Non-Invasive Methods of Human Embryo Quality Assessment: Exploring Morphokinetic, Metabolomic and Secretome Parameters That Predict Pregnancy Outcome in IVF

Parshvi Rajesh Vyas

A thesis submitted in conformity with the requirements for the degree of Masters of Science
Department of Physiology
University of Toronto

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Parshvi Rajesh Vyas

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University of Toronto
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Abstract

*In-vitro fertilization* (IVF) is a commonly used infertility treatment involving fertilization of the oocyte by sperm outside of the body. Morphological assessment is the primary non-invasive criteria used to select embryos for uterine transfer, however, using this method, the current success rate is around 30%. Thus, more non-invasive tools are needed to improve the process of embryo selection. The objective of this thesis is to explore and assess additional non-invasive methods, including morphokinetics, metabolic profiling and extracellular vesicle release, as biomarkers for selecting the embryo with the highest implantation potential. Results show some morphokinetic parameters are correlated with implantation and metabolomic analysis can provide further insight in understanding embryo physiology. Moreover, we show for the first time, the possibility of the release of extracellular vesicles from human pre-implantation embryos. This data provides validation that these techniques have the potential to be brought into routine clinical use for embryo selection.
Acknowledgments

I would like to sincerely thank my supervisors Dr. Clifford Librach and Dr. Hanna Balakier for their guidance and support. I would also like to thank Dr. Darcy Burns for his assistance with NMR data acquisition and analysis, and Dr. Battista Calvieri, Dr. Steven Doyle and Yan Chen for their assistance with electron microscopy. I would also like to extend my gratitude to the embryology staff at CReATe IVF for their assistance in sample collection and to the research staff for their support and encouragement.
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List of Abbreviations

ADP – Adenosine di-phosphate

ASRM – American Society for Reproductive Medicine

ATP – Adenosine tri-phosphate

BSA – Bovine serum albumin

cDNA – Complementary deoxyribose nucleic acid

CFAS – Canadian Fertility and Andrology Society

Ct – Cycle threshold

DNA – Deoxyribonucleic acid

ESED – Environmental secondary electron detector

eSET – Elective single embryo transfer

EV – Extracellular vesicle

GnRH – Gonadotrophin releasing hormone

hCG – human chorionic gonadotropin

hpi – Hours post insemination

HPLC – Higher performance liquid chromatography

ICM – Inner cell mass

ICSI – Intracytoplasmic sperm injection

IP – Implantation
iPSC – Induced pluripotent stem cell

IR – Infrared

IVF – *In-vitro* fertilization

LDH – Lactate dehydrogenase

MII – metaphase II

MKP – Morphokinetic parameter

MS – Mass spectrometry

MVB – Multivesicular body

NGS – Next Generation Sequencing

NIP – No Implantation

NIR – Near Infrared

NMR – Nuclear Magnetic Resonance

NPB – Nucleolar Precursor Body

NTA – Nanoparticle tracking analysis

PBS – Phosphate buffer saline

PDC – Pyruvate dehydrogenase complex

PFK – Phosphofructokinase

PGD – Pre-implantation genetic diagnosis

PGS – Pre-implantation genetic screening

PMSF – phenylmethylsulfonyl fluoride
PVS – Perivitelline Space

qPCR – Quantitative polymerase chain reaction

RNA – Ribonucleic acid

rpm – Rotations per minute

SART – Society for Assisted Reproductive Technologies

SEM – Scanning electron microscopy

SET – Single embryo transfer

STB – Syncytiotrophoblasts

TCA – Tricarboxylic acid

TE – Trophoderm

TEM – Transmission electron microscopy

TMS – Tetramethylsilane

TSP – Trimethylsilylpropanoic acid

VEGF – Vascular Endothelial Growth Factor

ZP – Zona pellucida
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Chapter 1
Introduction

1 Introduction

1.1 The Process of IVF

*In-vitro* fertilization (IVF) is the process by which an egg is fertilized by sperm outside of the body. Naturally, one egg is ovulated each month. Using superovulation, it is possible to inhibit the process of follicle atresia and allow more than one oocyte to grow to the MII stage. These oocytes are then retrieved from the ovaries and harvested in culture. Taking sperm from the partner or a donor, the oocyte can then be inseminated through intra-cytoplasmic sperm injection (ICSI) or by simple mixing (routine IVF). ICSI is a more invasive method than IVF as it involves direct injection of one spermatozoa into each oocyte. The procedure is typically performed in cases where there is male factor infertility or if the zona pellucida is difficult to penetrate. While there is an increased risk due to more handling time, reports have shown no increased risk in miscarriage rates, stillbirths or perinatal deaths in pregnancies conceived through ICSI compared to IVF (Bondeuelle et al. 2002). After insemination, the resulting embryos are then grown in culture for the duration of the pre-implantation phase. Transfer can occur either on day 3 or day 5 post insemination. Although multiple embryos are grown in culture, the American society for Reproductive Medicine (ASRM) and the Canadian Fertility and Andrology Society (CFAS) guidelines suggest that only one or two embryos be selected for uterine transfer in patients less than 37 years of age, to reduce the possibility of multiple births (CFAS report, 2006 ASRM report 2004)). Multiple births pose a greater threat to maternal and fetal health than singleton births and babies born through multiple gestations are at a higher risk for complications of prematurity, most commonly affecting the lungs and brain.

1.2 Current Methods for Embryo Selection for Uterine Transfer

Morphological assessment using light microscopy is typically the first line of approach when assessing embryos for uterine transfer. The method has been used since the inception of IVF
(Edwards et al. 1981) to identify the normal development of embryos and was later used as a tool to select embryos with the highest developmental potential (Hill et al. 1989). Human embryo development follows a specifically timed sequence of events, thus morphological assessment takes into consideration two major factors: 1) The developmental rate, assessing whether cellular divisions are occurring in a timely trajectory, and 2) The morphological characteristics including size, shape, fragmentation and positioning of the blastomeres at various stages of development.

Cleavage stage embryos are scored based on multiple characteristics. The most important indicator of embryo viability is cell number. Edwards et al. showed in 1981 that human embryos increase in cell number and the sequence of these events follows a specific timeline. Embryos that cleave too quickly or too slowly were found to have compromised development. (Giorgetti et al. 1995, Alikani et al. 2000). Additionally, the pronuclear zygote is most commonly assessed for the number and positioning of nucleolar precursor bodies (NPB) within each pronucleus. Studies have shown that the most viable zygotes have similarly sized pronuclei that are centrally located, and similarly sized NPB (Witterman et al. 2000, Montag and van der Ven 2001, Balaben et al. 2001). Cell fragmentation, is also assessed and has been found to be associated with metabolism abnormalities, cellular apoptosis (Jurisicova 1996, Perez et al. 1999), and/or chromosome segregation abnormalities (Pellestor et al. 1994). While several grading systems have been developed for assessing cell fragmentation, the most commonly used system is based on the percentage volume of the embryo that is composed of fragmentation. I.e. Score 1 = <10% fragmentation, Score 2 = 10-25% fragmentation and Score 3 = >25% fragmentation. (Ziebe et al. 1997, Racowsky et al. 2003). Additionally, blastomere symmetry, i.e. examining the size and shape of the blastomeres is important for normal cellular divisions and even distribution of organelles (Rienzi et al. 2005). Moreover, multinucleation, defined as the presence of more than one nucleus within individual blastomeres has also been shown to be related to chromosomal abnormalities (Kligman et al. 1996, Hardarson et al. 2001, Ambrogio et al. 2011).

For assessing embryos at the blastocyst stage (day 5-6 in humans) the first proposed grading system was published by Dokras et al. in 1991, where the blastocysts were divided into categories based on the stage (i.e. early, expanding, expanded, hatching or hatched), and the quality of the inner cell mass (ICM) and the trophectoderm (TE) were also taken into account (Dokras et al. 1991). Today, the most widely used grading system, developed by Gardner and Schoolcraft in 1999, is an improvement of the Dokas grading system (Balaben et al. 2006).
addition to the previously assessed factors, this newer grading system also takes into account the amount of volume occupied by the blastocoele, and the number and organization of the cells in the ICM and TE. The parameters of this grading system are outlined in Table 1.1. While this grading system is very thorough, many clinics find it to be too complex and impractical.

A simplified version of this grading system was issued by the Society of Assisted Reproduction (SART) where embryos were graded as ‘good’ (AA or AB), ‘fair’ (BA, BB or BC) or ‘poor’ (CB, CC). This system has been shown to be just as effective for predicting implantation and live birth rates which were correlated with the grade. Good quality embryos resulted in a 56% live birth rate in patients less than 35 years of age; 44% for patients between 35-37 years of age; and 35% for patients between 38-40 years of age. (Heitmann et al. 2013). In addition to providing a more suitable method for efficient clinical practice, this simplified system is also more comprehensible by patients for communicating treatment procedures.

In addition to morphology, pre-implantation genetic screening (PGS) and pre-implantation genetic diagnosis (PGD) can be utilized to assess aneuploidy and genetic mutations respectively. While these tests offer valuable information about the genetic integrity of each embryo, they involve the use of invasive biopsies, which are time consuming, pose some risk to the embryo, are very costly, and are not always readily available.
Table 1.1 Blastocyst grading system. Adapted from Matchtinger et al. 2013. Reproductive Biomedicine

<table>
<thead>
<tr>
<th>Blastocyst Stage Grade</th>
<th>ICM</th>
<th>TE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early blastocyst A</td>
<td>Tightly packed, many cells A Many cells forming a tightly knit epithelium</td>
</tr>
<tr>
<td>2</td>
<td>Blastocyst B</td>
<td>Loosely grouped, several cells B Few cells</td>
</tr>
<tr>
<td>3</td>
<td>Full blastocyst C</td>
<td>Very few cells C Very few cells forming a loose epithelium</td>
</tr>
<tr>
<td>4</td>
<td>Expanded Blastocyst</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Hatching Blastocyst</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Hatched Blastocyst</td>
<td></td>
</tr>
</tbody>
</table>

1.3 Morphokinetics

1.3.1 About Morphokinetics

Morphokinetics refers to the use of time-lapse technology to assess embryo development *in-vitro*. While morphological assessment is a static assessment of embryos at specific time points, morphokinetics allows for assessment of the dynamics of cellular divisions, enabling calculations of the rate of cellular divisions and the time points at which cellular stages are reached, in addition to a morphological assessment. As studies have shown that the status of an embryo can change dramatically in a span of only a few hours (Mio and Maeda 2008), the visualization of the entirety of the pre-implantation development period can provide invaluable information. Another advantage over static morphological assessment is that this technology does not require
that the embryos be removed from the incubator on each assessment day, avoiding changes in
temperature and pH of the media which could potentially be detrimental. Several published
studies have assessed various morphokinetic parameters and correlated them with blastocyst
development, implantation potential and live birth rates.

The current nomenclature for morphokinetic parameters are as follows:

The time from insemination (t0) to timing of each cell division is annotated with “t(cell stage)”.
For example, t2 is the duration of time from insemination to the two cell stage, t3 is the duration
from insemination to the three cell stage and so forth. The duration of each individual cell stage
is annotated with “(cell stage)C”. For example, the duration of the third cell stage (3C) is
reflective of the amount of time the embryo spent in the three cell stage and is calculated as t4-t3.
The duration of cell cycles are annotated as “cc(cell cycle)”. For example, cc2 is the duration of
time the embryo took to complete the second cell cycle and is calculated as t3-t2, and cc3 is t5-
t3. Finally, the synchrony of each cell cycle is annotated as s(cell cycle)”. This parameter
assesses how synchronously each of the individual blastomeres are dividing. For example, the
synchrony of the second cell cycle (s2), is calculated as t4-t3 and s3 is calculated as t8-t5.

