Analysis of HLA-G in Maternal Plasma, Follicular Fluid, and Preimplantation Embryos Reveal an Asymmetric Pattern of Expression

Valerie R. Shaikly, Ian E. G. Morrison, Mohamed Taranissi, Clare V. Noble, Anna D. Withey, Richard J. Cherry, Sandra M. Blois and Nelson Fernández

*J Immunol* 2008; 180:4330-4337; doi: 10.4049/jimmunol.180.6.4330

http://www.jimmunol.org/content/180/6/4330
Analysis of HLA-G in Maternal Plasma, Follicular Fluid, and Preimplantation Embryos Reveal an Asymmetric Pattern of Expression

Valerie R. Shaikly,†† Ian E. G. Morrison,* Mohamed Taranissi, † Clare V. Noble,† Anna D. Withey, † Richard J. Cherry,* Sandra M. Blois,* and Nelson Fernández2*  

Soluble HLA-G (sHLA-G) secretion by human preimplantation embryos in culture has been associated with successful embryo development, and therefore has potential to serve as a noninvasive marker of embryo viability. We have examined the spatial and temporal expression of HLA-G in embryos of varying developmental competence and the role of maternal factors in human embryonic HLA-G expression. Embryos that reached blastocyst stage on day 5 showed a higher frequency of sHLA-G secretion than those at morula or arrested stages (p < 0.05). There was no significant difference in sHLA-G secretion between normal embryos and those diagnosed as chromosomally abnormal by preimplantation genetic diagnosis. HLA-G detected in maternal plasma and follicular fluid did not appear to correlate with HLA-G expressed in the embryo or embryo supernatants. Confocal microscopy analysis indicated that HLA-G protein expression in embryos was not homogeneous; mostly, it was confined to blastocysts localized on trophoderm and trophectoderm projections. Single-particle fluorescent imaging analysis of HLA-G on the cell surface of JEG-3 cells showed that HLA-G particles were mostly monomeric, but dimeric and higher order oligomers were also observed. These results suggest that HLA-G play an important role in preimplantation embryo development. However, the observed expression of HLA-G in arrested and chromosomally abnormal embryos indicates that HLA-G testing should be used with caution and in conjunction with conventional methods of embryo screening and selection. The Journal of Immunology, 2008, 180: 4330–4337.

Defined as a “nonclassical” class I Ag, HLA-G is associated with allograft acceptance and masking of tumors or diseased cells from immune attack (1). HLA-G is also associated with implantation and protection of the allogenic fetus from the maternal immune system, since at the fetal-maternal interface HLA-G is expressed by placental cytotrophoblast cells. It has also been shown that HLA-G binds to inhibitory receptors on T lymphocytes and uterine NK cells (2). At this interface, HLA-G also modulates local immune cell cytokine secretion and plays a role in the remodeling of spiral arteries during embryo implantation (3).

Although HLA-G has been defined as a nonclassical class I Ag, it shares structural similarities with classical HLA class I A, B, and C Ags: it contains α structural domains and some isoforms are associated with the nonpolymorphic protein β2-microglobulin. Alternative splicing of the HLA-G primary transcript gives rise to four membrane-bound isoforms designated HLA-G1 to HLA-G4, and to three soluble isoforms HLA-G5 to HLA-G7. The full-length transcript HLA-G1 has all three extracellular domains, a transmembrane region, and a cytoplasmic tail. HLA-G5 has the same primary transcript but retains intron 4, which contains a premature stop codon, preventing the translation of the transmembrane region resulting in a soluble protein (denoted as soluble HLA-G (sHLA-G)3). The α-3 domain in the isoform HLA-G1 and its soluble counterpart sHLA-G5, which are investigated in this study, are noncovalently associated with β2-microglobulin. HLA-G can act as an Ag-presenting molecule but is thought to have a limited repertoire for binding peptides (4); at the cell surface, HLA-G has been shown to exist in disulphide-bonded dimers (5). Binding studies have shown that the HLA-G dimerization confers increased affinity to leukocyte Ig-like receptors, enhancing signaling at the cellular level (6).

