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Quantifying the Biomechanics of Conception: L-Selectin-Mediated Blastocyst Implantation Mechanics with Engineered "Trophospheres"

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An estimated 12% of women in the United States suffer from some form of infertility. *In vitro* fertilization (IVF) is the most common treatment for infertility encompassing over 99% of all assisted reproductive technologies. However, IVF has a low success rate. Live birth rates using IVF can range from 40% in women younger than 35 years to 4% in women older than 42 years. Costs for a successful IVF outcome can be upward of $61,000. The low success rate of IVF has been attributed to the inability of the blastocyst to implant to the uterus. Blastocyst implantation is initiated by L-selectin expressing cells, trophoblasts, binding to L-selectin ligands, primarily sialyl Lewis X (sLeX), on the uterine surface endometrium. Legal and ethical considerations have limited the research on human subjects and tissues, whereas animal models are costly or do not properly mimic human implantation biochemistry. In this work, we describe a cellular model system for quantifying L-selectin adhesion mechanics. L-selectin expression was confirmed in Jeg-3, JAR, and BeWo cell lines, with only Jeg-3 cells exhibiting surface expression. Jeg-3 cells were cultured into three-dimensional spheres, termed "trophospheres," as a mimic to human blastocysts. Detachment assays using a custom-built parallel plate flow chamber show that trophospheres detach from sLeX functionalized slides with 2.75×10^{-3} dyn of force and 7.5×10^{-5} dyn-cm of torque. This work marks the first time a three-dimensional cell model has been utilized for quantifying L-selectin binding mechanics related to blastocyst implantation.

Introduction

Infertility is a major medical condition affecting an estimated 12% of women in the United States.[1,2] The most common treatment is *in vitro* fertilization (IVF). Despite being the most common procedure for addressing infertility, IVF has a low success rate depending on the age and other health factors. The Society for Assisted Reproduction (SART) and the Center for Disease Control (CDC) report that live birth rates from IVF can range from 4% in those older than 40 years to 45% in those younger than 35 years, and these rates have remained relatively constant over the past 10 years[1–3] (sart.org). Furthermore, a single IVF cycle may cost $24–38,000 with an average cost of $61,000 per successful cycle or pregnancy.[4] The majority of this financial burden falls on those undergoing the treatment since an estimated 85% of all costs are paid out of the pocket by the patients.[5] It has been suggested that the main reason for the low success rate of IVF is a failure of the blastocyst/embryo to properly attach to the endometrial surface of the uterus.[6,7]

Blastocyst implantation involves a complex, chemically and temporally synchronized dialogue between an implantation competent embryo and a receptive uterus. The steps between fertilization and the initiation of implantation generally follow a well-conserved autonomous process resulting in the blastocyst.[8] In humans, endometrial receptivity is controlled by the ovarian steroids, estrogen (E2), and progesterone (P4).[9]

The cellular adhesion protein L-selectin mediates critical interactions in the earliest events of blastocyst attachment and implantation.[10] Upregulation of oligosaccharide-based selectin ligands, specifically sialyl Lewis X (sLeX), during endometrial receptivity and L-selectin expression on human trophoblasts have been shown.[11]

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Due to ethical and legal restrictions on obtaining and testing human tissue, animal models of implantation have been primarily investigated.[12] However, animal models all have limitations as substitutes for human implantation. Primates and guinea pigs are most similar to humans; however, primates are costly and produce few offspring[12–16] and guinea pig blastocyst implant with the zona pellucida intact.[17,18] Mice, rats, and rabbits are the easiest to obtain and most well defined, but do not exhibit interstitial implantation.[9,19,20] Pigs, sheep, and cows have prolonged implantation events, but also do not exhibit interstitial implantation.[21–24] Furthermore, the role of L-selectin and L-selectin ligands has yet to be determined in these animals.

There remains a clear need for a human blastocyst implantation model. Few attempts have been made using trophoblast-like choriocarcinoma cells, but these models are limited in their scope. JAR,[25] Jeg-3,[26] and BeWo[27] cells have been utilized since their protein expression profiles closely resemble the first trimester trophoblast cells.[28–41] Whereas these studies have added knowledge to this complex biological behavior, their focus was not to specifically investigate the L-selectin binding process. Additionally, these single-cell studies do not capture the large three-dimensional structure of blastocysts. Few three-dimensional models exist,[33,35,42,43] but they also do not quantify L-selectin binding.