For studying blastocyst development, Dal Canto et al. found that cleavage from 2 to 8 cells
occurs earlier in embryos that develop to blastocyst and achieve subsequent implantation. (Dal
Canto 2012). Additionally, Kirkegaard et al. reported that high quality blastocysts could be
predicted by a short duration of the first cell division, the absence of a 1 to 3 cell division and
duration of the three cell stage (Kirkegaard 2012). Moreover, Wong et al found that high quality
blastocysts reached the two cell stage (t2) between 0 and 33 minutes; had an s2 between 7.8 and
14.3 hours; and had a synchrony of the second and third mitosis to be less than 5.6 hours and
3.17 hours respectively (Wong 2010). Chamayou et al. reported on all morphokinetic parameters
up to the cleavage stage and found that embryos which reached the blastocyst stage had a t2
between 21.4 and 34.9 hours. Interestingly, this parameter did not hold significance when
assessing implantation rate as the outcome variable. (Chamayou et al. 2013). The most
comprehensive studies to assess morphokinetics have been conducted by the Meseguer group. In
2011, the group published an algorithm with a hierarchical classification system based on three
morphokinetic parameters that they found to be most significant for implantation: t5 between
48.8 and 56.6 hours, s2 of less than 0.76 hours and cc2 of less than 11.9 hours. Exclusion
criteria included uneven blastomere size at the 2 cell stage; direct division of 1 to 3 cells; and multinucleation at the four cell stage (Meseguer 2011). This algorithm has since been tested in a prospective, randomized, double blinded controlled study with 843 couples undergoing ICSI, resulting in a statistically significant improvement in ongoing pregnancy rates when embryos were selected based on the algorithm (51.4%) compared to standard morphology (41.7%) (Rubio et al. 2014). A summary of the major findings of various research groups, including significantly important MKP and comparison of study parameters is outlined in Table 1.2.
Table 1.2 - Comparison of literature findings for morphokinetic findings significantly associated with embryo development and implantation potential.

<table>
<thead>
<tr>
<th>MKP</th>
<th>Mean/Distribution (hpi)</th>
<th>Number of Embryos</th>
<th>Outcome Variable</th>
<th>Day of Transfer</th>
<th>Media</th>
<th>CO₂%, O₂%</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>t2</td>
<td>26.9 (21.4-34.8)</td>
<td>72</td>
<td>Blast Development</td>
<td>N/A</td>
<td>SAGE (Single step)</td>
<td>5%, 5%</td>
<td>Chamayou 2012</td>
</tr>
<tr>
<td></td>
<td>24.7 ± 3.6</td>
<td></td>
<td>Implantation</td>
<td>4</td>
<td>Irvine (Single step)</td>
<td>6%, 5%</td>
<td>Ciray et al. 2012</td>
</tr>
<tr>
<td></td>
<td>27.9 ± 3.4</td>
<td></td>
<td>Implantation</td>
<td>4</td>
<td>Irvine (Sequential)</td>
<td>6%, 5%</td>
<td>Ciray et al. 2012</td>
</tr>
<tr>
<td>t4</td>
<td>40.3 (33.1 - 57.2)</td>
<td>72</td>
<td>Blast Development</td>
<td>N/A</td>
<td>SAGE (Single step)</td>
<td>5%, 5%</td>
<td>Chamayou 2012</td>
</tr>
<tr>
<td>t5</td>
<td>52.3 ± 4.2</td>
<td>61</td>
<td>Implantation</td>
<td>3</td>
<td>SAGE (Single step)</td>
<td>5%, 20%</td>
<td>Meseguer 2010</td>
</tr>
<tr>
<td>t7</td>
<td>57.4 (46.1 - 82.5)</td>
<td>72</td>
<td>Blast Development</td>
<td>N/A</td>
<td>SAGE (Single step)</td>
<td>5%, 5%</td>
<td>Chamayou 2012</td>
</tr>
<tr>
<td>t8</td>
<td>61.0 (46.4 - 97.8)</td>
<td>72</td>
<td>Blast Development</td>
<td>N/A</td>
<td>SAGE (Single step)</td>
<td>5%, 5%</td>
<td>Chamayou 2012</td>
</tr>
<tr>
<td></td>
<td>54.9 ± 5.2</td>
<td>93</td>
<td>Blast Development</td>
<td>N/A</td>
<td>Origio (Sequential)</td>
<td>6%, 5%</td>
<td>Dal Canto 2012</td>
</tr>
<tr>
<td>cc2</td>
<td>11.8 ± 1.2</td>
<td>61</td>
<td>Implantation</td>
<td>3</td>
<td>SAGE (Single Step)</td>
<td>5%, 20%</td>
<td>Meseguer 2012</td>
</tr>
<tr>
<td>s2</td>
<td>0.78 ± 0.73</td>
<td>61</td>
<td>Implantation</td>
<td>3</td>
<td>SAGE (Single Step)</td>
<td>5%, 20%</td>
<td>Meseguer 2012</td>
</tr>
<tr>
<td>cc3</td>
<td>14.4 (9.7 - 21.0)</td>
<td>72</td>
<td>Implantation</td>
<td>5</td>
<td>SAGE (Single Step)</td>
<td>5%, 5%</td>
<td>Chamayou 2012</td>
</tr>
<tr>
<td>s3</td>
<td>8.0 (0.7-30.8)</td>
<td>72</td>
<td>Blast Development</td>
<td>N/A</td>
<td>SAGE (Single Step)</td>
<td>5%, 5%</td>
<td>Chamayou 2012</td>
</tr>
</tbody>
</table>
Currently, the two main platforms for assessing embryo morphokinetics are Eeva™ Test and the Embryoscope™. The Eeva Test™ is an automated computer based program that automatically assesses embryo quality without the need for manual annotations. The cell tracking software mainly tracks the time embryos spend for t3-t2 and t4-t3, using dark field microscopy, and provides a score that predicts the embryo’s chances of forming a useable blastocyst (Eeva Website). The efficiency of this system has been tested in four different clinics where a 30% higher implantation rate was observed between embryos scored as having a higher chance of forming a blastocyst compared with the rest of the cohort. (Kirkegaard 2014). The main drawback of the Eeva Test™, found in another study, is that 50.6% of embryos that resulted in a pregnancy were categorized as “non-viable” by the software, highlighting the importance of not discarding lower scoring blastocysts (Stecher et al. 2014). Conversely, the EmbryoScope™ uses bright field microscopy and the software requires the user to input annotations of cellular divisions. Unlike Eeva Test™ where the algorithm is pre-determined, the EmbryoScope™ time lapse imaging software allows the user to input their own algorithm for grading embryos, based on the individual morphokinetic parameters found to be significantly correlated with implantation in their own laboratory. (Embryoscope website).

Time-lapse imaging is a relatively new assessment tool and the current literature is not in full concordance as to which morphokinetic parameters are most important for implantation. This may be attributed to a number of factors including different culture media used, different incubators, gases used, laboratory air quality, user bias, stimulation protocols used, patient populations, etc. (Ciray et al 2013, Wale and Gardner et al. 2010, Kirkegaard et al.2013). The technique is most valuable for patients who have a large cohort of embryos, as the technology enables a better potential for selection. It may also be useful for poor prognosis patients who have had repeated IVF failures, advanced maternal age, and/or history of recurrent miscarriages, as the technology provides additional information to make an informed decision (ES website). While time-lapse imaging systems have drastically improved visualization of the pre-implantation development period and many studies have identified several morphokinetic parameters as potential biomarkers for pregnancy potential, it is important to note that success of a pregnancy is a multi-factorial. As such, time-lapse imaging alone is unable to fully assess the capacity of an embryo to implant, since aspects of internal physiology and genetics are not
determined by this technology. Thus studies on proteomics, genetics and metabolomics could provide additive value.

1.4 Metabolomics

1.4.1 Embryo Metabolism

Research has shown that morphological grading does not accurately reflect the internal physiological processes occurring in the embryo. Many aneuploid embryos are known to exhibit very high morphological appearance and conversely, many euploid embryos can exhibit poor morphology (Alfarawati et al. 2011). Additionally, embryos with similar morphological scores have been shown to have remarkably different metabolomics and proteomic profiles (Vergouw et al. 2008, Kratz-Jaffe et al. 2006, Gardner et al. 2001). Therefore, in order to identify embryos with the highest implantation potential, it may also be informative to assess the metabolic activity of an embryo. To date, much of the research on embryo metabolism has been undertaken with animal models, due to the invasive nature of these studies and the ethical limitations of performing functional studies on human embryos. However, it is possible to assess human embryo metabolism non-invasively by studying metabolites in spent embryo culture media.

Prior to the compaction stage, the embryo predominantly utilizes anaerobic glycolysis. When ovulation occurs naturally, the ovulated oocyte is surrounded by cumulus cells which actively convert glucose into pyruvate and lactate (Leese et al. 1985, Gardner et al. 1996). Thus, the early cleavage stage human embryo finds itself in an environment rich in lactate and pyruvate, but low in glucose. Furthermore, the early stage embryo has a high ATP to ADP ratio which negatively inhibits phosphofructokinase (PFK), thereby limiting the influx of glucose through the glycolytic pathway (Lane et al. 2000). Mouse studies have shown that the lactate dehydrogenase (LDH) complex comprises about 5% of total proteins in a mouse oocyte and embryo (Auerbachy et al. 1967). Up until the blastocyst stage, embryos utilize the LDH isoform I which favours pyruvate formation. At the blastocyst stage, LDH switches to isoform V which favours lactate formation (Gibbons et al. 2006). It is also known that glycine acts as an intracellular buffer and maintains cytoplasmic pH (Lane et al. 2005).
At the blastocyst stage, a low ratio of ATP to ADP activates PFK, increasing influx of glucose. NAD$^+$ is an important contributor to blastocyst metabolism as it regulates biosynthesis and reduces intracellular glutathione levels (Leese et al. 1984, Gardner et al. 1990).

NAD$^+$ is produced in two ways by the blastocyst: 1) the conversion of pyruvate to lactate produces NAD$^+$ as a by-product; 2) the conversion of oxaloacetate to malate in the malate aspartate shuttle also converts NADH to NAD$^+$. The pyruvate dehydrogenase complex (PDC) converts pyruvate from the cytoplasm to Acetyl CoA, a major substrate in the TCA cycle, in the mitochondria (Greenhouse et al. 1977) Factors regulating PDC include phosphorylation and the redox state of the cell and transcriptional regulation (Hardy et al. 2002). Additionally, citrate is a metabolite that is provided in the culture media and is also produced in mitochondrial metabolism. It is a precursor for lipid synthesis, and is essential for cellular proliferation (Hardy et al. 2002).

Due to ethical implications of performing functional metabolic studies on human embryos and the scarcity of research samples, our current understanding of human embryo metabolism is predominantly from animal studies, mainly murine, and the use of limited amount of substrates, primarily glucose, lactate, pyruvate and amino acids (Gardner et al. 2015) A major limitation in studying metabolism is that pre-implantation embryos exhibit metabolic plasticity, i.e. they adapt to the culture conditions they are put in. Therefore, standardization of embryo metabolism studies is not possible if they are cultured in different types of media. A greater understanding of human embryo metabolomics could enable us to optimize in-vitro culture conditions and potentially help the embryologist to select the healthiest embryos for uterine transfer.

1.4.2 Metabolomics: Technologies used to Assess Metabolites

There are several technologies that have been utilized to analyze metabolites in spent embryo culture media and they can be grouped into three main categories: vibrational spectroscopy, mass spectroscopy (MS) and nuclear magnetic resonance (NMR) spectroscopy. (Uyer et al. 2014)

Vibrational spectroscopy both Raman and Infrared (IR) and involves exposing the sample to electromagnetic radiation. (David et al. 2006) In Raman spectroscopy, light scattered by
vibrating molecules that absorb the electromagnetic radiation is measured and quantified. Conversely, for IR spectroscopy the sample is exposed to an infrared beam and energy absorption is measured and quantified. Although vibrational spectrometry has high accuracy, individual metabolites are not identified or quantified. Instead, a metabolic profile is produced which analyses the metabolites in the sample as a whole. The ‘pattern’ can then be compared between different samples (Uyer et al. 2014).

MS platforms generally work by employing three steps: 1) the sample is separated into individual ions; 2) the ions are separated according to their mass-to-charge ratio (m/z); and 3) the separated ions are detected by a detector (Warwick et al. 2005). MS is the most widely used analytical platform for metabolite studies and detects several hundred metabolites simultaneously with high sensitivity. However, due to the complexity of MS spectra, they are more difficult to analyze, and thus tend to be utilized for identifying novel metabolites, instead of quantifying known metabolites. Coupling MS with techniques such as chromatography and electrophoresis-based separation can further increase sensitivity and specificity.

