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Extracellular vesicles, microRNA and the preimplantation embryo: non-invasive clues of embryo well-being

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REVIEW

Extracellular vesicles, microRNA and the preimplantation embryo: non-invasive clues of embryo well-being

BIOGRAPHY

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KEY MESSAGE

Extracellular vesicles and microRNA are blastocyst secretory products found within spent media microdroplets after embryo culture. Blastocysts exhibiting either degeneracy or implantation failure release greater amounts of vesicles and microRNA into the media. These correlations support their use as non-invasive tools to aid clinical embryo selection for transfer.

ABSTRACT

Elective single embryo transfer is rapidly becoming the standard of care in assisted reproductive technology for patients under the age of 35 years with a good prognosis. Clinical pregnancy rates have become increasingly dependent on the selection of a single viable embryo for transfer, and diagnostic techniques facilitating this selection continue to develop. Current progress in elucidating the extracellular vesicle and microRNA components of the embryonic secretome is reviewed, and the potential for these findings to improve clinical embryo selection discussed. Key results have shown that extracellular vesicles and microRNAs are rapidly detectable constituents of the embryonic secretome. Evidence suggests that the vesicular population is largely exosomal in nature, secreted at all stages of preimplantation development and capable of traversing the zona pellucida. Both extracellular vesicle and microRNA concentrations within the secretome are elevated for blastocysts with diminished developmental competence, as indicated either by degeneracy or implantation failure, whereas studies have yet to firmly correlate individual microRNA sequences with pregnancy outcome. These emerging correlations support the viability of extracellular vesicles and microRNAs as the basis for a new diagnostic test to supplement or replace morphokinetic assessment.

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INTRODUCTION

A global shift towards elective single embryo transfer (eSET) has taken place among assisted reproductive technology (ART) since becoming recommended best practice for patients under the age of 35 years with a good prognosis (*The Practice Committee of the American Society for Reproductive Medicine and The Practice Committee of the Society for Assisted Reproductive Technology, 2013*). In the USA, the number of eSET ART cycles has increased from 21.4% in 2013 to 28.5% in 2014, and has now reached 42.7% as of 2016 for this age group (*Centers for Disease Control and Prevention, 2015*; *2016*; *2018*). This change reflects the growing requirement to minimize the serious health complications associated with a multiple pregnancy that adversely affects maternal and fetal health. Embryo cryopreservation has been pivotal in supporting widespread use of eSET by enabling sequential single embryo transfers in the event of a failed fresh cycle. Although the incidence of pregnancy resulting from a fresh IVF cycle is lower when eSET is used over a double-embryo transfer, once used in conjunction with frozen embryo transfer, eSET cumulative pregnancy incidence quickly becomes comparable to those from fresh double transfers (*McLernon et al., 2010*). This new reality has generated intense ongoing discussion about the current state of embryo selection practices and has launched substantial research efforts to improve this process.

Currently, clinics selecting an embryo for transfer based on morphometrics do so according to an assessment of morphology-based criteria at the pronuclear, cleavage or blastocyst stages (*Nasiri and Eftekhari-Yazdi, 2015*). Features such as timing of first cleavage, blastomere symmetry, cellular fragmentation, multinucleation, cell count and pronuclei definition are assessed to score each embryo using a predefined grading scheme (*Gardner and Balaban, 2016*). Morphological assessment continues to be universally used, as it is a rapid, technically simple, non-invasive and cost-effective technique. Although some new morphometrics are suggesting embryo morphology still has more to offer (*Ebner et al., 2016*; *Gazzo et al., 2020*), this approach has fallen under scrutiny owing to its modest ability to predict pregnancy after embryo transfer (*Stecher et al., 2014*) and the subjectivity between embryologists (*Baxter Bendus et al., 2006*). The addition of time-lapse systems has garnered mixed consensus about their benefit (*Adamson et al., 2016*; *Kieslinger et al., 2016*), and robust improvements in IVF metrics over conventional culture methods and morphometric evaluation have not been demonstrated (*Goodman et al., 2016*).

The prevalence of aneuploidy among preimplantation embryos continues to be a suspected cause of implantation failure and miscarriage. Although it remains an ongoing discussion, mounting evidence suggests identification of a euploid embryo for transfer significantly increases implantation, clinical pregnancy and live birth rates for patients with a range of indications, including advanced maternal age and recurrent miscarriage, as well as good-prognosis patients using single-embryo transfer (*Rubio et al., 2019*). Preimplantation genetic testing for aneuploidy (PGT-A) using blastocyst biopsy and next-generation sequencing (NGS) has now clearly been shown to have high predictive potential for forecasting failure to deliver for preimplantation embryos diagnosed with aneuploidy (*Tiegs et al., 2020*). Removal of a single blastomere for PGT-A at the cleavage-stage may decrease the conceptus' implantation potential (*Scott et al., 2013*; *Mastenbroek and Repping, 2014*); however, the implications of blastocyst biopsy are far less substantial (*Cimadomo et al., 2016*; *Tiegs et al., 2020*). Regardless, this assessment may still be confounded by the prevalence of genetic mosaicism leading to false euploid diagnoses (*Baart et al., 2006*). Additionally, for blastocyst-stage biopsy, the procedure requires a skilled embryologist with specialized equipment for laser ablation, which are the main contributors to the cost and accessibility of this service. The active development of non-invasive PGT-A (niPGT-A) has been promising and is showing high concordance with conventional methods without the need for embryo biopsy (*Leaver and Wells, 2020*).

Development of non-invasive diagnostics such as niPGT-A take advantage of accessible extracellular analytes within the spent blastocyst culture media and the blastocyst cavity. These extracellular sources have been extensively characterized by proteomics and metabolomics studies (*Hernández-Vargas et al., 2020*) in the search for correlators with aneuploidy and impending implantation failure. An explosion of interest in extracellular vesicles and microRNA sequences has been driven by their use as therapeutics (*Christopher et al., 2016*) and potential utility as biomarkers of abnormal cellular states (*Huang, 2017*). Advances in methods for isolation and detection have now extended this latter consideration into the realm of assisted reproduction in the hope of improving optimal embryo selection for embryo transfer. This review will cover recent studies that have characterized the extracellular vesicle and microRNA fractions of the preimplantation embryonic secretome. In this context, we use the term secretome loosely, referring to all extracellular components originating from the preimplantation embryo and not just the proteinaceous fraction. The aims of this review are to highlight inter-study trends and showcase results most capable of furthering the development of eSET diagnostics.

Extracellular vesicles

Extracellular vesicles are biological nanoparticles consisting of a lipid bilayer that originates from a wide range of host cells throughout the body. Extracellular vesicles are now renowned for their ability to carry significant phenotype altering cargo such as transcription factors and microRNAs (*Anand et al., 2019*). Encapsulated cargo is significantly more stable and may be readily internalized by recipient cells. Extracellular vesicles are classified according to their biogenesis; however, their origins are usually implied only after characterization of the vesicle's size and membrane composition. Secreted vesicles may bud directly from the plasma membrane (microvesicles, ectosomes), be released from within an intracellular precursor called the multivesicular body (MVB) after fusion with the cell membrane (exosomes) or form upon cell death, wherein apoptosis directs partitioning of residual cytoplasm to more sizeable apoptotic blebs or bodies. Exosomes are the smallest class of extracellular vesicles with a diameter measuring between 40 and 120 nm (*Vlassov et al., 2012*), whereas microvesicles range from 50 nm to 1000 nm and apoptotic bodies vary greatly, ranging anywhere from 500 nm to greater than a micron (*Andaloussi*

et al., 2013). Exosomal biomarkers are somewhat uninformative as their transmembrane protein constituency, a property routinely used for identification that relies heavily on the tetraspanins CD9, CD63 and CD81, is not specific to exosomal extracellular vesicles. The ubiquitous presence of the tetraspanins throughout the cell membrane enables other extracellular vesicle classes to also bear these markers, as the membrane is shared structurally. Residual exosome trafficking proteins involved in the formation of the MVB, i.e. ALIX and TSG101, are often found within exosomes, yet their presence is not guaranteed (Lötvall et al., 2014). In the absence of reliable exosome-exclusive markers, their presence is often inferred from a combination of these markers along with their less than 200 nm size distribution and, occasionally, observation of MVBs within their probable host cells. Apoptotic body composition is highly variable; however, the expression of phosphatidyl serine within the outer leaflet of the vesicle membrane is a reliable characteristic of these vesicles and may be used for identification. Recommendations for addressing these challenges, including proper isolation and reporting of extracellular vesicle populations present within limiting samples, such as preimplantation embryo conditioned culture media, are available and updated periodically (Théry et al., 2018).

