HB-EGF-loaded nanovesicles enhance trophectodermal spheroid attachment and invasion

Qi Hui Poh\textsuperscript{1}, alin rai\textsuperscript{1}, Jonathon Cross\textsuperscript{1}, and David Greening\textsuperscript{1}

\textsuperscript{1}Baker Heart and Diabetes Institute

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Abstract

The ability of trophectodermal cells (outer layer of the embryo) to attach to the endometrial cells and subsequently invade the underlying matrix are critical stages of embryo implantation during successful pregnancy establishment. Extracellular vesicles (EVs) have been implicated in embryo-maternal crosstalk, capable of reprogramming endometrial cells towards a pro-implantation signature and phenotype. However, challenges associated with EV yield and direct loading of biomolecules limit their therapeutic potential. We have previously established generation of cell-derived nanovesicles (NVs) from human trophectodermal cells (hTSCs) and their capacity to reprogram endometrial cells to enhance adhesion and blastocyst outgrowth. Here, we employed a rapid NV loading strategy to encapsulate potent implantation molecules such as HB-EGF (NVHBEGF). We show these loaded NVs elicit EGFR-mediated effects in recipient endometrial cells, activating kinase phosphorylation sites that modulate their activity (AKT S124/129, MAPK1 T185/Y187), and downstream signalling pathways and processes (AKT signal transduction, GTPase activity). Importantly, they enhanced target cell attachment and invasion. The phosphoproteomics and proteomics approach highlight NVHBEGF-mediated short-term signalling patterns and long-term reprogramming capabilities on endometrial cells which functionally enhance trophectodermal-endometrial interactions. This proof-of-concept study demonstrate feasibility in enhancing the potency of NVs in the context of embryo attachment and establishment.
Figure 2. Uptake of NVR-20 and NVs by endometrial HEC1A cells
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HB-EGF-loaded nanovesicles enhance trophectodermal spheroid attachment and invasion

Qi Hui Poh¹²³, Alin Rai¹³⁴, Jonathon Cross¹, David W Greening¹³⁴⁵*

¹Baker Heart and Diabetes Institute, Molecular Proteomics, Melbourne, Victoria, Australia. ²Department of Biochemistry and Chemistry, School of Agriculture, Biomedicine and Environment, La Trobe University, Bundoora, Victoria, Australia. ³Department of Cardiovascular Research, Translation and Implementation, La Trobe University, Melbourne, Victoria, Australia. ⁴Central Clinical School, Monash University, Melbourne, Victoria, Australia. ⁵Baker Department of Cardiometabolic Health, University of Melbourne, Melbourne, Victoria, Australia.

*To whom correspondence should be addressed:

David W. Greening
Molecular Proteomics, Baker Heart and Diabetes Institute
75 Commercial Road, Melbourne, 3004, Australia
Email: David.Greening@baker.edu.au

ORCID
Qi Hui Poh – 0000-0003-2620-2834; Alin Rai – 0000-0001-7994-5151; Jonathon Cross – 0009-0006-3057-8964; David W Greening – 0000-0001-7516-485X

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Abstract

The ability of trophectodermal cells (outer layer of the embryo) to attach to the endometrial cells and subsequently invade the underlying matrix are critical stages of embryo implantation during successful pregnancy establishment. Extracellular vesicles (EVs) have been implicated in embryo-maternal crosstalk, capable of reprogramming endometrial cells towards a pro-implantation signature and phenotype. However, challenges associated with EV yield and direct loading of biomolecules limit their therapeutic potential. We have previously established generation of cell-derived nanovesicles (NVs) from human trophectodermal cells (hTSCs) and their capacity to reprogram endometrial cells to enhance adhesion and blastocyst outgrowth. Here, we employed a rapid NV loading strategy to encapsulate potent implantation molecules such as HB-EGF (NV\textsuperscript{HBEGF}). We show these loaded NVs elicit EGFR-mediated effects in recipient endometrial cells, activating kinase phosphorylation sites that modulate their activity (AKT S124/129, MAPK1 T185/Y187), and downstream signalling pathways and processes (AKT signal transduction, GTPase activity). Importantly, they enhanced target cell attachment and invasion. The phosphoproteomics and proteomics approach highlight NV\textsuperscript{HBEGF}-mediated short-term signalling patterns and long-term reprogramming capabilities on endometrial cells which functionally enhance trophectodermal-endometrial interactions. This proof-of-concept study demonstrates feasibility in enhancing the functional potency of NVs in the context of embryo implantation.

Significance statement

Nanosized extracellular vesicles and a plethora of growth factors (i.e., HB-EGF) are critical signalling mediators during embryo implantation to the maternal endometrium – a cardinal event of pregnancy establishment. This study highlights a rapid and scalable cell extrusion method to load HB-EGF into trophectodermal cell-derived nanovesicles (NV\textsuperscript{HBEGF}). We report, through phosphoproteomics and proteomics analyses, NV\textsuperscript{HBEGF} short-term signalling and long-term reprogramming capabilities on recipient endometrial cells, including but not limited to EGFR-mediated phosphorylation patterns, downstream signalling events, and cellular processes intimately associated with embryo implantation and endometrial receptivity. Importantly, the application of NV\textsuperscript{HBEGF} stimulated heightened endometrial-trophectodermal attachment, and trophectodermal invasion – pivotal events in the early stages of pregnancy. We have thus harnessed trophectodermal NVs loaded with HB-EGF to orchestrate multifaceted signalling and cellular events in endometrial cells crucial for pregnancy establishment. Loaded NVs possess immense potential for therapeutic development and warrants further investigation.
**Introduction**

Embryo implantation is a multi-step process comprising blastocyst apposition and attachment to the maternal endometrial epithelium by its outer trophodermal layer and its subsequent invasion into the underlying tissue for intrauterine development\(^1\)\(^-\)\(^3\). Its failure accounts for ~75% of unsuccessful pregnancy outcomes in Assisted Reproductive Technologies (ART)\(^1\)\(^-\)\(^3\), presenting a significant hurdle for human reproduction. Paramount for successful implantation, reciprocal embryo-maternal communication\(^4\)\(^,\)\(^5\) mediated by secreted signalling players\(^6\)\(^-\)\(^8\) such as hormones (hCG\(^9\)), cytokines (LIF\(^10\), IL-18\(^11\)), and growth factors (GM-CSF\(^12\), G-CSF\(^13\)) remains an ongoing topic of investigation in reproductive biology, with efforts to develop them as diagnostic markers of uterine receptivity or therapeutic supplements to enhance implantation success, extending into clinical trials.