Several studies have reported the secretion of sHLA-G5 by embryos at cleavage stages into the culture microenvironment. This has been associated with increased cleavage rates and successful implantation of the embryo during in vitro fertilization (IVF) treatment (7–19). In IVF protocols, embryo selection for transfer to the uterus is based on visual assessment of embryo morphology and cleavage rate. It has been proposed that sHLA-G could be used as an additional marker to assess implantation potential and thus to increase IVF success rates. However, Van Lierop et al. and Noriko et al. (20, 21) were unable to detect sHLA-G in supernatants of embryos from cleavage to blastocyst stages of development and thus it has been suggested that detection of sHLA-G released by human embryos may be the result of artifactual detection. Indeed, quantitative measurements of sHLA-G protein expression reported in the literature vary between 10 and 20% higher than the total protein content of the embryo (22). If such quantitative measurements are real, the developing embryo would have to commit...
much of its energy into the production of sHLA-G. In contrast, HLA-G mRNA and protein products have been shown to be expressed by preimplantation embryos by other workers (23). Recently, Yao et al. (24) examined the expression of mRNA for each of the isoforms of HLA-G. These authors reported expression of HLA-G1 mRNA in some early cleavage embryos and concluded that each mRNA isoform is increased as the embryo develops. HLA-G5 mRNA was only expressed by 20% of morulae and blastocysts but embryos of all stages showed positive staining with the mAb MEM-G/9 that is specific for HLA-G1 and G5. The hypothesis that HLA-G observed in early embryos is derived from maternal oocyte protein was suggested to account for the disparity between observed mRNA and protein expression. In support of this suggestion, Rizzo et al. (25) reported that both membrane-shed HLA-G1 (sHLA-G1) and sHLA-G5 are present in follicular fluid derived from granulosa cells, and that this expression has a significant relationship with sHLA-G expressed by preimplantation embryos. However Lédée et al. (26) reported poor correlation between sHLA-G concentrations in follicular fluid, embryo supernatants, and implantation rates.

In this study, we have investigated sHLA-G in single cultured embryos over 24 h time periods and on embryos undergoing preimplantation genetic diagnosis (PGD). To investigate the feasibility of measuring the presence of sHLA-G for embryo selection, in a clinical setting, a commercial sHLA-G ELISA was used. Samples were also collected from a group of patients to test whether maternal sHLA-G protein in blood plasma and follicular fluid showed a “carryover” effect on observed embryo sHLA-G secretion and HLA-G protein expression. To gain insight as to the functional role of HLA-G expression, embryos donated to this study were examined for HLA-G topographical distribution using fluorescent probes in conjunction with confocal microscopy and single-particle fluorescent imaging (SPFI) analysis.

Materials and Methods

Collection of embryo and patient samples

Ethical approval for this work was obtained from the University of Essex Ethical Committee and the Human Fertilisation and Embryology Authority (HFEA; Project License R0165). Embryos were donated with informed consent by patients treated at the Assisted Reproduction and Gynaecology Centre (London, U.K.). Ovarian stimulation, oocyte retrieval, insemination, and PGD were conducted as previously described (27). Individual embryos were cultured in Vitrolife sequential medium and placed in fresh 50-μl droplets under oil every 24 h until day 4 of development. Conditioned culture dishes were stored at -20°C. Granulosa samples were isolated from follicular fluid aspirates by centrifugation before incubation with Abs.

In a separate cohort of 12 patients, blood samples were drawn on the day of egg collection into dry EDTA tubes and the plasma was separated by centrifugation and stored at -20°C. During the egg collection of this patient group, the fluid from up to five clear follicular aspirates was collected as sampling permitted and stored at -20°C. Protocols governing clinical and laboratory practice did not permit the traceability of individual oocytes from specific follicles, through the steps of fertilization, embryo culture, and either transfer or allocation to research. Laboratory protocols in place at this time did not permit the sampling of supernatant from individual embryos. Embryo supernatants from this group of patients available for research analysis were stored at -20°C. Embryos were allocated to this study on day 6 after embryo transfer and freezing of good quality blastocysts. Cryopreserved embryos donated to this research were thawed using Vitrolife Thaw kit and allowed to equilibrate in culture for minimum of 3 h before immunofluorescence studies.