NMR exploits magnetic moments. Some protons such as C\(^{13}\) and H\(^1\) have nuclear spins. When placed in the presence of a magnetic field, the nuclei themselves create a magnetic moment and either align against the magnetic field (high energy) or along with the magnetic field (low energy) (Roberts 1959). When the sample is irradiated with energy in the radio frequency range, atoms in the low spin state absorb energy and flip to the higher spin state. A detector senses the amount of energy absorbed by nuclei to achieve the higher spin state. The electronegativity of neighboring atoms creates a de-shielding effect as they attract electrons to themselves and away from the atoms experiencing magnetic moments. Thus atoms near highly electronegative atoms require more energy to flip spin states and appear downfield on a spectrum (Roberts 1959). For standardization purposes, a compound such as Tetramethylsilane (TMS) or Trimethylsilylpropanoic acid (TSP) is often used. Both these compounds have a tetrahedral structure with a silicon atom attached to three methyl groups. The silicon atom has a lower electronegativity than any other atoms found in most organic molecules, thus the hydrogen atoms in the methyl group are highly shielded and appear ‘up field’, and are assigned a reference peak of 0 ppm, to which all other peaks are standardized to (Roberts 1959).
Although NMR requires expensive equipment and extensive expertise for data analysis, the metabolite spectra in NMR is well characterized, and with protocol optimization, the spectra can be obtained in a shorter time relative to mass spectrometry (Uyer et al. 2014).

While MS is a more sensitive technique compared to NMR, it requires knowledge of the chemical composition of the sample and is limited by the chemical dynamic range and separation methodology. NMR is only limited by the magnetic field strength and no knowledge of the chemical composition of the sample is required as it does not involve separation of the sample (Uyer et al. 2014). In comparison to Raman and IR spectroscopies, NMR is able to determine relative abundance of metabolites present in the sample and is able to quantify all metabolites present in the sample simultaneously. (Leese et al. 1984, Brison et al. 1991, Botros et al. 2008)

While there is no single best technique for assessing metabolites, the type of metabolites to be analyzed, the required sensitivity, sample size, sample volume, time for acquisition, abundance of metabolites in the sample, cost and availability of equipment and expertise, all play a role when determining the ideal technique to use.

1.4.3 Utilizing Metabolomics to select Embryos for Uterine Transfer

In 1970, Menke and McLaren cultured mouse blastocysts in basic media, lacking amino acids, and realized that the embryos quickly lost their ability to oxidize glucose. (Menke and McLaren 1970). Following this, several studies have reported that changes in an embryo’s metabolism is associated with its ability to develop in-vitro. Further studies on bovine embryos reported that blastocysts that had better in-vitro development and subsequently implanted successfully, consumed glucose at a rate greater than 5μg/h (Renard et al 1980). In 1987, Gardner and Leese examined D4 mouse embryo and reported that blastocysts with higher viability consumed greater quantities of glucose than failed embryos (Gardner and Leese 1987).

The technique of using metabolites to assess embryos was first evaluated in humans by Gardner et al. using microfluidics. Human embryos that had a higher glucose uptake had a higher chance of forming a viable blastocyst and subsequent pregnancy (Gardner et al 2001). It was also found that female blastocysts consumed glucose at a higher rate than their male counterparts, an
interesting phenomenon, previously shown to be true in some other species (Tiffin et al 1991, Gardner et al 2010). In 2001, Seli et al used Raman spectroscopy and near infra-red spectroscopy (NIR) to analyze spent culture media of day 3 embryos. Failed implantations were compared to live births and an algorithm was generated that produced an ‘embryo viability score’. Another algorithm was developed by Vergouw et al, where droplets from single embryo transfer (SET) cycles were analyzed using NIR spectroscopy. This algorithm correlated with increased implantation potential (Vergouw et al. 2008). Additional studies have also reported similar findings (Seli et al. 2010, Ahlstrom et al. 2011). However, algorithms generated by NIR and Raman spectroscopy for embryo selection failed to result in improved outcomes in prospective trials (Scott et al. 2008). Moreover, Brison et al. assessed levels of amino acids using High Performance Liquid Chromatography (HPLC) and found that increased levels of asparagine and decreased levels of glycine and leucine were associated with a better pregnancy outcome. This study assessed spent media after only the first 24 hours, and embryos were transferred on day 2 post fertilization (Brison et al. 2004). In contrast, a study by Seli et al. 2008 using NMR reported that glutamate was the amino acid most associated with a successful pregnancy. Higher levels of glutamate in culture conditioned media correlated with a higher embryo viability and implantation rate. For this study, culture medium was collected on day 2 of culture and the transfer occurred on day 5. This study also reported that embryos which failed to implant had higher levels of alanine present in the culture media, consistent with a study by Houghton et al. (Seli et al 2008)

While multiple studies to date have examined spent embryo culture media for metabolite analysis, there are significant discrepancies between reports. This could be attributed to several factors including: different types of culture media, duration of culture before media collection (day 2,3 or 5), the day of transfer, the type of technology used, the parameters used for analysis, as well as patient demographics. Because the study design for each publication was remarkably different, it is not surprising to have discrepancies between these reports. Standardization of protocols and assessment is required in order to draw fair comparisons between the various studies, to interpret results, and to formulate accurate conclusions.
1.5 Extracellular Vesicles

1.5.1 Introduction to Extracellular Vesicles

Extracellular vesicles (EV’s) are small, double membrane bound vesicles that are released from many different cell types. They were first identified in reticulocytes over 30 years ago by two independent groups (Pan et al. 1983, Harding et al. 1984) For many years, EV’s were thought to be cellular debris and did not hold physiological importance. This changed in 1996 when Raposo et al. showed that they could stimulate an active immune response on their own (Raposo et al. 1996) Since then, numerous studies have identified a role for EV’s in cell-to-cell communication. (Ochman et al. 2000, de Gassart et al. 2004, Nabhan et al. 2012, Baj-Krzyworzeka et al. 2006) It is now known that secretion of EV’s is not a random occurrence, but rather a means of active communication between cells and requires energy input. Interestingly, the phenomena of EV release as a form of communication is not restricted to mammals, but is conserved throughout evolution and have been demonstrated in lower order eukaryotes and prokaryotes, suggesting that this is a robust and efficient process for intercellular communication (Andalouss et al. 2013). EV’s contain nucleic acids (RNA’s and DNA’s), proteins and lipids, are selectively packaged by the secreting cells, transfer their content to effectively change the physiology of recipient cells. EV’s have been shown to play a role in physiological regulation of processes such as stem cell maintenance, tissue repair and maintenance of the immune response. Additionally, EV release has been shown to be altered in some pathophysiological conditions underlying many diseases which has made them a popular avenue for biomarker discovery (Andalouss et al. 2013).

The three subtypes of EV’s are exosomes, microvesicles and apoptotic bodies and can be distinguished by their biogenesis, abundance of surface markers and size. Exosomes are 40nm to 120nm in size and are produced through the endolysosomal pathway. After formation, they are packaged within multivesicular bodies (MVB’s) which eventually fuse with the cell membrane releasing the exosomes into extracellular space. In contrast, microvesicles range in size from 50nm to 1000nm and release from the cell into the extracellular space by budding of the plasma membrane. Consequently, the surface architecture of microvesicles more closely resembles that of the parent cell than exosomes do (Andalouss et al. 2013). Regardless of their size and method of secretion, both types of EV’s carry contents that enable communication between cells and can
be characterized by surface marker proteins such as tetraspanins (i.e. CD9, CD63 and CD81), Integrins, Endosomal sorting complexes required for transport (ESCRT) components etc. The third type of EV’s are apoptotic bodies. These usually range from 500nm to 2000nm. While apoptotic bodies also have a bilayer membrane, they differ from exosomes and microvesicles in that they are not used for cellular communication purposes, but rather to fragment the cell when it is undergoing apoptosis. They can be distinguished from exosomes and microvesicles by absence of the above mentioned surface protein markers and their contents are typically limited to nuclear fragments and cell organelles (Andalouss et al. 2013)

1.5.2 Extracellular Vesicles as Biomarkers

In recent years the study of extracellular vesicles has been gaining increasing popularity, especially for their role as biomarkers in many different physiological and pathological processes. Regarding the immune system, exosomes are secreted from reticulocytes where they act as a method by which to eliminate unwanted molecules (Harding et al 2013). Additionally, exosomes can act as antigen presenting vesicles which induce antitumor immune responses (Bobrie et al 2011, Chaput and They 2011).

Vesicular research has also been extensively studied in different cancer types as tumour cells have been shown to release exosomes which aid in their progression by promoting angiogenesis and metastasis. (Rak 2010, Hood 2011). Moreover, vesicles released from tumour cells also have immunosuppressive properties that work to inactivate host T lymphocytes and natural killer cells.

With regards to epithelia, EV’s released by the intestinal epithelial cells have been shown to play a role in inflammatory conditions through antigen presentation, and they enable stationary epithelial cells to exert an affect at a distant location. EV’s in the bronchoalveolar fluid have been reported to carry protective molecules that induce tolerance for allergens and maintain airways (Prado 2008). In contrast, they have been shown to be promote the release of proinflammatory cytokines in asthma patients.
EV’s are also released by many cells in the nervous system, including neurons, oligodendroglial cells, and microglia and can mediate cross talk between the different cell types. (Faure et al 2006, Kramer Albers 2007, Lachenal 2011). They have also been shown to be involved in myelin formation (Bakhti et al 2011), neuronal growth and survival (Wang 2011).

The role of EV’s has also been studied in the field of reproduction. Membrane fusion of sperm and egg has been shown to be dependent on the release of CD9 positive vesicles by oocytes into the perivitelline space (PVS) where they bind to sperm, promoting membrane fusion (Miyado et al. 2008). Another study showed that sperm cultured in medium containing fluorescently labelled EV’s from oocytes acquired the fluorescent EV’s, indicating that EV’s may be a method of communication between the two gamete types. During the course of pregnancy, EV’s are present in the uterine environment, and are thought to be released from the apical surface of the endometrium. As these EV’s were found to contain miRNA’s that involve remodelling of the endometrium (i.e. extracellular matrix receptor interactions, adherens, tight junction proteins and VEGF signalling pathways), they are hypothesized to bind to the blastocyst and mediate the implantation process. (Ng et al. 2013) Additionally, EV’s released from syncytiotrophoblasts (STB EV’s) have been found in circulating maternal blood where the concentration of STB EV’s increases as pregnancy progression and recedes back to pre-pregnancy levels 48 hours after delivery (Sang et al. 2013).

To date, there has been no report of the release of EV’s from pre-implantation embryos, however, given that EV’s have been shown to be released from the oocyte during conception and from the endometrium during implantation, it is logical to assume that the embryo has the capacity to release them and they may be involved in the process of implantation.

1.6 Hypothesis and Objectives

Since its inception in 1978, the process of IVF has undergone many changes and improvements to enhance the quality of treatment, however, the success rate of IVF still remains relatively low. Consequently, physicians often transfer multiple embryos to increase the chances of attaining a pregnancy, risking multiple gestations, which can pose a great risk to the mother and her babies. Improving the embryo selection process has the potential to reduce the need to transfer multiple
embryos, without appreciably affecting the chances of a successful pregnancy. For this thesis, I studied the use of three non-invasive methods to assess embryo viability, with the goal to be able to select embryos with the highest implantation potential within a patient cohort, and to better understand human pre-implantation embryo physiology.

*I hypothesize that a defined subset of morphokinetic parameters, together with metabolic profiling and extracellular vesicle content of spent human embryo culture media will have predictive value for successful human embryo development and implantation potential.*

The first objective of this project was to assess morphokinetics of preimplantation embryo development and correlate this with metabolite levels. Observational morphokinetic parameters (t2, t3, t4, t5, t6, t7, t8+, tM, tSB, tB) and calculated morphokinetic parameters (s2, s3, cc2, cc3) were compared between embryos that resulted in a pregnancy to embryos that failed to implant. In order to identify whether these growth parameters correlated with internal physiology, NMR was used to assess metabolites levels in spent embryo culture media.

The second goal of this project was to identify and characterize extracellular vesicles in spent embryo culture media in order to 1) determine whether human pre-implantation embryos release EV’s, which in turn can cross the ZP, and then release into the surrounding culture medium using electron microscopy and nanoparticle tracking analysis, and 2) to determine if the contents of extracellular vesicles, (i.e. miRNA’s) may be suitable to be used as biomarkers for embryo selection during IVF; using qPCR and Next generation sequencing (NGS).
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Study Design and Embryo Culture

Ethics approval for this project was obtained from the University of Toronto’s Research Ethics Board (Ref 30251).