The goal of this work is to evaluate L-selectin expression of Jeg-3, JAR, and BeWo cells to understand surface expression and appropriateness for a model trophoblast in the study of the role of L-selectin in blastocyst attachment. A three-dimensional blastocyst model, termed "trophosphere," and a method for quantifying L-selectin/sLeX binding of trophospheres are also presented.

Materials and Methods

Cell and trophosphere culture

Jeg-3, BeWO, JAR, and U-937 (ATCC, Manassas, VA) cells were cultured in their recommended media supplemented with 5% fetal bovine serum (FBS; CellGro, Manassas, VA) in an incubator at 37°C with 5% CO_2. Jeg-3 cells were passaged twice per week at a 1:5 ratio; U-937 cells were passaged weekly at a 1:10 ratio. Cells are typically cultured for 4–8 weeks before being replaced.

Trophospheres were cultured using Jeg-3 cells at passage number 136–145, in a manner similar to previous studies.[33,42] Jeg-3 cells were seeded into PETG Erlenmeyer flasks at 10^6 cell/mL in 20 mL of MEM supplemented with 10% FBS. Seeded flasks were cultured on an orbital shaker at 50 rpm for 48–72 h.

Reagents

Immunoblot primary antibodies were LEAF purified anti-human CD62L DREG-56 (BioLegend, San Diego, CA); anti-actin rabbit antibody (Cell Signaling Technology, Danvers, MA). The immunoblot secondary antibody was the HRP goat anti-mouse IgG polyclonal antibody (BioLegend). Estradiol, progesterone, and dexamethasone (Sigma, St. Louis, MO) were used in western blot culture treatments. Flow cytometry primary antibodies were anti-human L-selectin (CD62L) monoclonal (Clone #4G8) antibody BBA24 (R&D Systems, Minneapolis, MN) or isotype-matched control (clone MOPC-1; BD Biosciences, San Jose, CA), and the secondary antibody was Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen, Carlsbad, CA). Immunoreagents for immunohistochemistry were mouse IgG1 (clone MOPC-21; BD Pharmingen), L-selectin antibody (Clone #4G8; R&D Systems), biotinylated goat anti-mouse IgG (H+L) (Vector Laboratories, Burlingame, CA), streptavidin/biotin blocking kit, and streptavidin-Texas Red (Vector Laboratories). The MECA-79 rat IgM antibody (BD Biosciences) and 3′-sLeX tetrasaccharide (Sigma) were used in flow detachment studies.

Western blot and immunoblot quantification

Western blotting was used to verify L-selectin expression of candidate cell lines. Jeg-3, BeWo, JAR, and U-937 cells were cultured normally, and total protein extracts were prepared using a lysis buffer described previously[44] and quantified by the BCA protein assay (Pierce Biotechnology, Rockford, IL). The *Drosophila* line Deg-5r (gifted by Noreen Robertson, DMD), was used as a negative control for L-selectin, while the U-937 monocytic leukemia cell line was used as a positive control.[45] For quantitative immunoblots, Jeg-3 cells were cultured normally, followed by a 2-day treatment of serum-free, phenol red-free medium and 10^{-8} M estradiol, 10^{-6} M progesterone, a combination of both estradiol and progesterone, or 10^{-7} M dexamethasone.

Sample lysates were separated on 4%–20% SDS-PAGE mini-gels (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ), blocked with 5% powdered milk in phosphate-buffered saline (PBS)-T (0.05% Tween-20), incubated with primary and secondary antibodies in the blocking buffer, and washed with PBS-T. Signals were detected using enhanced chemiluminescent reagents (Pierce) and captured on an X-ray film (Amersham). Signals were inactivated by incubating blots in the SG reagent (Vector Laboratories) and blots were then probed for actin.

