities.[10] Blocking the ability of the cross-linked fibronectin on the cancer cell MVs from interacting with integrins expressed by the recipient fibroblasts using the RGD peptide or inhibiting tTG's cross-linking abilities eliminated the growth- and survival-promoting effects of the cancer cell MVs. In the context of ES cell MVs, the fibronectin associated with the MVs was not cross-linked due to the fact that these cells do not express tTG (data not shown). Moreover, blocking the ability of the fibronectin expressed on the ES cell MVs to bind to and activate integrins on the trophoblasts was not sufficient to block the MVs' migration-promoting actions. However, the ES cells did express another extracellular matrix protein linked to early pregnancy, laminin,[13] and when inhibited together with fibronectin, the ability of the ES cell MVs to promote trophoblast migration was completely blocked. Thus, once again, fibronectin played an important role in mediating the effects of MVs. In the future, it will be interesting to see whether fibronectin works together with extracellular matrix proteins to mediate their effects in other non-cancerous cell types.

To complete the study, I then showed that fluorescently labeled MVs could be taken up by both trophoblasts in culture and when injected into blastocysts isolated from pregnant mice. Interestingly, the labeled MVs injected into the blastocysts consistently attached to the

trophectoderm while very few MVs could be detected on the inner cell mass (ICM), suggesting that the ES cell MVs might be preferentially taken up by trophoblasts.

Finally, I showed that when blastocysts were injected with ES cell MVs, they were able to implant ~20% more efficiently into pseudo-pregnant mice compared to blastocysts injected with vehicle alone. This suggested that the injected ES cell MVs aided the implantation process, potentially by further stimulating trophoblast migration as was demonstrated *in vitro*. Although a 20% increase in implantation efficiency may not seem like a large effect at first glance, it is worth mentioning that implantation is already a highly efficient process in the mouse. Additionally, these experiments were performed using only a single injection of ES cell MVs into the blastocyst lumen, and yet it was still enough to cause a significant change in implantation rates. These results are particularly exciting when taken in the context of *in vitro* fertilization. It is estimated that about 10% of couples will experience infertility problems, and many will turn to *in vitro* fertilization to increase their odds of conceiving despite the fact that it is costly and sometimes unsuccessful.[14] A major reason that *in vitro* fertilization fails is due to implantation failure. If couples could expect to see a 20% increase in their chances of conceiving by the injection of MVs derived from ES cells into blastocysts, this would represent a huge increase in their odds of becoming pregnant. Interestingly, in some fertility clinics outside of the United States, extra mitochondria are being injected into oocytes before *in vitro* fertilization to increase the quality of the oocytes and therefore the chances of conception.[15,16] Thus, the possibility of injecting MVs into blastocysts as a strategy to increase implantation rates is plausible.

While some recent studies have begun to link EVs to other aspects of reproduction, the majority of them have focused on the EVs shed by cells of maternal or trophoblast origin,[5,17,18]

and minimal work has been done regarding the function of EVs shed by embryonic cells. A lack of research in this area stems primarily from the fact that the implantation process is extremely difficult to study due to both ethical and technical limitations. For instance, early embryonic states are extremely transient in nature, and, in the mouse embryo, large changes can occur over the course of only a few hours. Thus, a blastocyst harvest from a mouse can potentially be useless for certain developmental studies due to a miscalculation in the conception time by as little as a few hours.[9] Moreover, implantation itself is very challenging to study because it is extremely difficult to monitor *in vivo* or develop *in vitro* models that fully recapitulate its complexity. This helps to explain why many complications that arise during pregnancy, such as preeclampsia and intrauterine growth restriction, are still of unknown origin.[19,20]

Trophoblasts also function to extensively remodel maternal spiral arteries to bring blood flow to the growing embryo.[6,7] These findings, combined with the fact that trophoblasts generate and shed MVs similar to ES cells, prompted me to investigate the effects of trophoblast MVs on endothelial cells. Other studies have investigated the effects of trophoblast EVs.[5,21,22] For example, one study showed that trophoblast EVs can influence the vascular smooth muscle cells to migrate away from maternal spiral arteries during the placental remodeling process.[23] Additionally, there have been suggestions that EVs derived from placental mesenchymal stem cells (MSCs) might influence endothelial cell function,[24] but no studies have looked at the function of EVs derived from more commonly used trophoblast cell lines in this context. Therefore, I set out to further explore the effects of HTR8/SVneo trophoblast MVs on endothelial cells.

I first showed that trophoblasts generate and shed large quantities of MVs. It is interesting to note that the trophoblast MVs can be visualized by staining the cells for

filamentous (F-) actin and tTG, similar to those MVs generated by some highly aggressive cancer cell lines (e.g. MDAMB231 breast cancer cells and U87 glioblastoma cells).[10,25] To date, trophoblasts are the only non-cancerous cell type that we have identified that produces MVs containing F-actin. Trophoblast migration and invasion into the uterus is considered an example of physiological metastasis, and since many of the same pathways are activated in invading trophoblasts compared to invasive and metastatic cancer cells,[26] perhaps the fact that trophoblasts exhibit cancer cell-like MVs is unsurprising.

