

RESEARCH ARTICLE

Embryo biosensing by uterine natural killer cells determines endometrial fate decisions at implantation

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Abstract

Decidualizing endometrial stromal cells (EnSC) critically determine the maternal response to an implanting conceptus, triggering either menstruation-like disposal of low-fitness embryos or creating an environment that promotes further development. However, the mechanism that couples maternal recognition of low-quality embryos to tissue breakdown remains poorly understood. Recently, we demonstrated that successful transition of the cycling endometrium to a pregnancy state requires selective elimination of pro-inflammatory senescent decidual cells by activated uterine natural killer (uNK) cells. Here we report that uNK cells express CD44, the canonical hyaluronan (HA) receptor, and demonstrate that high molecular weight HA (HMWHA) inhibits uNK cell-mediated killing of senescent decidual cells. In contrast, low molecular weight HA (LMWHA) did not attenuate uNK cell activity in co-culture experiments. Killing of senescent decidual cells by uNK cells was also inhibited upon exposure to medium conditioned by IVF embryos that failed to implant, but not successful embryos. Embryo-mediated inhibition of uNK cell activity was reversed by recombinant hyaluronidase 2 (HYAL2), which hydrolyses HMWHA. We further report a correlation between the levels of HYAL2 secretion by human blastocysts, morphological scores, and implantation potential. Taken together, the data suggest a pivotal role for uNK cells in embryo biosensing and endometrial fate decisions at implantation.

KEYWORDS

decidualization, embryo implantation, endometrium, hyaluronan, senescence, uterine natural killer cells

Abbreviations: BCM, blastocyst conditioned media; EnSCs, endometrial stromal cells; ECM, extracellular matrix; HA, hyaluronan (hyaluronic acid); HAS, hyaluronan synthase; HYAL, hyaluronidase; HMWHA, high molecular weight hyaluronic acid; LMWHA, low molecular weight hyaluronic acid; MPA, Medroxyprogesterone acetate; SA- β -Gal, senescence-associated β -galactosidase, TPM, transcripts per million; UCM, unconditioned media; uNK cells, uterine natural killer cells.

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1 | INTRODUCTION

The midluteal window of implantation constitutes an inflection point in the menstrual cycle, after which the endometrium either breaks down or becomes transformed into the decidua of pregnancy.¹ The fate of the endometrium depends foremost on implantation of an embryo capable of producing sufficient human chorionic gonadotropin (hCG), and other fitness hormones, to rescue the corpus luteum and maintain ovarian progesterone production.² Although progesterone exerts multiple functions, its primary role is to drive differentiation of endometrial stromal cells (EnSC) into specialized decidual cells,¹ which first encapsulate the implanting blastocyst,^{3,4} and then form a robust immune-protective matrix to accommodate invading placental trophoblast.^{1,5}

Recent studies demonstrated that decidualization is a multistep differentiation process that starts with an evolutionarily conserved acute cellular stress response,⁶ exemplified by a burst of free radicals, and release of inflammatory mediators.^{7,8} This inflammatory phase coincides with the implantation window,^{9,10} and is characterized further by the accumulation of proliferating uterine natural killer (uNK) cells.⁸ After a lag-period of approximately 4 days, phenotypic decidual cells emerge that are progesterone-dependent,¹¹ resistant to oxidative and metabolic stress,^{12,13} and highly responsive to embryonic signals.^{3,4,14} However, inflammatory reprogramming of EnSC also leads to the emergence of acute senescent cells.^{8,15} The topography of senescent decidual cells, namely their proximity to the luminal epithelium, suggests that they arise from cells subjected to significant replication stress during the proliferative phase.¹⁵ Although morphologically similar to decidual cells, senescent decidual cells are progesterone-resistant and secrete an abundance of extracellular matrix (ECM) proteins and proteinases, pro-inflammatory cytokines, and chemokines (designated senescence-associated secretory phenotype or SASP), which cause sterile inflammation and induce secondary senescence in neighboring decidual cells.^{8,15,16} Under continuous progesterone signaling in pregnancy, decidual cells secrete IL-15 and other factors involved in recruitment and activation of uNK cells, which in turn target and eliminate senescent decidual cells through perforin- and granzyme-containing granule exocytosis.^{8,15} In a non-conception cycle, however, falling progesterone in the late-luteal phase leads to a preponderance of senescent cells, influx of leukocytes, ECM breakdown, and menstrual shedding of the superficial uterine mucosa.¹⁷ These novel insights into the cellular dynamics in the stroma during the window of implantation place uNK cells at the center of endometrial fate decisions.

Human preimplantation embryos are genetically diverse and prompt elimination of embryos of low-fitness is paramount to limit maternal investment in failing pregnancies.^{18,19} Several studies have highlighted that decidual cells

are programmed to respond to embryos of different quality.^{3,4,14,20,21} Furthermore, growing evidence indicates that loss of this biosensing function of the endometrium causes recurrent pregnancy loss.^{18,22} Whether uNK cells are involved in embryo selection at implantation is not known, although there is evidence that hCG stimulates uNK cell proliferation.²³

Hyaluronan (HA), a ubiquitous ECM component, plays an important role in diverse biological processes that involve rapid tissue turnover and repair.²⁴ Not surprisingly, HA has been implicated in key reproductive events, including ovulation, fertilization, embryogenesis, implantation, and trophoblast invasion.²⁵⁻²⁷ HA, a linear glycosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, is expressed in various molecular sizes up to 10⁷ kDa.²⁵ It is produced by three different hyaluronic acid-synthase enzymes (HAS1-3), each generating polymers of different lengths. Turnover of HA at tissue level is rapid, mediated by a family of hyaluronidases (HYALs). The biological actions of HA depend on its size and concentration.^{24,25} High molecular weight HA (HMWHA) polymers are space-filling hydrating molecules that impede cell differentiation and exert anti-angiogenic and immunosuppressive activities. Moreover, low molecular weight HA (LMWHA; molecular mass \leq 250 kDa) activates a number of receptors, most prominently the cell-surface glycoprotein CD44 and hyaluronan-mediated mobility receptor (HMMR; otherwise known as RHAMM or CD168), involved in cell proliferation and migration, angiogenesis, and immunomodulation.^{24,25}

In this study, we report that downregulation of HAS2 expression upon decidualization of EnSC leads to loss of HA production. We exploited this observation to demonstrate that exogenous HMWHA, but not LMWHA, abrogates uNK cell-mediated killing of senescent decidual cells. Furthermore, we show that human IVF blastocysts of poorer morphological quality and those that failed to implant secrete lower levels of hyaluronidase 2 (HYAL2) when compared to their successful counterparts. Importantly, the secretome of unsuccessful embryos also strongly inhibited uNK cell activity, an effect reversed by recombinant HYAL2. In contrast, we found no evidence that successful embryos impede uNK cell-mediated clearance of senescence decidual cells.

2 | MATERIALS AND METHODS

2.1 | Endometrial sample collection

The collection of endometrial biopsies for research purposes was approved by the NHS National Research Ethics—Hammersmith and Queen Charlotte's & Chelsea Research Ethics Committee (REC reference: 1997/5065) and

Tommy's National Reproductive Health Biobank (REC reference: 18/WA/0356). Endometrial biopsies were obtained using a Wallach Endocell endometrial sampler following transvaginal ultrasonography from patients attending a dedicated research clinic at University Hospitals Coventry and Warwickshire (UHCW) National Health Service (NHS) Trust, Coventry, UK. Written informed consent was obtained prior to tissue collection in accordance with the guidelines of the Declaration of Helsinki, 2000. Endometrial biopsies were timed 5-10 days after the pre-ovulatory LH surge. A total of 93 endometrial biopsies were processed for EnSC cultures, uNK cell isolation, or both. Demographic details of the tissue samples used in different experiments are shown in Table S1.