Flow cytometry

Jeg-3, JAR, BeWo, and U-937 cells were tested for surface L-selectin expression using flow cytometry. Trypsinized cells were stained in the staining buffer (PBS with 0.2% bovine serum albumin [BSA]) with primary and secondary antibodies (2 μg/10^6 cells). Washes were performed with PBS-0.2% Tween with 3% FBS. Samples were acquired using a FACSORT flow cytometer (BD Biosciences) with 488 nm excitation from an argon ion laser at 15 mW. Alexa Fluor 488 log fluorescence was captured on the FL1 channel equipped with a 530-nm wavelength filter with 30-nm bandwidth. Data were acquired with Lysis II software (version 2.0; BD Biosciences) and analyzed with WinMDI software (Joseph Trotter; The Scripps Research Institute, La Jolla, CA, available online from http://facs.scripps.edu).

Trophosphere dimensional analysis

Trophospheres used for binding mechanics testing were also subjected to dimensional analysis for average size and measures of sphericity using ImageJ (NIH, Bethesda, MD). Images of trophospheres were made into binary images, and then analyzed using the built-in particle analyzer. Mean

diameters were determined by averaging both Feret's and minimum Feret's diameters. Both roundness (Major:Minor axes) and circularity (Perimeter^2:Area) measurements were tabulated to determine sphericity. In total, 130 spheres over 6 testing days were analyzed.

Trophosphere-sLeX detachment mechanics

To determine binding the mechanics of L-selectin expressing trophospheres to sLeX, a custom-built parallel plate flow chamber was utilized. sLeX tetrasaccharides were immobilized to glass slides at 0.1 μg/mL ($\sim 10^{12}$ molecules/cm^2) using basic carbodiimide chemistry. Briefly, glass slides were cleaned using 1:1 vol:vol methanol:hydrochloric acid solution, sulfuric acid, and finally, boiling. Slides were then coated using aminopropyltriethoxysilane (APTS). sLeX was activated using EDC/s-NHS chemistry and allowed to bind to the silanized slides for 2 h. Coated slides were rinsed with sterile PBS before testing. To block binding, MECA-79 was incubated on selected sLeX-coated slides for 2 h before testing. Polycarbonate plastic and 10% BSA-coated slides were used as negative controls. Coatings were verified using contact angle and immunochemical staining (results not shown).

Trophospheres were injected into the parallel plate flow chamber (PPFC) and allowed to bind to the coated slides for 2–5 min. Individual spheres were imaged under bright field microscopy. Fluid flow, using PBS at 37°C was increased incrementally until trophospheres were removed from the surface. Shearing forces and torques were calculated using estimations previously described by Pozrikidis[46] for spherical objects upon a plate in laminar flow.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. For all data sets, one-way ANOVA followed by Tukey's *post hoc* tests at 95% significance levels were used. All results are displayed as mean ± SEM.

Results

L-selectin expression and modulation in choriocarcinoma cells

Jeg-3, BeWo, and JAR cell lines were evaluated for L-selectin expression using western blotting and surface expression was determined using flow cytometry with the DREG-56 antibody. U-937 cells, which constitutively express L-selectin, were used as positive controls (western blot and flow cytometry)[45] and the *Drosophila* line Deg-5r was used as a negative control (western blot only). Western blots of total protein extracts show a specific band at ~90 kDa, as seen in Figure 1A. Although all cell lines express L-selectin, flow cytometry studies, Figure 1B, show that only the Jeg-3 cell line exhibits surface L-selectin expression. For these reasons, Jeg-3 cells were chosen for further testing.

The effect of estrogen, progesterone, and dexamethasone on L-selectin expression in Jeg-3 cells was examined. To

