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REVIEW ARTICLE

Exploring the potential of in vitro extracellular vesicle generation in reproductive biology

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Abstract

The interest in the growing field of extracellular vesicle (EV) research highlights their significance in intercellular signalling and the selective transfer of biological information between donor and recipient cells. EV studies have provided valuable insights into intercellular communication mechanisms, signal identification and their involvement in disease states, offering potential avenues for manipulating pathological conditions, detecting biomarkers and developing drug-delivery systems. While our understanding of EV functions in reproductive tissues has significantly progressed, exploring their potential as biomarkers for infertility, therapeutic interventions and enhancements in assisted reproductive technologies remains to be investigated. This knowledge gap stems partly from the difficulties associated with large-scale EV production relevant to clinical applications. Most existing studies on EV production rely on conventional 2D cell culture systems, characterized by suboptimal EV yields and a failure to replicate in vivo conditions. This results in the generation of EVs that differ from their in vivo counterparts. Hence, this review firstly delves into the importance of EVs in reproduction to then expand on current techniques for in vitro EV production, specifically examining diverse methods of culture and the potential of bioengineering technologies to establish innovative systems for enhanced EV production.

KEYWORDS
bioengineering, improved EV yield, in vitro EV production

1 | INTRODUCTION

EVs are a diverse group of membrane-bound structures that play crucial roles in intercellular communication and are involved in various biological processes. They can be classified into several subtypes based on their size and biogenesis as depicted in Box 1. EVs have been detected in almost all reproductive tissues such as the testis (Stewart et al., 2019), epididymis (Yanagimachi et al., 1985), uterus (Burns et al., 2016), ovarian follicles (de Ávila & da Silveira, 2019) and oviductal fluid (Almiñana et al., 2018). Growing evidence highlights the crucial role of EVs in facilitating functional modifications in recipient cells, making them significant players in intercellular communication within tissues and organs. Female reproduction, being intricately regulated by endocrine processes, may be influenced by EV-mediated communication, adding an additional layer to the complex regulatory network. As the content and function of EVs are specific to their donor cells and influenced by environmental factors, the roles of EVs in different female reproductive tissues are likely to vary.

In recent years EV research has gained considerable interest for their potential as biomarkers for diseases or as biocompatible delivery vehicles. However, for further research of EVs and their potential use in the clinic, more efficient systems that allow

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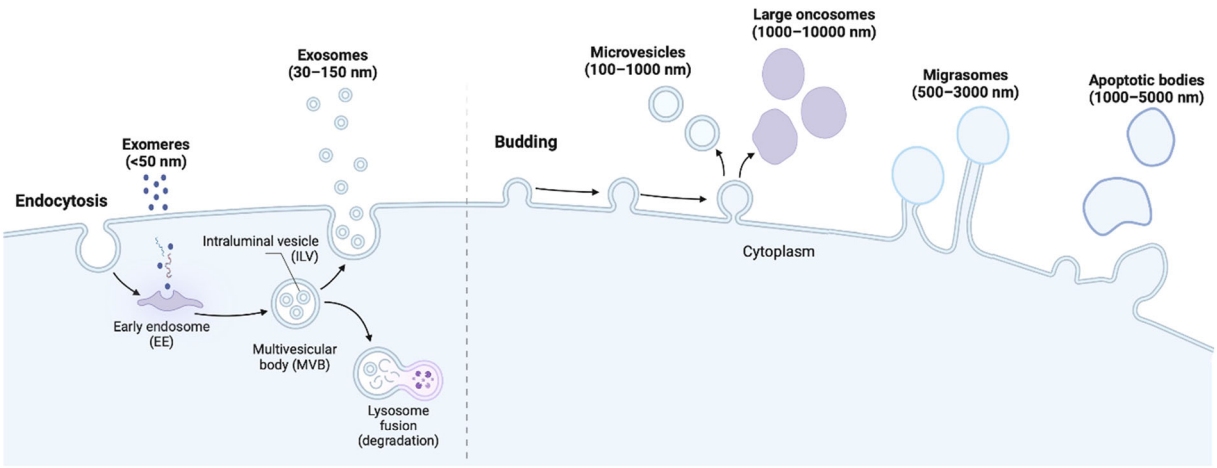

Box 1: Biogenesis of EVs

Extracellular vesicles (EVs) represent a diverse group of membrane-bound structures crucial for intercellular communication and various biological processes. They can be classified into several subtypes based on their size and biogenesis, as shown in the figure above. Exosomes are formed within multivesicular bodies (MVBs) as intraluminal vesicles (ILVs) (Parra et al., 2023). Upon MVB fusion with either lysosome for degradation or the plasma membrane for release, they are shed into the extracellular space (Machtinger et al., 2016). Ectosomes (such as microvesicles) are generated through outward budding from the plasma membrane and are subsequently released into the extracellular environment (Di Pietro, 2016). Apoptotic bodies represent a larger class of EVs that are released specifically during apoptotic cell death and contain molecular signals that attract phagocytes, promoting the clearance of apoptotic cells (Rodgers & Irving-Rodgers, 2010). In addition, other less-characterized subtypes include exomeres, migrasomes and large oncosomes. Exomeres are unique non-membranous nanoparticles, enriched in lipids, nucleic acids, amyloid precursor protein and metabolic enzymes, suggesting a potential role as molecular aggregates (Hennet & Combelles, 2012). Moreover, migrasomes, as implied by their name, are released by migrating cells and their formation is dependent on cell migration and actin polymerization (Almiñana & Bauersachs, 2020). The figure was adapted from Carver and Yang (2016) and Santonocito et al. (2014) and was made using Biorender.

large scale production of EVs are needed. Conventional 2D cell culture methods not only have a low yield but also have been shown to produce EVs that differ from in vivo-produced EVs in terms of their content (Rocha et al., 2018). For upscaled EV production, supplementation of media, mechanical stimulation of culture systems or a number of bioengineering technologies for different culture methods such as bioreactors and 3D culture systems are currently in use (Cao et al., 2020; Lim et al., 2023). Using bioengineering technologies has the potential to improve EV production as well as producing in vitro-derived EVs that better resemble their in vivo counterparts. Considering the impact of external factors on gamete and embryo development, there is a significant potential for the use of bioengineering technologies to study reproductive EVs and their importance in reproductive events.

Although seminal plasma-derived EVs are also present in the female reproductive tract and influence sperm function and therefore embryo production (Machtinger et al., 2016; Parra et al., 2023), the focus of the following review is mainly on EVs produced by female reproductive organs, oocytes and pre-implantation embryos. Therefore, in the initial section of this review, we examine the role of EVs in female reproductive tissues and their importance in crucial reproductive processes such as oocyte maturation, fertilization, sperm interaction, early embryo communication and implantation, as illustrated in Figure 1. Following that, we provide an overview of various techniques employed for in vitro EV production. Finally, we focus on bioengineering technologies that have the potential to enhance in vitro EV production.

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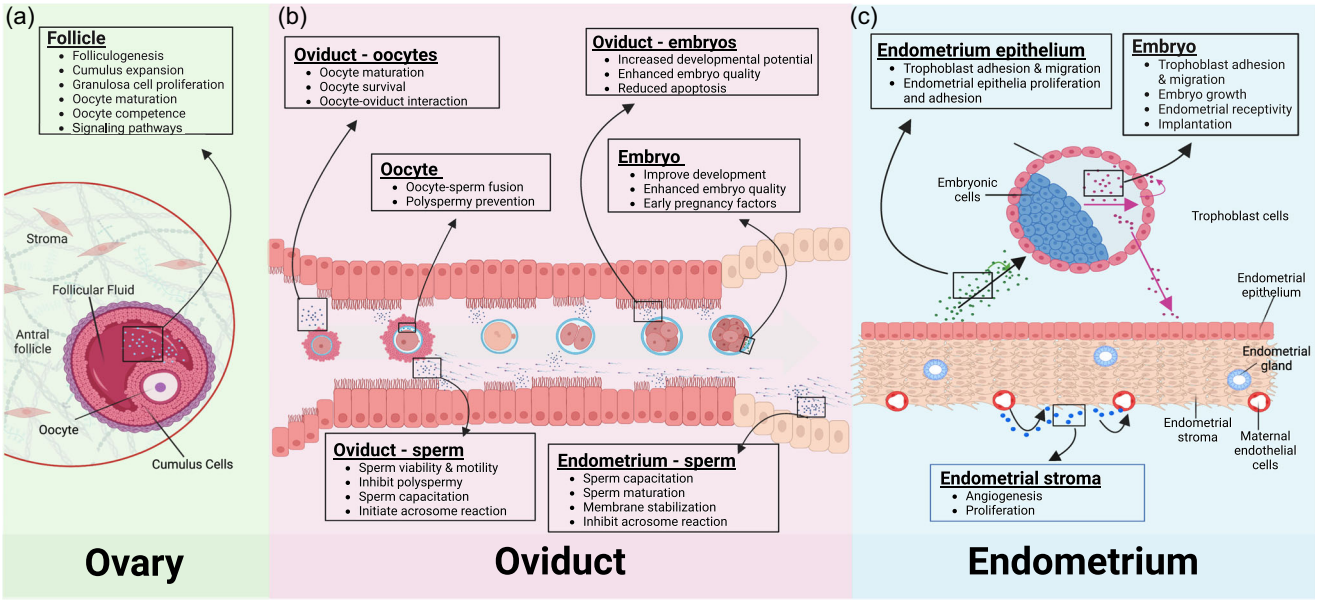

FIGURE 1 Roles of EVs in different female reproductive tissues. EVs differ in their molecular content and role in biological processes based on cell of origin and its environment. Hence, EVs produced in different reproductive tissues also have different roles influencing important reproductive events. In the ovary (a), follicular EVs are involved in supporting the progression of folliculogenesis, oocyte maturation and in several signalling pathways that reinforces oocyte competence. In the oviduct (b), EVs from oviductal cells, oocytes, sperm and embryos are present, driving key reproductive events such as oocyte maturation, sperm capacitation and early embryo development. Endometrial Evs (c) and their interaction with embryonic EVs are crucial for endometrial receptivity, trophoblast adhesion and angiogenesis. Figure was made using Biorender, adapted from Kurian and Modi (2019) and Qamar et al. (2020).