Controlled ovarian hyperstimulation was carried out using standard protocols (Griesinger et al. 2006). Transvaginal ultrasound-guided follicular aspiration was carried out approximately 36 hours after administration of a trigger shot of either hCG (Fresenius Kabi, Canada) alone, GnRH agonist alone (Abbvie, Canada) or a ‘dual’ trigger of both. Routine semen analysis and a two-step (40% and 80%) gradient separation technique (Sperm Filter, Gynotec, FertiliTech, Canada) were used to prepare sperm samples. Severe male factor cases, such as those involving samples from testicular biopsies were excluded from the study. Standard IVF or ICSI procedures were performed for fertilization of metaphase II oocytes 3-4 hours post retrieval. Injected oocytes were placed into micro-wells containing 25 μL of a continuous pre-equilibrated single culture medium overlaid with 1.5 ml paraffin oil (Vitrolife, USA). They were cultured in the EmbryoScope™ (VitroLife) time-lapse incubator in 6% CO₂, and 5% O₂ at 37°C until embryo transfer and/or cryopreservation.

The embryo transfer was performed 3 or 5 days after oocyte retrieval or in a future cycle after cryopreservation by vitrification. Selection criteria for embryo transfer and cryopreservation were based on the static morphology, a morphokinetic score and, in some cases, preimplantation genetic screening (PGS) using array-CGH, as applicable. Embryo static morphology was evaluated utilizing the SART simplified grading system (Heitmann et.al., 2013). Day 5 and 6 blastocysts were classified into three quality groups: good (AA, AB), fair (BA, BB or BC) and poor (CC or CB). Other embryos of inferior quality manifesting earlier developmental stages at day 6 post fertilization (morula and cleavage embryos) were classified as arrested embryos.
After embryo transfer, the spent media (approximately 20 µL) was collected and centrifuged at 300 x g for 10 minutes, 2000 x g for 10 minutes, 12000 x g for 30 minutes. Samples were frozen at -80°C until needed for experimentation.

2.2 Electron Microscopy

2.2.1 Negative Staining

For negative staining, samples were thawed for 30 minutes at room temperature and then pooled. Following preliminary centrifugation (300 x g for 10 minutes, 2000 x g for 10 minutes, 12,000 x g for 30 minutes), the supernatants were placed in polycarbonate thick walled ultracentrifugation tubes (Beckman) and centrifuged at 49 000 rpm for 3 hours at 4°C. After removing the supernatant, 10 µL of fixative was added to the pellet. In cases where there was no visible pellet, 5 µL of media collected from the bottom of the tube was fixed with 10uL of fixative after the rest of the supernatant was removed. After incubation overnight at 4°C, 1 µL of the solution was placed on a copper grid, along with 1 µL of negative stain. The grids were visualized with a Hitachi-H 700 TEM.

2.2.2 Scanning Electron Microscopy

ZP removal was performed using Tyrode’s Salt Solution (Sigma), according to the manufacturer’s instructions. The embryo was labeled with CD9 primary antibody for 30 minutes at room temperature (1:100), followed by labeling with 20nm gold IgG secondary antibody for 1 hour at room temperature (1:200). After a series of alcohol dehydration steps (50%,70%,80%,90%,95%,100%), the sample was processed in a critical point dryer and then carbon coated. Imaging was performed with a Hitachi S-570 SEM, equipped with the Environmental Secondary Electron Detector (ESED).

2.2.3 Transmission Electron Microscopy

Embryos for study were removed from the culture media and washed 3X with PBS and then fixed overnight with 0.15% gluteraldehyde and 4% paraformaldehyde in PBS. Embryos were then embedded in LR White Embedding Media (Electron Microscopy Sciences) and incubated
overnight at 60°C. Sections were cut using a microtome and placed on nickel grids. The grids were labeled with antibodies by placing them on top of a series of 100 μL droplets consisting of the following: 1) Blocking using 0.05% PBS-BSA for 3 X 5 minutes, followed by 3 X 30 second washes with ultra pure water; 2) primary antibody addition with CD9 at a concentration of 1:100 in 0.05% PBS-BSA for 1.5 hours, followed by 3 X 30 second washes with ultra-pure water; 3) Blocking using 0.05% PBS-BSA for 5 minutes, followed by 3 X 30 second washes with ultra-pure water; 4) Secondary antibody addition with 20nm gold particles for 45 minutes followed by 3 X 30 second washes with ultra pure water and; 5) 2% Uranyl acetate stain for 30 seconds followed by 3 X 30 second washes with ultra pure water; 5) osmium tetroxide vaporization for 30 seconds. The stained grids were then imaged using the Hitachi H-700 TEM.

2.2.4 Nanoparticle Tracking Analysis

Following preliminary centrifugation, vesicles were isolated using ExoQuick-TC™ kitsf (System Biosciences), according to manufacturer’s instructions. Since no pellet was visible using this small volume, the supernatant was removed, leaving behind 15 μL at the bottom of the tube. This was then re-suspended in 1mL of PBS and analyzed using the Nanoparticle Tracking Analysis (Malvern Instruments).

2.2.5 qPCR

Sample Preparation for RNA extraction

Spent embryo culture media samples were frozen at -80°C upon collection. Prior to RNA extraction, samples were thawed at room temperature for 30 minutes and subjected to serial centrifugation for 10 minutes at 300 x g, 10 minutes at 2,000 x g and 30 minutes at 12,000 x g. Proteinase-K and RNAse were added to remove all free floating miRNA. Vesicles were lysed by performing sonication for 15 minutes x 3, followed by addition of the lysis buffer provided in the microRNA Purification Kit (Norgene Biotek Corp.)

Extracellular microRNA and Carrier Protein Degradation

Extracellular protein carriers were degraded using Proteinase-K treatment as follows: Samples were incubated in 0.106 μg/mL of Proteinase-K for 30 minutes at 55°C, followed by Proteinase-K inhibition using 5μL of Phenylnmethysulfonyl fluoride (PMSF) (Sigma-Aldrich) for every
200μL of sample for 15 minutes at room temperature. Extracellular RNA species were degraded using 2μL of RNAse A1/T for every 100μL of sample for 30 minutes at 37°C. The RNAse was inhibited using 1 μL RNase inhibitor Superase for every 20 μL of sample for 1.5 hours at 37°C.

**Q-PCR**

RNA extraction was performed using the microRNA Purification Kit (Norgen Biotek Corp) according to manufacturer’s protocol, with a final elution volume of 20μL. For reverse transcription, the miScript II RT Kit (Qiagen); manufacturer’s protocol was altered for increased concentration of transcripts, by performing a 10μL reaction, instead of a 20μL reaction. Following cDNA synthesis, the sample was pre-amplified using the miScript PreAMP PCR Kit (Qiagen), according to manufacturer’s protocol, except the final product was not diluted. Q-PCR was performed using the miScript SYBR Green PCR Kit (Qiagen), according to manufacturer’s protocol, and results were analyzed using the Rotor-Gene Q Series Software (Qiagen).

### 2.2.6 Next Generation Sequencing

Frozen samples were thawed at room temperature for 30 minutes. Proteinase-K and RNAse were added to remove all microRNA’s not encompassed in extracellular vesicles. Vesicles were lysed by performing sonication for 15 minutes x 3, followed by addition of lysis buffer. RNA extraction was performed using the microRNA purification kit (Norgen). For Small RNA purification and library preparation for Next Generation Sequencing (NGS) the Ion Total RNA-Seq Kit v2 (Life Technologies) was used with a modification to manufacturer’s protocol: primers were diluted 4X and ligation was performed overnight.

### 2.3 Morphokinetic Analysis

Embryos were cultured in the EmbryoScope™ incubator. Digital images were taken every 20 minutes, creating a time-lapse movie. Cellular divisions were annotated using the EmbryoScope™ annotation software by the embryologists and/or researchers. The time of embryo subsequent mitotic divisions was expressed as hour’s post-ICSI or IVF procedure.
2.4 Metabolite Analysis

A stock of Deuterium Oxide (D\textsubscript{2}O) was made, with 0.25 mmol/L sodium phosphate dibasic (Na\textsubscript{2}HPO\textsubscript{4}) added as a pH buffer, along with 0.1mmol/L Trimethylsilylpropanoic acid (TSP), and 15.4mmol/L Calcium formate (Ca(HCOO)\textsubscript{2}) added as normalizers. The same stock was used for all samples run for NMR, and was kept in a desiccant for the duration of the experiments.

For NMR analysis, samples were thawed at room temperature for 30 minutes. A 22μm filter was washed with 400 mL ultrapure water, followed by 600 μL of D\textsubscript{2}O to remove all trace of water in the filter. Next, 180 μL of D\textsubscript{2}O was added to 20μL of sample and the mixture was filtered with the washed filter. The filtered solution was added to 3mm NMR tubes and analyzed using the Agilent DD2 700 MHz NMR. Samples were run in an Agilent Technologies VNMRS 800 MHz NMR spectrometer (Agilent Technologies, Santa Clara, CA) and metabolites were quantified by integrating peaks using MNova Software (Mestrelab Research), following standard phase shift and baseline corrections.
Chapter 3
Morphokinetics and Metabolomics of Embryo Growth

3  Morphokinetics and Metabolomics of Embryo Growth

3.1  Brief Introduction

Static morphology is the criteria currently used for embryo selection during IVF in most embryology labs around the world. However, information about the growth of embryos in between these assessment time points is not obtainable in a standard incubator, but may contain valuable information about embryo health and development. Additionally, while static morphological assessment provides information about the appearance of blastomeres, it does not accurately reflect the internal physiology, an important factor for the potential of the embryo to implant and sustain an ongoing pregnancy. Analysis of morphokinetic parameters, using the EmbryoScope™ Imaging system, allows for continuous surveillance of embryos in culture, enabling one to record key cellular divisions, and to potentially identify morphokinetic parameters MKP’s that may be correlated with embryo development and implantation potential. Morphology and morphokinetics are both observational in nature and therefore provide correlative results. In addition to the continuous observational benefits, the EmbryoScope™ culture system enables undisturbed observation without daily removal of embryos from the incubator to a microscope stage, which results in repeated transient changes in media temperature and pH that could potentially be stressful to the embryos.

Metabolites in embryo culture media were also analyzed, giving insight into the metabolic pathways employed by the embryos during growth in-vitro. Several prior studies have examined either metabolites in spent embryo culture media or morphokinetic parameters, independently, and related these to pregnancy outcome; however, no human study to date has looked at the two in combination.
Here, we investigated morphokinetic parameters that are indicative of blastocyst formation and implantation potential, and aimed to correlate them with metabolites in spent embryo culture media.

3.2 Brief Methods

**Definition of Morphokinetic Parameters**

Each morphokinetic parameter was annotated when the first sight of a cellular division was visible. For example, t2 as annotated at the first frame at which two cells were distinctly visible. The morula stage was annotated as the first frame where individual cell outlines were no longer visible. The start of blastulation was defined as the first sign of cavitation and the time to blastocyst (end of blastulation) was completed when the trophectoderm first made contact with the zona pellucida.

**Statistical Analysis**

Significant differences between groups for blastocyst development (tM, tSB, tB and 8 cell stage) was performed using the one-tailed, unpaired t-test with Welch correction. Differences between groups for implantation potential was determined using the F-test for variance in the case of t2 and tB, and the one-tailed, unpaired t-test for s2. Statistical differences between metabolites was determined using the one-tailed, un-paired t-test with Welch correction.

3.3 Morphokinetic Parameters related to Blastocyst Development

3.3.1 Rationale

During IVF treatment, transfer of embryos after *in-vitro* culture typically takes place on either day 3 or day 5. Although studies show better implantation rates after blastocyst transfer, this is very likely due to preselection of embryos able to form a blastocyst on day 5 or 6 (Blake et al. 2007). There is some speculation that a longer duration in culture may have some negative epigenetic or other effects on the growing embryo (Fernández-Gonzalez et al. 2004). Thus, the
benefits of the prolonged time in culture must be weighed against the theoretical or potential risks. Additionally, in patients where all of the embryos exhibit a poor morphology, a day 3 transfer may be beneficial because it is thought that the uterus may be a more nurturing or natural environment than the culture dish. In cases where embryo(s) are transferred on day 3, it is of great value to be able to predict which of these embryos will have the greatest chance of becoming a good grade blastocyst that will be more likely result in a pregnancy.