2.2 | Primary EnSC cultures

Human EnSC were isolated from endometrial biopsies as described previously.²⁸ Briefly, endometrial biopsies were subjected to enzymatic digestion using 500 µg/mL of collagenase type Ia (Sigma-Aldrich, Poole, UK) and 100 µg/mL of DNase I (Lorne Laboratories Ltd, Reading, UK) for 1 hour at 37°C. Digested tissue was filtered through a 40 µm cell strainer to remove glandular cell clumps, and the flow-through collected and cultured in DMEM/F12 (Thermo Scientific, Loughborough, UK) containing 10% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS), 1 × antibiotic-antimycotic mix, 10 µM of L-glutamine (Thermo Scientific), 1 nM of estradiol, and 2 µg/mL of insulin (Sigma-Aldrich). Cells were lifted with 0.05% trypsin and re-seeded as required. To induce decidual transformation, confluent EnSC monolayers were downregulated in phenol-free DMEM/F-12 media containing 2% DCC-FBS and decidualized with 10 µM of medroxyprogesterone acetate (MPA) and 0.5 mM of 8-bromo-cAMP (C+M treatment) (Sigma-Aldrich). To study the effect of exogenous HA, decidualizing EnSC were treated with either 100 µg/mL of HMWHA (molecular mass ~1320 kDa) or LMWHA (molecular mass ~33.0 kDa), purchased from Bio-Techne (USA), as described elsewhere.^{29,30} Medium was refreshed every 2 days. All experiments were performed at passage 2.

2.3 | Isolation and culture of uNK cells

uNK cells were isolated and cultured as described in detail elsewhere.^{8,15} Briefly, unattached cells from freshly digested endometrial biopsies were collected following overnight culture, and red blood cells depleted via Ficoll-Paque density medium centrifugation (400 ×g) for 30 min at room temperature (RT). uNK cells were isolated through magnetic-activated cell sorting (MACS) using microbeads targeting CD56 (Miltenyi Biotec, Bergisch Gladbach, Germany,

130-050-401), a specific marker for uNK cells. Cell density was adjusted to a maximum of 1×10^6 cells per 100 µL of wash buffer (0.5% BSA, 2 mM EDTA, and PBS) and incubated for 15 minutes at 4°C with CD56 microbeads (1:20 dilution). Unbound microbeads were removed by addition of 1 mL wash buffer, and cells were centrifuged (275 ×g) for 5 minutes at RT and then, resuspended in 500 µL of wash buffer. Cells were fed through MS columns within a magnetic field wherein CD56⁺ cells were retained in columns and the negative fraction passed through. The MS columns were rinsed three times with 500 µL of wash buffer within the magnetic field before the CD56⁺ cells were extracted by flushing the column with 1 mL of wash buffer away from the magnetic field. Isolated uNK cells were cultured in DMEM/F12 media supplemented with 10% of DCC-FBS, 1% of L-glutamine, and 1% of antibiotic/antimycotic, and containing 125 pg/mL of recombinant IL-15 (Sigma-Aldrich) to aid uNK cell maturation. uNK cells were cultured for no longer than 4 days.

2.4 | uNK-decidual co-culture assay

The uNK cells killing assay has been described in detail previously.^{8,15} Briefly, EnSC were seeded at a density of 50 000 cells/well in 96-well plates and decidualized for 6 days. Isolated uNK cells from whole endometrial tissue (n = 25 000) were then added to the cultures in the presence of C+M. Cultures continued for a further 2 days before harvesting for either RNA extraction or SA-β-Gal activity measurement. For experiments investigating the expression of *CLU* and *ILIRL1*, experiments were scaled to 6-well plates. Conditioned media was collected prior to lysis of cells. To block CD44, isolated uNK cells were incubated with the CD44 neutralizing antibody Hermes-1 (Thermo Scientific, MA4400, 10 µg/ml, 30 min, RT) prior to co-culture assay. Where appropriate, 100 µg/mL of HMWHA or LMWHA (Bio-Techne, MN, USA) or BCM were added simultaneously with uNK cells at the indicated timepoints. When indicated, HMWHA or BCM was pre-incubated with 300 IU/mL of recombinant HYAL2 (Sigma-Aldrich) for 30 minutes prior to the addition to co-cultures.

2.5 | Flow cytometry

The viability of isolated uNK cells was determined by incubation with Fixable Viability Stain 660 (FVS660, BD Biosciences, NJ, USA) diluted 1:1000 in PBS for 10 minutes at 4°C. For analysis of purity, cell suspensions were incubated with CD45-FITC (555482, 1:5, BD Bioscience) and CD56-PE (555516, 1:5, BD Bioscience) for 30 minutes in the dark at 4°C. After two washes with FACS buffer (0.5% BSA

and 2 mM EDTA in PBS), cells were resuspended in 500 μ L of FACS buffer prior to analysis on BD FACSMelody flow cytometer.

2.6 | Immunocytochemistry of EnSC and uNK cells

Confluent EnSC monolayers were fixed with 4% paraformaldehyde for 5 minutes at RT and then, incubated with 1% BSA in PBS-T (PBS plus 0.1% Tween 20) for 30 minutes at RT. Cells were stained with biotin-labeled hyaluronic acid-binding protein (HABP) (AMSBIO, Abington, UK, AMS.HKD-BC41, 1:125) overnight at 4°C and stained with fluorescein-conjugated streptavidin (Vector Laboratories, Peterborough, UK, 1:500). Cells were counterstained with ProLong Gold Antifade Reagent with DAPI for nuclei staining (Cell Signaling Technology, MA, USA) before image capture with an EVOS FL Auto fluorescence microscope (Life Technologies, Paisley, UK). A total of three images were captured for each biological repeat experiment, using matched microscope and contrast/brightness settings and quantified by fluorescence intensity using ImageJ. HABP was expressed as a percentage of DAPI fluorescence to normalize for cell number in field of view. uNK cell cytochemistry was performed with cytoSpin as described previously.⁸ Briefly, cell suspensions were loaded into EZ Single Cytofunnel (Thermo Scientific) and adhered to Surgipath X-tra microslides (Leica Biosystems, Wetzlar, Germany) by centrifugation (150 \times g) for 5 minutes at RT. Cells were fixed in 4% paraformaldehyde for 10 minutes, washed three times with wash buffer (0.05% Tween 20 in PBS) and blocked (1% BSA in PBS, 30 min, RT) to prevent nonspecific binding. Cells were incubated overnight at 4°C with antibodies directed against CD44 (Thermo Scientific, MA4400; 1:200) and CD56 (Leica Biosystems, NCL-L-CD56-504; 1:100). Unbound primary antibody was removed by washing and cells stained for 1 hour at RT with Alexa Fluor 488 anti-rat secondary antibody (Thermo Scientific, A11006, 1:1000) for CD44 and Alexa Fluor 594 anti-mouse secondary antibody (Thermo Scientific; A21205, 1:1000) for CD56. Cells were counterstained with ProLong® Gold and viewed using an EVOS FL microscope.

2.7 | Reverse transcription quantitative PCR (RT-qPCR)

Total RNA from human EnSC was extracted using STAT-60 (AMSBIO). Recovered RNA was quantified using a Nanodrop spectrophotometer and equal amounts of total RNA were transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Manchester, UK).

Analysis of target gene expression was performed through Power SYBR Green Master Mix (Life Technologies) on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). The expression level of each gene was calculated using the $\Delta\Delta$ Ct method and normalized against levels of the *L19* housekeeping gene. Primer sequences used were as follows: *PRL* sense 5'-AAG CTG TAG AGA TTG AGG AGC AAA C-3', *PRL* antisense 5'-TCA GGA TGA ACC TGG CTG ACT A-3', *IGFBP1* sense 5'-CGA AGG CTC TCC ATG TCA CCA-3', *IGFBP1* antisense 5'-TGT CTC CTG TGC CTT GGC TAA AC-3', *ILIRLI* sense 5'-TTG TCC TAC CAT TGA CCT CTA CAA-3', *ILIRLI* antisense 5'-GAT CCT TGA AGA GCC TGA CAA-3', *CLU* sense 5'-GGG ACC AGA CGG TCT CAG-3', *CLU* antisense 5'-CGT ACT TAC TTC CCT GAT TGG AC-3', *L19* sense 5'-GCG GAA GGG TAC AGC CAA-3', *L19* antisense 5'-GCA GCC GGC GCA AA-3', *HAS2* sense 5'-GAT GCA TTG TGA GAG GTT TCT ATG-3', *HAS2* antisense 5'-GTA GCC AAC ATA TAT AAG CAG-3', *HYAL2* sense 5'-CAC AGT TCC TGA GCT GGT G-3', *HYAL2* antisense 5'-ACC AGG GCC AAT GTA ACG GT-3'.