2 | THE ROLE OF EVS IN FEMALE REPRODUCTION

2.1 | EVs in the ovaries

Oogenesis takes place in the ovaries and depends on the interactions between oocytes and surrounding somatic cells. This crucial communication is established through gap-junctions, paracrine factors and follicular fluid (FF) components accumulated during follicle maturation (Di Pietro, 2016). FF contains various plasma components which are essential for the secretory activity of follicular somatic cells and oocyte growth and maturation (Hennet & Combelles, 2012; Rodgers & Irving-Rodgers, 2010). Recent studies have characterized EVs within FF, revealing their involvement in regulating pathways related to follicular growth, hormone response, oocyte maturation and meiosis resumption (Almiñana & Bauersachs, 2020; de Ávila & da Silveira, 2019; Tan et al., 2020).

Studies suggest that FF EVs can indirectly impact oocyte competence by improving cumulus cell function (Figure 1a) (Hung et al., 2017). In bovine and murine models, FF EVs promoted cumulus expansion and altered the mRNA expression of genes, such as prostaglandin-endoperoxide synthase 2 (PTGS2) and pentraxin 3 (PTX3) (Hung et al., 2015, 2017). Furthermore, FF EV-miRNAs have been shown to target genes involved in important pathways, including WNT, TGF-β, MAPK, neurotrophin, epidermal growth factor receptor and ubiquitin-mediated pathways, which are critical for various aspects of follicular development and oocyte competence (da Silveira et al., 2012; Santonocito et al., 2014; Sohel et al., 2013).

Notably, the cargo of follicular EVs changes throughout follicular development, and their content can be influenced by environmental factors such as stress. Under stress conditions, the secretion of EVs that enhance the defence system and prevention of cell death is induced (Carver & Yang, 2016). Moreover, the protective nature of follicular fluid EVs against oxidative stress has been demonstrated (Eldh et al., 2010). These findings highlight the dynamic and responsive nature of EVs in the follicular microenvironment.

2.2 | EVs derived from the oviduct

The mammalian oviduct, known as the fallopian tube in humans, plays a critical role in female reproduction by connecting the ovary to the uterus and facilitating the journey of the oocyte and embryo. It is divided into four regions: the infundibulum, ampulla, isthmus and utero-tubal junction. The infundibulum captures the released oocyte, while the ampulla is the site of fertilization. Subsequently, the preimplantation embryo undergoes significant development in the isthmus before passing

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through the utero-tubal junction to enter the uterus for implantation (Croxatto, 2002). In this context, oviductal EVs (oEVs) have emerged as essential mediators of communication between cells and gametes within the oviduct, and the cargo content of oEVs in various species contains miRNAs targeting pathways essential for oocyte viability and early embryonic development (Capra & Lange-Consiglio, 2020). Oviductal and oocyte-derived EVs aid the survival and maintenance of gametes in preparation for fertilization by supporting oocyte maturation and survival. When co-cultured with oviductal epithelial cells, oEVs promote optimal metaphase II oocyte maturation in dogs (which take place in the oviduct) (Franchi et al., 2020). The interaction between oEVs with embryos coupled with embryo-derived EVs have been shown to improve embryo quality and help with the release of early pregnancy factors (Figure 1b) (Alcântara-Neto et al., 2020).

During the pre-fertilization period, spermatozoa interact with oviductal cells and oEVs, undergoing capacitation and hyperactivation processes regulated by intracellular Ca^{2+} levels (Jin et al., 2011). Proteomic analysis of oEVs has revealed proteins involved in regulating sperm function and fertilization (Al-Dossary et al., 2013). Incubation of oEVs in in vitro culture systems has demonstrated improvements in sperm motility, fertilization rate and prevention of premature acrosomal reactions and polyspermy in several species (Alcântara-Neto et al., 2020; de Almeida Monteiro Melo Ferraz et al., 2020; Ferraz et al., 2019). As the fertilization site, the oviduct is crucial for embryo-maternal communication during early embryo development, leading to improved embryo quality and successful pregnancy (Wolf et al., 2003). Almiñana et al. (2017) demonstrated that in vivo-derived bovine oEVs could pass through the zona pellucida to be taken up into the cytoplasm of blastocysts, resulting in improved competence. Similarly, improved development, quality and cryo-tolerance of in vitro-produced embryos were observed upon supplementation with oEVs from both in vivo and in vitro conditions (Lopera-Vásquez et al., 2016, 2017). The effects of oEVs on early embryo development are attributed to specific miRNAs and mRNAs present in oEV samples from various species [mice (Fereshteh et al., 2018), chicken (Huang et al., 2017), cow (Almiñana et al., 2018) and human (Li et al., 2023)].

Notably, oEVs produced in different sections of the oviduct have distinct molecular cargos and physiological functions. For instance, oEVs from the isthmus have been found to increase embryo development to the blastocyst stage (Lopera-Vasquez et al., 2017), while those from the ampulla promote sperm intracellular Ca^{2+} levels for capacitation (Franchi et al., 2020). In summary, oEVs play critical roles in female reproduction, mediating essential processes such as oocyte maturation, fertilization and early embryo development. Their spatiotemporal regulation within different oviductal regions provides unique biological actions tailored for specific reproductive functions. Understanding the roles and molecular contents of oEVs is vital for advancing our knowledge of female reproduction and enhancing fertility-related therapies.

2.3 | EVs derived from the endometrium and placenta

The endometrium plays a pivotal role in female reproduction as the site of conceptus development and embryo implantation. Communication between the endometrium and conceptus is vital for successful implantation and placental formation. EVs derived from the endometrium (eEVs) have been extensively studied in various mammals, including sheep (Burns et al., 2016, 2018), human (Greening et al., 2016; Ng et al., 2013) and cattle (Kusama et al., 2018). Epithelial and stromal cell layers of the endometrium secrete distinct EVs that support trophoblast adhesion and migration as well as angiogenesis for a successful pregnancy (Figure 1c). The cargo contents of eEVs exhibit cyclic regulation during oestrous/menstrual cycles and adapt in response to a successful pregnancy (Kurian & Modi, 2019; Qamar et al., 2020), making them important regulators of endometrial receptivity and embryo implantation (Burns et al., 2018). The cargo contents of eEVs exhibit cyclic regulation during oestrous/menstrual cycles and adapt in response to a successful pregnancy (Greening et al., 2016; Ng et al., 2013). Notably, bovine eEVs from different oestrus stages showed varying protein abundances, with preimplantation stage eEVs having a higher number of proteins associated with cell apoptosis, while post-implantation stage eEVs had increased abundance of proteins involved in cell adhesion (Kusama et al., 2018). These findings are consistent with the physiological changes in the endometrial epithelium. Interestingly, treatment of epithelial cells with preimplantation stage eEVs activated apoptotic pathways, whereas post-implantation stage eEVs activated genes associated with cell adhesion, indicating a paracrine effect of eEVs in regulating receptivity and implantation (Kusama et al., 2018). eEVs have also been detected in trophectoderm cells of the conceptus in ewes, suggesting their involvement in embryo-maternal crosstalk (Burns et al., 2016). Studies have demonstrated that treatment with eEVs enhances adhesive behaviour in mouse blastocysts, human trophoblast cell lines and trophectodermal spheroids (Evans et al., 2019; Gurung et al., 2020; Vilella et al., 2015).

Following implantation, the placenta emerges as an important player, providing endocrine, nutritional and oxygen support crucial for foetal development. This underscores the significance of the placenta as a communication hub between the developing foetus and the mother (Adam et al., 2017; Tannetta et al., 2017). Placental EVs have garnered considerable attention in both normal physiological states and pathological conditions, significantly impacting placental function (Bidarimath et al., 2017). Previous research indicates that these EVs actively participate in processes such as angiogenesis within the trophectoderm. They stimulate the expansion of the cytotrophoblast layer into finger-like projections within the endometrium, while also inducing proliferation of maternal endothelial cells (Atay et al., 1989). As pregnancy advances, concentrations of exosomes and EVs released

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by the placenta increase, particularly in response to environmental cues like low oxygen tension and glucose concentrations. Such escalations further show their role as signalling mediators between the foetus and the mother (Adam et al., 2017).

Evidently, alterations in EV concentration, content and bioactivity are associated with various pregnancy complications, including gestational diabetes mellitus (GDM), pre-eclampsia (PE), foetal growth restriction (FGR) and preterm birth (PTB) (Nakahara et al., 2020). Notably, in conditions like GDM and PE, there's a marked increase in placental EVs detected in maternal circulation. Studies have revealed that placenta-derived exosomes contribute to insulin resistance and decreased glucose uptake in skeletal muscles, exacerbating GDM (Nair et al., 1979). In the case of PE, placental EV populations exhibit pro-inflammatory, anti-angiogenic and procoagulant activities, potentially leading to systemic inflammation, endothelial dysfunction and activation of the clotting system (Palma et al., 2021). Additionally, significant alterations in protein profiles within placenta-derived EVs from maternal serum have been observed in PTB (Menon et al., 2020).