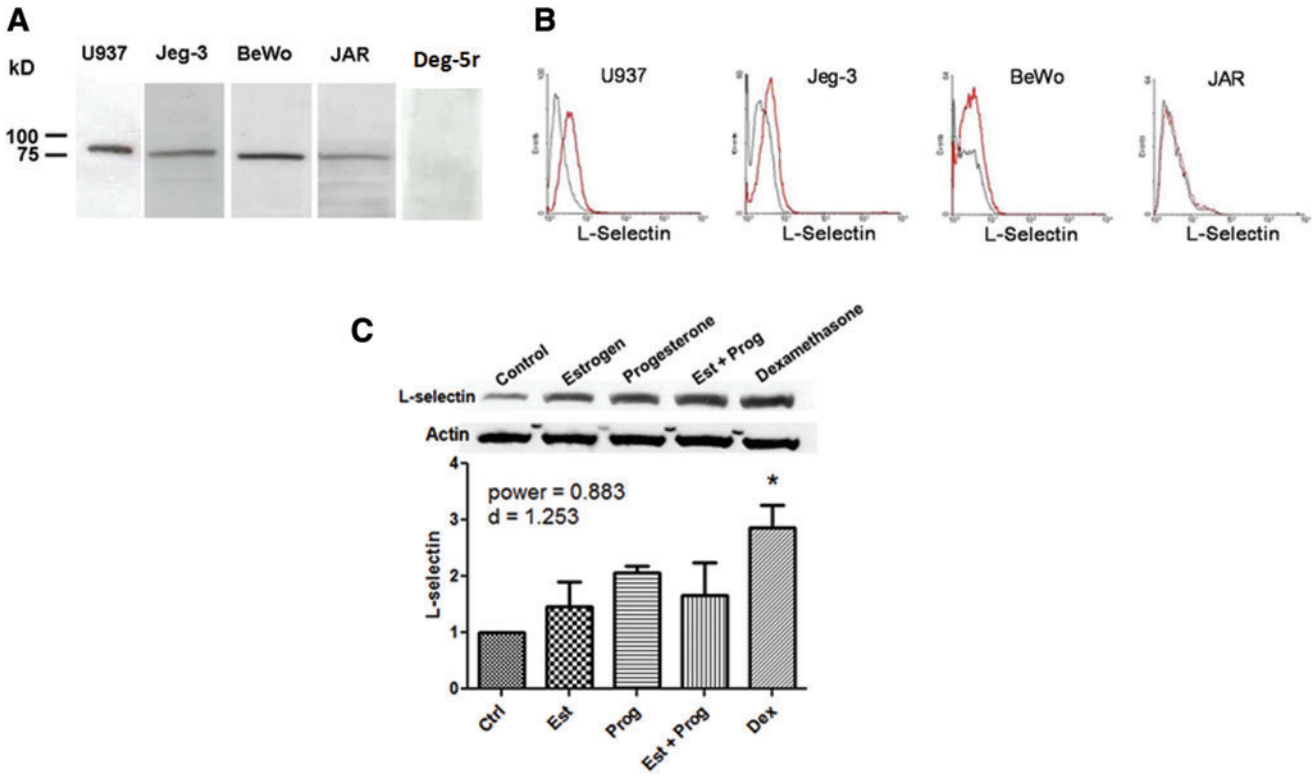

FIG. 1. L-selectin expression and modulation. **(A)** Western blotting shows Jeg-3, BeWo, and JAR cells express L-selectin. U937 monocytes are a positive control, while the *Drosophila* line Deg-5r is a negative control. **(B)** Only Jeg-3 cells exhibit surface L-selectin expression through flow cytometry. **(C)** Jeg-3 expression of L-selectin is upregulated by estrogen (Est), progesterone (Prog), and dexamethasone (Dex). Statistical analysis using one-way ANOVA with $p < 0.05$ and $n = 3$ for each group, *$p < 0.05$. Color images available online at www.liebertpub.com/tea

eliminate the effects of serum-derived growth factors and cytokines, cells were treated with the serum-free medium supplemented with physiological concentrations of hormones: 10^{-8} M estradiol (estrogen), 10^{-6} M progesterone, a combination of both estrogen and progesterone, or 10^{-7} M dexamethasone for 48 h. Control treatments were the serum-free media only. Figure 1C shows that L-selectin expression increased in response to all treatments: ~2-fold with progesterone, 1.5 times with estrogen, or a combination of both, and 3-fold with dexamethasone. However, only the dexamethasone treatment was statistically significant using one-way ANOVA. Immunostaining of trophospheres, Figure 2B, shows expression of L-selectin, although quantification and modulation by hormones were not yet performed.

Dimensional analysis of trophospheres

One hundred and thirty trophospheres over 6 days of testing were analyzed for size and sphericity, and results are displayed in Figure 3. Trophospheres were cultured with diameters of $278.4 \pm 4.6\,\mu m$. Two different sphericity measurements were taken. Average circularity, or the ratio between the area and squared perimeter, measured 0.73 ± 0.006. Roundness, or the ratio between the major and minor axes, measured 0.77 ± 0.009.