Of increasing interest as a signalling modality are extracellular vesicles (EVs)\(^14\)\(^,\)\(^15\); membrane-bound nanosized (30-1000 nm) vesicles that transport and deliver bioactive lipids, proteins, and genetic material to recipient cells, reprogramming and altering their molecular signature and phenotype\(^16\)\(^-\)\(^19\). Indeed, EVs from human embryos and trophodermal cells (hTSCs) harbour critical regulators of implantation that reprogram recipient endometrial proteome to enhance embryo-endometrial attachment\(^17\). However, their isolation procedures are tedious and time-consuming, prompting investigation into an EV-like alternative; nanovesicles (NVs), generated by serial extrusion of parental cells\(^20\)\(^-\)\(^23\). From hTSCs, NVs displayed similar biophysical and functional properties to EVs, significantly promoting trophoderm-endometrial attachment and embryo outgrowth (Proteomics, in review). As extrusion is recognised as an effective approach for drug loading into nanocarriers such as EVs and liposomes (4-fold higher than passive methods)\(^24\), this methodology enables opportunities for NV cargo modification. Indeed, loaded EVs and NVs are increasingly explored as fertility therapeutics\(^25\)\(^,\)\(^26\). For example, human chorionic gonadotropin (hCG), a potent embryonic signal, was loaded into uterine fluid EVs (UF-EV\(^{hCG}\)) and treated onto endometrial cells, enhancing their expression of receptivity markers\(^26\). Similarly, enrichment of NVs with known regulators of implantation may enhance or confer specific functions while retaining certain influential characteristics of parental cells, such as surface-expressed molecules that facilitate interaction with target recipient cells\(^20\)\(^,\)\(^27\), or natural composition of bioactive molecules that contribute to desired functional outcomes\(^22\)\(^,\)\(^23\)\(^,\)\(^28\).

Amongst the molecules investigated for facilitating embryo-maternal crosstalk that governs successful implantation, heparin-binding EGF-like growth factor (HB-EGF)\(^29\)\(^-\)\(^33\) remains one of the longest-standing and well-established. With potent embryotropic and endometrial reprogramming capabilities, HB-EGF is secreted by both the developing blastocyst and the receptive endometrium; importantly, both entities express its cognate receptors\(^34\), and are thus responsive to its role in mediating surface interactions, and downstream signalling cascades. Indeed, EGFR, MAPK, and PI3K-AKT signalling
pathways and their associated processes are indispensable for successful embryo and endometrial reprogramming\textsuperscript{[35-37]} during implantation and throughout pregnancy. In this study, we employed the extrusion methodology to enrich hTSC NVs with HB-EGF (NV\textsuperscript{HBEGF}) and investigated the response of low-receptive HEC1A endometrial recipient cells at a molecular level, including protein phosphorylation changes and global proteome reprogramming. Further, we assessed NV\textsuperscript{HBEGF} functional capacity to enhance trophectodermal spheroid attachment on stimulated endometrial cells and trophectodermal spheroid invasion into Matrigel™.

Materials and methods

Cell culture

\textit{Human trophectodermal cells (T3-TSC)} (kind gift from Prof. Susan Fisher, UCSF) were derived from individual blastomeres of donated human embryos.\textsuperscript{[38]} Cells were grown as a monolayer and routinely maintained as described\textsuperscript{[39]} in DMEM/F12 (Gibco, Invitrogen) supplemented with 1% v/v Penicillin-Streptomycin (P/S) and 10% v/v foetal calf serum (FCS, Gibco, Invitrogen), with addition of 10 ng/ml bovine fibroblast growth factor (bFGF, R&D Systems) and 10 μM SB431542 (#1614, Tocris Bioscience) to maintain a trophectoderm-like state. Cells were grown on flasks coated with 0.5% gelatin prior to experimental seeding and passaged using Trypsin-EDTA (Gibco). Spheroids were generated as described\textsuperscript{[39, 40]} with slight modifications. T3-TSC cells were seeded at 1500 cells per well in an ultra-low adhesion round-bottom 96-well plate in 100 μl of trophectoderm medium and incubated for 72 h.

\textit{HEC1A endometrial epithelial cells}

Human endometrial carcinoma HEC1A cells (HTB-112) were a kind gift from Professor Lois Salamonsen purchased from American Type Culture Collection (ATCC; Rockville, MD). Endometrial cells were routinely maintained in DMEM/F12 supplemented with 1% v/v P/S, and 5% v/v FCS and incubated at 37°C with 5% CO\textsubscript{2}. Cells were routinely passaged using 0.5% v/v trypsin-EDTA (Gibco). Prior to treatments used in this study, cells were cultured in basal media overnight comprising DMEM/F12 supplemented with 0.6% insulin transferrin selenium (ITS, Gibco) and 1% v/v P/S.

Generation of hTSC NVs and loaded NV\textsuperscript{HBEGF}

NV\textsuperscript{HBEGF} generation and purification were performed as described\textsuperscript{[20, 21, 28]} with modifications (N=3). Briefly, T3-TSC human trophectodermal cells (approximately 6.25 x 10\textsuperscript{6} cells per T-75 flask) were rinsed twice with PBS and detached with 10 mM EDTA (Sigma-Aldrich). The cell suspension was pelleted at 500 g for 5 min and re-suspended in ice-cold PBS containing 50 ng/ml human recombination human epidermal-like growth factor (HB-EGF) (#4266-50, Abcam). The cell suspension was sequentially extruded through 10, 5, and 1 μm pore-sized polycarbonate membranes (Nuclepore,
Whatman Inc., Clifton, NJ, USA) thirteen times across each filter using a mini extruder system (Avanti Polar Lipids, Birmingham, AL, USA). For unloaded NVs, the cell pellet was re-suspended in ice-cold PBS prior to sequential extrusion. Extruded NVHVBE GF and NVs were subsequently isolated using 10% OptiPrep™ (Stemcell Technologies) density cushion (step gradient formed by overlaying extruded sample on 10% and 50% iodixanol) and centrifuged at 100 000 g for 2 h at 4°C. Seven equal fractions were collected, diluted in PBS (to 1.5 ml), and ultracentrifuged at 100 000 g for 1 h at 4°C (TLA-55 rotor; Optima MAX-TL ultracentrifuge). NVHVBE GF and NVs pellets were resuspended in PBS and stored in 1 µg/ul aliquots at −80°C until use.

Co-culture attachment assay
HEC1A endometrial epithelial cells were used to model a low-receptive endometrium[41-44]. HEC1A cells were seeded at confluence onto round-bottom 96-well plates before overnight culture in basal media (DMEM/F12 supplemented with 1% v/v P/S), followed by a 24-h treatment with NVHVBE GF or NVs (50 µg/ml), HB-EGF (50 ng/ml), PBS (volume matched), Erlotinib (20 nM), or sequential Erlotinib (20 nM) for 2 h followed by NVHVBE GF. T3-TSC spheroids (1500 cells per spheroid, 1 spheroid per well) were transferred to stimulated endometrial cells and allowed to attach for 1 h, after which the media was aspirated and washed gently once with PBS. Spheroid adhesion (%) for each treatment was calculated by: [(number of attached spheroids/number of seeded spheroids) x 100] (n=12, N=5).

hTSC spheroid Matrigel invasion assay
hTSC spheroid invasion assays were performed with growth factor reduced Matrigel™ matrix (Corning) as previously described[45]. Briefly, hTSC spheroids were suspended in 100 µl DMEM/F-12 media containing 1% (v/v) Pen/Strep, 0.1% ITS, and either NVHVBE GF or NVs (50 µg/ml), HB-EGF (50 ng/ml), PBS (volume matched), Erlotinib (20 nM), or sequential Erlotinib (20 nM) for 2 h followed by NVHVBE GF (ErlonNVHVBE GF). The spheroid suspension (2-3/well) was overlaid onto Matrigel™ in 8-well microscopy chambers (Corning) and incubated for 24 h at 37°C. Subsequently, 50 µl media was removed from each well, mixed 1:1 with Matrigel™, then gently overlaid back onto the spheroids. Matrigel™ was then allowed to solidify for 30 min at 37°C prior to adding 200 µl of DMEM/F-12 [10% (v/v) FBS, 1% (v/v) Pen/Strep] containing the treatments as above. After 72 h, spheroids were imaged using Olympus FSX100. The extent of invasion (% increase) was quantified using ImageJ and calculated by: [(outer—inner circumference)/(inner circumference) x 100]. Data presented as a box plot was generated from individual points (n≥8) per treatment, providing the interquartile range and minimum, median, and maximum values of each treatment.