In summary, both endometrial and placental EVs play pivotal roles across various developmental stages, including endometrial receptivity, implantation, trophoblast invasion and immune modulation. The identification of changes in protein content within placental EVs positions them as promising biomarkers for identifying pregnancy complications, offering potential avenues for therapeutic interventions. Their significance in facilitating embryo-maternal communication and supporting successful reproduction underscores the importance of further research into these EVs for potential therapeutic and diagnostic targets.

2.4 | EVs derived from the embryo

As discussed, effective communication between the maternal environment and the embryo is essential for a successful pregnancy. Embryo-derived EVs are believed to play a crucial role in early embryo-maternal interactions, communication between different embryos, and further embryo development (Almiñana & Bauersachs, 2020). Studies have shown that concentrations of embryo-derived EVs from humans increase with developmental stage (Dominguez et al., 2014; Vyas et al., 2019) and can vary based on factors such as embryo sex and culture conditions in bovine embryos (Capalbo et al., 2016). Moreover, the miRNA profiles of embryonic EVs are associated with their implantation potential, with certain miRNAs linked to endometrial cell proliferation being more abundant in media from implanted human blastocysts compared to non-implanted ones (Bridi et al., 2021). These observations highlight the sensitivity and specificity of embryonic EV concentration and content in response to external conditions.

In vitro culture of embryos provides an opportunity to collect and analyze EVs released into the media. EVs isolated from spent media of bovine and human embryos have been found to be enriched in miRNAs related to pluripotency genes (Giacomini et al., 2017; Mellisho et al., 2017). Embryonic stem cells derived from the inner cell mass have also been found to release microvesicles that promote trophoblast migration when taken up by the trophectoderm (Desrochers et al., 2016). These findings suggest that embryonic EVs act in an autocrine/paracrine manner, influencing blastocyst quality, development and aiding in implantation. Embryonic EVs may also interact with maternal endometrial cells to facilitate successful implantation. Studies have demonstrated that dye-labelled embryonic EVs can interact with endometrial epithelial and stromal cells both in vivo (Burns et al., 2016) and in vitro (Giacomini et al., 2017). EVs and/or miRNAs secreted by embryos can target and alter cellular activities such as adhesion and migration, indicating their influence in implantation (Capalbo et al., 2016).

Embryo-derived EVs are also implicated in communicating with the maternal immune system (Giacomini et al., 2019; Montecalvo et al., 1950). Studies have shown upregulated genes in bovine oviductal epithelial cells in response to supplementation with good-quality embryos, particularly those belonging to the interferon-stimulated genes or interferon tau pathway (Dissanayake et al., 2021). These observations collectively emphasize the vital role of embryo-derived EVs in orchestrating early embryo-maternal interactions, implantation and embryo development, making them significant targets for further investigation and potential therapeutic applications.

3 | IN VITRO PRODUCTION OF EVS

The dynamic milieu within female reproductive tissues coordinates molecular interactions crucial for reproductive events. EVs emerge as key players in this complex balance, acting as carriers of bioactive molecules that influence critical cellular processes. When identified, such molecules within reproductive EVs could be used for improving current assisted reproductive technologies (ART). As our understanding of various roles of EVs expands, there arises a need for innovative approaches to enhance their in vitro production. The challenge lies in replicating the complex interplay of biochemical and biophysical cues that govern EV production in vivo. In order to bridge this gap between in vitro and in vivo conditions, it becomes essential to explore and employ innovative methods to enhance the yield and quality of EVs.

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3.1 | Potential uses of EVs in reproductive medicine

EVs have emerged as promising tools in reproductive medicine, offering new avenues for diagnostic tools, biomarker discovery and therapeutic intervention, particularly within ARTs. Studies focusing on reproductive EVs and their possible use to improve ART and other pregnancy/reproductive tissue-related conditions, are listed in Table 1. As EV cargo is dependent on the donor cell and its environment, EVs have great potential as non-invasive diagnostic tools for many pregnancy and other gynaecological conditions, also highlighted in Table 1. Bearing in mind the interactions of reproduction EVs in gamete development and maturation, certain biomarkers could be used for selecting good-quality oocytes by analysing spent in vitro culture media (Lange-Consiglio et al., 2017). Similarly, oviductal EVs are involved in sperm capacitation and their fertilizing ability. Supplementation of sperm washing or freezing solutions with factors found to affect this process could improve recovery of frozen-thawed sperm or improve sperm quality through priming before fertilization (Ferraz et al., 2019). This would also allow minimizing avoiding intra-cytoplasmic sperm injection (ICSI), a complex and sensitive procedure, and perform traditional IVF, decreasing the amount of manual handling and human error.

EVs secreted by pre-implantation embryos and uterine epithelium could serve as valuable, minimally invasive alternatives to traditional biopsies as diagnostic tools. Notably, EVs, with their ability to encapsulate the cellular heterogeneity of complex tissues more comprehensively than conventional biopsies, offer deeper insights into the endometrium or developing embryos (Abu-Halima et al., 2017; Simon et al., 2020). Moreover, their cargo of diverse co-expressed molecules enables the monitoring of qualitative and quantitative changes, facilitating a higher degree of personalization within ART practices.

IVF embryos often exhibit lower quality compared to in vivo counterparts, leading to reduced implantation and developmental success rates. Current IVF media attempt to compensate for the absence of physiological interactions in the oviduct environment but remain largely unchanged from formulations developed over five decades ago. The emerging recognition of EVs produced by male and female reproductive tracts and their effects on gametes and embryo development offers new possibilities. EVs could complement existing media supplementation or be analyzed to identify key effector molecules, potentially enhancing embryo quality and viability in ART procedures.

Moreover, current embryo selection protocols predominantly rely on morphological assessments, often supplemented by invasive biopsies and genetic screenings of biopsy cells. The analysis of the embryo secretome holds promise in identifying more reliable indicators of embryo quality (Zmuidinaite et al., 2021). Spent culture media from in vitro fertilized embryos, an easily accessible yet underutilized resource, presents an opportunity for assessing developmental competence. Furthermore, EVs derived from embryos offer a non-invasive alternative to biopsy analysis for pre-implantation genetic testing (Abu-Halima et al., 2017; Dissanayake et al., 2020; Zhang et al., 2019). Additionally, the molecular cargo of EVs from various embryo types may give valuable insights into embryo quality, as demonstrated by studies correlating the number and composition of EVs with implantation success (Liu et al., 2020; Tan et al., 2020). The development of EV-based assays for embryo quality not only addresses current ART challenges but also serves as a platform for pioneering high-resolution technologies capable of tracking multifaceted changes in embryos. This pursuit ultimately may enhance the precision and individualization of ART procedures.

For the assessment of window of implantation (WOI), current methods are subjective and invasive, relying on histological examinations and gene expression analyses of endometrial biopsy samples (Heger et al., 2012). However, the dynamic heterogeneity of endometrial cells poses challenges to these approaches (Suhorutshenko et al., 2018). EVs, co-expressing cell-specific markers and functional molecules, offer a promising substrate for tracking variations in cell composition and gene expression within the endometrium. Notably, EVs isolated from uterine flush solutions exhibit differential expression of key markers across the luteal phase, suggesting their potential as non-invasive biomarkers for WOI determination (Luddi et al., 2019). In addition, recent findings implicating EVs from endometrial cells in recurrent implantation failure underscore their potential as diagnostic biomarkers (Liu et al., 2020).

Beyond ART practices, EVs could also be utilized for diagnosing and treating reproductive and pregnancy-related pathologies, such as endometriosis, polycystic ovarian syndrome (PCOS), GDM, PE and PTB (Nair et al., 2024). Diagnosis of many of these pathologies often involves invasive methods or is only possible after symptoms arise; while, placental EVs have been isolated and characterized in maternal serum. In this regard, EVs could serve as targets for early and more definitive detection, paving the way for more personalized reproductive medicine practices (Kohli et al., 2016; Menon et al., 2020; Rooda et al., 2020; Shomali et al., 2020).

Despite ethical and legal constraints limiting experiments in human ART, ongoing advancements in EV analysis hold promise for refining reproductive medicine and ART protocols. Recent advancements in techniques for in vitro production of EVs present exciting opportunities to understand key mechanisms and enhance their therapeutic potential. By optimizing culture conditions and manipulating cell-derived EVs, researchers can tailor EV cargo and release profiles to target specific reproductive pathologies, bridging the gap between in vitro and in vivo conditions. For this, there are several methods developed for in vitro production of EVs.

Since the biogenesis, cargo and function of EVs depend on the cellular environment of their origin, in vitro-produced EVs often differ from those collected in vivo (Banliat et al., 2022; Bridi et al., 2021). As a result, EVs produced under classical in vitro

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TABLE 1 Studies focusing on in vivo-collected or in vitro-produced reproductive EVs, and their potential clinical outcomes based on main findings.