3.3.2 Results

Embryos in the blastocyst culture cohort were divided into two groups based on the quality of blastocyst on day 5 or 6 of culture. A total of 52 embryos were included in this analysis; however, some embryos were transferred before certain cellular stages were reached and were thus omitted from the analysis. Blastocyst static grading was performed by embryologists at the CReATe Fertility Centre and they were categorized them as “good grade blasts (1AB or higher) or poor grade blasts (2CC or lower). Out of all the MKP analyzed, a significant difference of means between good grade and poor grade embryos was observed in the time to morula formation (tM), the time to start of blastulation (tSB), and the time to blastocyst (tB) (Figure 3.1). While all three of these parameters were significant, it is important to note that tSB and tB are dependent on tM. In other words, if an embryo has a later tM, it will also have a later tSB and subsequent tB since these cellular stages are not independent.

Figure 3.1- A) Embryos that formed a good grade blastocyst on day 5 had a shorter time to reach the morula stage. n=14 for good grade blastocysts; n=20 for poor grade blastocysts. p=0.03 B) Good grade blastocysts started blastulation sooner than poor grade blastocysts. n=14 for good grade blastocysts; n=16 for poor grade blastocysts. p=0.003. C) Good grade blastocysts reached the blastocyst stage in a shorter time than poor grade embryos. n=13 for good grade blastocysts; n=13 for poor grade blastocysts. p=0.0013
Additionally, we also found a significant difference between the two groups for duration of the 8 cell stage (t9-t8) where embryos that formed a good grade blastocyst on day 5 spent significantly more time in the 8 cell stage (Figure 3.2).

**Figure 3.2** – Embryos that exhibited good morphology on day 5 (n=52) of culture spent a greater time in the 8 cell stage than embryos that exhibited poor morphology (n=27) on day 5. P<0.05

### 3.4 Morphokinetic Parameters related to Implantation Potential

#### 3.4.1 Rationale

Despite improvements over the years, the success rate for IVF remains low, suggesting that morphological assessment alone is insufficient for predicting embryo implantation success. While several countries around the world have mandated elective single embryo transfer (eSET), due to the lower chances of pregnancy, transferring more than one embryo is still common practice in many parts of the world, including North America (SOGC-CFAS report 2008). Studies have shown that there is an optimal time point for embryos to make cellular divisions (Dal Canto et al. 2012) and this can help identify the embryo with the highest implantation potential, alleviating the need to transfer multiple embryos which can reduce the chances
of multiple gestations, without significantly reducing the chance of a successful pregnancy.

3.4.2 Results

For correlation of MKP to implantation outcome, a total of 117 embryos transferred into 76 patients were used. Only samples from patients who had either 0% (n=88) or 100% (n=29) implantation were analyzed. Patient clinical data is shown in Tables 3.1 and 3.2.

When assessing morphokinetic parameters, cellular events that were not clearly distinguishable with the Embryoscope™ were excluded from analysis. The list of the average of MKP’s for each group is reported in Table 3.3.

Of all the morphokinetic parameters analyzed, the time to reach the two cell stage (t2), synchrony of the second cell cycle (s2), and time to reach the blastocyst stage (tB) significantly differed between the two groups.

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<tr>
<th>Table 3.1 - Clinical factors for patients included in this study. P-value for group comparison is based on a two-sample t-test.</th>
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<tr>
<td>Implantation (IP)</td>
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<td>Mean Age</td>
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<td>Mean Number of MII Oocytes</td>
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<th>Table 3.2 – Frequency count of number of transfers by pregnancy group. P=0.07, based on Fisher’s exact test</th>
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<td>Implantation (IP)</td>
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The means of each MKP were compared between the 0% implantation (NIP) and the 100% implantation (IP) groups and no significant differences were observed, however the distribution
of some cellular division timings showed significant differences between the two groups: A significant difference in variance was observed for the time to reach the two cell stage \((t_2)\). While all the embryos in the IP group fell between the narrow time frame of 21.26 to 30.38 hours post insemination (hpi), the range of embryos in the NIP group were spread out in a greater interval of 5.08 to 47 hpi (Figure 3.3)

Moreover, a significant difference in variance was also observed in the time to reach the blastocyst stage \((t_B)\) between the two groups, where all embryos in the IP group reached the blastocyst stage between 95.42 and 110.96 hpi. The embryos in the NIP had a wider range of 89.12 to 127.95 hpi (Figure 3.4)
Additionally, synchrony of the second cycle ($s_2=t_4-t_3$) was compared between the two groups. This parameter assesses how synchronously the two blastomeres are dividing, thus, a smaller $s_2$ suggests that the divisions of both blastomeres are occurring simultaneously, without one of the blastomeres lagging behind. In our data set, the average time for $s_2$ in the IP group was significantly lower ($0.991 \pm 0.16$ hpi) than the of the NIP group ($1.559 \pm 0.27$ hpi) and all of the embryos that implanted had a $s_2$ of 3.17 hours or less (Figure 3.5)

![Figure 3.5](image)

**Figure 3.5** – Distribution of synchrony of the second cell cycle ($s_2$) of embryos in the IP group compared to NIP group, n=29 for IP, n=84 for NIP. All embryos that implanted had an $s_2$ of less than 3.17 hpi.
Table 3.3 - All morphokinetic parameters analyzed are shown, with the number of embryos examined, mean and median in both the implantation and non-implantation groups.

| Parameter | Implantation (IP) | | | | No Implantation (NIP) | | | |
|-----------|------------------|---|---|---|------------------|---|---|
|           | Mean     | SD | n  | Mean     | SD | n   |
| tPNa      | 10.40    | 3.37 | 21 | 8.44     | 3.47 | 50 |
| tPNf      | 22.82    | 2.63 | 26 | 22.62    | 4.20 | 80 |
| t2        | 25.22    | 2.50 | 29 | 25.49    | 5.12 | 83 |
| t3        | 36.35    | 3.27 | 29 | 35.71    | 5.57 | 83 |
| t4        | 37.29    | 3.33 | 28 | 37.29    | 4.83 | 82 |
| t5        | 48.14    | 5.76 | 28 | 48.82    | 6.61 | 82 |
| t6        | 54.69    | 6.98 | 28 | 54.70    | 9.08 | 83 |
| t7        | 57.60    | 5.99 | 26 | 57.98    | 8.97 | 82 |
| t8        | 59.54    | 7.02 | 28 | 59.67    | 9.67 | 80 |
| t9+       | 69.60    | 8.12 | 25 | 71.28    | 10.08 | 80 |
| tM        | 82.81    | 4.79 | 27 | 86.41    | 8.73 | 85 |
| tSB       | 95.14    | 6.10 | 27 | 97.60    | 8.08 | 76 |
| tB        | 103.83   | 5.05 | 26 | 106.43   | 7.50 | 71 |
| tEB       | 113.18   | 3.89 | 19 | 114.95   | 5.15 | 38 |
| cc2       | 11.12    | 1.78 | 29 | 10.23    | 3.29 | 83 |
| cc3       | 11.86    | 3.23 | 28 | 13.10    | 3.76 | 82 |
| s2        | 0.991    | 0.163 | 29 | 1.56     | 0.272 | 84 |
| s3        | 11.04    | 5.61 | 27 | 11.28    | 9.03 | 80 |
3.5 NMR Optimization for Metabolite Analysis

3.5.1 Rationale

Since MKP studies are purely observational in nature, they do not give insight into the underlying physiological processes that account for the variation in growth rate of embryos, and the resultant impact of this variation on implantation, and subsequent establishment of a pregnancy.

Another aspect of embryo development that can be studied in a non-invasive way is metabolism. This relates to metabolically active compounds that are taken up or released by the embryo during its development. Metabolite analysis on spent embryo culture media enables the identification and quantification of metabolite uptake and release by the embryos during culture. This may provide insight into the type of specific glycolytic pathways the embryo is using, which may explain the rate at which the embryo is performing its cellular divisions, ultimately reflecting the overall health of an embryo and its potential for implantation.

3.5.2 Results

3.5.2.1 Optimization of NMR Scan Parameters

The metabolite spectra produced by NMR is highly dependent on the type of spectrometer and the parameters under which the scan is acquired. These parameters can change depending on sample type. Therefore, experiments were conducted to optimize the scan resolution for embryo culture media. Firstly, three different spectrometers were compared: the 600MHz, 700 MHz (equipped with a cryoprobe) and 800MHz. Although the 800MHz spectrometer is equipped with a stronger magnet, the 700MHz spectrometer produced the highest signal to noise resolution due to the presence of a cryoprobe (HFCN_COLD). A cryoprobe, consisting of the probe, a compressor, helium circulation regulation and temperature control units, works by cooling the radio frequency coil and sections of the preamplifiers in liquid helium to near absolute-zero temperatures. In this way, the sensitivity gain, or the signal-to-noise ratio is drastically increased compared to a room temperature probe by significantly reducing the background noise. A preliminary experiment was performed with this spectrometer, which demonstrated that metabolite levels differ between: (1) embryos that are at different developmental stages; and (2)
embryos at similar developmental stages, but exhibiting different morphologic grades (Figure 3.6)

![Metabolite Levels of Preliminary NMR Scan](image)

Figure 3.6 – Preliminary experiment demonstrating that 800MHz spectrometer is able to detect differences in metabolite levels of embryos at different developmental stages and of different developmental outcomes.

Additionally, as the goal of this project is to devise a diagnostic test, several other parameters needed troubleshooting to obtain the spectra with the highest resolution in the shortest amount of time. In order to accomplish this, the following parameters were changed: Sample concentration was increased from 1:30 to 1:20; Scan number was changed from 64 scans to 256 scans and the delay time between each scan was most optimal at 5 seconds. Additionally, water suppression was done at 4.79 ppm in order to increase the resolution of the metabolite peaks. After this optimization, each sample could then be run in 15 minutes and the spectra had a much higher signal to noise ratio.

3.5.2.2 Validation of Auto-Sampler Usage

To further enhance the efficiency of this protocol, the Agilent DD2 700 MHz NMR spectrometer has an auto-sampler which can be used to load 90 samples at a time, so that manual changing of the sample is no longer needed. However, this auto-sampler is not cooled, exposing the sample to room temperature prior to being scanned. In order to determine whether the metabolites in the spent embryo culture media samples are stable at room temperature for extended periods of time,
a sample was run at 0 hours, was kept at room temperature for 24 hours and then run again. The results revealed no significant changes in metabolites in the culture media after 24 hours at room temperature, suggesting that the metabolites are stable at room temperature (Figure 3.7)

**Figure 3.7** – NMR scan profiles are shown at 0 hours (a) and after 24 hours (b) at room temperature. Quantified levels of metabolites are shown in (c), no significant change in metabolite level is observed after 24 hours at room temperature. P=0.42
3.5.2.3 2D NMR

With the optimization performed above, the resolution of peaks was drastically improved; however, there were some samples where some metabolic peaks were too close together on the spectra to be identified with confidence. Thus, a correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) were performed, which enabled better resolution for identification of peaks in some samples.

3.6 Using Metabolomics to Explain Morphokinetic Observations

3.6.1 Rationale

While numerous studies have examined metabolites in spent embryo culture media or morphokinetic parameters, independently, in relation to pregnancy outcome; no study to date has reported on potential associations of morphokinetic and metabolomic parameters with each other and with IVF outcome. Here, the MKP that were described above in this chapter, were correlated with metabolite levels in spent embryo culture media.

3.6.2 Results

A table of metabolites analyzed and the corresponding NMR peaks can be found in Table 3.4. For some scans, peaks of some metabolites were distorted or indistinguishable and were omitted from analysis.
In order to establish a correlation between morphokinetic observations and metabolomics, metabolomic analysis was performed on each MKP where a significant difference in variance was observed between implantation groups. These were $t_2$, $t_B$ and $s_2$.

Morphokinetic analysis showed that all of the embryos in the IP group had a $t_2$ between 21.26 and 30.38 hpi. The spent embryo culture media was analyzed for metabolite differences between embryos that had a $t_2$ within this time frame and embryos that had a $t_2$ outside of the implantation window. A significant difference was seen in the levels of acetic acid and lactic acid, where embryos that had a $t_2$ between 21.26 and 30.38 hpi had lower amounts of acetic acid (Figure 3.8A) and lower amount of lactic acid (Figure 3.8B) in their spent embryo culture media than embryo that fell outside of this time frame.