Protein quantification and western blotting
All samples were lysed in 1% v/v sodium dodecyl sulphate (SDS), 50 mM triethylammonium bicarbonate (TEAB), pH 8.0, incubated at 95 °C for 5 mins and quantified by microBCA assay (Thermo...
Fisher Scientific) as described\textsuperscript{46}. Western blot sample buffer (4% w/v SDS, 20% v/v glycerol, and
0.01% v/v bromophenol blue, 0.125 M Tris-hydrochloride (Tris-HCl), pH 6.8) was added in a 1:1 v/v
ratio to lysed samples with 100 mM dithiothreitol (DTT, Thermo Fisher Scientific). Samples (10–20
μg) were resolved on Norvex 4–12% Bis–Tris NuPAGE gels with MES running buffer at 150 V for 1
h. Proteins on the gel were electrotransferred onto nitrocellulose membranes using iBlot\textsuperscript{TM} Dry 2.0
blotting system (Life Technologies) at 12 V for 8 min. The membranes were blocked with 5% w/v skim
milk powder in PBS-Tween (PBST) (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na\textsubscript{2}HPO\textsubscript{4}, 0.0018 M
KH\textsubscript{2}PO\textsubscript{4}, 0.05% w/v Tween 20) for 30 min at room temperature. The
membranes were washed and probed with primary antibodies (1:1000 dilution) for 24 h at 4°C in
PBST. Primary antibodies used include mouse monoclonal against CD44 (#119863, Abcam), and HB–EGF (#27450, Cell Signaling
Technology). Secondary antibodies used were: IRDye 800 goat anti-mouse IgG (#926-32210) or IRDye 680 goat anti-rabbit IgG (#926-68071) (1:15000, LI-COR Biosciences).

Biophysical particle analysis
Cryo-electron microscopy imaging (Tecnai G2 F30) of NV\textsuperscript{HBEGF} and NVs was performed as described\textsuperscript{47}. Briefly, NVs (~1 μg protein) were transferred onto glow-discharged C-flat holey carbon
grids (ProSciTech Pty Ltd., Kirwan, Australia). Excess liquid was blotted, and grids were frozen in liquid ethane. Grids were mounted in a Gatan cryoholder (Gatan, Inc.,Warrendale, PA, USA) in liquid nitrogen. Images were acquired at 300 kV using a Tecnai G2 F30 (FEI, Eindhoven, The Netherlands) in low dose mode.

Lipophilic dye labelling and uptake assay
For NV staining (NV\textsuperscript{HBEGF} and NV), NVs were incubated with Vybrant\textsuperscript{™} DiI Cell-Labeling Solution at 1:200 dilution (Invitrogen, V22885) at 1 μM concentration for 15 min at 37°C as described\textsuperscript{48}. Unbound dye was removed by subjecting labelled NVs (volume-matched DiI-PBS as label control) to
centrifugation at 100 000 g (1 h) on a 10% OptiPrep\textsuperscript{™} cushion. Pelleted DiI-NVs were resuspended in 50 μl of PBS. HEC1A cells grown to 70% confluency in 8-well glass chamber slide (Sarstedt) were
incubated with DiI-labelled NVs at 37°C for 2 h, then washed twice with PBS. Nuclei were stained with
Hoechst stain (10 μg/ml) for 10 min and fixed using 4% formaldehyde for 5 min and imaged with Nikon
A1R confocal microscope equipped with resonant scanner, using a 20x WI (1.2 NA); (Nikon, Tokyo,
Japan). Images were sequentially acquired. The XY image resolution was 1024 x 1024 at 0.033 FPS,
4x averaging, 2.4 dwell time. 3D images were taken by Z-stack of approximately 15 μm, 25 steps, at a
resolution of 1024 x 1024, 8x averaging 2.4 dwell time. NS studio was used to render images.

Proteomics: solid-phase-enhanced sample preparation
All samples, including NV\textsuperscript{HBEGF} and NVs (n=3), stimulated HEC1A cells for phosphoproteomics (n=3)
and global proteomics (n=4) were lysed in 1% v/v sodium dodecyl sulphate (SDS), 50 mM
triethylammonium bicarbonate (TEAB), pH 8.0, incubated at 95 °C for 5 mins and quantified by microBCA (Thermo Fisher Scientific) as described\[46\]. Proteomic sample preparation using single-pot solid-phase-enhanced sample preparation (SP3)\[49\] was performed on protein extracts (10 µg, 300 µg for phosphoproteomics) as previously described\[17\]. Briefly, samples were reduced with 10 mM DTT at RT for 1 h (350 rpm), alkylated with 20 mM iodoacetamide (IAA) (Sigma-Aldrich) for 20 min at RT (light protected), and quenched with 10 mM DTT. A Sera-Mag SpeedBead carboxylate-modified magnetic particle mixture (1:1 hydrophilic and hydrophobic mix, 651521050250, 451521050250, Cytiva) was added to protein extracts and incubated in 50% v/v ethanol for 10 min (1000 rpm) at RT. Beads were sedimented on a magnetic rack to remove the supernatant. Beads were washed three times with 200 µL 80% v/v ethanol, then resuspended in 100 µL 50 mM TEAB pH 8.0 and digested overnight with trypsin (1:50 trypsin: protein ratio; Promega, V5111) at 37 °C, 1000 rpm. The peptide and bead mixture were centrifuged at 20,000 g for 1 min at RT. Samples were then placed on a magnetic rack and the supernatant was collected, acidified to a final concentration of 1.5% formic acid, frozen at -80 °C for 20 min, and dried by vacuum centrifugation. Peptides were resuspended in 0.07% trifluoroacetic acid (TFA), quantified by Fluorometric Peptide Assay (Thermo Fisher Scientific, 23290) as per manufacturer’s instructions, and normalised to 0.5 µg/µl with 0.07% TFA.

**Phosphopeptide enrichment**

Peptide digests from each HEC1A cell treatment group (n=3) were lyophilised by vacuum centrifugation and reconstituted in Binding/Equilibration Buffer for phosphopeptide enrichment\[45\] using High-Select™ TiO₂ Phosphopeptide Enrichment kit (Thermo Fisher Scientific, A32993), as per manufacturer’s instructions. Briefly, peptide digests were transferred to a pre-equilibrated TiO₂ spin tip and centrifuged twice at 1000 g, 5 min. The column was washed twice with binding/equilibration buffer and subsequent wash buffer at 3000 g, 2 min, then with MS-grade water at 3000 g, 2 min. Phosphopeptides were eluted in 100 µl phosphopeptide elution buffer by centrifugation at 1000 g, 5 min, dried by vacuum centrifugation, and reconstituted in 0.07% TFA before quantification by Colorimetric Peptide Assay (ThermoFisher Scientific, #23275) as per manufacturer’s instructions.