Trophosphere-sLeX detachment mechanics

The binding strength of trophospheres to sLeX was determined by detaching trophospheres from sLeX-coated slides in a custom-built parallel plate flow chamber. Binding was blocked using MECA-79 antibodies, while polycarbonate plastic and 10% FBS-coated slides were used as controls. Average applied force and torque measurements are plotted in Figure 4. Trophospheres bound to sLeX were detached under 2.74×10^{-3} dyn of force and 7.5×10^{-5} dyn-cm of torque. MECA-79 blocking reduced detachment force and torque by 56% and 68%, respectively, however, these reductions were not significant. Binding to controls was virtually nonexistent.

Discussion

Millions of women worldwide suffer from infertility and rely on various medical treatments.[1,2] IVF is the most commonly used treatment for infertility; however, IVF leads to pregnancy ~30% of the time and costs upward of $10,000 per trial.[4,47] Low success rates of IVF have been attributed to limitations in the ability of blastocysts to properly implant to the uterus.[6,48–50] Due to cost, legal and ethical considerations, and insufficient supply of donor tissue, few studies of human blastocyst implantation exist. Animal studies, although important, are inadequate models for human implantation.[12–15,17,23,24,51,52] For these reasons, development of an *in vivo* implantation model is necessary.

L-selectin has been implicated as a primary molecule responsible for blastocyst implantation by promoting initial blastocyst attachment to the uterine endometrial epithelium.[11] It is believed that L-selectin is responsible for slowing