The 12 main studies covered in this section focus on the secretome of the whole preimplantation embryo; studies characterizing the secretome of embryo-derived cell lines were not included. Aspects of these main studies pertaining to the capture, identification and characterization of preimplantation embryo-derived extracellular vesicles and their relation to developmental competence are discussed. These main studies are presented in TABLE 1, and the review of their contents is supported by significant results found elsewhere.

Extracellular vesicles in the preimplantation embryonic secretome

Preimplantation embryo-derived extracellular vesicles are confirmed as secretory products in spent culture media collected during human clinical studies (Abu-Halima et al., 2017; Giacomini et al., 2017; Pallinger et al., 2017; Vyas et al., 2019) in addition to those involving various animal models: the cow (Mellisho et al., 2017; Qu et al., 2017; Pavani et al., 2019; Dissanayake et al., 2020), mouse (Pallinger et al., 2018; Simon et al., 2020) and pig (Saadeldin et al., 2014). Extracellular vesicles are released at all stages of human preimplantation development, including cleavage-stage embryos and zygotes (Vyas et al., 2019). Similarly, all stages of porcine (Saadeldin et al., 2014), bovine (Qu et al., 2017; Dissanayake et al., 2020) and mouse (Pallinger et al., 2018; Simon et al., 2020) preimplantation embryos have confirmed extracellular vesicle release. In all cases, the identified vesicles presented the hallmark cupped-shape, double membrane vesicle appearance and amplification of select embryonic-specific mRNA sequences (POU5F1 and NANOG [Giacomini et al., 2017]; Oct4, Sox2, Klf4, c-Myc and Nanog [Saadeldin et al., 2014]) from extracellular vesicle-enriched media fractions confirmed the embryonic origin of these vesicles. The media concentration of these vesicles is reported in the range of 10^8 and 10^{11} nanoparticles/ml (TABLE 1) and increases as development progresses towards the blastocyst stage (Giacomini et al., 2017; Vyas et al., 2019). The range in concentrations likely arises owing to the differences between studies in the number of embryos cultured per microdroplet, microdroplet volume, differing embryonic stage and conditioning time.

The extracellular vesicles present in spent culture media from day 3 and day 5 human embryos were positive for exosomal markers CD9, CD63 and ALIX (Giacomini et al., 2017). Similarly, cleavage (Qu et al., 2017) and morula-stage (Dissanayake et al., 2020) bovine embryo-derived extracellular vesicles are CD9+ and CD9+/CD81+, respectively. Generally, these extracellular vesicle populations consisted of vesicles within 50–200 nm in diameter and the extracellular vesicle population being actively released from preimplantation embryos throughout early development was concluded to be largely exosomal in nature. This is supported by the observation of membrane localized, CD9+ MVBs in fixed murine cell slices from oocyte, cleavage-stage and morula-stage embryos (Simon et al., 2020), including blastocyst trophectodermal cells (Shin et al., 2017) and within trophoblast cells of bovine blastocysts (Mellisho et al., 2017). Uptake of human preimplantation embryo-derived extracellular vesicles by primary human endometrial cells has been demonstrated, suggesting extracellular vesicles may be involved in the conceptus–uterus milieu that facilitates the initial attachment and growth of the extraembryonic lineages through the uterine epithelium (Giacomini et al., 2017).

Extracellular vesicles and the zona pellucida

It is now clear that embryonic-derived exosomes are capable of freely traversing the zona pellucida. In fact, the direct observation of exogeneous fluorescently labelled small vesicle internalization has revealed this flux is likely bidirectional (Saadeldin et al., 2014; Kim et al., 2019). Vyas et al. (2019) observed extracellular vesicles within the zona pellucida itself by electron microscopy (Vyas et al., 2019), which is corroborated by Simon et al. (2020). This raises the question whether the contents of the zona pellucida may provide sufficient embryo-derived material to be used as a non-invasive source of diagnostic information. Indeed, zona pellucida dissolution before transfer has no negative effect on implantation success (Urman et al., 2002). To the best of our knowledge, no studies have yet sought to correlate analytes trapped in the zona pellucida with embryo competence. Curiously, Vyas *et al*. (209) were unable to identify any extracellular vesicles within the zona pellucida of MII oocytes, suggesting that preimplantation embryo exosomal release may be a fertilization-dependent phenomenon (Vyas et al., 2019).

The pore size of the bovine zona pellucida ranges from 150 nm to 220 nm from the oocyte to the morulae stage with no apparent trend (Vanroose et al., 2000). Obligingly, the primary media extracellular vesicle populations are defined by an approximate 200 nm cut-off in vesicle size when conditioned with cleavage-stage embryos (TABLE 1), yet blastocyst media studies have reported a second, lesser extracellular vesicle population, with diameters reaching as high as 400–500 nm (Abu-Halima et al., 2017; Giacomini et al., 2017; Vyas et al., 2019; Simon et al., 2020). Microvesicle-sized extracellular vesicles matching this description of 400–500 nm in diameter have been detected throughout the perivitelline space (Vyas et al., 2019). It is

TABLE 1 STUDIES DESCRIBING THE EXTRACELLULAR VESICLE PRESENCE WITHIN THE PREIMPLANTATION EMBRYONIC SECRETOME

Reference	Species	Isolation method	Identification method	Developmental stage	Sample origin	Extracellular vesicle diameter, nm	Nanoparticle concentration, nanoparticle/ml	Significance
(*Simon et al., 2020*)	Mouse	UC	TEM, NTA	M, B, E	CM, BF, TE, ZP	20–500 (TEM), 50–310 (NTA)	1.74×10^7	Murine oocyte, morula, blastocyst, expanded blastocyst release extracellular vesicles; extracellular vesicles in perivitelline space from oocyte onwards; extracellular vesicles present in the blastocyst cavity.
(*Dissanayake et al., 2020*)	Bovine	Centrifugation, filtration, SEC	Microarray, NTA, SEM, TEM	C, M, B	CM	50–150 (TEM), 30–300 (NTA)	7.17×10^8 (degenerate) versus 5.68×10^8 (B)	Degenerate embryos left behind media with greater concentrations of nanoparticles.
(*Vyas et al., 2019*)	Human	Centrifugation	SEM, TEM, NTA	O, Z, C, M, B	TE, ZP, CM	50–500 (TEM), 100–200 (NTA)	7.89×10^8 (B), 7×10^8 (C)	Extracellular vesicles shown to be capable of passing zona at zygote, morula and blastocyst stages; extracellular vesicle subpopulation with >200 nm diameter identified.
(*Battaglia et al., 2019*)	Human	None	SEM, NTA, ELISA	B	BF	median: 78.4; mean: 94.0	2.46×10^9 (BF)	Human blastocyst cavity contains exosomes that are CD63+ and CD81+.
(*Pavani et al., 2019*)	Bovine	UC, Optiprep	NTA, TEM, Western	B	CM	25–250 mean: 134; mode: 97.5 (NTA)	2.4×10^{10}	Embryo-derived exosomes may be taken up by other embryos in culture.
(*Pallinger et al., 2018*)	Mouse	None	FC	B	CM	NA	~1.75×10^6 (annexin V+ subpopulation)	Mouse blastocysts release extracellular vesicles.
(*Qu et al., 2017*)	Bovine	Centrifugation, UC, BC	TEM, FM	C	CM	60–150	NA	Bovine exosomes increase blastocyst formation when reintroduced into culture media.
(*Pallinger et al., 2017*)	Human	Centrifugation	TEM, FC	B, E	CM	NA	~9×10^5 (pregnant), ~2.2×10^6 (not pregnant) (PI+ subpopulation)	Human blastocysts leading to implantation success release significantly less PI+ and extracellular vesicles than those that led to implantation failure after transfer.
(*Mellisho et al., 2017*)	Bovine	Centrifugation, UC, BC	FC, TEM, NTA	E	CM	median: ~110; mode: ~80	$3.9–8.5 \times 10^8$	Bovine embryos release extracellular vesicles; culture media of embryos with arrested development had higher vesicle counts (~40%) than parthenogenetic blastocysts.
(*Giacomini et al., 2017*)	Human	Centrifugation, UC	TEM, NTA, Western	C, B	CM	50–200 mean: 75	2.6×10^9 (C), 6.26×10^9 (B)	Human embryos release extracellular vesicles; extracellular vesicle subpopulation with >200 nm diameter identified.
(*Abu-Halima et al., 2017*)	Human	Centrifugation	NTA, TEM	B	CM	30–120 mode: ~145 (NTA)	3.8×10^9 (pregnant), 7.35×10^9 (not pregnant)	The extracellular vesicle concentration in positive pregnancy outcomes culture media was about 50% of that in negative outcome.
(*Saadeldin et al., 2014*)	Porcine	Centrifugation, UC, BC	TEM, FM, FC	C, B, E	CM	35–100	NA	Porcine embryos release exosomes that could be internalized by other embryos in culture after reintroduction.