**Liquid Chromatography–Tandem Mass Spectrometry**

Peptides were analysed on a Dionex UltiMate NCS-3500RS nanoUHPLC coupled to a Q-Exactive HF-X hybrid quadrupole-Orbitrap mass spectrometer equipped with a nanospray ion source in positive, data-dependent acquisition mode as described\[50\]. Peptides were loaded (Acclaim PepMap100 C18 5 µm beads with 100 Å pore-size, Thermo Fisher Scientific) and separated (1.9-µm particle size C18, 0.075 × 250 mm, Nikkyo Technos Co. Ltd) with a gradient of 2–80% acetonitrile containing 0.1% formic acid over 110 min at 300 nL min⁻¹ at 55°C (in-house enclosed column heater). An MS1 scan was acquired from 350–1,650 m/z (60,000 resolution, 3 × 10⁶ automatic gain control (AGC), 128 msec injection time) followed by MS/MS data-dependent acquisition (top 25) with collision-induced
dissociation and detection in the ion trap (30,000 resolution, 1 ×10^5 AGC, 60 msec injection time, 28% normalized collision energy, 1.3 m/z quadrupole isolation width). Unassigned precursor ions charge states and slightly charged species were rejected and peptide match disabled. Selected sequenced ions were dynamically excluded for 30 sec. The mass spectrometry-based proteomics data is deposited to the ProteomeXchange Consortium via the MASSive partner repository and available via MASSive with the identifier MSV000092562.

**Data Processing and Bioinformatics**

Peptide identification and quantification were performed as described previously\cite{39,50} using MaxQuant (v1.6.14) with its built-in search engine Andromeda\cite{51}. Tandem mass spectra were searched against *Homo sapiens* (human) reference proteome (74,811 entries, downloaded 12-2019) supplemented with common contaminants. Search parameters included carbamidomethylated cysteine as fixed modification and oxidation of methionine and N-terminal protein acetylation as variable modifications. Data was processed using trypsin/P as the proteolytic enzyme with up to 2 missed cleavage sites allowed. The search tolerance and fragment ion mass tolerance were set to 7 ppm and 0.5 Da, respectively, at less than 1% false discovery rate on peptide spectrum match (PSM) level employing a target-decoy approach at peptide and protein levels. Protein group or phosphorylation site tables were imported into Perseus (v1.6.7) for analysis, with contaminants and reverse peptides removed. Label free quantification (LFQ) algorithm in MaxQuant was used to obtain quantification intensity values and processed using Perseus as described\cite{52}. Cytoscape\cite{53} (v3.9.1) with STRING and EnrichmentMap plugins were used for functional enrichment analyses (KEGG, Reactome, Gene Ontology (GO) biological process) of proteins and to generate protein-protein interaction networks. The kinase-substrate database from PhosphoSite Plus was used to identify upstream kinases for phosphorylated proteins.

**Statistical Analysis**

Data clean up and analysis were performed using Perseus (MaxQuant computational platform) and Excel. Protein intensities were log_2 transformed and subjected to one-way ANOVA followed by Post hoc Tukey’s HSD test to identify significant differences between treatment groups. For stimulated HEC1A endometrial cells, proteins identified in ≥2 replicates (out of 3) or ≥3 replicates (out of 4) in each group were included in analysis. Phosphorylated sites (phosphosites) with a localisation probability of >75% and quantified in ≥2 out of 3 replicates per treatment group were included in the analysis. GraphPad Prism v9.4.1 and R (2022.02.3+492) were used for statistical analysis of functional data. One-way ANOVA for multiple comparisons or unpaired t-test was performed. All data is presented as mean plus/minus standard deviation (mean±SD). P-value<0.05 is considered statistically significant.
Results

3.1. Generation of HB-EGF-loaded NVs (NV\textsuperscript{HBEGF}) from human trophectodermal cells (hTSCs)

Cell-derived NVs were generated by serial extrusion of hTSCs (6.25 \times 10^6) suspended in PBS through microfilters of decreasing pore size (10-5-1 μm) as described\textsuperscript{[28]}. To generate NVs loaded with HB-EGF (NV\textsuperscript{HBEGF}), we serially extruded hTSCs in PBS containing 50 ng/ml of HB-EGF (Figure 1A). NVs were then isolated using density gradient separation\textsuperscript{[28]} (Figure 1A). NVs and NV\textsuperscript{HBEGF} displayed similar buoyant densities of 1.10-1.20 g/cm\textsuperscript{3}, and cryo electron microscopy revealed that NVs were spherical in shape and morphologically intact (Figure 1B), ranging 20-250 nm in diameter (mean 104.2 nm) (Figure 1C), consistent with NVs\textsuperscript{[28]} generated previously. We next questioned whether HB-EGF is successfully incorporated into NVs. We subjected NVs (NVs and NV\textsuperscript{HBEGF}, n=3) to mass spectrometry-based proteomic profiling (Figure 1D). Based on stringent peptide and protein identification criteria we quantified HB-EGF in all NV\textsuperscript{HBEGF} biological replicates, compared to unloaded NVs. We orthogonally validated loading of HB-EGF into NVs using a monoclonal antibody specific to human HB-EGF protein by Western blotting (Figure 1E).

3.2. NV\textsuperscript{HBEGF} uptake by recipient endometrial HEC1A cells

Previously, we have shown that hTSC NVs can be taken up by endometrial HEC1A cells to enhance their attachment to hTSC cell spheroids (in review, Proteomics). Here, we questioned whether loading of HB-EGF into NVs impacts their uptake. For this, NV\textsuperscript{HBEGF} were labelled with fluorescent lipophilic DiI dye (red) and incubated with HEC1A cells over a 2-hr period. Confocal fluorescence microscopy revealed that NV\textsuperscript{HBEGF}, similar to unloaded NVs, were readily taken up by HEC1A cells (Figure 2A). Imaging along the z-axis showed that NV\textsuperscript{HBEGF} were internalised and appeared as punctuate structures, typical of vesicle uptake by recipient cells\textsuperscript{[17, 45]} (Figure 2B).

3.3. NV\textsuperscript{HBEGF}-mediated phosphorylation is linked to intracellular signal transduction and EGFR signalling

HB-EGF activates various receptors (e.g., PRLR\textsuperscript{[54]}, CD44\textsuperscript{[55, 56]}) but their effect on receptor tyrosine kinases (RTKs)\textsuperscript{[57]} ERBB2/4 and especially EGFR, are more prominently studied. HB-EGF activation of EGFR\textsuperscript{[58]} induce receptor conformation changes, internalisation, and intracellular localisation; and downstream activation of the RAS-RAF-MEK-ERK, PI3K-AKT, STAT, and NF-kappa-B signalling pathways\textsuperscript{[58]} which have roles in modulating cell adhesion and motility. However, phosphorylation patterns, signalling dynamics, and functional outcomes downstream of EGFR activation remain poorly understood\textsuperscript{[59]}. For insights into whether the HB-EGF loaded into NVs are functional in recipient
HEC1A cells, we stimulated HEC1A cells with NV\textsuperscript{HBEGF} and NVs (5 min treatment) and performed phosphoproteomics analysis (\textbf{Figure 3A}, Table S1). Further, to investigate the dynamic cellular signalling events initiated by NV\textsuperscript{HBEGF}; Erlotinib\textsuperscript{[58]}, an EGFR inhibitor; was used as a pre-treatment to suppress NV\textsuperscript{HBEGF}-mediated EGFR signalling in HEC1A cells (ErloNV\textsuperscript{HBEGF}) (\textbf{Figure 3A}).