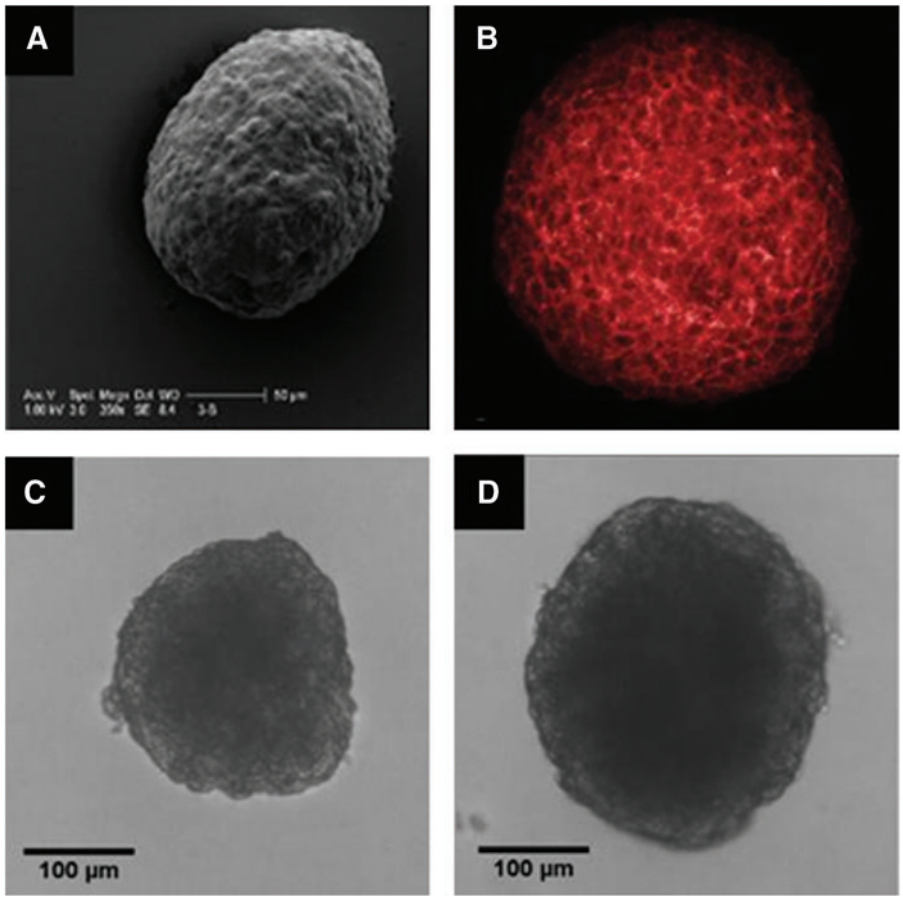

FIG. 2. Trophosphere imaging. **(A)** Scanning electron micrograph of a trophosphere. **(B)** L-selectin expression was analyzed by confocal microscopy with trophospheres stained with Dreg-56, biotinylated secondary antibody, and Texas Red-conjugated streptavidin (200×). **(C, D)** Representative light micrographs (100×) of trophospheres used for dimensional analysis. Diameter, circularity, and roundness measurements are **(C,** 238 µm, 0.77, 0.92**)** and **(D,** 299 µm, 0.84, 0.85**)**. Color images available online at www.liebertpub.com/tea

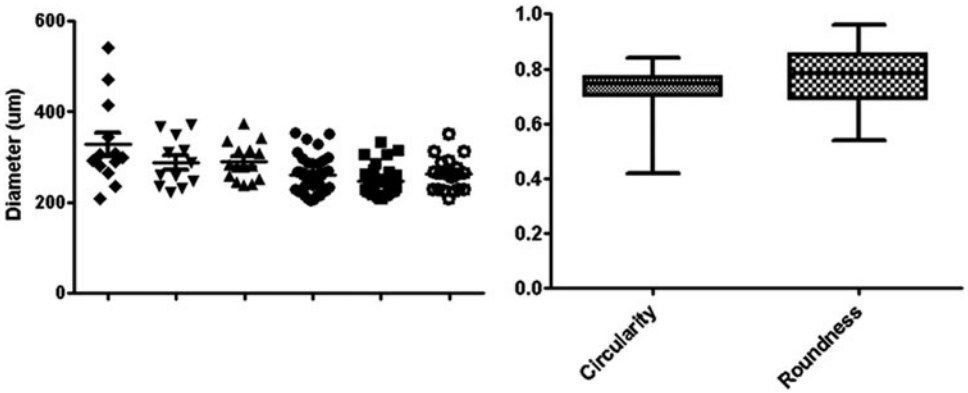

FIG. 3. Dimensional analysis of trophospheres. Using ImageJ software, light micrographs (100×) are processed and analyzed for sphere diameter, circularity (Area:Perimeter^2), and roundness (Major:Minor). Diameter measurements of 130 spheres over six trials are 278.4 ± 4.6 μm. Trophospheres are highly circular (0.73 ± 0.006) and round (0.77 ± 0.009). All values are mean ± standard error calculated using GraphPad Prism software.

and arresting the blastocyst during its travel from the Fallopian tube to the fundus of the uterus.[6,53,54] We hypothesize that a certain amount of bonding strength is necessary to achieve this initial attachment and that in the absence of L-selectin, there may be a reduction in blastocyst attachment to the uterine endometrial epithelium or that the attachment would have to go through a different pathway. *In vivo* implantation models have been previously reported,[35,42,43,55] however, these models are limited in scope in that they do not aim to quantify L-selectin binding mechanics.

Jeg-3, JAR, and BeWo cell lines, derived from first trimester trophoblasts, were evaluated for their L-selectin expression. This is crucial since protein expression profiles of trophoblast cells *in vivo* change significantly as pregnancy progresses, to the extent that, by 17 weeks of gestation, trophoblast staining for L-selectin is virtually absent.[11] Comparing the L-selectin expression profile by immunoblot, Figure 1A, and flow cytometry analysis, Figure 1B, L-selectin expression was verified in all three cell lines, but only on the surface of Jeg-3 cells. Therefore, Jeg-3 cells were selected for further studies as surface expression of L-selectin is critical for the attachment function.

L-selectin expression in Jeg-3 cells was investigated in response to hormones and corticosteroids: estrogen, which dominates in the follicular phase, progesterone, the primary hormone in the luteal phase of the menstrual cycle, and dexamethasone, a steroid used during oocyte retrieval in IVF. All treatments increased L-selectin expression in Jeg-3 cells with dexamethasone producing the most significant effect, Figure 1C. *Post hoc* power analysis confirms the significance of these results and suggests that a slight increase in the sample size ($N = 20$, $n = 4$ for each treatment) would result in significance for all treatments. Any effects on baseline expression in these experiments were avoided by using media free of steroid hormone mimetics and serum. These observations support that Jeg-3 cells have preserved responsiveness to endocrine regulators; nevertheless, caution should be used in trying to extend these results to primary human trophoblast cells.