2.8 | Enzyme-linked immunosorbent assay

Levels of secreted clusterin and sST2 in cell culture supernatant were quantified using DuoSet Enzyme-linked immunosorbent assay (ELISA) kits (Biotechne, MN, USA). EnSC in 96-well plates were decidualized for 8 days, with spent media collected and refreshed every 2 days. Conditioned media was centrifuged (10 000 \times g) for 5 minutes at 4°C to remove cellular debris and stored at -20°C for assay. Quantitation of secreted factors proceeded exactly as per manufacturer's instructions with the absorbance value determined on a PHERAstar FS plate reader (BMG Labtech, Ortenberg, Germany) at 450 nm. Samples were interpolated from known standards using a four parameter logistics fit.

2.9 | SA- β -Gal quantitation

Levels of cellular SA- β -Gal were determined using the 96-well Quantitative Cellular Senescence Assay kit (Cell Biolabs Inc; CA, USA), using a modified version of the manufacturer's protocol. Decidualizing EnSC in 96-well plates were washed with ice-cold PBS and lysed in 50 μ L of ice-cold assay lysis buffer containing protease inhibitors (cComplete Protease Inhibitor Cocktail, Roche). Lysates (25 μ L) were transferred to black-walled, black-bottomed 96-well plates, and 25 μ L of 2 \times assay buffer was added. Plates were sealed and the reaction was incubated for 1 hour at 37°C in a non-humidified, non-CO₂ incubator. The reaction was terminated

by the addition of 200 μL stop solution and fluorescent intensity unit (FIU) determined on a PHERAstar FS plate reader (BMG Labtech) at 360/465 nm. Assays were normalized to seeding density.

2.10 | Collection of blastocyst conditioned media

Blastocyst conditioned media (BCM) from IVF embryos cultured in individual droplets were obtained from two different units: Centre for Reproductive Medicine, UHCW NHS Trust, and Eugin Clinic, Barcelona, Spain. Following embryo transfer, BCM obtained from embryos that resulted in a positive or negative pregnancy test were designated positive (BCM⁺) or negative (BCM⁻), respectively. Parallel droplets without embryos were also obtained and designated unconditioned media (UCM).

Collection of BCM at UHCW NHS Trust was approved by the Research Ethics Committee West Midlands, Coventry and Warwickshire (REC Reference: 12/WM/0094). Fertilized zygotes were cultured in Cleav medium (Origio, Denmark) for 3 days, followed by Blast medium (Origio) for culture to the blastocyst stage. Cultures were performed in an atmosphere of 6% CO₂, 5% O₂, and 89% N₂, in an Embryoscope time-lapse incubator, with each embryo in an individual well containing 25 μL of culture medium under mineral oil. Embryo transfer was performed on day 5 after egg collection with the medium frozen in situ the following day and stored at -80°C until use.

Collection of BCM at Eugin Clinic, Barcelona, was approved by the Comité de Ética de la Investigación con Medicamentos (CEUGIN-2019-08-KILLDROP) on 22/10/2019. Embryos were cultured in SAGE 1-Step media (CooperSurgical, Denmark) covered with Ovoil (Vitrolife, Sweden). Cultures were performed in conventional incubators at 37°C under an atmosphere of 6% CO₂, 5% O₂, and 89% N₂. BCM (10 μL) was collected after embryo transfer on day 5 and transferred from the culture plate to a 0.6 mL Eppendorf tube. Drops were collected individually. In the same way, UCM were collected from the sample plate. BCM and UCM were stored at -80°C until use. At both centers, the collection of BCM was fully anonymized in accordance with the approvals of the respective Ethics Committees.

2.11 | HYAL2 expression and secretion in human embryos

The research use of human cryopreserved embryos that were surplus to patient requirements was approved by the Research Ethics Committee, Coventry and Warwickshire (REC Reference: 04/Q2802/26), and the Human Fertilisation

and Embryology Authority (Reference: R0155). All patients provided informed written consent. To examine the expression of HYAL2, vitrified human blastocysts were warmed using the Vit Kit thawing protocol according to the manufacturer's instructions (Irvine Scientific, Co. Wicklow, Ireland). Blastocysts were transferred to PBS solution and fixed in 2% PFA, 4% BSA in PBS solution at RT for 2 hours. Where appropriate, the zona pellucida was removed by brief incubation in acid Tyrode's solution prior to fixation. Fixed blastocysts were washed in 4% BSA in PBS before permeabilization in 0.5% Triton X-100 in PBS at RT for 2 hours and then, washed in 0.2% Tween 20 in PBS for 15 minutes. Nonspecific antibody-binding sites were blocked with blocking solution (4% BSA, 5% goat serum, and 0.2% Tween 20 in PBS) at RT for 1 hour. Immuno-detection proceeded by overnight incubation of rabbit anti-HYAL2 primary antibody (Abcam, ab68608, 1:100) in blocking solution at 4°C . Unbound primary antibody was removed the following day by washing in 0.2% Tween 20 in PBS and blastocysts incubated for 2 hours at RT with goat anti-rabbit Alexa Fluor 488-conjugated secondary antibodies (Abcam, ab150077). Following washing, blastocysts were transferred to 5 μL Vectashield mounting medium containing DAPI on a glass slide. To protect the 3D integrity of the blastocyst, a small pinhead-sized drop of paraffin wax was placed in each corner of a 10 \times 10 mm coverslips before setting over the sample. HYAL2 immunofluorescence in blastocysts was viewed on an Olympus 1X81 microscope using 490 nm excitation and 525 nm emission wavelengths. DAPI was detected at 359/461 nm.

To measure secreted HYAL2 levels, embryo conditioned media was diluted 1:10 with blastTM medium (Origio) with HYAL2 levels quantified by ELISA as per the manufacturer's protocols (ABIN841408, Antibodies Online). Additional information on these embryos is presented in Table S2. Morphological assessment of embryos was based on standardized criteria.³¹ Briefly, the inner cell mass and trophectoderm of embryos were assessed morphologically and graded separately on a scale of A-E, with A being the best and E the worst.³¹ Blastocysts graded <BB were deemed low quality.

2.12 | Statistical analysis

Because of intrinsic variability between primary cultures, the data are presented as fold-change relative to an informative comparator and analyzed using paired one-way ANOVA with a Dunnett's multiple comparison test. Normal distribution was confirmed by the Shapiro-Wilk test of normality. The level of embryonic HYAL2 secretion between groups was compared using an unpaired *t* test. Individual data points are shown for biological replicates and repeat experiments. $P < .05$ was considered significant with different letters above the bar graphs indicating significant differences between groups