B, blastocyst; BC, bead capture; BF, blastocyst fluid; C, cleavage-stage; CM, culture media; E, expanded; FC, flow cytometry; FM, fluorescence microscopy; M, morula; NTA, nanoparticle tracking analysis; O, oocyte; PI+, propidium iodide-positive; SEC, size exclusion chromatography; SEM, scanning electron microscopy; TE, trophectoderm; TEM, transmission electron microscopy; UC, ultracentrifugation; Z, zygote; ZP, zona pellucida.

possible that larger extracellular vesicles are breaching the zona pellucida at the time of blastocyst hatching, making this a possible viable strategy to generate greater amounts of accessible material for non-invasive assessment. Zona pellucida damaging procedures, namely intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT), cannot be ruled out either. Although the implications of such procedures on the extracellular vesicle population remains unclear, *Saadeldin et al. (2014)* observed no major increases in various embryonic mRNA when comparing porcine SCNT embryos with non-SCNT controls. *Pallinger et al. (2017)* detected a significant portion of extracellular vesicles by flow cytometry in the spent media of hatched blastocysts after staining with either annexin V or propidium iodide. The size distribution was not reported, yet the successful detection by conventional flow cytometry suggests the vesicles were over

200 nm in diameter and the presence of externalized phosphatidyl serine along with DNA cargo points towards apoptotic bodies (*Pallinger et al., 2017*).

Presence of extracellular vesicles in the blastocyst cavity

Extracellular vesicles with exosomal character have now been reported within the blastocyst cavity of human blastocysts (*Battaglia et al., 2019*), and mouse blastocyst cavity extracellular vesicles have also been observed (*Simon et al., 2020*). In humans, *Battaglia et al. (2019)* estimated the blastocyst cavity extracellular vesicle concentration to be about 2.5×10^9 nanoparticles/ml, a value on par with the typical 10^8–10^9 nanoparticles/ml reported for culture media samples. The primary extracellular vesicle population diameters were consistent with exosomes, being no greater than 175 nm with a median of 75 nm. The exosomal tetraspanin presence of CD63 and CD81 in these vesicles was confirmed (*Battaglia et al., 2019*). The contents of the blastocyst cavity may prove a more reliable diagnostic source than the secretome, as it is impervious to media changes and is sheltered from contaminating factors such as residual maternal cumulus cells. A blastocyst rupturing strategy wherein its DNA contents are deliberately released into the culture media has improved the concordance of niPGT (*Kuznyetsov et al., 2018*; *Li et al., 2018*; *Jiao et al., 2019*), and such a strategy may be worth investigating to improve extracellular vesicle detectability. The blastocyst cavity naturally contracts to varying degrees repetitively during expansion and, in some cases, complete collapse may occur (*Sciorio et al., 2020*). With certainty, this collapsing phenomenon has been correlated with ongoing clinical pregnancy rate (*Huang et al., 2016*; *Marcos et al., 2015*; *Sciorio et al., 2019*; *2020*). Only 37.5% of transferred human blastocysts that exhibited at least one total collapse resulted in pregnancy, compared with 51.9% resulting from those with minor or no collapse (*Sciorio et al., 2020*). The ejection of these contents at a time when the zona pellucida has thinned or ruptured lends plausibility to the notion that such a release mechanism could be a major blastocyst-specific mechanism expediting release of extracellular vesicles and other analytes, such as microRNAs into the spent culture media.

Extracellular vesicle isolation and analysis

An overnight or 24-h period seems to be sufficient to condition medium sufficiently for reliable detection. In each study in TABLE 1, culture media samples were frozen at –80°C before extracellular vesicle isolation and characterization. Freeze–thaw cycles can diminish total extracellular vesicle yield by as much as 50% per cycle (*Cheng et al., 2019*), and it is likely the sensitivity of extracellular vesicles detection in conditioned media sample may be improved substantially by analysing fresh samples. The toolkit available to investigators looking to isolate a scarce vesicle population from small volumes of starting material is limited and depends highly on the requirements of the downstream analysis; common isolation techniques, such as ultrafiltration and size exclusion chromatography, are generally not compatible with single embryo culture media samples. The bovine has proven instrumental in this regard as high numbers of oocytes may be fertilized and cultured in a single experiment to bolster biomaterial availability within conditioned media (*Kropp et al., 2014*; *Kropp and Khatib, 2015*; *Gross et al., 2017*; *Mellisho et al., 2017*; *Qu et al., 2017*; *Pavani et al., 2019*; *Dissanayake et al., 2020*).

Of the 12 papers listed in TABLE 1, extracellular vesicle isolation was conducted by numerous means: eight used differential centrifugation, six used ultracentrifugation, three used bead capture, one used size exclusion chromatography, one used filtration and two proceeded to analyse crude media (non-summative). The choice of vesicle purification method is a factor that significantly affects the interpretation of the resulting extracellular vesicle population (*Brennan et al., 2020*). Differential centrifugation is used to remove larger cell debris, such as apoptotic bodies, and is often used in tandem with ultracentrifugation to purify and isolate the exosomal fraction. This purification regimen can be repeated multiple times to increase the purity of the isolate and is adequate for subsequent vesicle analysis by electron microscopy. Bead capture was used successfully when vesicles needed to be clustered to meet the minimum threshold for detection by fluorescence microscopy and flow cytometry. Size exclusion chromatography enables vesicle 'washing' to remove salts and other contaminants that may confound downstream analysis. Even the smallest commercial columns, however, produce elution fractions that are a greater volume than embryo culture microdroplets limiting its use in this application to large volumes of pooled media (*Dissanayake et al., 2020*). Material losses make these approaches inappropriate for media samples that have been conditioned by a single embryo. When it comes to single embryo resolution, currently crude media works best when paired with compatible analytics (*Abu-Halima et al., 2017*; *Pallinger et al., 2017*).