ELISA for sHLA-G

sHLA-G levels were assayed using a specific sandwich enzyme ELISA kit (Exbio) which uses MEM-G/9-specific mAb MEM-G/9. When embryos are transferred between 50-μl droplets, they are transferred with a small amount of medium. To ensure that equal volumes of embryo supernatant were tested, 10 μl of dilution buffer were then added to the microplate test wells. Forty microliters of embryo supernatant from each droplet were loaded manually into the wells and the assay was then programmed into and performed using a Triturus automated ELISA analyzer (Grifols). sHLA-G standard was diluted to give a calibration curve within the range of expected sHLA-G expression of the embryos and the results were adjusted for the sample dilution. Follicular fluid and plasma samples were tested in duplicate; however, embryo culture supernatants could not be run in duplicate as only one 50-μl droplet was available for testing.

Immunofluorescence labeling

Embryos were fixed with 1% paraformaldehyde, then placed in acid Tyrode’s solution (Sigma-Aldrich), to thin the zona pellucida, and permeabilized with 0.1% Triton X-100 unsub diluted for fluorescent HLA-G detection. Cells were then washed and blocked with PBS supplemented with 2% BSA. Embryos that were labeled with biotin conjugate and Qdot-streptavidin were also blocked with an avidin/biotin blocking kit (Vector Laboratories).

All incubation steps were conducted in microdroplets under mineral oil. Embryos were incubated with primary Abs at a dilution of 20 μg/ml for 1 h, washed with 1% BSA PBS, then incubated with secondary Abs. The primary and secondary Ab combinations used were: HLA-G-specific mAb MEM-G/9 (Exbio)/goat mouse-specific IgG FITC (Serotec), or MEM-G/9 biotin conjugate (Exbio)/Qdot-streptavidin 585 nm (Molecular Probes). For positive immunofluorescence controls, rabbit-human-specific actin Ab (Sigma-Aldrich) was used with mouse rabbit-specific IgG FITC (Serotec). Some embryos were labeled directly with MEM-G/9 PE (PhyE) conjugate (Exbio) or MEM-G/9-FITC conjugate (Exbio). To detect lipid rafts and HLA-G on the cell membrane, embryos were incubated with MEM-G/9-PhyE (20 μg/ml) and cholera toxin B conjugate with Alexa Fluor-488 (10 μg/μl; Molecular Probes). After incubation, the embryos were washed through sequential droplets, mounted in DABCO, and stored at 4°C before analysis. Granulosa cells were blocked and incubated in a similar manner before fixation. Visualization of immunofluorescence labeling was conducted using a Bio-Rad Radiance 2000 Scanning Laser Confocal Microscope. SPFI was performed with an Olympus IX70 microscope with a ×40/NA0.85 UPlanApo objective and 100 W mercury lamp. Images were recorded by a Wright Instruments camera, with a back-illuminated Peltier-cooled couple-charged-device, attached to the microscope Keller port. Images of particles were identified as diffraction limited spots and analyzed by fitting with a two-dimensional Gaussian function using specialized software as previously described by our laboratory (28).

Controls

The unique nature of each embryo and the droplet in which it was cultured did not allow for direct parallel controls for individual embryos; thus, it was necessary to use a reference HLA-G-positive cell type for comparative purposes. The human choriocarcinoma cell line JEG-3 (American Type Culture Collection) was used; this cell line is known to express detectable levels of HLA-G (29). JEG-3 cells were cultured in MEM (Sigma-Aldrich) supplemented with 10% FCS. Supernatant fluid from JEG-3 cultures was collected 72 h after passaging the cells. Medium droplets that contained no embryos but had been exposed to the same culture conditions were used as negative controls. For immunohistochemistry, a random sample of embryos were selected during the course of this study and then incubated with nonspecific mouse IgG in place of the primary Ab as a negative control. JEG-3 cells cultured for 72 h after passaging were labeled as a positive control for MEM-G/9; the nuclei were located by incubation with DRAQ5 (Biostatus). The HLA class I-negative Jar cell line was incubated with MEM-G/9 to serve as a control for Ab cross-reactivity. Embryos incubated with an actin-specific Ab served as a control for immunofluorescence methodology.