3 | RESULTS

3.1 | Loss of HA synthesis upon decidualization of EnSC

We examined HA expression in decidualizing primary EnSC cultures using biotin-labeled hyaluronic acid-binding protein (HABP).³² HABP reactivity was prominent in undifferentiated EnSC but downregulated upon decidualization with 8-bromo-cAMP and the progestin medroxyprogesterone (C+M) (Figure 1A). Quantitative analysis confirmed that HA reactivity declines by 74% and 94%, respectively, after 4 and 8 days of decidualization (Figure 1B). To explore the mechanisms accounting for the loss of HA production upon decidualization, we examined the expression of the three hyaluronic synthases (HAS1-3) and five catabolic hyaluronidases [HYAL1-4 and sperm adhesion molecule 1 (SPAM1)].^{25,33,34} First, we mined publicly available RNA-seq data of undifferentiated EnSC and cells decidualized for 4 days (GEO accession number: GSE104721). *HAS1*, *HAS3*, *HYAL1*, and *HYAL4* mRNA levels were generally below three transcripts per million (TPM) in both undifferentiated and decidualized cells, suggesting little or no expression (Figure S1). *SPAM1*, a testis-specific gene,³⁵ is not expressed by EnSC and *HYAL3* does not encode for a classical hyaluronidase, although it may play a role in augmenting *HYAL1* activity.^{36,37} In contrast, the average (\pm SEM) expression of *HAS2* and *HYAL2* in undifferentiated EnSC was 20.5 (\pm 11.4) and 48.0 (\pm 4.3) TPM, respectively. Upon decidualization, *HAS2* was downregulated, whereas *HYAL2* transcript levels remained unchanged (Figure S1). To confirm these observations, *HAS2* and *HYAL2* mRNA levels were measured by RT-qPCR in five independent primary EnSC cultures decidualized for 4 or 8 days. In agreement with the RNA-seq data, *HYAL2* expression was maintained throughout the decidual time-course, whereas *HAS2* mRNA levels declined by 56% and 82% after 4 and 8 days of decidualization, respectively (Figure 1C; $P < .005$, Dunnett's multiple comparisons test).

We postulated that loss of endogenous HA production may render decidualizing cells responsive to HA derived from other sources, for example the implanting blastocyst.³⁸ To test this hypothesis, three independent primary EnSC cultures were first decidualized for 4 days to downregulate endogenous HA production and then, decidualized for four additional days in the presence or absence of LMWHA or HMWHA (Figure 1D). As shown in Figure 1E, HA had no effect on the induction of the canonical decidual marker genes *PRL* and *IGFBP1*, irrespective of its molecular weight. Expression of *CLU* and *IL1RL1*, marker genes of senescent decidual cells and decidual cells, respectively,¹⁵ were also not affected by HA. *CLU* codes the secreted molecular chaperone clusterin (also known as apolipoprotein J), whereas both the transmembrane and soluble IL-33 receptors (termed ST2L

and sST2, respectively), are encoded by *IL1RL1*. In keeping with the observations at transcript level, we found no evidence that HA impacted on either clusterin or sST2 secretion in decidualizing cells (Figure 1F). To confirm that HA does not affect decidual subpopulations, senescence-associated β -galactosidase (SA- β -Gal) activity was measured in parallel cultures. As expected, decidualization was associated with a robust increase in SA- β -Gal activity but addition of HA had no effect on basal or induced levels (Figure 1G).

3.2 | HMWHA inhibits uNK cell clearance of senescent decidual cells

To explore if there is a role for HA signaling in the peri-implantation endometrium, we examined the expression of putative HA receptors using recently generated single-cell RNA-seq data from endometrial biopsies obtained 8 and 10 days following the pre-ovulation LH surge (GEO accession number: GSE127918).¹⁵ Several genes coding HA receptors were expressed prominently in endometrial endothelial cells (Figure 2A), including *ICAM1* (coding intercellular adhesion molecule 1), *LAYN* (layinin) and *TLR4* (toll-like receptor 4), pointing toward a potential role for HA signaling in vascular remodeling in pregnancy. The most striking observation, however, was the high expression of CD44, the principal HA receptor, in uNK cells (Figure 2A). CD44 regulates cytotoxicity of circulating NK cells and potentially their trafficking to the endometrium.^{39,40} These observations prompted us to investigate if HA-CD44 interactions modulate uNK cell-dependent clearance of senescent decidual cells. To test our hypothesis, uNK cells were isolated from midluteal endometrial biopsies using magnetic-activated cell sorting (MACS). Flow cytometry analysis of uNK cells isolated from three independent biopsies showed an average viability (\pm SEM) of 80.8% (\pm 2.5) (Figure 2B). Furthermore, 95% (\pm 1.4) of CD56⁺ cells co-expressed the pan-leukocyte marker CD45 (Figure 2C) and 79.7% (\pm 1.9) the canonical HA receptor CD44 (Figure 2D). Expression of CD44 on CD56⁺ uNK cells was confirmed by immunocytochemistry (Figure 2E). To validate that uNK cells selectively target senescent decidual cells, co-cultures were established with EnSC first decidualized for 6 days. After 48 hours, the co-cultures were processed for SA- β -Gal activity measurement or RNA extraction (Figure 2F). Spent medium was also collected and secreted clusterin and sST2 levels measured by ELISA. As expected, the induction of SA- β -Gal activity upon decidualization was abrogated in the presence of uNK cells, as was the induction and secretion of clusterin (Figure 2H). In contrast, *IL1RL1* mRNA expression and sST2 levels were unaffected (Figure 2I), confirming that uNK cells selectively target and eliminate senescent decidual cells.

Next, we treated seven independent decidual-uNK cell co-cultures with either HMWHA or LMWHA. As shown in Figure 3A, HMWHA inhibited uNK cell-mediated clearance of senescent decidual cells, exemplified by the lack of

downregulation of SA- β -Gal activity and clusterin secretion. sST2 secretion was unaffected by HMWHA. Furthermore, addition of LMWHA had no effect on either SA- β -Gal activity or clusterin and sST2 secretion in decidual-uNK cell