Regarding $t_B$, morphokinetic analysis showed that all of the embryos in the IP group had a $t_B$ between 95.42 and 110.96 hpi. While looking at metabolites in the spent embryo culture media, the embryos that had a $t_B$ in the implantation window had significantly higher levels of 3-aminoisobutyrate in the spent culture media than embryos that had a $t_B$ outside of the implantation window (Figure 3.9B)

### Table 3.4 – The parts per million (ppm) shift and type of peak used to identify each metabolite on NMR spectra

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Shift (ppm)</th>
<th>Peak Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactate</td>
<td>4.1</td>
<td>q</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.65</td>
<td>d</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>2.341</td>
<td>m</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.233-5.223</td>
<td>m</td>
</tr>
<tr>
<td>Oxaloacetate</td>
<td>2.38</td>
<td>s</td>
</tr>
<tr>
<td>3-hydroxyisovalerate</td>
<td>1.26</td>
<td>s</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.91</td>
<td>s</td>
</tr>
<tr>
<td>3-aminoisobutyrate</td>
<td>1.19</td>
<td>d</td>
</tr>
</tbody>
</table>
Figure 3.8 - Lactic acid (B) and acetic levels (C) are lower in spent embryo culture media of embryos that were within the implantation range for t2, outlined with a dotted line in (A). Metabolites were quantified as a ratio to TSP. For Lactic acid analysis, 42 embryos fell within range and 4 outside range, p<0.05. For acetic acid analysis, 43 embryos fell within range and 4 outside range, p<0.05.

Interestingly, higher levels of 3-aminoisobutyrate was also observed in embryos that had a s2 of less than 3.17 hours, reflective of the embryos that had successful implantation (Figure 3.10B)
Figure 3.9 – (B) Higher levels of 3-aminoisobutyrate are present in the spent media of embryos that had a tB within the implantation range; 11 embryos fell within range and 27 fell outside of range, outlined with a dotted line in (A). p<0.05

Figure 3.10 – (B) Higher levels of 3-aminoisobutyrate are present in the spent media of embryos that had a s2 of less than 3.17 hpi; 37 embryos fell below this threshold while 10 embryos had a s2 above the threshold. The threshold is outlined with a dotted line in (A). P<0.05
### 3.7 Summary and Discussion

When comparing MKP to blastocyst development, significant differences in means were found for \( t_M \), \( t_{SB} \) and \( t_B \). Embryos that developed into a good grade blastocyst on day 5 of culture reached all three of these stages sooner than embryos that developed into a poor grade blastocyst on day 5. Although all three of these parameters showed significant differences, it is important to note that these are sequential events thus the \( t_{SB} \) is dependent on \( t_M \) and the \( t_B \) is dependent on both the \( t_M \) and \( t_{SB} \). To our knowledge, these parameters have not been previously reported to be associated with embryo viability.

Additionally, significant differences were observed in the time spent in the 8 cell stage where good grade blastocysts spent a greater time in this cell cycle than poor grade blastocysts. The 8 cell stage is crucial for development because it is the time at which gap junctions are formed between each blastomere. Prior to compaction, each of the individual blastomeres is autonomous and can each give rise to separate pregnancies; however, after the blastomeres start communicating, this is no longer the case. A shorter time spent at this stage may indicate inadequate time to form proper gap junctions between cells, potentially compromising cell-to-cell communication. (Hardy et al. 1996)

With regards to implantation, significantly different MKPs were found between implanted (IP) and non-implanted (NIP) embryos for time to reach the two cell stage, time to reach blastocyst and synchronicity of the second cell cycle. Embryos in the IMP group reached the two cell stage between 21.26 and 30.38 hpi. In comparison, Chamayou et al., found that a \( t_2 \) between 21.4 and 34.8 hours was associated with blastocyst development \textit{in-vitro}, however, these findings did not hold significance when comparing implantation potential (Chamayou 2013). Metabolic analysis showed lower levels of lactic acid and acetic acid in the spent media of embryos that fell within the implantation window for \( t_2 \). Prior to the compaction stage, embryos primarily use the anaerobic pathway for energy. In the absence of oxygen, glucose is converted into pyruvate and lactic acid is expelled. (Wilding et al. 2009). Thus, lactic acid levels in the spent culture media reflect the levels of anaerobic glycolysis and our results suggest that embryos that have a lower rate of anaerobic glycolysis have a higher chance of implantation. This is consistent with the “Quiet Embryo Hypothesis”, coined by Leese and Houghton, which stems from the notion that embryos under stress have increased energy demands, thus, healthier embryos have a quieter
metabolism (Leese et al. 2002). However, after the compaction stage, embryos dramatically increase their energy intake to fulfill the demands of active biosynthesis and increased cell divisions, and turn to aerobic glycolysis as their main energy source (Leese et al. 2002). In this pathway, acetic acid is used as a substrate. Our results indicate lower amounts of acetic acid in the spent culture media of embryos that fell within the implantation window for t2, suggesting that embryos which have a higher implantation potential uptake more acetic acid, potentially operating at a higher rate of aerobic glycolysis after compaction.

For tB, all of the implanted embryos fell between 95.42 and 110.96 hpi. Metabolomic analysis revealed that embryos within the implantation window had significantly higher levels of 3-aminoisobutyrate in their culture media. 3-aminoisobutyrate is a by-product of Succinyl-CoA, an intermediate in the Krebs cycle. Thus, higher levels of 3-aminoisobutyrate suggests higher rates of aerobic respiration in these embryos.

Interestingly, we also found higher levels of 3-aminoisobutyrate in the spent media of embryos that had a greater synchrony of the second cell cycle. The importance of cleavage synchrony was first addressed in 2008 by Lemmen et al, and has been reported in multiple studies following their report (Meseguer et al. 2011, Chaamyou et al. 2013). This parameter assesses whether the two blastomeres that are present after the first cellular division are dividing synchronously for subsequent divisions. Consistent with our results, studies typically show that healthier embryos divide synchronously and spend less time between the three and four cell stages. Chamayou et al. observed a mean s2 of 2.0 hpi in their study population; however, no significant difference was observed between pregnancy groups (Chamayou et al. 2013). Alternatively, Meseguer et al. observed a significant difference for s2 between pregnancy groups, where the implanted embryos had a mean s2 of 0.78 hpi. Consistent with the published literature, our results indicate that a lower s2 correlates with a higher implantation potential. In our dataset, all of the implanted embryos had an s2 of 3.17 hpi or lower, consistent with the hypothesis that healthier embryos divide synchronously. Metabolomic analysis showed that embryos that had an s2 below that of the implantation threshold had a greater amount of 3-aminoisobutyrate in their culture media, suggesting a higher rate of aerobic respiration.

To our knowledge, only one study has previously identified 3-aminoisobutyrate in spent embryo culture media. Wallace et al. examined levels of various metabolites using NMR spectroscopy in
day 2 culture media of human embryos. Higher levels of formate/3-aminoisobutyrate ratios were found in patients with a successful pregnancy. Interestingly, embryos with a successful pregnancy also had lower levels of 3-aminoisobutyrate/acetoacetate (Wallace et al. 2014). Many studies have reported conflicting results when examining metabolites in spent embryo culture media. The differences seen between groups may be attributable to several factors: 1) different brands of culture media have been shown to alter the growth rate of embryos (Ciray et al. 2012). Embryos are very plastic, and make use of the metabolites that are available to it. Thus, the composition of the culture media can drastically change the rate of embryo growth along with the metabolites that are found in spent culture media; 2) slight variations in oxygen content can also drastically change growth patterns (Wale and Gardner et al. 2010); 3) variation in the time from injection of the sperm during ICSI from the first oocyte to the last (especially when there are a large number of oocytes) could also impact morphokinetic results. For example, t0, also known as time of ICSI, begins at the time the embryo dish is placed inside the incubator, however, the embryos that were inseminated first have already begun their growth by the time the last embryos are inseminated. Thus, the number of oocytes that need to be inseminated and the speed at which they are inseminated is a confounding factor when analyzing morphokinetic results.

A major limitation of this study is that the outcome data for implantation is based on embryos that were already pre-selected within their patient cohort for uterine transfer. In other words, the blastocyst with the highest morphology score was the one that was transferred. Thus, the implantation potential of the remaining blastocysts with sub-optimal morphology was not assessed. This is a difficult limitation to overcome because it would not be ethically appropriate to transfer poor grade embryos in the presence of good grade embryos. Performing studies on patients who have had multiple IVF cycles from frozen embryos can address this concern to a certain extent.

To our knowledge, this is the first study to investigate the connection between metabolomics and morphokinetics in human pre-implantation embryos. Elucidating the internal physiology of embryos in relation to these parameters can help us understand these morphological and morphokinetic observations. There has been only one other study, on murine embryos, that examined the correlation of morphokinetics of cleavage stage embryos and blastocyst metabolism and subsequent embryo viability: Lee et al. categorized blastocysts as fast (mean
first cleavage timing of 15.1± 0.08 hpi) or slow (mean first cleavage timing of 17.6 ±0.1 hpi) growing embryos. Faster embryos were reported to have a larger inner cell mass, increased glucose consumption, a decreased glycolytic rate (percentage of glucose converted to lactate), higher aspartate uptake and a lower global amino acid turnover. Faster embryos were also more likely to successfully develop into a fetus. The major limitation of this study is the use of the first cleavage event to classify embryos. The use of additional parameters to categorize embryos can provide a more comprehensive platform for the study of embryo metabolomics. In addition to being used as a biomarker for selection of embryos with the highest implantation potential, metabolomic studies on spent culture media also helps us gain a better understanding of the physiology of the human pre-implantation embryos, which may lead to intervention/rescue strategies, leading to an increased success rate of IVF treatment.
Chapter 4
Extracellular Vesicles

4 Extracellular Vesicles

4.1 Brief Introduction

With respect to secretomics, the study of EV’s and their contents has recently become a subject of intense study in many fields, such as cancer and reproductive biology. EV’s are thought to be involved in cell-to-cell communication and have been found in almost all bodily fluids (Tetta et al. 2013). Previous studies have confirmed that exosomes, a sub-type of extracellular vesicles, are released from oocytes and bind to sperm, potentially enhancing the fertilization process (Barraud-Lange et al. 2013). Exosomes have also been found to be released from the endometrial wall in mice, and it is suggested that they bind to the embryo and are involved in the process of implantation (Ng et al. 2013). Although exosomes have been shown to be released from many different cell and tissue types in the body, whether EV’s are released by human pre-implantation embryos and cross the zona pellucida has yet to be reported. Here, I characterize the release of extracellular vesicles from various stages of embryo development, and examine the microRNA species contained within those EV’s with the aim to identify microRNA candidate(s) that could be used as a biomarker for selecting embryos of high developmental potential during IVF treatment.

4.2 Brief Methods

RNA Kit Optimization

Three methods were tested for extracellular vesicle enrichment: 1. ExoQuick (SBI); 2. Total Exosome Isolation Solution (Life Technologies); and 3. No pre-enrichment. For no pre-enrichment, the culture media was placed directly into the RNA isolation kits after preliminary centrifugation spins. RNA isolation optimization was carried out using four different kits,
according to manufacturer’s protocols: 1. Total Exosome RNA and Protein Isolation (Life Technologies); 2. RNeasy Micro Kit (Qiagen); 3. miRCURY RNA Isolation Kit for Biofluids (Exiqon) and 4. microRNA Purification Kit (Norgen Biotek Corp). Results were analyzed using a small RNA chip in an Agilent Bioanalyzer.

4.3 Characterization of Extracellular Vesicles

4.3.1 Rationale
Extracellular vesicles have not previously been shown to be released by human pre-implantation embryos. With the ultimate goal of developing a diagnostic test that can select embryos of the highest chances of becoming a successful pregnancy, release of EV’s must first be demonstrated. This involved identifying whether EV’s are released from embryos, whether they can cross the zona pellucida and whether embryo-derived EV’s are present in spent embryo culture media.