In the 12 extracellular vesicle papers in TABLE 1, extracellular vesicles were analysed by the following methods: 10 used transmission electron microscopy (TEM), seven used nanoparticle tracking analysis (NTA), four used flow cytometry, three used scanning electron microscopy, and two used fluorescence microscopy (non-summative). Media conditioned with a single day-5 human blastocyst must be analysed 'on-the-fly' within a matter of hours to be used as a diagnostic for embryo selection from a fresh cycle. Electron microscopy remains the gold standard for characterizing individual extracellular vesicles by enabling visualization of morphological features, such as the bilipid membrane in tandem with immunolabelling for markers such as tetraspanins. Embryo-derived extracellular vesicles may be visualized by fluorescence microscopy; however, both groups that used this method used aldehyde/sulfate bead capture to first concentrate extracellular vesicles from the media (*Saadeldin et al., 2014*; *Qu et al., 2017*). In a clinical setting, these microscopic methods are unable to easily resolve population level 'macro characteristics', such as size distribution or total number of extracellular vesicles. Traditional Western blotting of exosomal fractions from embryo conditioned media has been demonstrated (*Giacomini et al., 2017*; *Pavani et al., 2019*); however, the biomass requirements call for substantial starting media volumes that are simply not clinically feasible. Nanoparticle tracking analysis allows nanoparticles to be rapidly observed in suspension with minimal upstream sample purification. It has a minimum nanoparticle concentration requirement of about 10^6 nanoparticles/ml in a 1-ml loaded sample, an acceptable limitation for this application. It is capable of fully resolving

the nanoparticle population within pooled embryo culture media samples (*Giacomini et al., 2017*; *Battaglia et al., 2019*; *Pavani et al., 2019*; *Vyas et al., 2019*; *Simon et al., 2020*). In fact, the technique effectively characterized single microdroplets conditioned with a small cohort of six embryos (*Giacomini et al., 2017*) and even single embryo resolution has been achieved after conditioning with both cleavage-stage and blastocyst-stage embryos with varying degrees of success (*Abu-Halima et al., 2017*; *Mellisho et al., 2017*; *Dissanayake et al., 2020*). Of these single embryo studies, *Abu-Halima et al. (2017)* had no background contaminants in their unconditioned culture media; however, both *Mellisho et al. (2017)* and *Dissanayake et al. (2020)* encountered difficulties reliably differentiating between embryo-derived extracellular vesicles and the pre-existing nanoparticle populations within the commercial media before embryo exposure. This is an issue that has troubled nearly all NTA analysis of spent embryo culture media to date. Instruments used for NTA can be fitted with laser diodes capable of exciting labelled extracellular vesicles that may make distinguishing between the pre-existing nanoparticle population much easier (*Dragovic et al., 2011*). Of the four published papers using flow cytometry, two used aldehyde–sulfate latex beads to first bind all extracellular vesicles and bring these vesicles above the minimum size threshold required for detection by flow cytometry. Using flow cytometry, two clinical studies analysed pooled samples (*Mellisho et al., 2017*; *Pallinger et al., 2018*). *Saadeldin et al. (2014)* used group culture and *Pallinger et al. (2017)* conducted a single study and successfully analysed single blastocyst media samples. Flow cytometry is an expensive investment for an ART clinic; however, media samples may be sent to a local flow cytometry facility for analysis in a matter of hours (*Pallinger et al., 2017*). Both NTA and flow cytometry offer accurate, rapid, single event resolution with little to no processing of the initial sample, avoiding both enrichment bias and material loss, major factors that would likely change substantially from clinic to clinic. Specially tuned flow cytometers, known as a nanoflow cytometers, can resolve nanoparticles as small as 40 nm, significantly lower than the typical 200 nm cut-off for conventional flow cytometers (*Lian et al., 2019*). Nanoflow cytometry may resolve smaller populations among media borne extracellular vesicles, providing a more comprehensive analysis of spent culture media microdroplets conditioned with single blastocysts. No publications using nanoscale flow cytometry to this end have yet been published.

Pre-existing nanoparticles in culture media

Media-borne nanoparticles have been reported within most culture media before embryo exposure (*Giacomini et al., 2017*; *Pavani et al., 2019*; *Vyas et al., 2019*; *Dissanayake et al., 2020*), likely originating from natural media components such as human or bovine serum albumin. *Vyas et al. (2019)* reported pre-exposure media concentration of nanoparticles 7.33×10^8 nanoparticles/ml, a value that dropped to 7.0×10^8 nanoparticles/ml after exposure, suggesting embryo uptake of these particles may have occurred (*Vyas et al., 2019*). The population has been reported to have extracellular vesicle character (*Giacomini et al., 2017*) whereas, in other studies, it did not (*Pavani et al., 2019*; *Dissanayake et al., 2020*). A pre-existing nanoparticle population (6.65×10^{10} nanoparticles/ml) within the media used by *Pavani et al. (2019)* was not determined to have extracellular vesicle character. *Giacomini et al. (2017)* reported that blank human serum albumin supplemented culture media did not contain substantial particulate numbers, and those that were present did not exhibit extracellular vesicle character; however, media supplemented with serum substitute supplement contained CD9+ / CD63+ extracellular vesicles, ranging from 50–200 nm in diameter with an average size of 70 nm (*Giacomini et al., 2017*). In fact, in cases in which a pre-existing nanoparticle population was identified, it was orders of magnitude greater than the net increase contributed by the embryo during culture (*Vyas et al., 2019*; *Dissanayake et al., 2020*; *Simon et al., 2020*).

Extracellular vesicle and preimplantation embryo developmental competence

Embryo-derived exosomes are now believed to serve as paracrine factors and contribute to the 'group culture' effect, the observation that embryos of all species experience higher blastocyst rates after in-vitro culture when cultured in groups (*Lane and Gardner, 1992*; *Dai et al., 2012*). A significant increase in developmental capacity was seen when isolated vesicles were reintroduced into the culture media of both murine (*Kim et al., 2019*) and bovine zona-intact embryos (*Qu et al., 2017*; *Pavani et al., 2019*). Studies have sought to link extracellular embryonic extracellular vesicles to developmental competence, and data thus far have been promising. Media conditioned with day-2 and day-8 bovine embryos that later degenerated had significantly elevated extracellular vesicles compared with those that developed to the blastocyst stage with normal morphology (8.3×10^8 versus 5.9×10^8 and 7.2×10^8 versus 5.7×10^8 nanoparticles/ml, respectively) (*Dissanayake et al., 2020*). An initial clinical pilot experiment by *Abu-Halima et al. (2017)* suggested that significantly lower extracellular vesicle concentrations were present in media samples from single blastocyst transfers that led to a pregnancy compared with those that did not (3.8×10^9 versus 7.35×10^9 nanoparticles/ml, respectively). A subsequent study by *Pallinger et al. (2017)* confirmed this result and impressively revealed the predictive power of a DNA-containing extracellular vesicle subpopulation in correctly forecasting clinical pregnancy outcome after transfer of late-stage blastocysts that had otherwise indistinguishable embryo morphometrics (*Pallinger et al., 2017*). This study, conducted using conventional flow cytometry, was able to propose a 'cut-off' propidium iodide-positive (PI+) extracellular vesicles concentration of about 1×10^6 PI+ extracellular vesicles/ml; implantation outcome could be predicted with a sensitivity of 0.90 and a specificity of 0.86 depending on whether blastocyst conditioned media samples had more or less than this number of PI+ extracellular vesicle in the microdroplet. It is notable that *Pallinger et al. (2017*; *2018*) successfully analysed crude human and mouse blastocyst culture media by traditional flow cytometry without bead capture. The analysed population was an annexin V+ and PI+ subpopulation, suggesting externalized phosphatidylserine and DNA content, characteristics traditionally associated with an apoptotic subpopulation. This is reinforced by results from *Simon et al. (2020)* that showed about 16% of murine blastocyst derived extracellular vesicles contain DNA (*Simon et al., 2020*).

The connection between blastocyst developmental competence and the prevalence of extracellular vesicles in

spent culture media has now been established. Animal model studies independently concluded degenerate embryos release more extracellular vesicles alongside independent clinical claims connecting elevated extracellular vesicles to implantation failure. From a diagnostic perspective, extracellular vesicles are meeting the critical requirement that the extent of extracellular vesicle release from the blastocyst is more sensitive to its developmental competence than to any other uncontrollable variable, such as mild deviations in its developmental pace. Investigators have considerable instrumentation at their disposal to develop this connection further, including targeting of exosomal, microvesicle and apoptotic body subpopulations for protein, DNA and RNA characterization. We postulate artificial blastocyst cavity rupturing will improve extracellular vesicle detectability and invite the idea that a natural, foreboding collapse may even be the defining event that elevates extracellular vesicles in the media of compromised blastocysts. The avoidance of sample freezing and additional manipulation, such as zona pellucida removal, may further amplify these trends.