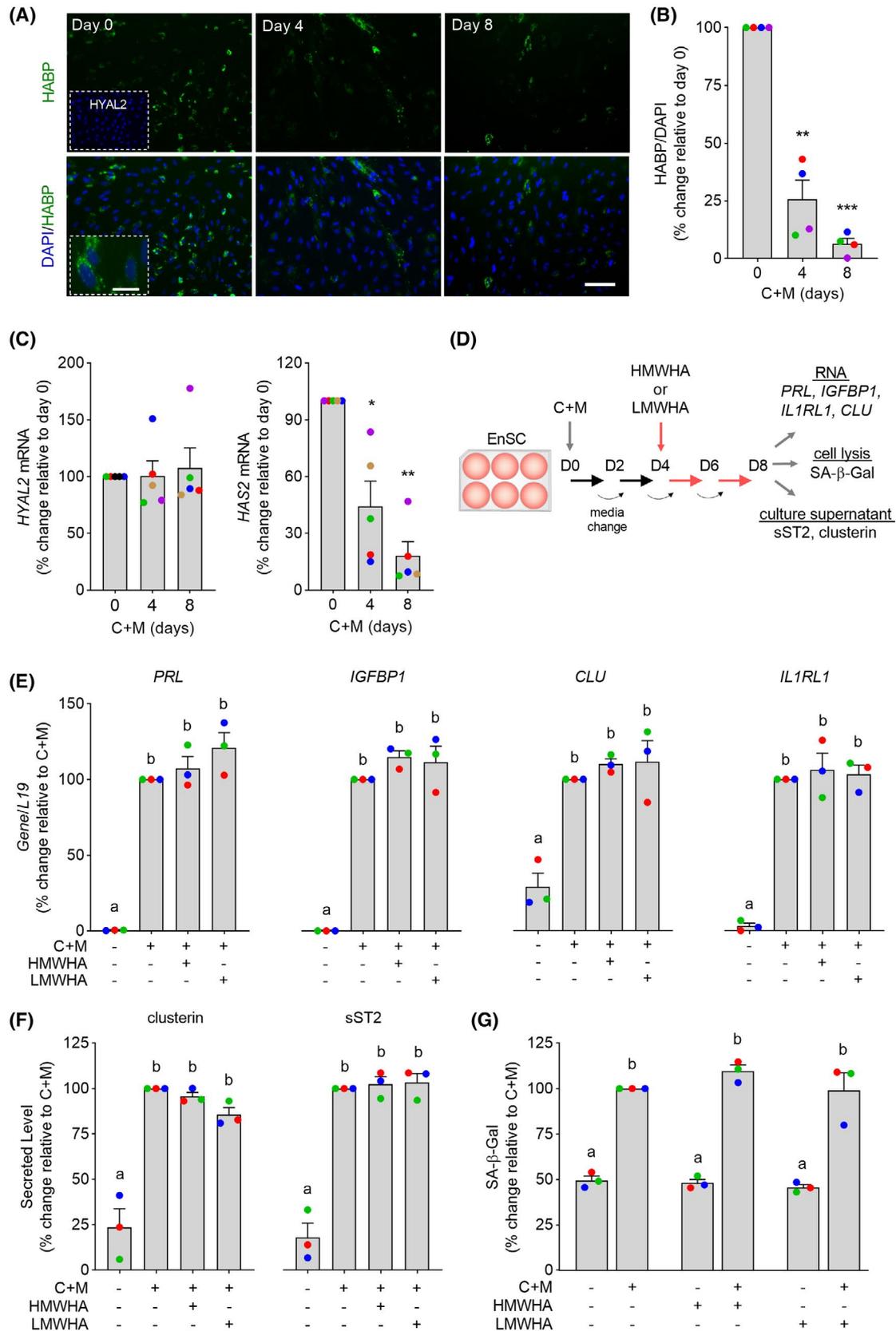


FIGURE 1 Loss of HA production upon decidualization. A, Representative images of HABP immunofluorescence in undifferentiated EnSC (day 0) and cells decidualized with 8-br-cAMP and MPA (C+M) for 4 or 8 days. Scale = 200 μ M, insert = 50 μ M. Cultures treated with recombinant *HYAL2* to digest HA were used as control (*insert, upperpanel*). B, Quantification of HABP⁺ cells. The data show the relative change in HABP⁺ cells (mean \pm SEM) upon decidualization in four independent primary cultures. ** indicates $P < .01$ and *** $P < .001$, ANOVA with Dunnett's multiple comparison test. C, RT-qPCR analysis of *HYAL2* mRNA (left panel) and *HAS2* mRNA (right panel) expression in undifferentiated EnSC (day 0) and cells decidualized for 4 or 8 days. The data show the relative change in transcript levels (mean \pm SEM) upon decidualization of five independent primary cultures at the indicated timepoints. * denotes $P < .05$ and ** $P < .01$, ANOVA and Dunnett's multiple comparison test. D, Experimental design of decidualizing EnSC treated with HA. E-G, HMWHA and LMWHA do not impact decidual marker gene expression (E), clusterin and sST2 secretion (F), or SA- β -Gal activity (G). Individual data points from three biological repeat experiments are shown together with bar graphs denoting mean \pm SEM. Different letters indicate significance between groups at $P < .05$ (one-way ANOVA and Dunnett's multiple comparison test)

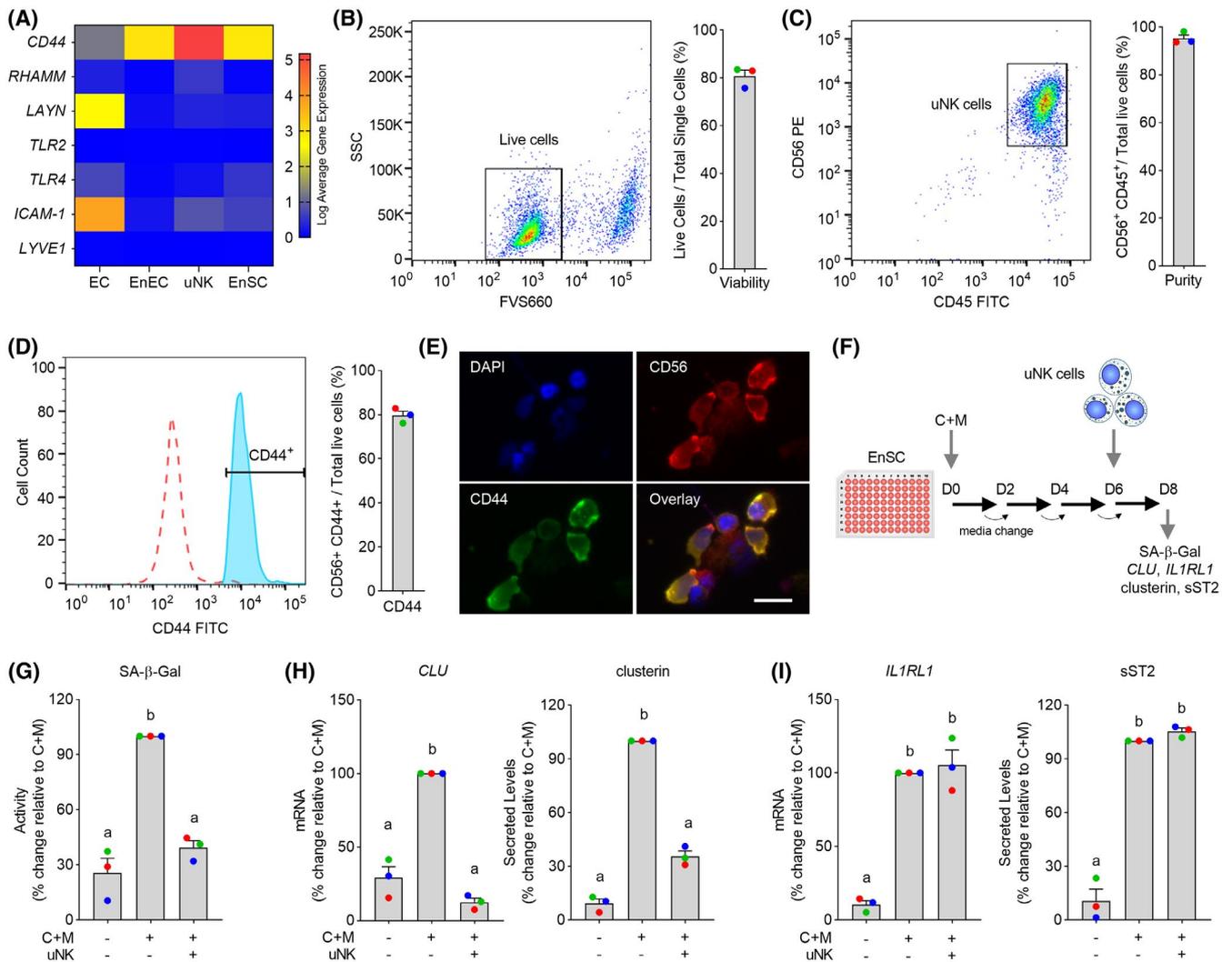


FIGURE 2 Decidual-uNK cell co-cultures. A, Heatmap showing relative expression of genes coding putative HA receptors in different endometrial cell types in vivo. EC, endothelial cells; EnEC, endometrial epithelial cells. B-D, Flow cytometric analysis. B, Viability of uNK cells following MACS isolation was determined by FV660 exclusion. C, Purity of uNK cells after MACS isolation was determined by gating cells for CD56 and CD45. D, Histogram depicting CD56⁺/CD44⁺ uNK cells after MACS isolation (*filled histogram*) vs negative control (*open histogram*). Representative dot plots or histogram are shown together with bar graphs (mean \pm SEM) showing individual data points of three biological repeat experiments. E, Cytospin preparations of isolated uNK cells stained for DAPI (nucleus), CD56 and CD44. Co-localization of CD56 and CD44 is shown in bottom right panel. Scale = 100 μ M. F, Schematic representation of the decidual-uNK cell co-culture killing assay. G-I, Analysis of SA- β -Gal activity (G), senescent decidual cell markers (H), and decidual markers (I) in decidualizing EnSC co-cultured with or without uNK cells. The data show relative change to the indicated measurements in three biological repeat experiments compared to cultures decidualized with C+M for 8 days in the absence of uNK cells. Different letters above the bar graphs (mean \pm SEM) indicate statistical differences ($P < .05$) between groups (one-way ANOVA and Dunnett's multiple comparison test)