NV\textsuperscript{HBEGF} treatment, compared to NVs, resulted in unique phosphorylation of 303 proteins and identification of 396 phosphopeptide sites in HEC1A cells, including EGFR signalling regulators ERRFI1 S273\textsuperscript{[60]}, PRKCD S304\textsuperscript{[61]}, RALBP1 S99, RICTOR S21\textsuperscript{[62]}, and SHC1 S139\textsuperscript{[63]} (Table S2). Following treatment on target cells, NV\textsuperscript{HBEGF} also upregulated (log2fc≥0.5) 705 phosphoproteins and 1218 phosphopeptide sites compared to NV, include those downstream of EGFR activation (\textbf{Figure 3B, Figure S1, Table S2}). However, Erlotinib pre-treatment attenuated phosphorylation of SH3KBP1 S210 and AKT1 S124\textsuperscript{[64]} and S129\textsuperscript{[65]}, potentially limiting its response to activation and kinase activity.

Additionally, phosphorylation of MAPK1 T185 and Y187 (mediated by EGFR\textsuperscript{[66]}) were not detected, along with MAP3K4 S1198, PEBP1 S52, and PTPN12 S449 (\textbf{Figure 3B and S1, Table S2}); indicative of NV\textsuperscript{HBEGF}-mediated activation of EGFR signalling in HEC1A cells. Interestingly, Erlotinib also reduced expression of phosphorylated proteins associated with endometrial receptivity\textsuperscript{[67, 68]} (MAPK1, ANK3, GPRC5C, KIF4A, NDRG1, BAG3, FMNL2, KANK2, LNPK, LIMCH1, MVB12A, NAB2, TBC1D1, UIMC1) and embryo implantation\textsuperscript{[68]} (PEBP1, CARMIL1, PHLDB2, EPB41L1, REPS1, NDRG1, SCML2, SEMA6A, SHROOM2, STX, WWC1), which were upregulated by NV\textsuperscript{HBEGF} compared to NVs (\textbf{Figure 3B, Table S2}). Inhibition of EGFR-mediated signalling may thus result in altered expression and activation of proteins/phosphoproteins critical for endometrial function.

For insights into the downstream cellular processes and signalling pathways affected by EGFR inhibition following NV treatment, we performed functional enrichment analysis on 421 proteins which phosphorylation were inhibited by Erlotinib (\textbf{Table S3}). From this subset of proteins, we identify various networks enriched including intracellular signalling, gene expression, cytoskeleton organisation, and AKT1, BRAF and GTPase activity – processes downstream of EGFR activation, were amongst those downregulated (\textbf{Figure 3C}). Subsequent NV\textsuperscript{HBEGF} treatment induced phosphorylation of 261 out of 421 Erlotinib-inhibited proteins, which are associated with GTPase activity, AKT1 and intracellular signal transduction, and the VEGF-VEGFR2 signalling pathway (\textbf{Figure 3C, Table S4}), indicative of an alternative signalling mechanism to EGFR activation.

From this profiling analysis we demonstrate that NVs loaded with HB-EGF can mediate rapid (5 min) and dynamic changes in the phosphorylation landscape of HEC1A endometrial cells, including regulators of intracellular signal transduction and EGFR signalling networks, as well as known regulators of endometrial receptivity.
3.4. \(NV^{HBEGF}\) treatment on recipient HEC1A endometrial cells significantly increased expression of proteins upregulated at the embryo-maternal interface

Embryo implantation into the maternal endometrium takes approximately 1 to 2 days\[^{69}\]. To define the influence of earlier \(NV^{HBEGF}\)-mediated phosphorylation and signalling events on endometrial cell proteome at the time of implantation, we investigated the proteome landscape of HEC1A endometrial cells following 24 hr stimulation with \(NV^{HBEGF}\), NVs, HB-EGF, Erlo\(NV^{HBEGF}\), or PBS (vehicle) (Figure 4A, Table S5). Compared to vehicle, 67 proteins were uniquely identified and significantly upregulated following \(NV^{HBEGF}\) treatment (Figure 4B), including proteins present either at the embryo-maternal interface\[^{68, 70}\] (S100A16/6/4\[^{71}\], TAGLN2\[^{72-74}\], PTGFRN\[^{75}\], CKAP4, TPD52L2, UFL1, NDUFB10, GALNT2, RAPH1), in the endometrium during pre-attachment (CSTB\[^{76}\]), or associated with placental development (FTL\[^{77}\], LAMP1\[^{78-80}\], LRP1\[^{81}\]). Of these, 6 proteins were similarly upregulated in \(NV\) treatment (CKAP4, LAMP1, GALNT2, NDUFB10, RPL38, RPS19); while 26 proteins may be attributed to HB-EGF function in \(NV^{HBEGF}\) (Figure 4B).

Erlotinib treatment disrupted phosphorylation of ERBB/EGFR signalling players – a potential mechanism by which \(NV^{HBEGF}\) and NVs reprogram HEC1A cells. We analysed the proteome of HEC1A cells following Erlo\(NV^{HBEGF}\) treatment and compared with \(NV^{HBEGF}\) treatment. Indeed, of 127 proteins downregulated (107 absent, 20 significantly downregulated) by Erlo\(NV^{HBEGF}\) treatment compared to \(NV^{HBEGF}\) (Figure 4C) included 3 proteins associated with ERBB/EGFR signalling: (i) MTOR, a protein synthesis regulator that forms a positive feedback loop to AKT signalling; (ii) GRB2, upstream regulator of MAPK and PI3K signalling pathways; and (iii) RPS6KA1, a gene expression regulator. Processes associated with the downregulated proteins include vesicle-mediated transport, symbiotic process, organelle organisation, and cellular localisation; with 86 proteins categorised as ‘KW-0597: phosphoprotein’ (Figure 4C, Table 6). Indeed, the phosphorylation expression levels of their 13 associated kinases were decreased following Erlo\(NV^{HBEGF}\) treatment compared to \(NV^{HBEGF}\), including AKT1, CDK1/9/16, CHEK1, CSN1K1A1, IKBKB, LIMK1, MAPK1, MET, PRKCD, RPS6KA1, SRC (Figure 4D).

To correlate how cellular changes are altered in HEC1A cells by \(NV^{HBEGF}\) and its influence on the endometrium at the time of implantation, we identified upregulated and downregulated proteins in \(NV^{HBEGF}\) compared to Erlo\(NV^{HBEGF}\) and vehicle (Figure 4E). We note that compared to \(NV^{HBEGF}\), Erlo\(NV^{HBEGF}\) treatment downregulated players involved downstream of the EGFR signalling pathway (MAPK1/3/14, BCAR1, IQGAP1, CRKL, INPPL1, CAV1, AP2A1, CAMK2G, GSK3B, PLCG2, NRAS), highlighting EGFR signalling as a central mechanism of \(NV^{HBEGF}\)-mediated endometrial reprogramming. Interestingly, proteins upregulated by \(NV^{HBEGF}\) have been shown to be also upregulated in expression at the embryo-maternal interface\[^{68, 70}\] (GSTO1, FKBP1A, ISG15, MAP2K1, AHCYL2,
SWAP70, PPP1CB, LAMA3, RPS20, RPL14). In this study, these identified differentially expressed proteins are involved in symbiotic process, membrane trafficking, and intracellular localisation and transport (Figure 4F, Table S7). Contrastingly, processes relating to metabolism (nitrogen, carbon, small molecule) and RNA splicing and biogenesis were associated with ErloNV<sup>H</sup>H<sup>E</sup>G<sup>F</sup> and PBS treatment respectively (Figure 4F, Table S8/9).