Organ/tissue	Source of EVs	Main findings	Clinical relevance	References
Ovary	In vivo	Follicular EVs improve meiotic resumption of domestic cat vitrified oocytes	Supplementation of follicular EVs to vitrification solution could improve ART success rate	de Almeida Monteiro Melo Ferraz et al. (2020)
	In vitro	Identified HGSOC-associated sEV protein markers	Use as biomarker for the diagnosis of HGSOC	Yokoi et al. (2023)
Oviduct	In vivo	Supplementation with oviductal EVs during IVC improves developmental competence of IVF embryos	Use oviductal EV supplementation to improve IVF embryo quality	Lopera-Vasquez et al. (2017), Qu et al. (2019)
		Oviductal EVs are important for sperm motility and survival	Improve quality of sperm used for ARTs and improve ARTs	Alcantara-Neto et al. (2020), Ferraz et al. (2019)
		Oviductal EVs improve gamete recovery and quality following freeze-thaw procedures	Use oviEV supplementation for better recovery of gametes	de Almeida Monteiro Melo Ferraz et al. (2020)
	In vitro	Supplementation with oviductal EVs during IVC improves developmental competence of IVF embryos	Use oviductal EV supplementation to improve IVF embryo quality	Fang et al. (2023)
		BOEC-CM and EVs isolated from BOEC culture improved the quality of in vitro produced bovine embryos	Use oviductal EV supplementation to improve IVF embryo quality	Lopera-Vasquez et al. (2016)
		BOEC-produced EVs improve oocyte cryotolerance	Supplement the freezing media with oviEVs to improve gamete recovery	Sidrat et al. (2022)
Uterus	In vivo	miRNA markers identified for receptivity	Use as biomarker for determining patient-specific WOI	Vilella et al. (2015), Luddi et al. (2019), Giacomini et al. (2021)
		miRNA profiles of EVs from EC and healthy patients show differential expression	Use as non-invasive and early diagnostic tool for EC	Roman-Canal et al. (2019)
		Unique miRNA-lncRNA signature detected in EVs from endometriosis patients	Endometriosis diagnostics	Khalaj et al. (2019)
	In vitro	Different protein profiles of EVs from EcESCs and EuESCs	Understand underlying mechanisms of endometriosis for better diagnosis and treatment	Hsu et al. (2021)
		Endometrial exosomes modulate invasive capacity of trophoblast and embryo implantation	Identify mechanisms involved in adhesion and implantation to improve clinical outcomes.	Greening et al. (2016), Gurung et al. (2020), Segura-Benitez et al. (2022)
Placenta	In vivo	Different EV profiles in full term and preterm births	Early and non-invasive diagnosis of PTB	Menon et al. (2020)
		Identified specific miRNAs only present in GDM	Early and non-invasive diagnosis of GDM	Nair et al. (2018), Kandzija et al. (2019)
		Differences in the number and content of EVs in PE	Early and non-invasive diagnosis of PE	Salomon et al. (2017), Motta-Mejia et al. (2017), Lok et al. (2009), Kohli et al. (2016)
Oocyte/Blastocyst/ Embryo/Fetus	In vivo	Fetal trisomy detected from fetal EVs in maternal plasma	Non-invasive detection of genetic and/or developmental abnormalities from maternal plasma	Zhang et al. (2019)

(Continues)

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TABLE 1 (Continued)

Organ/tissue	Source of EVs	Main findings	Clinical relevance	References
	In vivo vs in vitro	EVs from in vivo-collected or in vitro-produced embryos have different EV profiles	Identify which signals are missing in in vitro conditions and adjust culture systems for improved ARTs	Bridi et al. (2021), Aguilera et al. (2023)
	In vitro	Blastocysts secrete EVs containing genome-wide sequences of DNA to the medium	Spent media could be used for assessing embryo quality before transfer in ARTs	Simon et al. (2020), Abu-Halima et al. (2017), Dissanayake et al. (2020)

Abbreviations: ARTs, assisted reproductive technologies; BOEC-CM, bovine oviductal epithelial cell conditioned media; EC, endometrial cancer; EcESCs, ectopic endometrial stromal cells; EuESCs, eutopic endometrial stromal cells; GDM, gestational diabetes mellitus; HGSOC, high-grade serous ovarian cancer; IVC, in vitro embryo culture; IVF, in vitro fertilization; PE, pre-eclampsia; PTB, pre-term birth; WOI, window of implantation.

conditions may lack clinical relevance. In the subsequent section, we explore strategies and techniques for refining the in vitro production of EVs, providing an overview of current technologies used based on the latest literature.

A key limitation in this area of research is the differing goals of ART practices and EV studies. ARTs primarily aim to enhance procedure success rates, leading to increased pregnancy rate and live births. In contrast, EV research in the reproductive field focuses on characterizing EV content and their role in cellular communication. Reproductive EV studies typically maintain established culture conditions for gametes, limiting modifications that could improve EV yield or quality. Techniques for enhancing EV yield often involve introducing stressors or altering culture conditions, which are not easily applicable to gamete culture protocols. Given that most studies on improving in vitro EV production seldom focus on reproductive EVs, our analysis centres on studies utilizing different tissues/cells of origin, as summarized in Table 2. Here, we aim to show current technologies available in the EV field that could also be used for reproductive EVs more extensively.

3.2 | 2D cell culture for EV production

To address the need for a more reliable and predictable EV production, in vitro cell culture models have been explored for various tissues. Traditional 2D cell culture systems are commonly used for reproductive EV studies due to their ease of manipulation and cost-effectiveness (Fang et al., 2023). Nonetheless, traditional 2D culture not only leads to dedifferentiation and loss of function in cell populations (thereby changing EV composition), but also leads to low EV yield (Haraszti et al., 2018; Ng et al., 2019). To overcome this limitation and facilitate their use in preclinical and clinical studies, researchers have investigated strategies to upscale EV release from 2D cultured cells. These strategies include chemical and physical stimulation, as well as physiological modifications. Chemical stimulation involves supplementing culture media with specific inhibitors, such as the glycolysis inhibitor iodoacetate and oxidative phosphorylation inhibitor 2,4-dinitrophenol (Ludwig et al., 2020) or small chemicals, namely norepinephrine and forskolin (Wang et al., 2020). Additionally, physical stimulation, such as stress-induced mechanisms, has been shown to enhance EV yield (Piffoux et al., 2019). Studies have demonstrated increased EV production through electrical (Fukuta et al., 2019) or ultrasound stimulation (Zhang et al., 2022) of various cell types. In addition, a number of studies also employed 2D culture systems with micropatterned surfaces to increase EV yield (Hisey et al., 2021; Ji et al., 2021). Considering the sensitivity of gametes and early embryos to chemicals and factors in media, to our current knowledge, such techniques have not been tested for these specialized reproductive cells. There are, however, some systems that implement other physical stimulations during embryo culture, such as tilting platforms and electrical stimulation (Hara et al., 2013; Matsuura et al., 2010). Although such culture methods have shown increased blastocyst rates and developmental potential, how such mechanical stimulation may influence EV characteristics have not been studied.

Despite the potential success in increasing EV yield in 2D cultures with these techniques, these systems still fall short in mimicking natural behaviours due to the requirement of a 3D architecture and interaction with the extracellular matrix (ECM) for regulating complex biological functions (Weigelt et al., 2014; Xu et al., 2009). A noteworthy study by Almiñana et al. highlighted clear differences between in vivo-collected and in vitro-produced (following 2D cell culture) oviductal EVs in terms of their secretion dynamics and content (Almiñana et al., 2017). This study identified 186 differentially expressed proteins between the two groups, with functional analysis revealing the absence of some proteins involved in sperm-oocyte binding, fertilization and embryo development in the in vitro-produced EV group (Almiñana et al., 2017). This is in line with findings from Milazzotto et al. where clear differences in RNA profiles between in vivo and in vitro produced embryos were shown (Milazzotto et al., 2022). Specifically, 85% of metaboloepigenetic pathways were differentially expressed between in vivo and in vitro embryos (Milazzotto et al., 2022), that could explain different EV profiles being secreted by these embryos. Such substantial differences in EV

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TABLE 2 Overview of studies from the last 6 years that focus on improving EV production using different culture methods with different mechanical stimuli. Twelve of the 27 publications listed above were studies using bioreactors, with 2 of them combining bioprinting and bioreactor technologies. Eleven publications listed used 3D culture methods with only 1 combining bioprinting methods for 3D culture. Four studies used 2D culture methods, of which 1 study combined microfluidics, 2 studies applied electrical or ultrasound stimulation and 2 studies used patterned surfaces.

Culture method	Culture method specifics	Cell type	Isolation technique	EV yield /concentration	Size of EVs	Biological implications	Reference
2D	2D culture with low-intensity pulsed ultrasound (LIPUS) treatment at 90 mW/cm^2	hMSCs, SCAP	DUC	1.5-fold increase (concentration and protein) in the LIPUS group	200 nm	SCAP-EVs following LIPUS exhibited stronger inhibition of bone resorption in periodontitis and increased promotion of osteogenic differentiation and anti-inflammation in vitro	Zhang et al. (2023)
2D	2D culture on micropatterned PCL surfaces	Rat ASCs	DUC	Grooved substrates yielded 1.8-folds more sEVs than flat substrates	100–200 nm	Microgrooved substrates upregulated the expression of Hrs/Alix/Rab35 in rASCs and also altered the content of rASC-sEVs to have higher angiogenic capacity. Supplementation of HUVECs with G-EVs promoted their angiogenic properties	Ji et al. (2021)
2D	2D culture in microtrack patterned culture dishes	MDA-MB-231 triple-negative breast cancer cells	DUC	2-folds increase on the microtracks compared to flat surface. As the microtrack spacing and width decreased, the number of sEVs/cell produced also increased 1.5 folds	100–200 nm	Microtracks significantly increase cell density, aspect ratio, alignment to the substrate and single-cell migration speed for triple-negative breast cancer cells	Hisey et al. (2021)
2D	2D culture of cells with low-level electric treatment (ET) at 0.34 mA/cm^2	Murine melanoma and fibroblast cell lines	DUC	ET increased the particle number of EVs 1.26- and 1.7-folds in both B16F1 and 3T3 Swiss albino cell, respectively	100 nm	Quality and the uptake of EVs did not change following ET	Fukuta et al. (2020)
2D + microfluidics	Modularized perfusion cell culture platform with three individual chambers for media, culture and collection	HeLa cells	Centrifugation and filtration	Modular perfusion system produced 5-folds more EVs than the static culture	50 nm	The perfusion system replicated the biological behaviour of cells, similar to that in vivo, enabling real-time analysis of cells and high production of tdEVs to interpret the high quality of parental cells	Kim et al. (2023)
3D	3D culture in PEG hydrogel cylindrical microwell arrays with inverted-pyramidal openings, shaking	hMSCs	DUC	3D shaking group produced 100-folds higher MV. Protein content 10-fold higher with shaking	300 nm	MVs produced were used for supplementation and resulted in angiogenic and neurogenic stimulation	Cha et al. (2018)