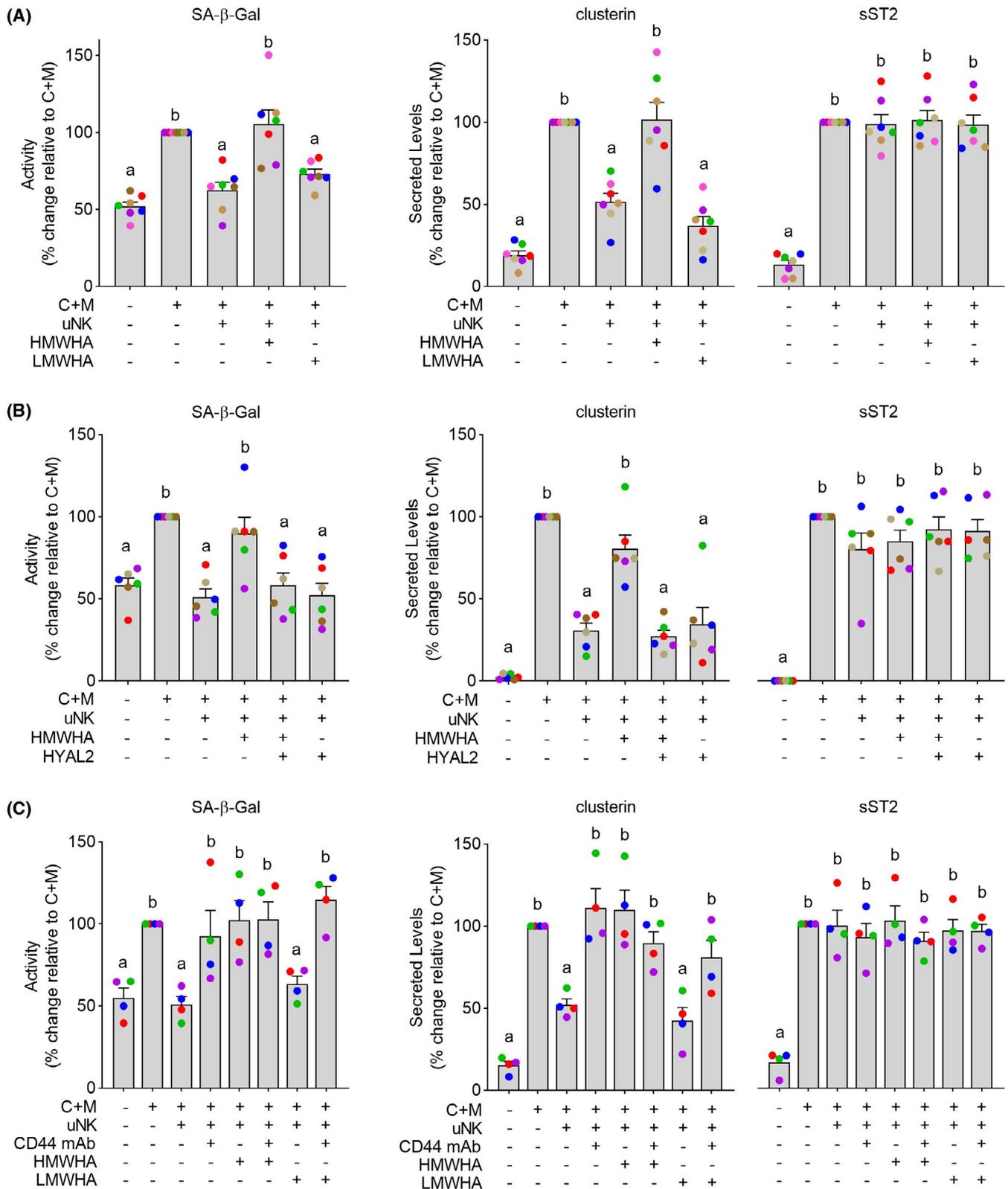


FIGURE 3 HMWHA inhibits uNK cell-dependent killing of senescent decidual cells. A, Impact of HMWHA and LMWHA on uNK cell-dependent killing of senescent decidual cells in seven biological repeat experiments. Killing of senescent decidual cells was monitored by SA-β-Gal activity and secreted clusterin levels. sST2 is selectively secreted by decidual cells. B, Impact of HMWHA or HMWHA pre-incubated with HYAL2 on uNK cell-dependent killing of senescent decidual cells in six biological repeat experiments. C, Impact of CD44 inhibition using a monoclonal antibody (CD44 mAb) on uNK cell-dependent killing of senescent decidual cells in the presence or absence of HMWHA or LMWHA in four biological repeat experiments. The data show relative change in the indicated measurements compared to cultures decidualized for 8 days in the absence of uNK cells. Different letters above the bar graphs (mean ± SEM) indicate statistical differences ($P < .05$) between groups (one-way ANOVA and Dunnett's multiple comparison test)

co-cultures. To substantiate these observations, six additional co-cultures were established and treated with either HMWHA or HMWHA pre-incubated with recombinant HYLA2, the enzyme that selectively degrades HMWHA into smaller fragments.⁴¹ As demonstrated in Figure 3B, HMWHA inhibition of uNK cell-dependent clearance of senescent decidual cell markers, monitored by SA- β -Gal activity and clusterin secretion, was completely lost upon pre-incubation with recombinant HYLA2. Addition of HYAL2 alone to the co-cultures had no effect on uNK-dependent clearance of senescent decidual cells (Figure 3B). Degradation of HA by HYAL2 into smaller fragments initiates CD44 cell signaling.^{24,25,42} To explore the role of CD44, co-cultures were treated with Hermes-1, a monoclonal antibody that recognizes the N-terminal HA-binding domain of CD44.⁴³ Incubation of co-cultures with this blocking antibody inhibited uNK cell-mediated clearance of senescent decidual cells (Figure 3C), irrespective of treatment with LMWHA or HMWHA. Taken together, the data indicate that HMWHA is a potent inhibitor of uNK cell activation, an effect that likely involves silencing of CD44 signaling.

3.3 | uNK cells are biosensors of embryo quality at implantation

The HA system is tightly controlled in preimplantation embryos and trophoblast cells.^{25,27} We speculated that embryo-derived HA signaling may determine endometrial responses at the implantation site by modulating uNK cell activity. To investigate this possibility, we adapted our co-culture system as depicted in Figure 4A. Briefly, in two different IVF centers, blastocyst conditioned medium (BCM) was pooled from individual fully anonymized day-5 IVF embryos, which resulted either in an ongoing pregnancy following single-embryo transfer (BCM⁺, ie, positive pregnancy test 15 days after embryo transfer; n = 28) or failed implantation (BCM⁻, negative pregnancy test; n = 38). Each pool consisted of 80 μ L of spent media recovered from seven blastocysts. We also collected UCM from parallel embryo-free droplets. Next, we decidualized nine independent primary EnSC cultures for 6 days and then co-cultured uNK cells in UCM, BCM⁺ or BCM⁻ diluted 1 in 7.5 with decidualization medium. Co-cultures were harvested after 48 hours. Strikingly, uNK cell-mediated killing of senescent decidual cells, as measured by SA- β -Gal activity and secreted clusterin levels, was unaffected by UCM or BCM⁺ but markedly inhibited in response to soluble signals emanating from unsuccessful blastocysts (BCM⁻) (Figure 4B). Pre-incubation of BCM⁻ with recombinant HYLA2 fully restored uNK cell activity against senescent decidual cells (Figure 4C). Notably, exposure of decidualizing EnSC to either UCM, BCM⁺ or BCM⁻ had no effect on either SA- β -Gal activity or clusterin and sST2

secretion in the absence of co-cultured uNK cells (Figure S2). Taken together, the data strongly infer a central role for uNK cells in embryo recognition and selection upon implantation. Furthermore, the level of embryo-derived HYAL2 secretion appears to determine uNK cell activity.

Expression of HYAL2 in human blastocysts was confirmed by immunocytochemistry (Figure 4D). We also collected BCM from 43 human IVF embryos transferred on day 5 and quantified HYAL2 levels by ELISA in individual droplets (Table S2). As shown in Figure 4E, human embryos deemed of low-quality based on morphological criteria,³¹ secreted lower levels of HYAL2 when compared to good-quality (grade: \geq BB) embryos ($P = .003$, unpaired t test). Similarly, embryos that implanted successfully following transfer secreted higher levels of HYAL2 when compared to their unsuccessful counterparts ($P = .042$, unpaired t test; Figure 4F).