Statistical analysis

The profiles of sHLA-G levels for each embryo at different stages of development were annotated and statistically investigated using SPSS version 12. To investigate trends in sHLA-G expression, taking into account the SE of the ELISA and the variability observed in sHLA-G expression, embryos were categorized as sHLA-G positive or negative for some statistical analysis. A supernatant sample was categorized as negative if below the lowest standard of 3.12 U/ml. Such categorization of samples does not reflect the true biological nature of sHLA-G expression. As some observations were close to the detection limit, some embryos with sHLA-G expression below 3.14 U/ml would be categorized as false negatives. The analysis is therefore based on the assumption that the sample numbers in this study were sufficient to compensate for false-negative categorization and allow the observation of trends in sHLA-G expression. Observed trends were examined for significance using χ² analysis.
Results

Expression of sHLA-G in embryo culture supernatant

Embryo culture supernatants from single cultured embryos were tested and scored as either sHLA-G positive or negative on a daily basis as clinical sampling permitted. Results from 498 culture supernatants from 166 embryos showed that embryos that reached cavitating and blastocyst stages on day 5 were more likely to secrete sHLA-G than those at the morula or other arrested stages (p < 0.05, Fig. 1A). One hundred and five samples were tested from embryos that had undergone PGD for chromosome abnormalities; no significant difference was observed in the patterns or frequency of sHLA-G secretion between normal embryos and embryos that had been transferred back with biotinylated MEM-G/9-labeled with streptavidin Qdots. Marked immunofluorescence labeling was observed on trophoderm, hatching trophoderm, and trophoderm projections. (Fig. 2B, 4–6).

Embryos labeled with isotype control in place of the primary Ab, FITC secondary, or streptavidin Qdots only showed a low background signal that did not differ from autofluorescence control images (Fig. 2B7). HLA-G was observed on the cell surface of JEG-3 cells, the positive control for this work, using both MEM-G9/anti-mouse FITC, MEM-G9-PhyE conjugate, and biotinylated MEM-G9/streptavidin Qdot 585 (Fig. 2B8). The HLA class I-negative Jar cell line, the control for Ab cross-reactivity, showed no immunofluorescence with MEM-G9 (Fig. 2B9). Actin detection in embryos confirmed that the labeling protocol was effective (Fig. 2B10).

Maternal and embryo expression of sHLA-G

Confocal microscopy analysis identified a subpopulation of HLA-G-expressing granulosa cells in the four patients from whom granulosa cells were collected via ovarian follicle aspiration. Expression was not homogenous among granulosa cells and was only observed in occasional cell clusters and cells (Fig. 2A, 1–4). Table 1 shows sHLA-G detection in maternal and embryonic samples from a group of 12 patients undergoing IVF treatment. In maternal blood plasma, sHLA-G was detected in all 11 samples tested, with an average value of 33 U/ml and a range of 4–60 U/ml (where 100 U/ml corresponds to 40–50 ng/ml according to the 2004 Soluble HLA-G Workshop, Essen, Germany). The second set of maternal samples investigated for sHLA-G expression was the follicular fluid samples obtained at the time of oocyte retrieval. Forty-seven percent were found to be positive for sHLA-G with a range of 3.5–8 U/ml. Conditioned embryo supernatants from this patient group were also tested for sHLA-G expression. Twenty-one percent were positive with a range of 3.5–7 U/ml. Supernumerary embryos (i.e., those embryos not used for patient treatment) were allocated for this study on day 6 of development and examined for HLA-G protein expression using confocal microscopy. Twenty-four embryos were labeled for HLA-G; 50% of those showed positive labeling, however, the distribution and intensity of fluorescent detection observed in embryo blastomeres was variable. Analysis of sHLA-G expression in maternal blood plasma and follicular fluid did not appear to show a carryover effect on the expression of sHLA-G and HLA-G protein in the embryos available for testing.
SPFI to investigate the spatial distribution of HLA-G in preimplantation embryos