To mimic the size and shape of a human blastocyst, Jeg-3 cells were cultured into three-dimensional structures, which we coined "trophospheres." Trophospheres were cultured in a manner similar to Grümmer *et al.*[42] We extended this technique by performing a parametric study of shaker speed,

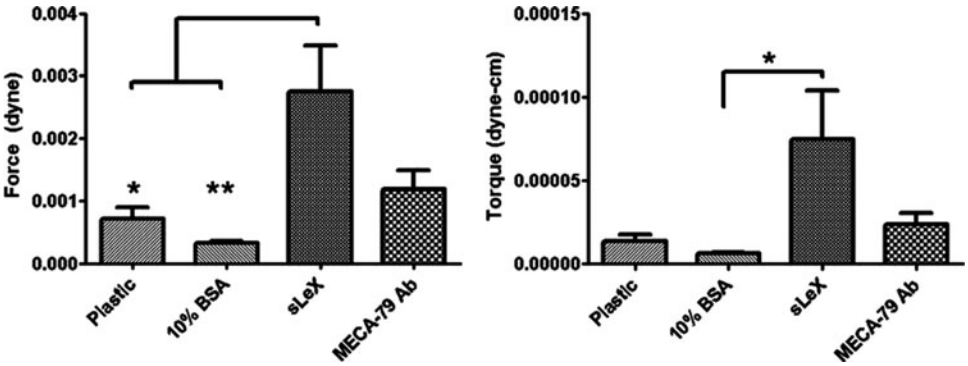

FIG. 4. Trophosphere detachment mechanics. Trophospheres are detached from sialyl Lewis X (sLeX)-coated slides ($n = 16$) under 2.74×10^{-3} dyn of force and 7.5×10^{-5} dyn-cm of torque. Blocking with MECA-79 ($n = 17$) decreases necessary detachment force and torque by 56% and 68%, respectively. Bare polycarbonate ($n = 10$) plastic and 10% bovine serum albumin ($n = 12$)-coated slides are used as controls. Statistical analysis performed using one-way ANOVA, with Tukey *post hoc* tests of significance signified by stars, *$p < 0.05$ and **$p < 0.01$.

seeding density, and the serum level revealed which culture conditions were most suitable in achieving the desired trophosphere diameter and sphericity (data not shown). Trophospheres were cultured with diameters of $278.4 \pm 4.6\,\mu m$, which are slightly larger than the average diameter of a human blastocyst, 150–250 μm.[56] Since the trophosphere shape is variable, both roundness and circularity measurements were taken. Figure 3C shows a trophosphere with average circularity, 0.77, and a high degree of roundness, 0.92. This difference is due to the slightly undulating surface of the trophosphere causing a larger perimeter and thus decreasing the circularity. Figure 3D shows a trophosphere with comparable degrees of circularity, 0.84, and roundness, 0.85. These differences can be attributed to the methods of calculating circularity, (Area/Perimeter^2) and roundness (Major/Minor axis). Although roundness calculations are more robust than circularity, both average greater than 70% and show little variation suggesting that trophospheres can be cultured reproducibly. For comparison, we tested 20-μm latex microspheres, which resulted in average circularity, 0.91, and roundness, 0.95, to be only slightly higher compared with trophospheres. Immunostaining, Figure 3B, shows trophospheres express L-selectin. Quantification and modulation of L-selectin expression of trophospheres had not been performed at this time.

Few similar studies on three-dimensional trophoblast constructs binding to endometrial cells exist. However, these studies differ from ours and do not attempt to quantify L-selectin/sLeX binding. Spheroids have been cultured using JAR[43,57,58] or BeWo[35] cells and subsequently bound to monolayers of RL95-2 endometrial-like cells. However, we have shown that neither JAR nor BeWo cells exhibit surface L-selectin expression. Additionally, these studies incorporate binding times upward of 30 min and utilize centrifugation assays that impart large forces. Although these studies provide some insight into binding events in general, it is questioned as to what specific mechanisms are at work as well as what the binding mechanics are. Grümmer *et al.*[42] use Jeg-3, JAR, and BeWo cells, but their spheroids range from 200 to 1000 in diameter. Our work eliminates nonspecific cell–cell interactions by binding directly to sLeX-coated slides and allows for quantifying cellular detachment force and torque.