Collectively, we highlight the capacity of NV-mediated reprogramming of endometrial cells to modulate proteome dynamics associated with EGFR signalling and changes in the endometrium associated with embryo attachment. We next questioned whether HB-EGF-loaded NVs from human trophodermal cells could regulate endometrial function. Our data suggests that HB-EGF-loaded NVs potentially display the capacity to enhance cell attachment/adhesion and invasive capacity, as previously reported in trophodermal cell-derived NVs and secreted EVs. This hypothesis was tested.

3.5. NV<sup>H</sup>H<sup>E</sup>G<sup>F</sup> treatment significantly enhances endometrial-trophodermal adhesion following uptake by recipient endometrial cells

Using a co-culture attachment assay as an in vitro proxy measure of adhesive capacity<sup>[82, 83]</sup>, we assessed whether NV<sup>H</sup>H<sup>E</sup>G<sup>F</sup> treatment onto HEC1A cells enhances their adhesion to trophodermal spheroids (Figure 5A). Low-receptive HEC1A endometrial cells (monolayer) were stimulated with treatments for 24 hrs, then incubated with hTSC spheroids and allowed 2 hrs for attachment. Unattached spheroids were removed, remaining attached spheroids were counted, and the attachment rate assessed (Figure 5A). NV<sup>H</sup>H<sup>E</sup>G<sup>F</sup> treatment demonstrated the highest significant increase in spheroid attachment rate to HEC1A cells (%) at 65±10 – almost 40% higher than PBS control (27±6, p<0.005) (Figure 5B) and 20% higher than NVs (46±7, p<0.005). However, in ErloNV<sup>H</sup>H<sup>E</sup>G<sup>F</sup> (21±6, p<0.005), NV<sup>H</sup>H<sup>E</sup>G<sup>F</sup> did not restore the attachment capabilities of spheroids pre-treated with Erlotinib (to PBS levels); lastly, HB-EGF treatment alone performed similarly to unloaded NVs (42±25, p>0.05) (Figure 5B).

3.6. NV<sup>H</sup>H<sup>E</sup>G<sup>F</sup> treatment with trophodermal spheroids significantly enhances their invasive capacity into Matrigel™ matrix

Trophoblast invasion and outgrowth into the endometrium is a hallmark of successful implantation and placentation<sup>[73, 84-86]</sup> and assessed in vitro using the Matrigel™ matrix invasion assay<sup>[39, 45, 83]</sup> (Figure 5C). Here, trophodermal spheroids were incubated with corresponding treatments for 2 hrs prior to seeding into Matrigel™. A second dose of treatment in media was supplemented after 24 hrs and the level of invasion monitored across 72 hrs using light microscopy (Figure 5E, F). Increase in invasion was measured by subtracting the area of the original spheroid from the final measured area of invasion.
(Figure 5E, F). NV\textsuperscript{HBEGF} treatment displayed the highest significant increase in spheroid invasion (%) at 248.7±75.1 – approximately 1.5-times higher than PBS (185±32.6, p<0.0005), while NV (237.9±76.9, p>0.05) and HB-EGF (210.5±79.5, p<0.05) treatment performed similarly (Figure 5D).

From our observations with ErloNV\textsuperscript{HBEGF} (80.9±36.4, p<0.0005), EGFR inhibition with erlotinib diminished the invasive capacity of spheroids which could not be restored by subsequent NV\textsuperscript{HBEGF} treatment (Figure 5D).

Our findings demonstrate the enhanced functional impact of HB-EGF loading into NVs by demonstrating increased (i) attachment of low receptive endometrial cells to trophectodermal spheroids and (ii) invasion of trophectodermal spheroids into Matrigel™ matrix, compared to unmodified NVs.

In doing so, we highlight EGFR signalling as a critical mediator of NV\textsuperscript{HBEGF} function.

Discussion

Nanosized extracellular vesicles and a plethora of growth factors (i.e., HB-EGF) are critical signalling mediators during embryo implantation to the maternal endometrium – a cardinal event of pregnancy establishment. This study highlights a rapid and scalable cell extrusion method to load the implantation regulator HB-EGF into trophectodermal cell-derived nanovesicles (NV\textsuperscript{HBEGF}). Our study employs phosphoproteomics and proteomics analysis to demonstrate NV\textsuperscript{HBEGF} short-term signalling and long-term reprogramming capabilities on recipient low receptive HEC1A human endometrial cells. We highlight that NV\textsuperscript{HBEGF} elicit EGFR-mediated effects in recipient endometrial cells. Importantly, these protein phosphorylation activities and signalling patterns, including the activation of kinases and phosphorylation sites that regulate their function (i.e., AKT1 S124\textsuperscript{[64]} and S129\textsuperscript{[65]}, MAPK1 T185 and Y187\textsuperscript{[66]}); and signalling processes (i.e., AKT signal transduction, GTPase activity) downstream of EGFR activation; induce functional changes in recipient cells to enhance endometrial attachment to the trophectoderm, and trophectodermal invasion into Matrigel™ matrix.

At the implantation site, trophectodermal cells of the blastocyst release EVs enriched with bioactive molecules that reprogram itself\textsuperscript{[87-91]} and the endometrium\textsuperscript{[16-18, 92]} to support embryo-maternal crosstalk and implantation. NVs derived from hTSCs\textsuperscript{[17]} therefore retain a high proportion of bioactive proteins innate to trophectodermal cells, including those implicated in embryo-maternal interactions (ANXA2\textsuperscript{[93-95]}, DPP4\textsuperscript{[96, 97]}, CTB\textsuperscript{[98-100]} and trophoblast invasion (TAGLN2\textsuperscript{[73]}, CTBS/D\textsuperscript{[99]}, LGALS3\textsuperscript{[85]}). Indeed, we show that hTSC NV\textsuperscript{HBEGF} and NVs, enriched in these molecules, are effective supplements for promoting endometrial adhesion to trophectodermal cells and trophectodermal invasion into Matrigel™ (Figure 5). Similarly in various applications, NV composition can be tailored to suit various therapeutic purposes, such as the selection of macrophages for spinal cord\textsuperscript{[27]} or tumour\textsuperscript{[20]} targeting, stem cells for their regenerative properties\textsuperscript{[22, 23, 28]}, and insulin-producing cells for diabetes management\textsuperscript{[101]}.
However, the parental cells’ natural composition can often limit their function, requiring dose titrations and functional assays\textsuperscript{15, 102} to determine an effective dose, although selection of the appropriate functional assays and their standardisation remains an area of active discussion\textsuperscript{15}.