Taken together, our results implicate uNK cells as pivotal biosensors of embryo quality and identified the level of embryo-derived HYAL2 secretion as a major determinant for successful implantation. Figure 5 summarizes the proposed mechanism of embryo-dependent modulation of uNK cell activity in endometrial fate decisions at implantation.

4 | DISCUSSION

Based on sensitive urinary hCG measurements, several prospective observational studies reported miscarriage rates of approximately 30% in young women trying to conceive.⁴⁴⁻⁴⁷ Most of these losses involve menstruation-like shedding of the endometrium 1-2 weeks after implantation and are, therefore, often not recognized clinically. The high attrition rate in the very early stages of pregnancy is widely attributed to embryonic aneuploidy, caused either by age-dependent meiotic errors in oocytes or age-independent erroneous mitotic divisions during preimplantation development.^{48,49} A recent analysis of published single-cell RNA-seq data concluded that 80% of human embryos harbor aneuploid cells.⁵⁰ This intrinsic genetic instability of human embryos, which gives rise to a vast array of chromosomal errors, poses a major challenge to the endometrium; how to eliminate embryos of low-fitness without compromising implantation of high-quality embryos?

Evidence from multiple mammalian species indicates that the endometrium is capable of mounting an implantation response tailored to the developmental potential of individual embryos.^{21,51-53} In humans, directed migration of decidualizing EnSC and active encapsulation of the conceptus constitutes an important initial step in the implantation process.⁵² In vitro studies demonstrated that high-quality human embryos stimulate migration of decidual cells, whereas low-quality embryos fail to do so. Conversely, migration of

undifferentiated EnSC is actively inhibited by high-quality embryos, but not low-quality embryos.^{3,4,20,54} Furthermore, cross-species experiments demonstrated that flushing of the

mouse uterus with spent culture medium from human IVF embryos deemed of insufficient quality for transfer triggers an acute cellular stress response; whereas uterine flushing

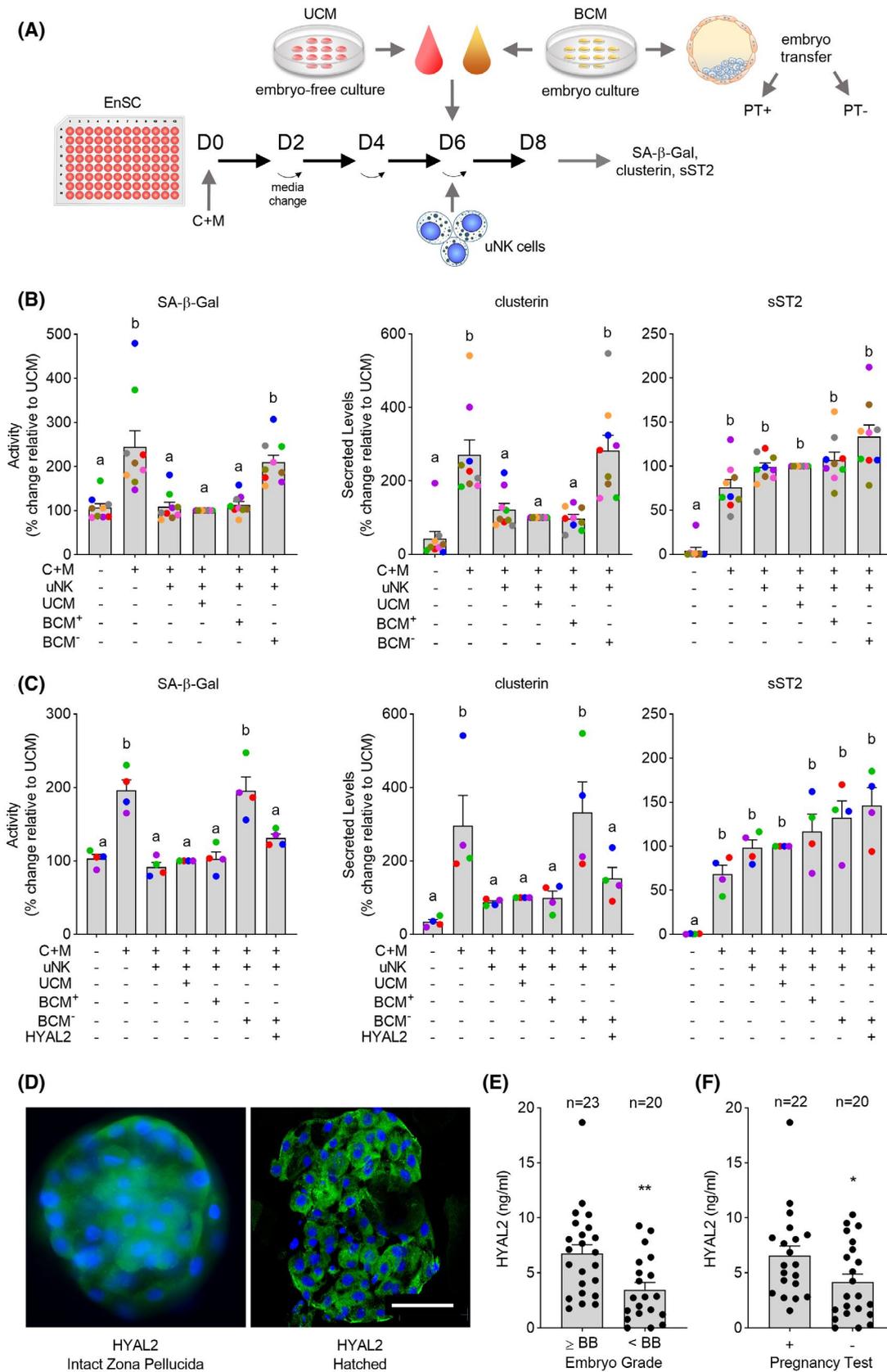


FIGURE 4 uNK cells are biosensors of low-fitness human embryos. A, Schematic depiction of experimental design. BCM, blastocyst conditioned medium; UCM, unconditioned medium; and PT, pregnancy test. B, Impact of UCM, BCM⁺, and BCM⁻ on uNK cell-dependent killing of senescent decidual cells in nine biological repeat experiments. Killing of senescent decidual cells was monitored by SA- β -Gal activity and secreted clusterin levels. sST2 is selectively secreted by decidual cells. C, Impact of BCM⁻ or BCM⁺ pre-incubated with HYAL2 on uNK cell-dependent killing of senescent decidual cells in four biological repeat experiments. The data show relative change in the indicated measurements compared to decidual-uNK cell co-cultures exposed to UCM. Different letters above the bar graphs (mean \pm SEM) indicate statistical differences ($P < .05$) between groups (one-way ANOVA and Dunnett's multiple comparison test). D, HYAL2 immuno-reactivity in unhatched and hatched human blastocysts (left and right panels, respectively). Scale = 100 μ M. E, secreted HYAL2 levels in conditioned media from embryos graded morphologically as good (grade \geq BB) or poor ($<$ BB). F, Secreted HYAL2 levels in culture medium conditioned by embryos that either resulted in a positive or negative pregnancy test following single-embryo transfer. * indicates $P < .05$, ** indicates $P < .01$ (unpaired t test)