4.3.2 Results
4.3.2.1 EV’s are Present in Spent Embryo Culture Media
Nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM) were used to determine whether EV’s are present and detectable in spent embryo culture media. For TEM, pooled samples of day 3 (n=19) and day 5 (n=55) spent media and unconditioned control media were stained with a negative stain. Images show vesicular like structures present in both the day 3 and day 5 samples. The vesicles observed in the day 5 spent media appear to be slightly larger than the vesicles observed in the day 3 spent media. With this technique, no vesicles were observed in the control media incubated without embryos. (Figure 4.1)
Figure 4.1 – TEM images with negative staining of (A) control media, (B) day 5 spent media and (C) day 3 spent media. Vesicular-like structures are visible in both day 3 and day 5 spent media, and appear to be larger in size in day 5 media compared to day 3. Higher magnification of vesicles in (D) day 3 media and (E) day 5 media show the appearance of a rigid structure and a double membrane surrounding the vesicles.

Additionally, NTA was performed on day 3 spent media and unconditioned control media. Results show the presence of particles in day 3 spent media along with unconditioned control media. The majority of particles in the day 3 media appear to be in the size range of 100 to 300 nm, consistent with the size of exosomes and extracellular vesicles. Comparing the size distribution of particles between day 3 and control media, there appears to be a greater concentration of particles that are less than 200 nm in the day 3 media, and a greater concentration of particles that are greater than 300 nm in the control media (Figure 4.2). These findings indicate that particles are present and detectable in spent embryo culture media, however, there are also particles present in the control, unconditioned media. The next step was to determine whether the particles detected in the spent media were released by the embryo during in-vitro culture.
4.3.2.2 EV’s are Present on the surface of Embryonic Membrane

To determine whether EV’s are released from human pre-implantation embryos, the zona pellucida was removed and the surface of the embryonic membrane was visualized using scanning electron microscopy (SEM). Embryos were incubated with anti-CD9 antibodies conjugated to 20nm gold particles. Images show CD9 positive vesicular-like structures that appear to be released from the surface of the embryo (Figure 4.3)
Figure 4.3 - SEM images of CD9-positive immuno-gold labeling on the surface of the embryonic membrane of a day 3 embryo. The embryonic membrane is shown in green while the red shows CD9 labeling. Images represent two different areas on the same embryo.

4.3.2.3 Potential for EV’s to Cross the ZP

For the duration of culture, embryos are surrounded by the zona pellucida (ZP), a thick glycoprotein layer that serves multiple functions, one of which is to protect the growing embryo. In order to detect extracellular vesicles in embryo culture media, the vesicles must be able to cross the ZP after they are secreted from the embryonic blastomeres into the perivitelline space.

To determine whether extracellular vesicles that are released by embryos are able to cross the ZP, scanning electron microscopy (SEM) images were taken of the outer surface of the ZP. Similar to the SEM of the surface of the embryonic membrane, the embryos were stained with CD9 and then conjugated to 20nm gold particles. Images of the surface of the ZP show staining of CD9, however, due to poor dehydration of the zona pellucida, no spherical vesicles are seen (Figure 4.4, 4.5)
Figure 4.4 – SEM images of the surface of a day 3 embryo with the zona pellucid intact. Shown at two different magnifications. Porous morphology of the ZP suggests potential areas of release of EV’s.

Figure 4.5 – CD9+ immune-gold labelling (20nm) on the surface of the zona pellucida of a day 3 embryo. Images represent different areas of the ZP on the same embryo. Due to poor resolution, vesicular structures are not visible.
Since visualization of EV’s through SEM was unsuccessful, transmission electron microscopy (TEM) was then performed, with the goal to be able to visualize EV’s being released from the embryonic membrane and going through the ZP. For the first experiment, a 3PN 1-cell zygote was visualized. TEM images show that vesicles appear to be released from the surface of the membrane, and are seen throughout the ZP, suggesting that they are traveling through the ZP, in the perivitelline space, into extra-cellular space (Figure 4.6)

![Figure 4.6](image.png)

**Figure 4.6** – TEM images of a day 3 abnormal embryo labeled with 5nm gold antibodies against CD9. The architecture of the embryo is shown in figure A). Higher magnification of the ZP shows the appearance of CD9 positive vesicles. Immuno-gold labelling is highlighted with arrows.

### 4.3.2.4 Characterization of EV Release at different Developmental Stages

To further understand the release of EV’s throughout development, embryos at different developmental stages were imaged using TEM. Embryos, donated for research, were imaged at the following stages: 1 cell fertilized zygote, 2 cell stage, 4 cell stage, 8 cell stage, 10-16 cell stage, morula stage, blastocyst stage and an empty vesicle (an abnormal blastocyst that does not have an inner cell mass and is only comprised of trophectoderm cells). Additionally, to determine whether vesicles that are visible in the ZP of embryos were produced by the embryo itself and were not remnants from the oocytes stage, prior to fertilization, 6 metaphase II oocytes were also imaged. TEM images show vesicles present in the zona pellucida at all embryonic
stages including an empty vesicle. Interestingly, the presence of vesicles is not observed in the ZP of MII oocytes. Representative images are shown in Figure 4.7.

4.3.3 Summary and Discussion

Negative staining shows the presence of EV’s in both day 3 and day 5 spent embryo culture media. The vesicles in the day 3 spent media appear to be smaller than the vesicles in day 5 spent embryo culture media, suggesting that the size of vesicles released changes throughout development. Although we did not observe any vesicles in the control media with negative staining TEM, other studies have shown the presence of EV’s in various unconditioned cell culture media (Tanetta et al. 2014, Kropp et al 2014) The source of the vesicles is suggested to be from albumin, which is an additive in most media. The media used for this study, Global Total, is supplemented with human-serum albumin, so it was surprising that vesicles were not observed in the control media using negative staining TEM. However, the absence of vesicles in the control media may be due to low abundance of vesicles in the control media, as vesicles were observed in NTA analysis. It is likely that there are a greater number of vesicles in the spent media, than the control media because the spent media has the vesicles that are already in the basal media, along with additional vesicles that are potentially secreted from the embryo. Therefore, it is more likely, due to the sensitivity of the methodology used, that vesicles will be detected in the spent media than the control media.

In contrast to the lack of observed vesicles with negative staining, NTA analysis revealed the presence of vesicles in the control media. Comparing the size distributions of the particles in the control media and the day 3 media, the control media had a higher concentration of particles larger than 2000nm. Since NTA analysis confirmed the presence of particles in the spent and the control media, the next step was to determine whether these particles are released from the surface of the embryo and whether they are extracellular vesicles.

To determine if the vesicles observed in spent embryo culture media samples were from the embryo, SEM microscopy was used to visualize the surface of the embryonic membrane. Images showed the presence of CD9 positive vesicles on the surface of the cell membrane, suggesting that vesicles are released from the embryo during culture. CD9 is a tetraspanin protein that is characterized as an extra-cellular vesicle marker (Harding et al. 2013). The
positive staining observed on the surface of the embryonic membrane suggests that these spherical vesicles are extra-cellular vesicles, and not apoptotic bodies.

Embryos that are grown in culture are encompassed in a thick glycoprotein layer called the zona pellucida. Although SEM images show the release of vesicles from the surface of the membrane, in order to be detected in the embryo culture media, the vesicles must be able to cross the zona pellucida actively or passively. The next goal was to determine if this was occurring. SEM of the embryo with the ZP intact was performed; however, the dehydration step of the zona pellucida was difficult, resulting in images of very low resolution. Although positive CD9 staining was observed, vesicle morphology was not distinguishable. Additionally, it is possible that any vesicles present before processing were washed away during sample preparation. As SEM was not successful, TEM was performed to visualize vesicles instead. TEM images of a one-cell embryo shows that CD9 positive vesicles were present in all the layers throughout the ZP, suggesting that they are released from the embryonic surface and travel through the ZP into the culture media.

A major consideration, was to differentiate whether the vesicles observed in the ZP were released prior to fertilization, by oocytes, or whether the vesicles were remnants from cumulus cell projections. TEM of the oocyte was used to resolve this. While vesicles are visualized in the images of embryos at different stages, there were no vesicles seen in the ZP of MII oocytes, suggesting the vesicles observed in the ZP of embryos were produced by the embryo, and not prior to fertilization.

Visualization of EV’s in all layers of the ZP at all embryonic stages suggests that the vesicles are released throughout development. Interestingly, EV’s were also observed in the ZP of an empty vesicle, which has no inner cell mass, suggesting that the trophectoderm is a contributor to the EV population seen at the blastocyst stages. Alternatively, the vesicles in the empty vesicle may be remnants from previous growth stages.
4.4 Contents of Extracellular Vesicles

4.3.4 Rationale

A good diagnostic tool is one that is non-invasive, imposes minimal or no threat to the health of the embryo and can be performed in a relatively short period of time. Detection of specific microRNAs in spent embryo culture media meets all of these criteria and therefore, may be a potential diagnostic tool. A two-step approach was used to identify a biomarker. I first adopted a candidate approach to determine if microRNA detection in single microdroplets of 20μl embryo culture media was possible. I then moved on to try to identify novel microRNA’s that have not been previously characterized in spent embryo culture media.

As the study of extracellular vesicles is fairly new, there is currently no gold standard for vesicle enrichment, vesicle lysis and miRNA extraction. Previous work at the Librach Lab, on seminal fluid, follicular fluid and serum, has shown that different populations of extracellular vesicles (based on size, phospholipid concentration and surface proteins) contain different contents of proteins and miRNA’s. It is also known that some exosome enrichment buffers and RNA isolation kits tend to be biased in their extractions by favouring the isolation of certain sub-types of extracellular vesicles (Alvarez et al. 2012). To find a suitable biomarker, it was important to know that the relative abundance of candidate miRNA’s is a true representation of the culture media contents, and not an artifact of the extraction process. Therefore, I performed optimization techniques at each step in order to find the most suitable methods for the isolation.

After optimization, I then moved on to the candidate approach. At the time these experiments were conducted, two studies were published that assessed microRNA species in spent embryo culture media. A study by Rosenbluth et al. found high levels of microRNA-372 and microRNA-645 in culture media with human blastocysts with failed IVF outcomes (Rosenbluth et al. 2014). Additionally, another study found additional microRNA species in embryo culture media of bovine blastocysts (Kropp et al. 2014). Both of these studies identified the presence of
microRNA species in spent embryo culture media; however, the methods involve pooling several microdroplets of spent media from embryos that had a similar outcome after embryo transfer (i.e. successful pregnancy, unsuccessful pregnancy). Additionally, these studies do not address whether these microRNA species were encompassed within extracellular vesicles, or whether they were free floating, or both. For the purpose of this study, it was important to determine whether microRNA’s are detectable in a single microdroplet and whether these microRNA’s are in EV’s. Therefore, I optimized a protocol for qPCR that allows detection of microRNA species in single culture media microdroplet.

Next Generation Sequencing (NGS) was performed on spent embryo culture media to try and identify novel microRNA’s that may be indicative of embryo developmental potential; however, due to the limited amount of culture media available for analysis, the NGS analysis was unable to detect microRNA species.

4.3.5 Results

4.3.5.1 Method Optimization

4.3.5.1.1 Extracellular Enrichment Optimization

Three different methods to isolate EV’s from spent culture media were compared: 1) ExoQuick Exosome Isolation Buffer (SBI), 2) Total Exosome Isolation Solution (Life Technologies) and 3) No pre-enrichment treatment, where the culture media was used as-is, without a pre-enrichment kit. Additionally, four different miRNA extraction kits were compared to each other. The results were quantified using the Agilent Bioanalyzer. Using no pre-enrichment for EV’s, combined with using the Norgen microRNA Extraction kit to extract RNA, resulted in the highest yield of both total and short RNA’s. (Figure 4.8)
Figure 4.8 – Optimization of RNA isolation and extracellular vesicle enrichment. EV’s were enriched using one of three different methods: ExoQuick (EQ); Total Exosome Isolation Solution (TE) or No pre-enrichment (NT). RNA was extracted using three different commercial kits: Qiagen, Norgen and Exiqon.