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TABLE 2 (Continued)

Culture method	Culture method specifics	Cell type	Isolation technique	EV yield /concentration	Size of EVs	Biological implications	Reference
3D	3D culture on the surfaces of spherical support matrix beads and distributed in medium by stirring in a spinner flask	WJ-MSCs or MSCs from adipose tissue or bone marrow	DUC or TFF	TFF improved exosome yield by 27-fold and 3D culture 20-fold, compared to 2D cell culture-UC, respectively. 3D-TFF-exosomes was a 140-fold increase of exosome yield compared with 2D-UC-exosomes	120 nm	Both 3D cell culturing method and the TFF exosome isolation method contributed to improved siRNA transferring and gene silencing activity of exosomes	Haraszti et al. (2018)
3D	3D printed titanium scaffolds coated with hydroxyapatite, with different pore sizes and shapes. Scaffolds were cultured dynamically on a tube rotator	MC3T3 murine pre-osteoblasts and hBMSCs	DUC	EV protein content was 1.32-folds higher when cultured on scaffolds vs. 2D. The T500 and T1000 groups exhibited a 1.93- and 2.22-fold increase when compared to the S500 and S1000 scaffolds, respectively	200 nm	Osteogenic differentiation of hBMSCs following treatment with primed EVs was evaluated. Triangle pore scaffolds significantly increased osteoblast mineralisation (1.5-fold) when compared to square architectures	Man et al. (2021)
3D	3D microwell array culture in the form of aggregates	Human gastric cancer cell lines	DUC	On average, number of EV/cell was doubled in 3D culture when compared to 2D culture	85-180 nm	RNA profiles of EVs from 3D or 2D culture were similar. Downregulation of proteins in EVs from 3D cultures was observed. Spatial cellular architecture affects dynamic co-regulation of microRNAs and proteins	Rocha et al. (2019)
3D	3D culture in peptide hydrogel (PGmatrix DMEM kit)	HeLa cells	DUC + filtration	Decreasing secretion rate in 2D culture from ~8000 particles/cell at 6 h of culture to ~2000 at 48 h of culture. In 3D culture, increasing EV secretion from ~250 particles/cell on Day 5 to ~1700 particles/cell on Day 11 of culture	60–120 nm	Secretion profile in 2D and 3D cultures differ as well as the size of EVs secreted. 3D-EVs show a highly similar RNA profile to in vivo EVs	Thippabhotla et al. (2019)

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TABLE 2 (Continued)

Culture method	Culture method specifics	Cell type	Isolation technique	EV yield /concentration	Size of EVs	Biological implications	Reference
3D + bioprinting	3D culture on microfiber fabricated by coaxial bioprinting	hBM-MSCs	DUC	Microfiber samples had ~32- and ~46-fold more protein mass than 2D cultures. EV numbers followed the same trend, with microfibers enriching EVs by 164- and 1009-folds	90–250 nm	Signature proteins of MSC-EVs are preserved between microfiber and conventional 2D culture. EV produced by microfibers display angiogenic properties	Chen et al. (2021)
3D	3D spheroids on a rotary shaker	WJ-MSCs	DUC + OptiPrep DG	Compared to the 2D system, 2.5- to 3.5-folds and 4.5- to 6.5-folds more EVs produced with a3D or T-a3D system, respectively. A comparison of total number of EVs revealed that a 6-fold (3D system) and 11-fold (T-a3D system) increase was observed compared to the 2D group	100–200 nm	Proteomic analysis showed T-a3D culture conditions enhance TGF-b3-related ECM organization, cell–cell adhesion and SRP-dependent co-translational protein, inducing EV production. Proteomic analysis confirmed that the expression of CD9 and TGFb3 was increased in T-a3D spheroids at Days 5 and 7, compared to 2D MSCs The system also improved regeneration capacity of T-a3D-EVs	Min Lim et al. (2023)
3D	3D graphene scaffold	hUMSCs	DUC	N/A	50–150 nm	3D-Exo reduced Aβ secretion in the Alzheimer's disease model, improved cognitive functions in APP/PS1 transgenic mice and inhibited inflammatory and oxidative stress responses	Yang et al. (2020)
3D	3D cell culture in collagen hydrogel	hPDLSCs and hBMSCs	Centrifugation + Ultra15 centrifugal filter unit (100 kDa)	The total protein output of the 3D-Exos was 2.9-fold higher than that of the 2D-Exos	20–500 nm	The 3D-Exos enhanced osteogenesis and migration of hBMSCs. The 3D culture microenvironment modulated exosomes bioactivity through activation of the YAP signalling pathways. The 3D-Exos improved bone defect in SD rats	Yu et al. (2022)

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TABLE 2 (Continued)

Culture method	Culture method specifics	Cell type	Isolation technique	EV yield /concentration	Size of EVs	Biological implications	Reference
3D	Static 3D spheroid culture	BM or lung tissue MSCs	DUC	No significant differences between 2D and 3D culture systems	<100 nm	Aggregation of MSCs into 3D spheroids did not provide the necessary bio-instructive cues to maintain and direct their therapeutic potential. EVs isolated from static 3D cultures are not a viable alternative to 2D monolayer as 3D EVs did not retain MSC anti-fibrotic and anti-immunomodulatory properties both in vitro and in vivo	Kusuma et al. (2022)
Bioreactor	3D culture on engineered 3D tissues placed in bioreactor for flow stimulation or mechanical stretching	hDPSCs, MSCs or SkMCs	DUC	Compared with the 3D static group, groups with 0.5 or 1.0 mL/min of perfusion had 24-fold and 37-fold higher EV production in DPSCs. For MSCs scaffold with 0.5 mL/min flow stimulation, EV production was 40.7- and 3.4-folds higher than the 2D and 3D static counterparts. The engineered tissues under stretching produced 11-fold higher EV than unstretched tissues	200 nm	Mechanical stimulations boosted EV production from engineered tissues. This increase is likely mediated by YAP mechanosensitivity	Guo et al. (2021)
Bioreactor + bioprinting	3D culture in 3D-printed scaffold perfusion bioreactor	hMSCs	DUC + Nanosep spin column	EV production under 5 mL/min flow rate was 83-, 28-, 3- and 2.5-folds higher than flask, 0, 1 and 10 mL/min, respectively	40–200 nm	Enhanced EV production in perfusion bioreactor culture is not specific to MSCs, as HUVECs culture also resulted in a similar trend. Supplementation of bioreactor MSC-EVs increased the angiogenic activity of HUVECs in vitro and in vivo diabetic mouse wound-healing model	Kronstadt et al. (2023)
Bioreactor	Flat-plate bioreactor with 0.1 and 1 mL/min flow rate[Md2]	hBM-MSCs	DUC + DG UC	4×10^8 particles/cell and 1 μg protein/cell	NA	Increased EV yield promoted by the calcium induction mechanism of EV biogenesis under bioreactor conditions	Kang et al. (2022)
Bioreactor	3D culture in 2 L-scale controlled stirred tank reactor (STR) operated under fedbatch (FB) or fed-batch combined with continuous perfusion (FB/CP)	hBM-MSCs	Total Exosome Isolation Kit (Invitrogen)	1.25-fold increased concentrations of EVs in FB/CP compared to other groups	162–137 nm	Results suggest that the size, concentration and the purity of EVs produced during the STR cultures do not differ between FB and FB/CP conditions	Fernandes-Platzgummer et al. (2023)

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TABLE 2 (Continued)

Culture method	Culture method specifics	Cell type	Isolation technique	EV yield /concentration	Size of EVs	Biological implications	Reference
Bioreactor	Stirred suspension bioreactors (SSB)	Human MPCs	DUC	EV/cell ratio doubled with 40 rpm perfusion compared to static; and showed 1.5-fold increase with 80 rpm compared to 40 rpm	20–100 nm	The MPC-EV fraction increased proteoglycan and Type II Collagen deposition in murine cartilage defect model. SSB culture of MPCs showed increased chondrogenic gene expression and reduced MMP production in the EV fraction. EVs from both static and SSB groups induced Type II Collagen deposition in MPCs	Phelps et al. (2022)
Bioreactor + bioprinting	3D-printed scaffold-perfusion bioreactor	HDMECs	DUC + Nanosep 300 kDa MWCO spin column	Approximately 100-fold (Day 1) and 10,000-fold (Day 3) increased EV production in bioreactor group compared to static scaffold and flask controls	40–200 nm	Ethanol conditioning enhanced vascularization bioactivity of EVs in both static and dynamic conditions, demonstrating that the therapeutic potential is retained following ethanol conditioning	Patel et al. (2019)
Bioreactor	3D culture in hollow fibre reactor	hMSCs from umbilical cord	DUC	19.4-fold higher exosome yield in 3D than 2D	120 nm	Enhanced renoprotective and anti-inflammatory efficacy in 3D-EVs	Cao et al. (2020)
Bioreactor	Vertical-Wheel Bioreactor	hMSCs from BM, AT or UCM	Total exosome Isolation (Invitrogen)	Fold increase of 5.7 in 3D MSC-EVs compared to 2D MSC-EVs. When analyzed individually, a fold increase of 4.0 for BM MSC, 4.4 for AT MSC and 8.8 for UCM MSC was observed when produced in the bioreactor	<200 nm	Improved MSC-EV production in a bioreactor system with high purity	de Almeida Fuzeta et al. (2020)