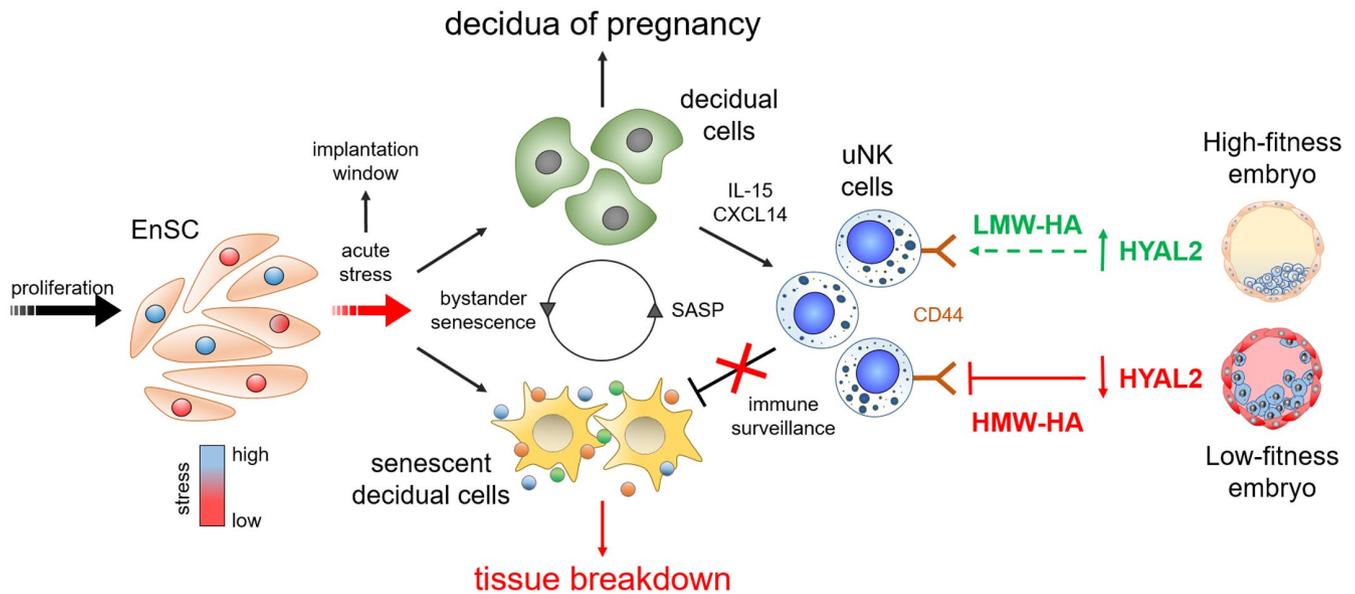


FIGURE 5 Schematic summary of the role of uNK cell sensing of embryos in endometrial fate decisions at implantation. For detailed explanation, see text

with spent medium of successfully implanted human embryos (ie, resulting in ongoing pregnancies) enhances endometrial expression of multiple metabolic and implantation genes.²¹ These observations indicate that human embryos are subjected to both negative and positive selection at implantation.²² The nature of embryonic “quality” signals decoded by decidual cells is largely unknown, although recent studies have provided some insights. For example, compelling experimental evidence implicated embryo-derived microRNAs, more specifically hsa-miR-320a, in stimulating directional migration of decidualizing EnSC.²⁰ Interestingly, hsa-miR-320a also acts in an autocrine manner to promote development of preimplantation embryos.⁵⁵ Moreover, heightened secretion of tryptic serine proteinases by low-quality embryos, perhaps reflecting proteotoxic stress induced by aneuploidy, induces endoplasmic reticulum stress in decidualizing EnSC,²¹ with loss of secretion of pro-implantation modulators such as LIF, IL-1 β , and HB-EGF.¹⁴

Despite these emerging insights, it remains unclear how a local embryo-induced endometrial stress response

triggers a concatenation of events that ultimately leads to breakdown of the entire superficial endometrium, even in women receiving progesterone supplements. Several lines of evidence indicate that endometrial fate decisions at implantation pivot on the surveillance and clearance of senescent decidual cells by uNK cells. First, senescent decidual cells are progesterone-resistant and produce a cocktail of factors involved in ECM remodeling, including multiple proteases, and inflammation.¹⁵ Second, unlike undifferentiated EnSC, decidual cells are highly susceptible to secondary senescence (also termed “bystander” senescence), enabling rapid propagation of the senescent phenotype throughout the tissue.^{8,15} We further demonstrated that recurrent pregnancy loss is associated with a pro-senescence decidual response in non-conception cycles, linked to loss of clonogenic progenitor cells and uNK cell deficiency.^{15,56-58} Here, we show that uNK cell-dependent clearance of senescent decidual cells is actively suppressed by low-fitness embryos, which is predicted to promulgate secondary senescence and lead to tissue breakdown. We

also demonstrate that HYAL2 restores killing of senescent decidual cells in the presence of cues from low-quality embryos. Combined with other lines of evidence presented in this study, this observation firmly implicates HMWHA-silencing of CD44 activity in abrogating uNK cell cytotoxicity. Indeed, HMWHA possesses multivalent sites to bind CD44, promotes clustering of the receptor, and inhibits downstream pathways, including Rac-dependent signaling.²⁴ Interestingly, both HA production and HYAL2 expression have been shown to be critical for embryo development in animal studies.²⁵ For example, inhibition of HA synthesis by 4-methylumbelliferone suppresses bovine blastocyst formation,⁵⁹ whereas HA supplementation of culture media improves embryo development in multiple species.²⁵ The effects of HA on embryo development are dependent on HYAL2 as LMWHA promotes development, whereas HMWHA exerts the opposite effect.⁶⁰ *HYAL2* transcripts are constitutively expressed in human preimplantation embryos.²⁶ Here, we showed by immunocytochemistry that this glycosylphosphatidylinositol-anchored enzyme localizes to both the trophectoderm and inner cell mass of human blastocysts. Importantly, HYAL2 was also detectable in BCM, with levels correlating to both embryo morphology and implantation competence. Thus, as reported for hsa-miR-320a,²⁰ embryonic HYAL2 expression appears critical for both preimplantation development and endometrial responses upon implantation.

Increased deposition of HA into the ECM, specifically LMWHA, is a hallmark of inflammatory processes.²⁴ In the postovulatory endometrium, HA deposition first peaks during the midluteal window of implantation but then declines rapidly in the stroma.⁶¹ These observations are congruent with the decline in HA production in EnSC decidualized *in vitro* for 4 days or longer. Furthermore, loss of HA expression in decidual cells surrounding the conceptus has also been reported in mice.⁶² Although we demonstrated that HA production declines in parallel with transcriptional silencing of *HAS2*, decidualization is also associated with loss of O-linked N-acetylglucosamine (GlcNAc) transferase (OGT),¹³ an enzyme that strongly increases *HAS2* activity and stability through O-GlcNAcylation of serine 221.⁶³ Despite the lack of HA, uNK cell-dependent killing of senescent decidual cells was attenuated upon CD44 inhibition and addition of LMWHA did not enhance uNK cell cytotoxicity in co-cultures. These observations suggest that decidual cells either continue to produce low levels of HA fragments or express alternative CD44 ligands. Two plausible candidates are osteopontin and serglycin,⁶⁴ both of which are strongly up-regulated upon decidualization, at least at transcript level.¹⁵

In summary, we report for the first time that uNK cells are biosensors of low-fitness human embryos. We demonstrated that the embryo-sensing function of uNK cells relates to HYAL2 expression in the blastocyst and its ability to degrade

HMWHA. Furthermore, the role of uNK cells in embryo selection at implantation may depend on an adequate decidual response, creating a permissive local environment devoid of HMWHA production. We posit that loss of uNK cell activity at the implantation site propagates decidual senescence and facilitates menstruation-like shedding of the endometrium.

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CONFLICT OF INTERESTS

The authors declare no Competing Interests.

AUTHOR CONTRIBUTIONS

Conceptualization, J.J. Brosens; Investigation, C.-S. Kong, P.J. Brighton, A.A. Ordoñez, S. Turner, T. Tremaine, J. Muter, E.S. Lucas, and E. Salisbury; Manuscript writing, P.J. Brighton, C.-S. Kong, and J.J. Brosens; Funding Acquisition, J.J. Brosens and G. Hartshorne; Resource acquisition, J.J. Brosens, G. Hartshorne, A.A. Fouladi-Nashta, G. Tiscornia, and R. Vassena; Supervision, J.J. Brosens, G. Hartshorne, G. Tiscornia, R. Vassena, and P.J. Brighton.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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