MicroRNA

Of the diverse extracellular vesicle cargo, encompassing major classes of cellular molecular constituents, including proteins, DNA and RNA (*Doyle and Wang, 2019*), preference towards a class of small RNA transcripts, known as microRNA (miRNA), has arisen in translational research owing to their suitability as biomarkers as a result of their enhanced stability and rapid detectability (*Kreth et al., 2018*). Extracellular miRNAs may exist encapsulated within any class of extracellular vesicles (*Sohel, 2016*), with increasing evidence supporting targeted packaging among the exosomal fraction (*Anand et al., 2019*). miRNA sequences are small (~22 nt) non-coding RNA sequences that serve to regulate post-transcriptional gene expression in animals, plants and viruses (*Rana, 2007*). Currently, an estimated 2300 true human miRNA sequences exist (*Alles et al., 2019*), many of which are highly conserved across species, including invertebrate model organisms (*Ibáñez-Ventoso et al., 2008*). About one-half of these sequences are expressed from proximal genes, referred to as clusters, and are predisposed to unified expression (*Kim and Nam, 2006*). miRNAs are processed to their mature form from much lengthier precursor transcripts and ultimately function by directing the RNA-induced silencing complex to initiate endonucleolytic cleavage of mRNA in a process known as RNA silencing (*Kim et al., 2009*). This complex comprises numerous proteins and includes the miRNA-primed endonuclease belonging to the Argonaute (Ago) protein family (*Kim et al., 2009*). Dependent only on the nominal requirement of a tolerant 7 nt complementary base pair match within the 3′ untranslated region of the mRNA, a single miRNA sequence may regulate hundreds of different mRNA targets; it is believed as many as one-third of human genes may be potential targets for miRNA (*Kim et al., 2009*). A single miRNA copy can initiate the cleavage of many copies of each mRNA, further underscoring the potency of this means of transcriptional regulation. miRNAs are transcribed ubiquitously across cell types, including embryonic stem cells, and in a cell- or tissue-specific manner (*The FANTOM Consortium et al., 2017*); in most cases, the top five miRNAs account for about one-half of the total miRNAs (*The FANTOM Consortium et al., 2017*; *O'Brien et al., 2018*). The extensive variety of miRNA expressed in a given cell or tissue has empowered comprehensive expression profiling, and has led to the realization that miRNA profiles may be significantly skewed as a result of status changes, including differentiation (*Kulcenty et al., 2019*) or pathology (*Peng and Croce, 2016*). Comprehensive miRNA profiling has enabled the prioritization of previously characterized cellular pathways based on their enrichment with proteins prone to transcriptional regulation according to the current expression profile. miRNAs are found extracellularly in many biofluids (*Weber et al., 2010*) and the conditioned culture media of many cell types, including differentiating stem cells (*Zhang et al., 2016*). It is most frequently bound to Argonaute proteins (*Turchinovich et al., 2011*), whereas encapsulation within extracellular vesicles, such as exosomes, microvesicles and apoptotic bodies, is less common (*Zhang et al., 2015*). miRNAs are detectable by polymerase chain reaction (PCR), offering rapid and highly sensitive detection in a matter of hours. miRNAs are resistant to degradation after days at room temperature (*Mall et al., 2013*), after numerous freeze–thaw cycles (*Matias-Garcia et al., 2020*) and even when total RNA integrity is compromised (*Jung et al., 2010*). Together, these attributes have led to intense investigation of their utility as non-invasive diagnostic biomarkers, spurring the detection of cancer (*Paranjape et al., 2009*), diabetes (*Jiménez-Lucena et al., 2018*), cardiovascular disease (*Zhou et al., 2018*) and other pathologies.

miRNAs in the preimplantation embryo

The miRNA presence within the preimplantation embryo represents sequence contributions from the oocyte and the spermatozoa, many of which are actively degraded by the two-cell stage (*Tang et al., 2007*), alongside concomitant zygotic expression that begins shortly after fertilization (*Yang et al., 2016*) and steadily increases towards the blastocyst stage (*Byrne and Warner, 2008*; *Yang et al., 2008*; *Ohnishi et al., 2010*; *García-López and del Mazo, 2012*; *Rosenbluth et al., 2013*; *Berg and Pfeffer, 2017*). By the blastocyst stage, as many as 135 mature miRNA sequences have confirmed expression in the human, many of which are embryonic-specific (*Houbaviy et al., 2003*; *Suh et al., 2004*; *Rosenbluth et al., 2013*) and span numerous predominant clusters including: miR-15a/16-1, miR-17-92, C19MC, miR-106a-363, miR-106b-25, miR-302-367 and miR-371-373 (*Houbaviy et al., 2003*; *Svoboda and Flemr, 2010*; *Battaglia et al., 2019*). In fact, the miR-290 cluster, homologues of the human miR-371-373 cluster, account for more than 60% of all miRNA sequences within murine embryonic stem cells (*Yuan et al., 2017*). miRNAs are highly conserved between species with homologous sequences and clusters bearing identical seed sequences, validating the use of animal model preimplantation embryos as informative alternatives to the limited human embryos available for research. Multiple reports have already confirmed distorted miRNA profiles within blastocysts derived from donors with a history of infertility (*McCallie et al., 2010*) and those with differing chromosomal status after PGT-A (*Rosenbluth et al., 2013*). Indeed, even male and female blastocysts likely have differentially expressed miRNAs (*Rosenbluth et al., 2013*).

miRNAs in the preimplantation embryonic secretome

Extracellular miRNAs are now confirmed within culture media microdroplets used

to condition human, cow and mouse embryos, as well as within the blastocyst cavity of human preimplantation embryos (TABLE 2). These reports will be covered within this subsection with focus on elements most relevant to supporting diagnostic application. Major revelations on the embryonic miRnome will be detailed according to the binary presence or absence of a particular sequence, the total number of unique mature sequences detected (referred henceforth as the variety) and differences in the concentration of each detectable sequence between compared groups. In all cases, only the mature forms of the miRNA sequences are discussed and no emphasis is placed on novel miRNA sequences that have been most recently proposed based on deep sequencing experiments and are yet to be accepted as bona-fide sequences.

Initial 'miRnome' studies did not detect a miRNA signature in the media (*Katz-Jaffe and McReynolds, 2013*); however, secretomic screening studies now consistently report at least several dozen detectable sequences, aided by preamplification technology and improved techniques. The miRnome variety strongly overlaps with that of the trophectoderm (*Capalbo et al., 2016*) and likely represents the entire inner cell mass and trophectoderm (*Cimadomo et al., 2019*). The total variety of miRNAs reported between studies has differed according to the analytical depth of each, i.e. from panels of single quantitative PCR assays to next-generation sequencing). The most strongly expressed blastocyst miRNAs are most abundant in the culture media and the blastocyst cavity, i.e. the miR-371-373 cluster (*Rosenbluth et al., 2013*; *Battaglia et al., 2019*; *Cimadomo et al., 2019*). These sequences have received the most attention, in addition to sequences put forth by early clinical reports that may correlate with ploidy status or implantation outcome: miR-20a (*Capalbo et al., 2016*), miR-30c (*Capalbo et al., 2016*), miR-142-3p (*Borges Jr. et al., 2016*) and miR-191 (*Rosenbluth et al., 2014*). Affirmatively, whole bovine blastocysts with a history of slow development underwent an approximate 18-fold increase in miR-30c and an approximate 30-fold increase in miR-10b expression compared with a faster cohort (*Lin et al., 2019a*). This was accompanied by an approximate 13-fold increase in miR-30c and an approximate 21-fold increase in miR-10b within their respective conditioned media microdroplets. As total miRNA expression increases within the embryo from the two-cell stage onwards (*Tang et al., 2007*), greater amounts of miRNA are found in the media: blastocysts released much more miRNA than earlier stages (*Capalbo et al., 2016*) and a 12-fold increase in miR-372 and 1.9-fold increase in miR-191 were seen in day-5 versus day-4 ICSI-inseminated embryo culture media samples (*Rosenbluth et al., 2014*). Conditioning time is also an important parameter, as media collected when human embryos reached the blastocyst stage on day 6/7 had significantly more miRNA than from faster developing blastocysts collected on day 5 (*Cimadomo et al., 2019*). If the media sampling occurred later on day 6/7, a significantly greater variety of miRNA panel members were detected (6.7) compared with media collected on day 5 (4.2).