Modifying NV composition is a method of fine-tuning their function; for example loading of chemotherapeutic drugs\textsuperscript{20, 103} for cancer therapy or drug-specific investigations, or antioxidative enzymes\textsuperscript{24, 104} for oxidative stress-related diseases; it may thus be explored further to achieve a range of outcomes in different contexts. The extrusion strategy described in this study, for example, can be amended to load other factors to enhance implantation, such as those explored in clinical trials (i.e., hCG (NCT01786252\textsuperscript{105}, NCT01030393\textsuperscript{106})), without genetically modifying parental hTSCs\textsuperscript{107}.

While HB-EGF was selected for enrichment into NVs for its indispensable roles in pregnancy establishment\textsuperscript{30-33, 36, 108-111}, its well-researched mechanism of action makes it a suitable target for functional validation and for dissecting the embryo-maternal interface. HB-EGF interacts with receptor tyrosine kinases (RTKs) EGFR and ERBB4 expressed on target cells to initiate multiple downstream signalling cascades\textsuperscript{35, 112} (i.e., MAPK, PI3K-AKT/PIP, small GTPase) (reviewed\textsuperscript{113}). Furthermore, HB-EGF may perform synergistically with the high expression of heparan sulfate proteoglycans\textsuperscript{114} expressed in NVs from their trophodermal source, as this enhances their binding to high-affinity receptors (i.e., ERBB4\textsuperscript{109}), potentially augmenting its influence in recipient cells. However, given the variety of signalling patterns initiated by EGFR, this can induce variable phenotypic responses and outcomes in cells\textsuperscript{115-118}; for example, GTPase activity regulates cytoskeletal remodelling and cell polarity\textsuperscript{119, 120} in endometrial cells to enhance their adhesive capacity\textsuperscript{93, 121, 122}; in embryos, however, it influences transcription activity and signalling (CREB, WNT, JNK)\textsuperscript{123, 124} to modulate cell differentiation\textsuperscript{124} and embryo size\textsuperscript{123}. We have thus assessed the temporal effects of NV\textsuperscript{HBEGF} treatment; from the early phosphorylation-mediated signalling events occurring in recipient cells, to its molecular landscape and function at the approximate time of embryo attachment (1 to 2 days\textsuperscript{69}).

The proteome of recipient HEC1A endometrial cells indicates expression of 5 other RTKs (AXL, DDR1, MET, MST1R, EPHA2), which may interact with corresponding ligands enriched in NVs (i.e., LGALS3, collagens, HGF) to activate signalling cascades that converge with the EGFR-mediated pathway\textsuperscript{125}. For example, proteins phosphorylated by NV\textsuperscript{HBEGF} and NVs (i.e., GAB1, NCK2, and AKT1), while categorised as EGFR signalling players, are also contributors of MAPK, PI3K-AKT, and MTOR signalling – all present downstream of RTK activation\textsuperscript{126}. Indeed, upon EGFR inhibition, subsequent NV\textsuperscript{HBEGF} treatment induced EPHA2 phosphorylation and downstream signalling modulators (i.e., BRAF, MAP3K2, PAK4, PXN, SH3KBP1) (Figure 3B). NV\textsuperscript{HBEGF} may also activate cell-surface receptor CD44\textsuperscript{55} expressed on HEC1A endometrial cells, which interaction with HB-EGF\textsuperscript{56, 127} was previously implicated in endometrial tissue remodelling\textsuperscript{55}. CD44 is integral for endometrial decidualisation\textsuperscript{127} and adhesion to the embryo\textsuperscript{128}; with its expression linked to implantation.
success\textsuperscript{127} and female fertility status\textsuperscript{129}. Upon binding to compatible ligands, CD44 phosphorylates GAB1\textsuperscript{130} to initiate AKT signalling, and activates downstream effectors including RhoGTPases\textsuperscript{131-133}, to induce cytoskeletal reorganisation and cell migration and adhesion. Interestingly, despite EGFR inhibition, NV\textsubscript{HBEGF} induced the phosphorylation of GAB1 (S163) (Figure 3B), and other proteins implicated in the regulation of GTPase activity, supporting NV\textsubscript{HBEGF}-CD44 interaction as another pivotal driver of endometrial reprogramming. At the site of embryo implantation, GTPase activity exerts influence on PI3K-AKT signalling and RhoA in mouse embryos to mediate their implantation\textsuperscript{134}, endometrial cell contraction/migration\textsuperscript{120, 135}, and focal adhesion\textsuperscript{119, 135-137}; it is thus an indispensable mediator of embryo-endometrial interactions\textsuperscript{93, 121, 122}. Compared to endometrial cells, hTSCs and their derived EVs were enriched in GTPases\textsuperscript{17}; the latter’s treatment onto recipient endometrial cells upregulated cytoskeletal organisation and cell polarity processes, potentially through GTPase activity as a trophectoderm-mediated signalling strategy. Indeed, supplementation of our unloaded NVs significantly augmented the adhesive capacity of HEC1A endometrial cells to trophectodermal spheroids, as well as the invasive capacity of trophectodermal cells (Figure 5). Whether the latter observation is attributed to PI3K-AKT signalling\textsuperscript{124} still warrants investigation.

We have demonstrated marked functional influence of NV\textsubscript{HBEGF} on HEC1A endometrial cells compared to HB-EGF and NVs; which significantly augmented their adhesion to trophectoderm cells by ~40% from baseline (PBS) – double the capacity of HB-EGF and NVs (Figure 5). Given that NV\textsubscript{HBEGF} and HB-EGF share a higher proportion of upregulated proteins in endometrial cells compared to NVs, and the well-studied role of HB-EGF\textsuperscript{30-33, 36, 108-111} and ERBB/EGFR\textsuperscript{116, 138-140} signalling at the embryo-maternal interface, we posit that the latter has substantial influence on our functional observations. Indeed, with the erlotinib targeted inhibition of EGFR\textsuperscript{141}, NV\textsubscript{HBEGF} treatment could not restore endometrial or trophectodermal cell function to baseline (PBS) levels. Moreover, amongst the phosphorylation of kinases and expression of their corresponding proteins downregulated by EGFR inhibition (Figure 4D), the most dysregulated proteins include those upregulated at implantation sites\textsuperscript{142} (Figure 4E). Even so, the functional capacity of HB-EGF was inconsistent, and at best comparable to NVs; a similar phenomenon was observed in hCG-loaded EVs from human uterine fluid\textsuperscript{143}, which demonstrated the enhanced capacity to induce expression of receptivity markers in recipient endometrial cells compared to hCG alone, EVs alone, or co-supplementation of hCG with EVs. Prior attempts to develop signalling mediators (i.e., hCG\textsuperscript{9}, LIF\textsuperscript{10}, and G-CSF\textsuperscript{113}) with strong links to fertility and endometrial receptivity as fertility-enhancing supplements have also been unsuccessful in clinical trials. Taken together, these observations allude to a multi-faceted signalling mechanism by engineered EVs or NVs that encompass properties of their enriched molecule and their biological source, thereby enhancing their functional benefit and potential therapeutic utility. NVs thus represent a feasible and adaptable method of large-scale generation of therapeutic vesicles for tuning endometrial phenotype and function. This proof-of-concept study demonstrate feasibility in enhancing the potency of NVs in
the context of embryo attachment and pregnancy establishment. Whether these loaded NVs improve implantation rate \textit{in vivo} warrants future investigation.
Supporting Information

Supporting information is available from the Wiley Online Library or from the author.