Quantification of trophosphere detachment is modeled after the work by Prozirkidis,[46] who made mathematical predictions of flow forces and torques on spherical objects in laminar flow. The shear force and torque are related to the particle radius and fluid shear rate under the assumptions that the particles are perfectly spherical, nondeforming, and subject to a fully developed laminar flow. In this study, a custom-built PPFC ensured laminar flow and trophospheres have been shown to be nearly spherical.

Trophospheres detached from sLeX functionalized slides under 2.74×10^{-3} dyn of force and 7.5×10^{-5} dyn-cm of torque. Blocking with MECA-79[11,59,60] decreased detachment force and torque by 56% and 68%, respectively. The next logical step is to evaluate the effects that hormonal treatments have on trophosphere binding. However, effects of hormonal treatments are only detectable in cells cultured in the serum-free medium, based on our results with Jeg-3 monolayer cultures, while the serum-free medium does not support trophosphere culture. Decreasing serum levels below 10% results in sporadic and irregular spheres due to extensive cell death. There is currently much debate on the effects of hormone therapies, specifically progesterone, on IVF outcomes. Most recent evidence suggests that high progesterone serum levels results in lower IVF success and most likely lowered endometrial receptivity.[61–63] Although our work does not address this body of evidence, the authors do have ongoing studies regarding trophospheres binding to model endometrial cell lines. Preliminary data suggest that hormone treatments to endometrial cell lines result in decreases to L-selectin ligand expression and trophosphere/endometrial detachment forces.

Although improper blastocyst implantation is thought to be the cause for the limited success rates of IVF, very little is known about the mechanics of implantation. It has been shown that during the secretory phase, uterine contractions occur at a rate of 1–5/min causing a uterine pressure of 5–25 mm Hg.[64] These uterine contractions are directed from the cervix to the fundus, which may help explain why implantation generally occurs near the fundus. Eytan *et al.*[65,66] have modeled uterine contractions showing directionality of fluid flow within the uterus. However, neither the forces imparted on the blastocyst by uterine contractions nor the uterine fluid flow is known. As our results show, L-selectin-mediated binding requires little force to be broken. Thus, it is within the realm of possibility that mechanical conditions within the uterus are sufficient to prevent the initial stages of implantation. Curiously, Eytan *et al.*,[64–66] have also modeled instances of slow motion near the fluid–wall interface and trapping, where the blastocyst would become stuck within a regional vortex. These conditions may allow for blastocysts to initiate the implantation process and for enough time for stronger adhesions to occur.

Conclusions and Future Works

To the authors' best knowledge, this work presents the first time Jeg-3 cells have been shown to exhibit surface L-selectin expression. Additionally, this is the first time that L-selectin/sLeX bonding in trophoblastic cells has been quantified by culturing Jeg-3 cells into three-dimensional blastocyst mimics or trophospheres. Quantification of this binding system is a crucial step in advancing the field of blastocyst implantation and provides groundwork for determining the forces experienced by blastocysts during the implantation process.

The work presented here may be useful in both the laboratory and clinic settings as either a research tool, or clinically, either to optimize implantation by pretreatment of blastocysts, or as part of a diagnostic test. In the short term, this model will be utilized in the laboratory environment. We have ongoing studies investigating the effect of various environmental factors on binding between trophospheres and a model uterine epithelium with hopes to transition into testing primary tissues. Patients undergoing IVF are introduced to extreme hormone therapies, yet the effects of these hormone therapies on the aspect of blastocyst implantation are not well known. Our model system may have direct impacts into understanding the role of hormonal effects on implantation mechanics. In the long term, model results may provide a metric for maximizing implantation capacity of blastocysts generated by IVF or provide diagnostic parameters to aid in the selection of

implantation-competent blastocysts and, thus, increase the efficiency of IVF.

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Disclosure Statement

No competing financial interests exist.

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