4.3.5.2 EV Lysis and RNA Extraction Optimization

Two different methods were performed on spent embryo culture media. Three droplets were pooled together and then aliquoted into the three groups: 1) No treatment; 2) Sonication of samples for 3 x 15 seconds; and 3) sonication for 3 x 15 seconds and lysis buffer. Sonication reduced the number of particles detected in the media, however, the addition of a lysis buffer further reduced this number (Figure 9). There was an increase in particles between 5 and 25nm, which further confirmed that the combination of lysis buffer and sonication effectively lysed the vesicles (Figure 4.9b).
Figure 4.9 – A) Nanoparticle tracking analysis of extracellular vesicle size and concentration with no treatment, following sonication only and following sonication and lysis buffer addition. B) Enlargement shows an increased number of particles between 5-25nm when sample is treated with both sonication and lysis buffer, indicating vesicle lysis.

4.3.5.3 Candidate Approach

QPCR was done on three different microRNA species previously reported to be present in spent embryo culture media: miR-181a, miR-373 and miR-645. The experiment was performed on a sample size of 10 different embryo culture media droplets. In order to distinguish vesicular microRNA’s from free floating, Proteinase-K and RNase treatment were used prior to vesicle lysis, to degrade all free-floating microRNA species and their carrier proteins, so that
microRNA species that are protected within vesicles could be analyzed separately. Using absolute Ct values as a reference, miR-181a and miR-372 were readily detectable in RNA extractions of single culture media microdroplets (Figure 4.10) Micro-RNA-181a and microRNA-372 had a lower Ct value than miR-645. This is consistent with other reports, where microRNA-645 was shown to be present at a lower concentration than microRNA-372 in spent embryo culture media (Rosenbluth et al. 2014)

![Figure 4.10](image)

**Figure 4.10** – Absolute Ct values of miR-181, miR-372 and miR-645 in spent embryo culture media. Results show average of 10 embryo culture media droplets analyzed separately.

**4.3.5.4 Novel MicroRNA’s in Spent Embryo Culture Media**

NGS was performed twice on pooled samples of embryo culture media. 10 pregnant and 10 non-pregnant samples were pooled for analysis. For the first run, the loading density was 94%, which is very high so the run was repeated with a 2X sample dilution. Although the second run was diluted, the loading density was still fairly high, at 93%. Despite the high loading density, 90% of the final library were adaptor dimers and only 5% of the final library were potentially readable fragments, equating to 308, 512 reads. After analysis of the final library, 0% of the reads aligned to the human microRNA reference library. Upon sequencing these reads, it was
found that they were a combination of adaptors, adaptor dimers and barcode sequences with no useable reads.

4.3.6 Summary and Discussion

The study of EV’s is relatively new and there is no established ‘gold standard’ for the isolation and lysis of EV’s. Due to the lack of standardization of techniques, it was critical to perform experiments that would both optimize the exosome yield and lysis methodology. Previous studies have reported superiority of certain kits over others; however, this is particular to the sample type, sample volume and the downstream application.

To establish an EV enrichment method that would enable efficient isolation of EV’s from embryo culture media, two commercial kits were compared, together with no pre-enrichment. Although ultracentrifugation is a commonly used method for the enrichment of EV’s, the small volume available did not lend itself to the use of ultracentrifugation, which requires at least 1mL of volume. Each of the two kits employ a different method for isolating EV’s. While the exact method of extraction is proprietary, and therefore not disclosed, the general method of isolation for ExoQuick™ Exosome Precipitation Solution is a Polyethelene glycol (PEG) based polymer that attracts EV’s through hydrophobic interactions. Conversely, the Total Exosome Isolation Solution works by occupying water molecules in the sample, allowing the less-soluble components, such as EV’s, to be precipitated out of solution and collected at a low-speed centrifugation. It is unclear which EV enrichment method is most effective because the final yield is also dependent on the method of RNA isolation.

When comparing different methods of RNA isolation, three commercial kits were compared: The RNeasy Mini Kit by Qiagen, the microRNA purification kit by Norgen, and the miRCURY™ RNA Isolation Kit by Exiqon. In the case of both day 3 spent embryo culture media and day 5 spent embryo culture media, the Exiqon kit had the lowest yield of RNA and the Norgen kit had the highest. The company does not disclose the specific technology used in the Norgen Kit; however, it is known that this kit has a different filter than most other kits, which has a higher affinity to RNA. Moreover, the highest yield was when the Norgen Kit was used without any prior pre-enrichment of EV’s, which was the combination used for downstream qPCR analysis.
At the time the experiments were conducted, two previous reports had examined microRNA’s in spent embryo culture media; however, it is unclear as to whether these microRNA’s are free floating or are within extracellular vesicles, and the results were obtained by pooling multiple microdroplets of similar developmental stages or of implantation outcome. For the purpose of this study, the ultimate goal is to devise a diagnostic test, thus it was critical to be able to detect the microRNA’s in a single microdroplet. In order to do this, the optimization steps for EV enrichment and RNA extraction mentioned above were critical. Moreover, a pre-amplification step was performed following cDNA synthesis. This step involves the addition of primers of interest which are then used to amplify the transcripts. In effect, this dramatically increases the concentration of the transcripts of interest prior to qPCR. Using this method, microRNA-181a, miR-372 and miR-645 were successfully detected in single microdroplets of spent embryo culture media. miR-181a was found to be elevated in embryos and in spent media of degenerated bovine blastocysts. miR-645 has been shown to inhibit nucleoplasmin 2, which is involved in nuclear organization and reprogramming of the cell (Kropp et al. 2014). This microRNA species was also found to be elevate during the 4-8 cell stage by (Lingenfelter et al. 2011), and then declined for the remainder of pre-implantation development.

miR-372 and miR-645 are two microRNA’s that were identified by Rosenbluth et al. in human spent embryo culture media. The average cycle threshold (CT) value was found to be 25.5 for miR-372 and 39.5 for miR645. This is in concordance with our results, as we report similar CT values for these microRNA’s, including the finding of a higher CT value for miR-645 than miR-372. MiR-372 has been shown to play a role in the MAP3K/CDK6 pathway, which is involved in cell cycle regulation and apoptosis (Maragkakis et al. 2009). It has also been shown to be involved in the reprogramming of human fibroblasts to induced pluripotent stem cells (iPSC’s) (Subramanyam et al. 2011).

After being able to successfully identify microRNA’s in single microdroplets, I then moved on to identifying novel microRNA’s that may be suggestive of embryo developmental and implantation potential. NGS was used for this process; however, the levels of microRNA transcripts were below the detection threshold for NGS. The only detectable transcripts were the ones that arose due to primer dimers and adaptor sequences. Due to the very low input volume, the concentration of primers and adaptors was much higher than any transcripts present in the sample. The success of qPCR over NGS suggests that qPCR has a higher detection threshold for
transcripts. Alternatively, the pre-amplification step could be the primary reason why detection using qPCR was possible while NGS was not. Based on these findings, I conclude that qPCR is a more appropriate technique for detection of microRNA species in spent embryo culture media than NGS, particularly when a pre-amplification step is incorporated into the protocol.
5 General Discussion

The objective of this study was to identify whether morphokinetics and metabolomics of embryo development, together with extracellular vesicular miRNA content, could be used as potential tools for the selection of embryos that have the highest chance of successful implantation.

My results show that the time to reach tM, tSB, tB and the duration of the 8 cell stage are predictive of blastocyst development in-vitro. Additionally, the distribution of t2, tB and s2 are significantly different between implanted and non-implanted embryos. The group of non-implanted embryos had a much wider distribution for the timing of these morphokinetic parameters. However, there was also a lot of overlap of the data, which highlights the importance of using these parameters as exclusion criteria, rather than selection criteria, to ensure that no viable blastocyst is excluded. It is known that different research studies have reported variability in the findings of significant MKP’s related to embryo development and implantation potential. The t2 in this study for implanted embryos ranged from 21.26 – 30.38 hpi; however, Chamyou et al. reported a range of t2 between 21.4 to 34.8 hpi, for embryos that formed a viable blastocyst. Both studies used a single step media; however, the embryos analyzed in Chamayou’s study were incubated at 5% CO₂, compared to 6% CO₂ in the present study. Given that incubator conditions, culture media, and patient population varies from clinic to clinic, it is critical that each clinic perform their own validation studies for significant MKP.

Furthermore, this is the first human study to correlate metabolite levels in spent culture media with morphokinetics. In particular, lactate acid and acetic acid levels correlated with t2; and 3-aminoisobutyrate correlated with tB and s2. Assessing metabolite levels provides another tool for enhancing embryo selection in addition to morphology and morphokinetics, but it also enables one to study the metabolic pathways employed by the embryo in culture and further understand the physiology of pre-implantation embryos. A major limitation of this study is that metabolite levels were measured as an endpoint analysis. Effectively, the embryonic stage at which each quantified metabolite is released or taken up is not known, only the collection of metabolites
present at the end of the culture are measured. The embryonic genome is activated at the compaction stage when a lot of metabolic changes are also known to occur: while the pre-compaction embryo mainly employs anaerobic respiration, aerobic respiration is the main energy source for post compaction embryos. Separately assessing metabolites in spent media of pre- and post-compaction embryos can allow for more accurate assessment of metabolic processes occurring at different stages. This is possible with sequential step media, where the embryo is cultured in a basic media for the first 3 days of culture and then is transferred to a different droplet for the remaining duration of culture.

EV’s have been heavily studied for their role as biomarker in various physiological and pathological states, but the presence of EV’s released from human pre-implantation embryos has not been previously reported, to my knowledge. Electron microscopy results showed the presence of EV’s throughout the ZP, in the perivitelline space and in the culture media of pre-implantation embryos at various developmental stages. Moreover, these results suggest that vesicles are released by human embryos and that they are detectable in spent culture media. Furthermore, I was able to develop a protocol that allowed the detection of vesicular microRNA’s in single microdroplets of spent culture media, suggesting that the microRNA’s contained within vesicles has the potential to be a biomarker for the prediction of embryo success.

While this study focused on non-invasive methods for assessing embryo competency, another area of consideration is invasive genetic testing including PGS. Not all embryos analyzed in this study had known ploidy status, thus, there is a possibility that some of the transferred embryos, in patients that did not opt for PGS, may have been aneuploid. Including only embryos with known euploid status would strengthen the impact of the study.

In an ideal setting, each parameter used to assess embryo competency (morphology, morphokinetics, metabolomics, PGS, extracellular vesicles, etc.) would be performed on each embryo and combining all of these methods together would provide additive value to enable selection of the most competent embryo. However, not every technology is available at every clinic or is not feasible for every patient, thus informed decisions have to be made based on the information available at hand, keeping in mind that the information can often be limited.
The studies presented here indicate that non-invasive biomarkers, including morphokinetics, metabolomics and extracellular vesicles, provide valuable information that could be very useful for improving the process of embryo selection. However, it is important to keep in mind that infertility is multifactorial, thus a ‘one-size-fits-all’ solution may not be feasible. Nevertheless, these studies improve our knowledge of human embryo physiology and could enable us to increase the success of IVF treatment for infertility by improving embryo selection.
6 Future Directions

The results of these studies provide valuable information about the practicality and efficiency of using morphokinetics, metabolomics and extracellular vesicles to assess embryo health. Additional studies that could further enhance our understanding of embryo physiology and also enable us to discover biomarkers for embryo selection that could be utilized in routine clinical practice, are outlined below

1. Identification of vesicular miRNA candidate indicative of implantation potential

   In this study, a qPCR protocol was optimized that allowed for miRNA detection from single microdroplets. This protocol can be used to separately analyze miRNA’s in spent embryo culture media, comparing embryos with successful and unsuccessful implantation.

2. Determine if metabolite profile can provide additive information in combination with morphokinetics

   When comparing the distribution of morphokinetic parameters between implanted and non-implanted embryos, there is a lot of overlap between the two groups. Many embryos that did not achieve implantation had MKP values similar to embryos that did implant. Comparing the metabolite profiles of embryos that had similar morphokinetic values but different implantation outcomes may provide additive value to distinguish good embryos from poor embryos.

3. Metabolic analysis of embryo ploidy status

   Metabolites in spent culture media can provide insight into the internal processes in the embryo. PGS is a very invasive, time consuming and expensive process that is not always readily available. Aneuploid embryos have been shown to have
different levels of amino acid consumption compared to euploid embryos (Picton et al. 2010). Comparing metabolite signatures of euploid and aneuploidy embryos to develop a signature that is indicative of ploidy status could potentially provide a non-invasive alternative to PGS testing.
References


Baj-Krzyworzeka, Monika, et al. "Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes." Cancer Immunology, Immunotherapy 55.7 (2006): 808-818.


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