Extracellular miRNA study design

Inter-study preimplantation miRnome comparisons must be made cautiously owing to the significant differences in experimental designs and data normalization. miRNA profiling depth differed significantly and included panels of quantitative reverse transcription PCR and digital droplet PCR-based assays, quantitative reverse transcription PCR arrays and, most recently, NGS (TABLE 2). The clinical studies interrogated media conditioned with a single fresh embryo either to the cleavage-stage on day 3 or to the blastocyst stage between days 5–7 before both biopsy and vitrification, with the exception of a single study that examined media conditioned with embryos that had been cryopreserved at the pronuclear stage (*Rosenbluth et al., 2014*). Culture conditions varied significantly across animal studies, i.e. media selection, additives, microdroplet sizes, embryo culture density and culture times. In all studies, human and animal models alike, data were presented as either unnormalized (*Gombos et al., 2019*), normalized to an exogeneous spike-in (*Kropp et al., 2014*; *Borges Jr. et al., 2016*; *Abu-Halima et al., 2017*; *Gross et al., 2017*; *Cimadomo et al., 2019*), normalized to an endogenous control: snU6 (*Rosenbluth et al., 2014*; *Cuman et al., 2015*; *Heidari et al., 2019*; *Lin et al., 2019a*), B2M (*Heidari et al., 2019*), SNORD96 (*Sánchez-Ribas et al., 2019*), GAPDH (*Kropp et al., 2014*), miR-16-2 (*Abu-Halima et al., 2017*) or miR-372 (*Battaglia et al., 2019*), or a combination of these strategies. The choice of normalization strategy may dictate the outcome of a secretomics study (*Abu-Halima et al., 2017*). Data normalization is a particularly troubling issue with preimplantation embryo secretomics studies for two reasons: selection of suitable endogenous controls is already an ongoing issue itself for preimplantation embryos (*Mamo et al., 2007*; *Jeong et al., 2014*; *Mahdipour et al., 2015*); and such controls may no longer be valid in an extracellular context owing to the additional complexity added by the release mechanism that may violate their status as experimentally insensitive variables. This may be especially true if the 'housekeeper' is located elsewhere than the cytosol, i.e. nuclear, where mature miRNA resides or, if the housekeeper is a separate molecular class entirely such as an mRNA or even protein. The appropriateness of the chosen normalization strategy is dependent on the importance of the release mechanism in defining the secretome, a truth that remains unknown.

Extracellular miRNAs from non-embryonic sources

In line with extracellular vesicle studies, an miRNA presence has been continuously reported within commercial conditioned media before embryo exposure, possibly originating from a number of sources, including natural supplements added to commercial media (*Kropp et al., 2014*; *Rosenbluth et al., 2014*; *Cuman et al., 2015*; *Kropp and Khatib, 2015*; *Capalbo et al., 2016*; *Sánchez-Ribas et al., 2019*). Persistent cumulus cells have plagued interpretation of extracellular genomic DNA collected during ongoing development of niPGT and efforts to retrieve mitochondrial DNA (*Hammond et al., 2017*). Cumulus cells also express miRNAs (*Andrei et al., 2019*), and it is foreseeable these cells may be similarly contributing to miRnome studies. Even an NGS analysis of such media has reported many extraembryonic miRNA sequences (*Sánchez-Ribas et al., 2019*). For clinical prospective studies using donated embryos, it may be worthwhile formulating media using a synthetic human serum albumin alternative to avoid the background that has complicated many of the miRnome studies (TABLE 2); similarly, animal research studies may wish to culture in medium

TABLE 2 STUDIES RESPONSIBLE FOR THE CURRENT KNOWLEDGE OF THE PREIMPLANTATION EMBRYO 'MIRNOME'

Reference	Species	Culture media purification method	Detection method	Developmental stage	Sample source	MicroRNAs identified, n	Significance of study
(*Sánchez-Ribas et al., 2019*)	Human	Centrifugation, filtration, miRNeasy Kit	NGS, single assays (2, qPCR w/ PreAmp)	C	CM	53	miR-181b-5p and miR-191-5p were most commonly detected; miR-191-5p was higher in euploid versus aneuploid embryo media before normalization to SNORD96; many miRNAs were also detected in control media.
(*Lin et al., 2019a*)	Bovine	miRNeasy Serum/Plasma Kit	NGS, single assays (5, qPCR)	B	CM	114	miR-30c and miR-10b elevated in slow versus intermediate cleaving embryos and their accompanying microdroplets; miR-10b elevated in degenerate versus blastocyst media.
(*Heidari et al., 2019*)	Mouse	None	Single assays (3, qPCR)	B	CM	3	Vitrified blastocysts showed reduced expression of miR-16-1 and miR-let-7a compared with fresh blastocysts, whereas, conversely, let-7a, miR-16-1 and miR-15a were upregulated in associated media samples at both the blastocyst and outgrowth stages.
(*Gombos et al., 2019*)	Human	miRNeasy Serum/Plasma Kit	Single assay (1, ddPCR	C	CM	1	miR-191-3p was the single sequence investigated; sequence was significantly higher in competent embryos than those leading to miscarriage.
(*Cimadomo et al., 2019*)	Human	miRCURY RNA Isolation Kit	Array (qPCR with PreAmp)	B	CM	29	Higher numbers of miRNAs from poor-quality CM; association with developmental competence was not determined.
(*Battaglia et al., 2019*)	Human	None	Array (qPCR with PreAmp), single assays (3, ddPCR)	B	BF	89	miRNA sequences found in BF; miR-372 was notably more abundant than other miRs.
(*Gross et al., 2017*)	Bovine	miRNeasy Serum/Plasma Kit	FC (FireFly Panel), single assays (3, qPCR)	B	CM	8	Eight miRNAs were differentially expressed between male and female CM; all were significantly up-regulated in female media: miR-15b, miR-16, miR-16-2, miR-17, miR-22, miR-30b, miR-122, miR-320a.
(*Abu-Halima et al., 2017*)	Human	miRNeasy Micro Kit	Microarray, single assays (qPCR)	B	CM	621	A total of 102 miRNA sequences were significantly different between pregnancy versus non-pregnancy groups with miR-29c-3p being the most different; miR-634 and miR-30c were highly expressed CM sequences; competent embryos have less miRNA variety in CM; 101 miRNAs overlapped with those found within spermatozoa.
(*Capalbo et al., 2016*)	Human	BC	Array (qPCR)	C, M, B	CM, TE	59	Blastocysts released the greatest amount of miRNA; 57 secretome sequences overlapped with those in the TE.
(*Borges Jr. et al., 2016*)	Human	None	Single assays (7, qPCR)	C	CM	4	miR-142-3p was significantly more expressed in CM from non-implantation versus implantation groups.
(*Kropp and Khatib, 2015*)	Bovine	miRNeasy Serum/Plasma Kit	miRNA-seq	B, E	CM	11	Media conditioned with degenerate embryos had higher concentrations of miRNAs; many miRNAs in control media were absent in CM.
(*Cuman et al., 2015*)	Human	miRCURY RNA Biofluids Isolation Kit	qPCR array, single assays (1, qPCR)	B	CM	47	A total of 19 sequences were exclusive to the implanted group, whereas 22 were exclusive to the non-implanted group; miR-661 was the highest differentially expressed sequence; 22 miRNAs were only present in control media.
(*Rosenbluth et al., 2014*)	Human	None	qPCR array	B	CM	10	miR-191-5p was 3.7-fold higher in CM conditioned with aneuploid versus euploid embryos.
(*Kropp et al., 2014*)	Human, Bovine	miRNeasy Serum/Plasma Kit	Single assays (6, qPCR)	B	CM	1	miR-25 was detected in CM; miR-196a2 in control CM miRNAs was decreased after embryo exposure.