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TABLE 2 (Continued)

Culture method	Culture method specifics	Cell type	Isolation technique	EV yield /concentration	Size of EVs	Biological implications	Reference
Bioreactor	3D culture on low concentration Synthemax II Microcarriers in a Vertical-Wheel™ Bioreactor	BM-MSCs	UC	28-fold increase of EVs/mL in the 3D versus 2D culture	130–140 nm	3D-derived EVs induced a significant 7-fold higher elongation in TG neurons compared to 2D-derived EVs after 5 days	Jalilian et al. (2022)
Bioreactor	Seesaw-motion bioreactor (SMB)	hNK	DUC	2-fold increase in EV production in SMB compared to static culture	50–350 nm	EVs produced were functionally active in killing melanoma and liver cancer cells in both 2D and 3D culture conditions in vitro, as well as in suppressing melanoma growth in vivo	Wu et al. (2022)
Bioreactor	3D culture in aggregates placed on a rocking base of a WAVE bioreactor	hBM-MSCs	PEG precipitation and UC + DG UC	2-fold increase in EV numbers from 3D culture compared to 2D culture in a 2-day culture	180 nm	3D dynamic aggregates promote therapeutically relevant miRNA and protein cargo in EVs. Significant improvement in wound healing properties of EVs from 3D culture vs. 2D culture. Decreased ROS production in cells supplemented with 3D-EVs when compared to no supplementation or supplementation with 2D-EVs	Yuan et al. (2022)
Bioreactor	Vertical-Wheel bioreactors	hiPSCs	DUC	The microcarrier cultures had 17–23 fold higher EV secretion, and EV yield in mTeSR media was 2.7–3.7 fold higher than HBM medium.	112–163 nm	Microcarrier culture with mTeSR showed a smaller EV size than other groups, and the cargo was enriched with proteins and miRNAs reducing apoptosis and promoting cell proliferation. hiPSC-EVs stimulated proliferation and M2 polarization of microglia in vitro. HiPSC expansion on microcarriers produced much higher yields of EVs than hiPSC aggregates in VWBRs.	Muok et al. (2024)

Abbreviations: ASCs, adipose-derived stem cells; AT, adipose tissue; BM, bone marrow; DPSCs, dental pulp stem cells; DG, differential gradient; DUC, differential ultracentrifugation; hBM-MSCs, human bone marrow MSCs; hDMECs, human dermal microvascular endothelial cells; hiPSCs, human-induced pluripotent stem cells; hNK, human natural killer cells; hPDLSCs, human periodontal ligament stem cells; hUMSCs, human umbilical cord MSCs; MPCs, mesenchymal progenitor cells; MSCs, mesenchymal stem cells; SCAP, stem cells from apical papilla; SkMCs, skeletal muscle cells; TFF, tangential flow filtration; UC, ultracentrifugation; UCM, umbilical cord matrix; WJ-MSCs, Wharton's Jelly-derived MSCs.

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content based on the production method underscore the need for new in vitro cell models to better study EV functions in a 3D environment that closely simulates physiological conditions.

3.3 | Bioreactors for EVs production

Bioreactors enable long-term cell growth through continuous media flux and the supply of nutrients, gases and the removal of waste and metabolites, leading to high-scale EV production (Haraszti et al., 2018; Watson et al., 2016). The main advantage of bioreactors is the ability to maintain a high number of cells in culture for an extended period within the same device. Different types of bioreactors include microcarrier-based (Kang et al., 2022), stirred tank (Fernandes-Platzgummer et al., 2023; Phelps et al., 2022), hollow fibre (Cao et al., 2020), vertical-wheel (Muok et al., 2024), seesaw-motion bioreactor (Wu et al., 2022) as well as 3D-printed bioreactors (Kronstadt et al., 2023; Patel et al., 2019). Such studies focusing on EV production in bioreactors more commonly use mesenchymal stem cells (MSCs) derived from various tissues (Guo et al., 2021; Kang et al., 2022; Kronstadt et al., 2023). Although other cell types (Guo et al., 2021; Patel et al., 2019; Wu et al., 2022) have been tested for improved EV production and therapeutic efficiency, reproductive cells have not been used in such platforms to the best of our knowledge.

Most of the aforementioned studies do in fact show higher EV yield with higher therapeutic effects. For example, Cao et al. cultured MSCs in hollow fibre bioreactors to produce MSC-derived exosomes for potential use in treating acute kidney injury (Cao et al., 2020). While surface markers, morphology and exosome size did not differ between the bioreactor and 2D-produced exosomes, the total exosome production was increased in the bioreactor system. Additionally, the uptake of bioreactor-produced exosomes by tubular epithelial cells was more efficient, demonstrating a greater anti-inflammatory effect (Cao et al., 2020). Furthermore, Patel et al. utilized a 3D-printed scaffold-perfusion bioreactor to assess the impact of dynamic culture on EV production from endothelial cells, showing a significant enhancement of EV production through this method (Patel et al., 2019). Despite the advantages of bioreactors for highly scalable EV isolation, challenges remain in monitoring donor cell viability and controlling cell growth, potentially leading to uncontrollable contamination of EVs with apoptotic bodies or stress vesicles.

3.4 | 3D cell culture for EV production

In recent years, the development of 3D cell cultures has gained traction as a viable alternative to the traditional 2D models (Bahcecioglu et al., 2020; Brancato et al., 2020; Lau et al., 2020). Non-adherent techniques, such as hanging-drop and liquid overlay methods, allow cells to self-assemble into aggregates, resembling solid tissues and promoting the secretion of ECM and growth factors, which regulate proliferation and differentiation. Studies comparing these non-adherent techniques for EV production using MSC cell culture have shown higher EV yields compared to traditional 2D culture (Lim et al., 2023). While these methods are applicable for EV isolation, they come with limitations in terms of controls and scalability. Another non-adherent culture alternative involves the use of agarose matrices adapted for EV production. Conical agarose microwell arrays prevent cell attachment to the surface, promoting controlled and homogeneous cell aggregation (Thomsen et al., 2017). This method has been well-characterized in mostly cancer cells for increased EV production (Rocha et al., 2018; Thomsen et al., 2017). These studies showed the effects of 3D cellular architecture in microRNA and protein cargo of EVs, as well as highlighting agarose microwell arrays as a good model for mimicking cancer cell metastatic behaviour compared to 2D culture methods.

However, it is essential to develop new cell models that better mimic the spatial architecture of tissues to study EV functions in a 3D environment that more accurately represents the physiological state (Kim et al., 2020; Kretzschmar & Clevers, 2016). Models that rely on cell adherence during culture to enhance EV yield, such as solid scaffolds, bioreactors and hydrogels, have emerged. Solid scaffolds provide a rigid structure to cells, allowing for a controllable and reproducible 3D distribution of cells in vitro (Edmondson et al., 2014). Various materials like ceramics, glass, metals and polymers can be used to create solid scaffolds with 3D printing technologies, offering cells a porous structure with a homogenous interconnected pore network (Edmondson et al., 2014). Although solid scaffolds support cell growth and differentiation, they are rarely used for EV isolation mainly due to their high cost, difficulties in EV release and low EV yield. Few studies that do use this method show the effects of pore size and shape in cell behaviour and EV release (Chen et al., 2021; Man et al., 2021; Yang et al., 2020).

Hydrogels have emerged as a highly promising technique for 3D cell culture due to their ability to mimic the mechanical properties of the ECM that sustain cells in their physiological state (Zhao et al., 2020). The mechanical properties of hydrogels, such as swelling and mesh size, significantly influence the behaviour of embedded cells, as these properties define the material's stability in culture. Natural hydrogels, such as collagen, gelatine, fibrin and alginate, are commonly used to culture cells in the form of organoids or spheroids. However, they exhibit limitations in terms of low stiffness, low long-term stability, high susceptibility to protease degradation and high batch-to-batch variability (Caliari & Burdick, 2016). Synthetic hydrogels, on the other hand, are composed of fabricated molecules that imitate the structure of natural polymers. This offers users the ability to design gel properties, control adhesive moieties and tailor mechanical properties, such as material stiffness or pore size. Examples of synthetic hydrogels include polyacrylamide (PA), polyethylene glycol (PEG) and hyaluronic acid (HA) (Ahmed, 2015).