Next, I showed that trophoblast MVs could transfer their contents to human umbilical vein endothelial cells (HUVECs) and promote their survival and tubulation. This suggests that trophoblast MVs might play a role in communicating with endothelial cells during spiral artery remodeling. Additionally, I found that trophoblast MVs activated numerous signaling proteins in HUVECs. Of these, the phosphorylation (activation) of ERK is especially noteworthy since ERK activity is important for endothelial cell tubulogenesis.[27] It was surprising, however, to see that JNK was also activated in response to trophoblast MVs, and that the inhibition of JNK signaling prevented endothelial cell tubulation. While there are studies implicating JNK signaling in angiogenesis, there is no consensus as to whether JNK has a positive or negative role in the process.[28] My data suggest that JNK may in fact promote MV-mediated endothelial cell tubulation.

What are the contents in trophoblast MVs that are important for promoting endothelial cell tubulation? I showed that trophoblast MVs do not contain VEGF, unlike EVs collected from some cancer cell lines, ruling out the possibility that trophoblast MVs induce endothelial cell tubulation by directly activating VEGF receptor signaling. However, I did notice that trophoblast MVs contained both fibronectin and laminin. Interestingly, neither the RGD and YIGSR peptides

alone, nor the combination of the two inhibitory peptides could decrease trophoblast MV-mediated signaling in HUVECs as dramatically as in the system described in chapter 2. Specifically, in chapter 2, I determined that the fibronectin and laminin present on ES cell MVs were necessary for promoting trophoblast migration. However, trophoblast MVs did not promote trophoblast migration. This suggests that there are potentially other factors in the ES cell MVs that promote trophoblast migration, or, once again, MVs derived from a specific cell type are more likely to influence other cell types rather than their cell type of origin.

This led me to perform mass spectrometry analysis on the trophoblast MVs. I was excited to discover that the trophoblast MVs contained a wide variety of metabolic proteins. An immunoblot analysis of the trophoblast MV lysates confirmed that many metabolic proteins were present at levels that were comparable to those in the whole cell lysates, including glutaminase (GLS1), pyruvate kinase M2 (PKM2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and lactate dehydrogenase A (LDHA). To form new blood vessels, endothelial cells have to undergo dramatic metabolic changes to transform from a quiescent cell to a rapidly growing and migrating cell.[29,30] In fact, the metabolic changes that endothelial cells undergo are similar to those changes that occur during cellular transformation (e.g. the Warburg effect).[30] Similarly, other members of the Cerione group have observed that MVs from different cancer cells also contain metabolic proteins. This is especially noteworthy in the context of trophoblast MVs containing F-actin rings, and trophoblasts themselves being a highly migratory and invasive cell type. These findings led us to hypothesize that the trophoblast MVs could be transferring metabolic enzymes to HUVECs to boost their metabolism and allow them to form blood vessels.

In fact, further analysis showed that inhibiting glutaminase with BPTES or glycolysis with 2-deoxyglucose blocked trophoblast MV-stimulated tubulation. This suggested that

metabolism is definitely a player in mediating HUVEC tubulation. Additionally, inhibiting signaling proteins activated by the trophoblast MVs, including JNK and ERK activation, also inhibited trophoblast MV-stimulated tubulation, supporting the idea that signaling activated by the trophoblast MVs is also necessary for HUVEC tubulation to occur. It is possible that the trophoblast MVs initiate a combination of both signaling and metabolic changes that promote endothelial cell tubulation or that the signaling changes also stimulate the metabolic changes. In the future, it would be interesting to see whether trophoblast MV-stimulated metabolic changes in HUVECs could be observed. For instance, one could measure whether trophoblast MVs stimulated HUVECs to secrete more lactic acid into the medium compared to controls.

Finally, while my thesis has centered around MVs and their functions in early development, it would be interesting to see what function, if any, exosomes shed by ES cells or trophoblast cells might perform. During the course of investigating the first study, presented in chapter 2, we noted that exosomes did not stimulate trophoblast migration (data not shown). However, other studies have focused on ES cell exosomes or a combination of ES cell exosomes and MVs, suggesting that exosomes might play a role in early developmental processes as well.[31,32]

Furthermore, I determined over the course of my studies that ES cell EVs contain a wide variety of nuclear proteins and transcription factors, including Oct3/4, Sox2, KLF4 and c-Myc. When these four transcription factors are ectopically expressed in fully differentiated cells, they can reprogram cells to de-differentiate into a stem-like state, creating induced pluripotent stem cells (iPSCs).[33] While the process of inducing pluripotency is still extremely inefficient,[33,34] the potential to be able to create stem cells from an individual's somatic cells raises exciting new possibilities in the field of regenerative medicine.[35] Since ES cell EVs contain these transcription

factors, it would be interesting to see whether ES cell EVs could be harnessing to aid in inducing pluripotency or perhaps, delaying differentiation. Alternatively, since EVs often contain markers specific to their cell of origin,[36] EVs from somatic cells could potentially be used to differentiate stem cells into specific lineages.

The fact remains that the study of EVs is emerging as an exciting and novel area in intercellular communication. The more that we learn about EVs, the more it seems that they exert an influence on almost every aspect of cell biology. While the field is currently focusing on the roles of EVs in cancer biology, biologists are beginning to look at EVs in other contexts. In this thesis, I have further expanded our knowledge of the roles of one type of EV in the context of early development.

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