B, blastocyst; BC, bead capture; BF, blastocyst fluid; C, cleavage-stage; CM, culture media; ddPCR, digital droplet polymerase chain reaction; E, expanded blastocyst; FC, flow cytometry; M, morula; NGS, next-generation sequencing; qPCR, quantitative polymerase chain reaction; TE, trophectoderm.

free of bovine serum albumin or in medium supplemented with a synthetic macromolecular alternative, such as polyvinylpyrrolidone (*Pavani et al., 2019*) or polyvinyl alcohol (*Biggers, 1997*). Spermatozoa are also carriers of mature miRs, some of which are critical for the initiation of preimplantation development (*Liu et al., 2012*), and it is conceivable that unsuccessful zona-bound spermatozoa may contribute to the secretome in non-ICSI studies, such as those reliant on IVF or animal studies using naturally fertilized oocytes. *Rosenbluth et al. (2014)* reported a lack of correlation with pregnancy outcome for ICSI-inseminated embryos that otherwise held for those regularly inseminated (*Rosenbluth et al., 2014*). It is, however, difficult to conclude whether this difference is attributable to accessory

spermatozoa or instead due to the physically damaging ICSI procedure itself causing leakage into the media; the two significant correlative miRNAs identified within this study, miR-191 and miR-372, were both elevated in ICSI versus non-ICSI blastocyst culture media.

Exogeneous miRNA uptake

The existence of miRNAs before the arrival of the embryo has not been completely detrimental, however, as it has led investigators to consider that embryos may also be taking up miRNAs. Pre-existing preimplantation embryonic miRNA levels drop within the media after exposure in a handful of studies (*Kropp et al., 2014*; *Rosenbluth et al., 2014*; *Kropp and Khatib, 2015*; *Lin et al., 2019b*), much like purported extracellular vesicle uptake (*Saadeldin et al., 2014*; *Vyas et al., 2019*). *Cuman et al. (2015)* reported a population of 22 control exclusive miRNAs when comparing controls with blastocyst conditioned media (*Cuman et al., 2015*). Kropp *et al*. reported miR-196a2 was detected at significantly reduced amounts in culture media after bovine blastocyst culture compared with controls (*Kropp et al., 2014*). In a follow-up study, *Kropp and Khatib (2015)* reported that eight pre-existing miRNAs were downregulated in groups with blastocysts compared with only two in groups with degenerate embryos (*Kropp and Khatib, 2015*). *Rosenbluth et al. (2014)* reported that a single pre-existing sequence, miR-645, was downregulated to a greater extent in media exposed to embryos with confirmed implantation success than those that failed; in fact, a six-fold increase in this sequence was associated with implantation failure for non-ICSI blastocysts (*Rosenbluth et al., 2014*). It is possible that healthier embryos absorb more miRNA. miRNA uptake could, in part, be due to the miRNAs existing in an encapsulated state; however, studies introducing unprotected exogeneous miRNAs into the culture media of bovine preimplantation embryos (*Kropp and Khatib, 2015*; *Lin et al., 2019a*; *2019b*) and trophoblast cell lines (*Cuman et al., 2015*) have also reported uptake. Neither miR-30c or miR-10b media supplementation affected bovine blastocyst development from the zygote stage (*Lin et al., 2019a*; *2019b*), whereas miR-24 supplementation significantly reduced blastocyst development from 75.6% to 48.3% when introduced at the morula-stage (*Kropp and Khatib, 2015*).

Unravelling the mechanism of miRNA release has been approached using trophoblast cell lines and has received little attention from studies using whole embryos that instead have focused on correlating miRNA profiles with embryo quality. *Cuman et al. (2015)* used a trophoblast cell line to condition media and characterized the presence of miR-661 (a sequence identified previously within this study from a media study using whole human preimplantation embryos). The results showed that most miR-661 was protein-bound to Ago2, with the balance likely being encapsulated. This conclusion agrees with the observation that two sequences of interest (miR-181b-5p and miR-191-5p) were present within a high molecular weight protein fraction of the embryo conditioned media (*Sánchez-Ribas et al., 2019*).

miRNA presence in the blastocyst cavity

miRNA profiling of the human blastocyst cavity fluid identified 89 miRNA sequences after sampling nine human blastocysts, representative of most of the known miRNA clusters expressed at this stage (*Battaglia et al., 2019*). Among the most prevalent miRNAs, miR-372 of the miR-371-373 cluster is a sequence with similarly high expression at the blastocyst stage (*Rosenbluth et al., 2013*) and is regarded as one of the most prevalent miRNAs comprising the extracellular secretome (*Rosenbluth et al., 2014*; *Cimadomo et al., 2019*). Similar high expression of the homologous miR-290-295 cluster is seen in later stages of preimplantation development in numerous model organisms (*Yang et al., 2008*; *Maraghechi et al., 2013*). *Battaglia et al. (2019)* recognized that 80% of these sequences were previously categorized as 'exo-miRs' (sequences with a predisposition for exosomal packaging) and 40 overlapped with those observed in spent blastocyst media previously (*Capalbo et al., 2016*). When considered along with the observation of exosomes and other extracellular vesicles within the blastocyst cavity of multiple species, the possibility for these sequences to be encapsulated warrants further investigation.

Extracellular miRNAs and preimplantation embryo developmental competence

Clinical studies have sought to correlate the miRnome with pregnancy outcomes after transfer and association with ploidy status according to PGT-A data, whereas studies involving animal models have sought correlation with morphology, developmental pace, ability to form outgrowths (*Heidari et al., 2019*) and cryopreservation (**TABLE 2**). Ailing blastocysts presenting degenerating morphology confirmed that implantation failure or even a history of vitrification release greater amounts and variety of miRNA sequences into the spent culture medium; however, the correlation with ploidy status remains unclear. miR-16-1 and let-7a, despite being downregulated in whole vitrified murine blastocysts compared with fresh blastocyst controls (~5-fold and ~1.5-fold, respectively), showed an increase in the concentration of both sequences (~16-fold each) in the conditioned medium (*Heidari et al., 2019*). Given the transcriptional overlap between the secretome and the trophectoderm and even whole embryo, this may suggest that vitrified embryos have a much more active release mechanism, i.e. apoptosis, and that other sequences may have been similarly elevated. Comparing euploid and aneuploid blastocyst conditioned media, *Rosenbluth et al. (2014)* detected a 4.7-fold increase in miR-191-5p within the aneuploid media; however, *Sánchez-Ribas et al. (2019)* did not detect any significant differences.

Abu-Halima et al. (2017) used implantation success as the metric of developmental competence, and reported that media samples from blastocysts leading to implantation failure had an average of 163 different miRNAs, whereas those that led to pregnancy had 149 (*Abu-Halima et al., 2017*). *Cuman et al. (2015)* observed a 19-miRNA exclusive subset within media of day-5 blastocysts that led to an ongoing pregnancy and a 22-miRNA exclusive subset within the non-implanted group. A single, highly expressed miRNA sequence (miR-661) was repeatedly expressed exclusively and at significant levels in the media of five non-implanted, individually cultured blastocysts. Furthermore, miR-372 was only observed within media of embryos that failed to implant (*Cuman et al., 2015*). *Rosenbluth et al. (2014)* reported a significant increase in U6 normalized miR-191-5p expression in media of aneuploid embryos compared with those that were euploid (*Rosenbluth et al., 2014*); additionally, a 7.1-fold increase in miR-372 and a 5.1-fold increase in miR-191-5p were seen in