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Recent studies have shown that the impact of hydrogel features on EV release and diffusion is influenced by cell lines, with greater rates observed under less flexible matrix conditions. Notably, EV diffusion was not determined by the mesh pore size but rather by the hydration of the hydrogel. The water permeation of EVs via aquaporin allows them to become more deformable, facilitating EV transport through the hydrogel (Lenzini et al., 2020; Worthington et al., 2015). Several recent studies have successfully employed the aforementioned techniques to improve in vitro EV production. For instance, a study by Thippabhotla et al. involved culturing patient-derived cervical cancer cells in a 3D environment, embedded in peptide hydrogels (Thippabhotla et al., 2019). The EVs produced in this 3D model were compared to those obtained from conventional 2D patient-derived cells, as well as in vivo-derived EVs from cervical cancer patients' plasma. The findings revealed that EVs obtained through 3D culture exhibited a much higher similarity to in vivo circulating EVs in terms of their small RNA profile compared to 2D-derived EVs (Thippabhotla et al., 2019). This study underscores the significant impact of culture and growth conditions on EV content and emphasizes the superior ability of 3D culture to mimic in vivo conditions. In summary, the in vitro production of EVs in 3D systems represents a promising avenue for studying their functions within a more physiologically relevant environment. These advancements may have profound implications for future clinical applications. Nonetheless, further research and optimization are necessary to improve scalability, reproducibility and control over EV production in these innovative systems.

In the context of reproductive biology, organoid and spheroid culture techniques have been employed for reproductive cell culture, especially for oviductal (Kessler et al., 2015; Xie et al., 2018) and endometrial (Shibata et al., 2024; Yamauchi et al., 2003) cell cultures. Although these culture conditions showed a more in vivo-like cell behaviour, these systems have not been tested for EV production. Most studies on reproductive EVs rely on the flushing of organs and collection of reproductive organ fluids for further analysis (O'Neil et al., 2020; Piffoux et al., 2019; Salas-Huetos et al., 2019). These studies often investigate the impact of EVs on reproductive events. However, relying on in vivo collected EVs has its limitations, as it necessitates access to patient or animal tissues, leading to variations between EVs from different sources and lacking scalability and consistency for potential clinical studies. Considering the biomechanical and biochemical cues in female reproductive organs, adopting bioengineering technologies could not only enhance EV yield but also facilitate the production of EVs that closely resemble those found in vivo. It is important to note that the majority of current studies rely on 2D cell culture systems. However, it's crucial to recognize the suboptimal nature of these conditions, as they fail to replicate the in vivo interactions, mechanical stimulations and other characteristics of these tissues. Consequently, transitioning toward more physiologically relevant 3D culture systems is imperative for a more precise representation of the complex in vivo environment in future studies on reproductive EVs.

4 | BIOENGINEERING TECHNOLOGIES FOR THE IN VITRO PRODUCTION OF EVS

Bioengineering technologies have emerged as valuable tools in addressing challenges in biology and medicine by using engineering principles (Naegeli et al., 2022). Over the past decade, significant progress in bioengineering, such as the development of diverse biomaterials, improvements in 3D printing technologies and fabrication of microfluidic devices, has enabled deeper insights into biological systems and diseases (Dey & Ozbolat, 2020), including some reproductive tissues (Almeida et al., 2023; Hellström et al., 2017; Li et al., 2011). These advances not only allow for the creation of 3D printed scaffolds (bioprinting) that better mimic the in vivo reproductive environment, but also facilitate the development of organ-on-a-chip models using microfluidics (De Bem et al., 2020; Ferraz, Henning, Costa, et al., 2017; Ferraz, Henning, Stout, et al., 2017; Ferraz et al., 2018; Park et al., 2020). When combined with stem cell research, intelligent biomaterials and three-dimensional biofabrication strategies, these technologies enable the engineering of highly mimicked tissues or organs, showing great promise in regenerative medicine and translational applications in reproductive medicine (Laronda et al., 2017).

Bioprinting, akin to 3D printing, employs an additive manufacturing process that uses a digital blueprint to print objects layer by layer (Dey & Ozbolat, 2020). However, unlike conventional 3D printing, bioprinters work with cells and biomaterials to create organ-like structures that can support cell culture. On the other hand, microfluidics involve the fabrication of micromanufactured devices containing chambers and tunnels through which fluids flow or are confined (Le Gac & Nordhoff, 2017). The use of microfluidic devices allows for constant perfusion of media during cell culture, mimicking the vasculature in vitro. Given their ability to replicate in vivo tissues and vascular flow while facilitating 3D cell culturing, bioengineered models have been employed in the field of EV research to upscale production, sorting (Paniushkina et al., 2020; Patel et al., 2019; Thippabhotla et al., 2019) and to better study EV biology in various tissue models such as prostate (Padmyastuti et al., 2023), muscle (Vann et al., 2022) and liver-kidney (Tian et al., 2020). An overview of different techniques used for EV production on a scale from high throughput to mimicking physiology are shown in Figure 2.

As previously discussed, bioreactors are widely used for cell culture to achieve increased EV yield. These platforms also allow for the investigation of different treatments and culture conditions, enabling the identification of mechanisms involved in EV production on a larger scale. For instance, in a study by Patel et al., human dermal microvascular endothelial cells were cultured in a scaffold perfusion bioreactor system and subjected to ethanol treatment (Patel et al., 2019). This treatment not only increased EV yield but also enhanced vascularization bioactivity (Patel et al., 2019). In another report, EV secretion was improved in human dental pulp stem cells, skeletal muscle cells and hMSCs cultured in a perfusion bioreactor system by applying physical force onto

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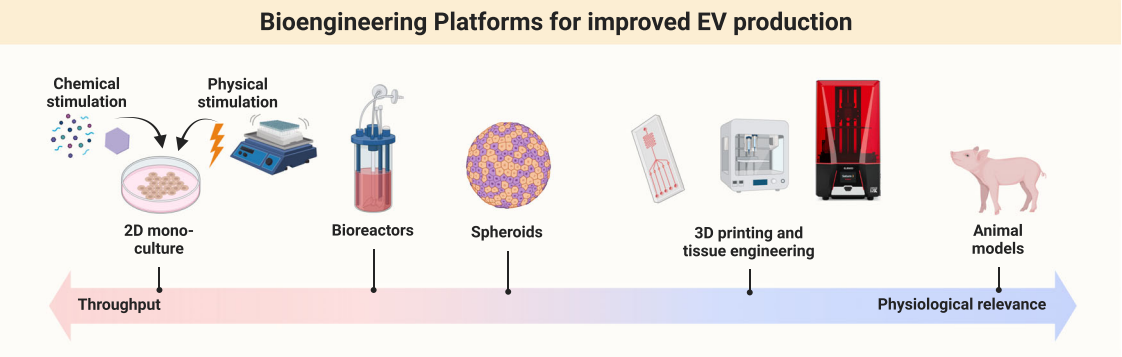

FIGURE 2 Bioengineering platforms for improved EV production. The figure illustrates diverse bioengineering approaches employed in the in vitro production of extracellular vesicles (EVs), showcasing the continuum between throughput and physiological relevance. Each technology is strategically positioned on this spectrum to highlight its characteristics in terms of scalability and mimicry of natural physiological conditions. Figure was made using Biorender.

the cells (Guo et al., 2021). The induction of physiological mechanical stimulation in the form of shear stress increased EV yield, and the Yes-associated protein (YAP) mechanosensitivity was identified as a mediator of this behaviour (Guo et al., 2021).

In other studies, 3D printing technologies have been used to create more complex culture environments to upscale EV production. For instance, Jeske et al. 3D-printed a microchannel bioreactor system to dynamically form hMSC spheroids and promote cell growth (Jeske et al., 2022). This dynamic aggregation of MSCs not only promoted autophagy and altered cell metabolism towards glycolysis but also stimulated EV production (Jeske et al., 2022). Similarly, Chen et al. used coaxial bioprinting to 3D print long fibres with human bone marrow mesenchymal stem cells (hBM-MSCs), resulting in an enrichment of EVs by three orders of magnitude compared to the 2D culture control group (Chen et al., 2021). Bioprinting technologies have also been adapted for reproductive cells/tissues, albeit not for EV production. Although we have these models for uterine contractility (Souza et al., 2017), follicle culture (Wu et al., 2022) and fertility restoration (Laronda et al., 2017), we need more in depth studies to evaluate the EVs produced in these systems and test their relevance for clinical uses.

Moreover, microfluidic devices have been fabricated using 3D printing technology for cell culture. Hao et al., for example, utilized PDMS-based microfluidic devices with squeezing ridges to stimulate small EV (sEV) secretion from hBM-MSCs (Hao et al., 2022). Their results indicated that allowing the passage of cells through the channels of the microfluidic device increased sEV secretion approximately four-fold compared to the control group, with no implications on the functionality of the hBM-MSC-induced EVs (Hao et al., 2022). In the context of reproductive biology, many microfluidic systems have been used for modelling endometrial (Ahn et al., 2021; De Bem et al., 2020; Gnecco et al., 2017), ovarian tissues (Nagashima et al., 2018; Weng et al., 2018) and as models for a connected reproductive system (Han et al., 2010; Xiao et al., 2017), but no information about EVs were provided in these studies.

While numerous studies have primarily focused on using bioengineering technologies to increase EV yield and enhance therapeutic efficacy, a notable gap remains in exploring the impact of the culture environment on the composition of produced EVs, particularly in comparison to in vivo-generated EVs. In the context of reproductive biology, the application of bioengineering has predominantly centred on the development of microfluidic or other more complex systems for sperm selection (Li et al., 2016; Phiphattanaphiphop et al., 2020; Sharma et al., 2022; Son et al., 2017; Vasilescu et al., 2021), embryo culture (Esteves et al., 2013; Ferraz et al., 2017; Yuan et al., 2019) and as models for reproductive tissues (Chen & Schoen, 2019; Ferraz et al., 2018; Mastrorocco et al., 2020, 2021; Shea et al., 2014). However, none of these models studied how such culture conditions effect EV biogenesis and content. Furthermore, despite notable advancements in increasing the efficiency of in vitro embryo production through these systems, they still fall short in replicating the comprehensive cellular milieu, encompassing crucial cell-to-cell interactions and mechanical forces found in vivo. The versatility of microfluidic devices enables the fabrication of more bio-mimetic systems for various organs and tissues. Employing such devices, which not only emulate the overall structure of the target tissue but also incorporate continuous fluid flow and other mechanical forces, holds the potential to facilitate more in vivo-like production of EVs on a larger scale. This innovative approach goes beyond current limitations and opens avenues for exploring the effects of these dynamic conditions on EV content in the context of reproductive events.