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Author Contributions

QHP, AR, and DWG conceived and designed experiments. QHP carried out majority of experiments. JC assisted with proteomic sample preparation. QHP, AR, and DWG wrote, reviewed, and edited the manuscript. All authors approved the final manuscript.

Conflicts of interest: The authors declare no competing interests.

Data and Software Availability: All mass spectrometry data and spectral identifications have been deposited in the ProteomeXchange Consortium via the MASSive partner repository with the identifier MSV000092562 (NV composition, reprogrammed cell phosphoproteomics, reprogrammed cell global proteomics).
Figure 1. Production and characterisation of NV\textsuperscript{HBEGF}. A) NV\textsuperscript{HBEGF} were generated by serial extrusion (10, 5, 1 μm filters, 13 times per membrane) of human trophectodermal cells (T3-TSCs) with either 50 ng/ml of HB-EGF or PBS and purified using density-cushion ultracentrifugation to obtain 7 fractions (F1-7) of increasing density. NV-containing fraction (F5) was obtained. B) Cryo-electron microscopic image of NV\textsuperscript{HBEGF} displayed spherical and morphologically intact structures; scale 100 nm. C) Size distribution of NV\textsuperscript{HBEGF} based on cryo-electron microscopic images (n=4) reveal enrichment of particles 50-150 nm in diameter. D) Abundance of HB-EGF using mass spectrometry analysis; normalised LFQ intensities (log\textsubscript{2}) of HB-EGF between NV\textsuperscript{HBEGF} and NVs generated using the same workflow from hTSCs and mouse embryonic fibroblasts. E) Western blot display of HB-EGF enrichment in NV\textsuperscript{HBEGF} compared to NVs (n=3).

Figure 2. Uptake of NV\textsuperscript{HBEGF} and NVs by HEC1A endometrial cells A) Confocal fluorescent microscopy images demonstrating uptake of NV\textsuperscript{HBEGF} or NVs labelled with Dil lipophilic fluorescent dye labelled (red) by HEC1A endometrial cells after a 2-h incubation (n=3). B) Fluorescent Z-stack image displaying intracellular distribution of Dil-labelled NV\textsuperscript{HBEGF} (red). Nuclei of HEC1A endometrial cells were stained with Hoechst (blue). Scale bar 10 μm.

Figure 3. NV\textsuperscript{HBEGF} remodel the phosphoproteome landscape in HEC1A endometrial cells. A) Workflow for NV\textsuperscript{HBEGF} and NV treatment onto recipient HEC1A endometrial cells, including a 2-step treatment of erlotinib (EGFR inhibition) followed by NV\textsuperscript{HBEGF} stimulation, and subsequent cell phosphoproteome preparation and analysis. B) Heatmap expression (log\textsubscript{2}) of phosphorylated proteins and phosphosites of players of the EGFR signalling pathway, which are downregulated when EGFR is inhibited by erlotinib (white corresponds to missing values). C) (Top) Erlotinib inhibited the phosphorylation of 421 proteins (compared to PBS), while subsequent NV\textsuperscript{HBEGF} treatment induced phosphorylation of 261 of the inhibited proteins; (Bottom) bubble plot displaying key biological processes and pathways corresponding to the 421 and 261 proteins respectively.

Figure 4. NV\textsuperscript{HBEGF} remodel the proteome landscape and EGFR signaling network at the time of implantation. A) Workflow employed for proteomic analysis of stimulated HEC1A endometrial cells. B) Proteins uniquely identified and significantly upregulated in NV\textsuperscript{HBEGF} or NV-treated HEC1A cells compared to PBS. C) Pre-treatment of HEC1A cells with erlotinib followed by NV\textsuperscript{HBEGF} downregulated the expression of 127 proteins compared to NV\textsuperscript{HBEGF}, which are categorised into related biological processes. D) NV\textsuperscript{HBEGF} and ErloNV\textsuperscript{HBEGF}–mediated phosphorylation levels of 13 kinases that are matched to downregulated proteins. E) Comparative analysis of HEC1A cellular proteome treated with NV\textsuperscript{HBEGF} compared to ErloNV\textsuperscript{HBEGF} and PBS, and a two-way scatter plot highlighting top dysregulated proteins.
proteins in the presence of EGFR inhibitor, erlotinib. F) Bubble plot display of biological processes and pathways associated with proteins significantly upregulated (including unique) by NV$^{\text{HBEGF}}$ treatment and proteins significantly downregulated (including absent) in NV$^{\text{HBEGF}}$ compared to ErloNV$^{\text{HBEGF}}$ and PBS.

**Figure 5.** NV$^{\text{HBEGF}}$ enhances attachment to endometrial cells and outgrowth and invasion in Matrigel™ of trophectodermal spheroids. A) Experimental workflow for co-culture attachment assay. B) Box plot indicating percentage of spheroid attachment to HEC1A endometrial cells following treatment with PBS, NV$^{\text{HBEGF}}$, NV, HB-EGF, or ErloNV$^{\text{HBEGF}}$ (n=5), where rate of spheroid attachment (%) is the number of attached spheroids divided by the number of seeded spheroids expressed as a percentage. C) Experimental workflow for TSC spheroid outgrowth and invasion into Matrigel™. D) Box plot indicating quantified area of TSC spheroid outgrowth and invasion into Matrigel™ 72 hr following treatment with PBS, NV$^{\text{HBEGF}}$, NV, HB-EGF, or ErloNV$^{\text{HBEGF}}$ (n=8). E) Bright-field microscopic images of TSC spheroids outgrowth and invasion into Matrigel™ 72 hr following treatment with PBS, NV$^{\text{HBEGF}}$, NV, HB-EGF, or ErloNV$^{\text{HBEGF}}$. Scale bar 100 µm. F) Area of outgrowth extending from spheroid taken for measurements is shaded in grey and quantified using ImageJ. *p<0.05, **p<0.005, ***p<0.0005, ****p<0.001

**Figure 1.** Production and characterisation of NV$^{\text{HB-EGF}}$.
Figure 2. Uptake of NV^{HR-EGF} and NVs by endometrial HEC1A cells
Figure 3. NV^R^B^E^G^F remodel the phosphoproteome landscape in HEC1A endometrial cells
Figure 4. NVBEGF remodel the proteome landscape and EGFR signaling network at the time of implantation.
Figure 5. NVHB-EGF enhances attachment to endometrial cells and outgrowth and invasion in Matrigel of trophodermal spheroids.
References


and its receptors mediate deciduization and potentiate survival of human endometrial stromal cells. 


**[60] Liu, N., Matsumoto, M., Kitagawa, K., Kotake, Y., et al., Chk1 phosphorylates the tumour suppressor Mig-6, regulating the activation of EGF signalling. *EMBO J* 2012, 31, 2365-2377.**


**[76] Lu, L., Chen, Y., Yang, Z., Liang, S., et al., Expression and Regulation of a Novel Decidual Cell-Derived Estrogen Target during Decidualization. *Int J Mol Sci* 2022, 24.**


[125] Sudhesh Dev, S., Zainal Abidin, S., Farghadani, R., Othman, I., Naidu, R., Receptor Tyrosine Kinases and Their Signaling Pathways as Therapeutic Targets of Curcumin in Cancer. *Front Pharmacol* 2021, 12, 772510.