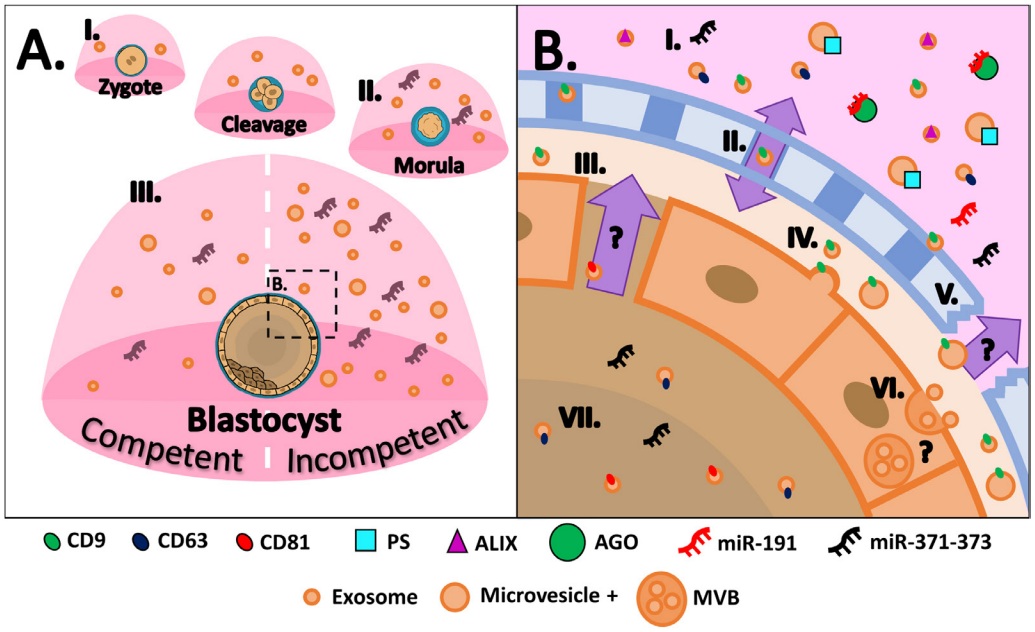

FIGURE 1 Current view of the extracellular vesicle and microRNA (miRNA) contributions to the human preimplantation embryo secretome (conceptual, not to scale). (A) Trends in the secretomic extracellular vesicles and miRNA presence throughout preimplantation development and their known association with developmental competence; (I) preimplantation embryos release extracellular vesicles at all stages of development; (II) culture media extracellular vesicles and miR-372 are increasing towards the blastocyst stage; (III) competent embryos release fewer extracellular vesicles and fewer miR-371-373 sequences; (B) known presence of extracellular vesicles and miRNAs within the blastocyst cavity, perivitelline space, zona pellucida and secretome, including possible major release mechanisms; (I) blastocyst-conditioned media contains extracellular vesicles positive for tetraspanins CD9 and CD63, in addition to a secondary, larger vesicle population that may be microvesicles or apoptotic bodies. Competence-associated miR-191 and miR-371-373 sequences are prevalent and miR-191 is believed to be bound to a >100 kDa protein, possibly to Argonaute; (II) exosomes have bidirectional movement across the zona pellucid; (III) natural breakdown of trophectoderm and rupture of blastocyst cavity may release contents into perivitelline space and eventually culture media; (IV) CD9+ vesicles are present within the perivitelline space; (V) breach of the zona pellucida during blastocyst hatching may be responsible for the observation of media extracellular vesicles >200 nm in diameter; (VI) possible exosome release from fused multivesicular bodies based on their confirmed presence within the murine trophectoderm; (VII) blastocyst cavity contains exosomes that are CD63+ and CD81+, in addition to prevalent miRNA sequences from the miR-371-373 cluster. The presence of multiple tetraspanins per vesicle is possible and not depicted. AGO, Argonaute protein; PS, phosphatidyl serine.

media from failed non-ICSI blastocyst transfers compared with those that led to a pregnancy (*Rosenbluth et al., 2014*). The link between developmental competence and miR-191-5p is agreed upon; in contrast, direct counting of miR-191-5p without normalization to an endogeneous housekeeper was shown to be significantly higher in media from euploid versus aneuploid embryos (*Sánchez-Ribas et al., 2019*). A study dedicated to resolving miR-191-3p media expression (the complementary mature sequence to miR-191-5p) reported an approximate 10-fold higher non-normalized expression in media of competent blastocysts leading to a live birth (*Gombos et al., 2019*). Three other sequences displayed a significantly different detection rate (present versus not present) between not pregnant versus pregnant: miR-182-5p, miR-302-3p, miR-519d-3p; in all cases, these sequences were more often found in media from non-pregnant embryos (*Cimadomo et al., 2019*). In this study, each of the 10-sequence panel of miRNA sequences were more abundant in the non-pregnant media and four reached significance: miR-302a-3p, miR-372-3p, miR-373-3p, miR-518a-3p; however, none of these were significant after accounting for the confounding variable that the non-pregnancy media group contained more day 6/7 blastocyst samples than day 5 samples. The increased conditioning time required for the blastocysts of the non-pregnant group may be the reason for this. Contrary to this study, miR-20a and miR-30c were both higher in media of euploid implanted versus non-implanted blastocysts (*Capalbo et al., 2016*). Comparison of media from poor-quality euploid blastocysts with confirmed implantation failure with that conditioned with day-matched controls, the mean average number of miRNAs detected on a 10-miR panel was significantly higher (8.5 versus 6.7) in the poor-quality media. Additionally, the media from these substandard embryos contained a significantly elevated amount of miR-373-3p than the day-matched blastocyst control media (*Cimadomo et al., 2019*).

In an attempt to establish a correlation with morphometrics, Lin *et al*. (2019) probed media conditioned with either slow-cleaving, degenerate-containing bovine embryo cohorts or more moderately paced groups of embryos with less degenerate embryos for select sequences (*Lin et al., 2019a*). Medium conditioned with degenerate embryos had a nearly three-fold increase in miR-10b and a 30-fold increase in miR-30c compared with medium conditioned by healthy looking blastocysts. *Kropp and Khatib (2015)* conducted miRNA-sequencing analysis on media conditioned with bovine embryos from the morula to blastocyst stage and pooled according to whether normal or degenerative development occurred. Three miRNA sequences showed significant differential expression, each of which were higher in the degenerate

media: miR-24, miR-191 and miR-148a. miRNAs are undoubtedly present in the spent embryo conditioned media. It is also clear that embryos of different status, including sex (*Rosenbluth et al., 2013*; *Gross et al., 2017*), have different intrinsic and extracellular miRNA profiles, a promising first step towards their use as biomarkers. The blastocyst cavity is an equally viable source of miRNA and is also enriched for highly expressed blastocyst sequences. While undesirable, the presence of pre-existing miRNAs within commercial media may not pose a significant obstacle to embryo diagnostics given the availability of embryo-specific miRNAs transcribed and released by the blastocyst. Embryos exhibiting degeneracy or with a history of vitrification are releasing a greater variety and amounts of miRNAs into the culture media. Differences in miRNA release between euploid and aneuploid embryos exist, yet a decisive correlation has yet to be found. Despite overlap in some differentially expressed media sequences between studies, no individual sequences have yet been repeatedly identified across multiple independent studies that hold adequate predictive power to forecast a single blastocyst transfer outcome. Currently, the abundant miR-371-373 cluster and miR-191 are the most promising prospects for such a correlation with developmental competence. Maintenance of consistent culture conditions between embryos both intra- and interstudy are critical owing to the effect of conditioning time on the miRnome. Variability in embryo developmental pace and the rapidly increasing miRNA production as development moves towards the blastocyst stage are other variables that must be managed, along with additional factors such as fertilization method and cryopreservation that seem to be having an effect. Moving forward, it would be beneficial to establish a common normalization strategy for extracellular sequences and supplement datasets with non-standardized data until the release mechanisms are better understood.

In conclusion, extracellular vesicles are proving to be a robust metric of embryonic health. Multi-study agreement exists that embryos exhibiting degeneracy and with confirmed implantation failure are shedding greater numbers of vesicles into the culture media. The potential for developing a diagnostic tool based on an assessment of the blastocyst's extracellular vesicle secretome is especially promising, as subpopulations bearing distinct markers and cargo are only just being explored. The picture is not yet so clear for microRNA. MicroRNA studies have been faced with the arguably greater challenge of reliably snapshotting a complex network of regulators that are being actively transcribed with ongoing development. Although some correlation between the amplitude of the miRnome with aneuploidy and degeneracy is agreed upon, the miRnome currently seems equally or more sensitive to environmental variables. The primacy of ending culture when a fully developed blastocyst is ready for transfer is an example of an uncontrollable environmental variable that seems capable of impeding reliable miRnome profiling. A lack of consensus about the appropriate control variables further clouds interstudy comparison. The sheltered blastocyst cavity may prove to be a solution to some of these issues. The currently conflicting results, particularly regarding miR-191, may shift to overwhelming agreement once the most clinic-specific environmental variables are better understood and controlled.

FIG 1.

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