The use of bioengineering techniques, as highlighted in this review, has demonstrated efficacy in enhancing EV production. Integrating these techniques into studies focused on reproductive EVs not only promises to expand our understanding of key contributors in reproductive events but also presents an opportunity to unravel potential underlying causes of infertility. The acquisition of new data through the use of these advanced devices holds promise for advancing research in this field and carries significant clinical implications, potentially leading to improved practices in reproductive clinics. This comprehensive

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exploration of bioengineering and biomechanical approaches will undoubtedly contribute to both research and clinical applications in reproductive biology.

5 | IMPACT OF IN VITRO PRODUCTION METHODS ON EV CARGO COMPOSITION

The cargo of EVs, which includes proteins, lipids, RNA and other biomolecules, is highly influenced by the cellular environment and culture conditions. Traditional 2D cultures, commonly used for their ease and cost-effectiveness, often produce EVs with cargo profiles that differ from those derived from in vivo conditions. These differences can be attributed to factors such as cell de-differentiation and loss of function in 2D cultures, which lead to altered EV composition and reduced yield. The different advanced culture methods described above can also significantly alter EV cargoes.

For instance, a study comparing the secretion of EVs from hiPSCs grown on microcarriers versus as aggregates in vertical-wheel bioreactors, confirmed similar pluripotency and stemness properties for both methods (Muok et al., 2024). Nevertheless, the protein and miRNA cargo of hiPSC-EVs were influenced by the culture environment. Compared to aggregate cultures, microcarrier-based cultures involved more cell–matrix interactions, leading to enriched pathways related to cell adhesion, ECM organization and cell cycle regulation. Microcarrier cultures also showed higher miRNA diversity, with miRNAs linked to cell proliferation, metabolism and immune modulation, indicating that the culture method significantly impacts the EV cargo profile. This suggests that optimizing culture conditions can enhance the functionality and therapeutic potential of hiPSC-EVs (Muok et al., 2024).

When hMSCs were cultured in dynamic 3D aggregation cultures they produce more EVs compared to 2D cultures (Yuan et al., 2022). Transcriptome analysis of the EVs in this study showed upregulation of neuroprotective and anti-apoptotic miRNAs in 3D hMSC-EVs, which are beneficial for immunomodulation and neural protection. Proteomic analysis revealed the upregulation of proteins related to cell adhesion, ECM-receptor interactions and signalling pathways that support cell viability and migration. The study suggests that the enhanced EV biogenesis in 3D cultures results from improved cell–cell interactions and ECM enrichment, alongside specific sorting mechanisms for EV cargo, leading to EVs with therapeutic potential for immunomodulation and neural protection (Yuan et al., 2022).

Similarly, when MSCs from bone marrow were cultured in 3D spheroids compared to 2D cultures, 787 common proteins, 466 unique to 2D EVs and 242 unique to 3D EVs were identified (Kusuma et al., 2022). Functional enrichment analysis showed key differences in immune-based and fibrosis-related functions, with 3D EVs enriched in proteins involved in inflammatory response, cell differentiation and leukocyte migration. In contrast, 2D EVs were linked to processes such as elastic fibre formation and ECM disassembly. Differential expression analysis identified specific proteins significantly upregulated in each condition, highlighting the unique protein cargo profiles dependent on the culture environment (Kusuma et al., 2022).

Exosomes isolated from the supernatants of hUMSCs cultured on both 3D scaffolds and 2D films, contained differentially expressed miRNAs, with 68 upregulated and 127 downregulated miRNAs in 3D compared to 2D cultures (Yang et al., 2020). KEGG pathway analysis revealed these miRNAs were involved in neurodegenerative diseases, immune response and environmental adaptation. Additionally, 3D exosomes showed higher levels of proteins such as neprilysin (NEP), insulin-degrading enzyme (IDE) and heat shock protein 70 (HSP70), which are associated with Aβ degradation and neuroprotection (Yang et al., 2020).

On the other hand, a study by Wu et al., comparing NK-92MI-derived EVs produced by shaking culture in a seesaw-motion bioreactor or in static culture, showed that, despite the similarities in physical properties, the shaking culture conditions led to increased EV production compared to static culture (Wu et al., 2022). Nevertheless, the concentrations of key cytotoxic proteins (perforin, granzyme A and granzyme B) were similar between the two methods, indicating that shaking culture maintained EV morphology, structure and protein composition while only enhancing EV yield (Wu et al., 2022).

Although such studies are informative, they mainly compare different cell types and not demonstrate how going beyond 2D culture could improve EV outcomes. In that regard, Thippabhotla et al. (2019) was the only one to demonstrate such comparisons. Their study compared EV secretion dynamics from 3D and 2D cultured HeLa cells, using peptide hydrogel scaffolds for 3D culture to mimic in vivo conditions (Thippabhotla et al., 2019). EVs from 3D cultures showed a more active secretion rate and a denser distribution of smaller EVs compared to 2D cultures (Thippabhotla et al., 2019). Further analysis indicated that the RNA profiles of EVs derived from 3D cultures were significantly similar to those from cervical cancer patient plasma, while EVs from 2D cultures were less comparable. This suggests that the 3D culture environment better replicates the in vivo physiological conditions, making it a more accurate model for studying EV production and function. Additionally, this study highlighted the importance of culture conditions in determining EV cargo, with 3D-cultured EVs showing enriched miRNAs associated with cancer diagnostics and other regulatory functions (Thippabhotla et al., 2019). The findings support the use of 3D culture systems for generating in vivo-like EVs, which could be crucial for accurate biomimetic tissue modelling and drug screening applications.

Regarding reproductive EVs, a study form Alminana et al. (2017) has shown the difference between in vivo versus 2D culture-derived oviductal EVs. Proteomic analysis identified 315 proteins in oviduct EVs, with 97 unique to in vivo, 47 unique to in vitro and 175 common to both (Almiñana et al., 2017). Differential expression analysis showed 186 proteins with significant

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expression differences between in vivo and in vitro EVs. Functional analysis revealed that many identified proteins are involved in metabolism, cellular processes and cell-to-cell communication. Key reproductive proteins like OVGP1, MYH9 and lactadherin were found in different patterns between in vivo and in vitro EVs, highlighting the influence of the culture environment on EV content. The study demonstrated that EVs from both sources can be internalized by embryos, enhancing development and quality during in vitro culture (Almiñana et al., 2017). These findings underscore the importance of using in vivo-like culture conditions to better replicate physiological environments for EV production.

While 3D culture systems appear to produce EVs with cargo profiles that more closely resemble in vivo conditions, there are still relatively few studies directly comparing these 3D-cultured EVs with in vivo EVs. This gap in the research needs to be addressed to better validate the use of 3D-cultured EVs for therapeutic applications. Comprehensive comparisons and in-depth analyses are necessary to confirm the potential of 3D culture systems in producing EVs that are functionally equivalent to those derived from natural physiological environments. Such studies will enhance our understanding and improve the reliability of using these EVs in clinical and therapeutic settings.

6 | CONCLUSION

In conclusion, the growing field of EV research holds great promise in revolutionizing various aspects of reproductive biology, from biomarker discovery to therapeutic interventions and advancements in assisted reproductive technologies. This review has focused on the crucial role of EVs as potential biomarkers and therapeutic agents in reproductive contexts. However, significant challenges, such as low yield and non-scalable production methods, hinder their clinical translation.

Central to overcoming these challenges is the adoption of innovative bioengineering approaches to bridge the gap between in vitro and in vivo conditions, thereby enhancing the yield and quality of reproductive EVs. Bioprinting, microfluidics and bioreactors technologies represent just a few examples of bioengineering tools that have demonstrated promise in upscaling EV production and elucidating EV biology.

While much progress has been made in increasing EV yield and therapeutic efficacy, a critical knowledge gap persists regarding how the culture environment influences EV composition, particularly in comparison to in vivo-generated EVs. Addressing this knowledge gap not only will deepen our understanding of reproductive events but also holds the potential to unravel the underlying causes of infertility. Integration of bioengineering techniques into studies focused on reproductive EVs presents a transformative opportunity to advance both research and clinical applications within reproductive biology. These innovative devices promise to not only expand our understanding of key contributors in reproductive events but also pave the way for improved clinical practices.

In essence, this comprehensive exploration of bioengineering and biomechanical approaches represents a promising trajectory for future advancements, bringing us closer to unravelling the complexity of reproductive biology and addressing the unmet clinical needs in infertility management and assisted reproduction.

AUTHOR CONTRIBUTIONS

Roksan Franko: Conceptualization (equal); data curation (equal); formal analysis (equal); methodology (equal); writing—original draft (lead); writing—review and editing (equal). **Marcia de Almeida Monteiro Melo Ferraz**: Conceptualization (lead); funding acquisition (lead); project administration (lead); supervision (lead); writing—original draft (supporting); writing—review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

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