The SPFI methodology has previously been used to investigate molecules on the cell surface, in adherent cell lines grown in culture, where it is possible to locate the focal plane of single molecules at the cell surface (30, 31). When adapting SPFI methodology for this study, it was found that focusing on single particles on embryo blastomeres was difficult due to the large three-dimensional shape of the cells. Also the high autofluorescence of the embryo blastomeres did not allow signals from single particles to be identified above background fluorescence (Fig. 2C1). In previous studies, HLA-G has been found colocalized in lipid rafts in JEG-3 cells (32). To facilitate focus on the cell membrane of the embryo and locate any HLA-G that may be present in low copy number, we double surface stained some embryos with cholera toxin B conjugated with Alexa Fluor 488 and MEM-G/9-PhyE.
Lipid rafts were observed on embryo blastomeres but no HLA-G staining was seen at this focal plane (Fig. 2C2). In JEG-3 cells, lipid rafts and HLA-G were observed simultaneously. However, analysis of confocal images and wide-field couple-charged-device imaging showed that colocalization was very low and HLA-G was not predominantly located in lipid rafts (Fig. 2C3). SPFI analysis was performed on JEG-3 cells stained with MEM-G/9-PhyE (Fig. 3B), and the signal intensity compared with that of MEM-G/9-PhyE images on a polylysine-coated glass slide (Fig. 3, A and C). Results indicated that expression of HLA-G1 at the cell surface was not uniform on the population of cells analyzed. Some cells showed a very high density with clustering of HLA-G. On lower-expressing cells, the majority of particles were monomeric, but dimeric and higher aggregates of HLA-G were also observed (Fig. 3, D and E).

### Discussion

sHLA-G expression has been reported in culture medium that have contained embryos for 48 or 72 h. In this study, we report variable expression between different embryos and between the same embryos over 24-h periods. Criscuoli et al. (15) found that patients in whom no sHLA-G was detected in embryo supernatants often had sHLA-G detected in embryo supernatants in a subsequent IVF cycle. sHLA-G expression has not been previously studied in embryos beyond day 3 of development. In this study, we extend these observations and report sHLA-G expression in embryos cultured until day 5 of development. Our results show that embryos which develop to blastocysts are more likely to express sHLA-G during their development. Desai et al. (16) also reported a significantly higher sHLA-G detection in day 3 culture medium of spare embryos (not suitable for freezing or transfer) on day 3 that developed to blastocyst during extended culture before discard. It could be argued that sHLA-G association with blastocyst potential may be a result of the progression in culture of the higher cleavage rate, associated with sHLA-G-positive embryos, noted by other groups at earlier stages of embryo development. As only embryos that reach the blastocyst stage were transferred during this study, this also increased the number of sHLA-G-positive embryos transferred. Thus, culturing embryos to blastocyst stage may inadvertently select sHLA-G-positive embryos. The extended culture of embryos has unknown long-term effects and has been reported to be detrimental to fetal development in other species (33). Our results have shown that a significantly higher proportion of embryos that express sHLA-G develop to blastocysts in culture. Thus, the

### Table I. Summary of results to show sHLA-G detection in maternal and embryo samples

<table>
<thead>
<tr>
<th>Mean Difference of sHLA-G Detected in Blood Plasma on Day of Egg Collection (Mean of Group = 33 U/ml)</th>
<th>sHLA-G Detected in Follicular Fluid on Day of Egg Collection</th>
<th>HLA-G Detected by IHC in Supernumerary Embryos on Day 6</th>
<th>Outcome of Treatment Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>In Group</td>
<td>In Group</td>
<td>In Group</td>
<td>In Group</td>
</tr>
<tr>
<td>Patient 1</td>
<td>0.7</td>
<td>0/4</td>
<td>0/4</td>
</tr>
<tr>
<td>Patient 2</td>
<td>12.0</td>
<td>1/3</td>
<td>1/4</td>
</tr>
<tr>
<td>Patient 3</td>
<td>–2.8</td>
<td>2/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Patient 4</td>
<td>11.3</td>
<td>2/3</td>
<td>2/4</td>
</tr>
<tr>
<td>Patient 5</td>
<td>–11.2</td>
<td>0/3</td>
<td>0/4</td>
</tr>
<tr>
<td>Patient 6</td>
<td>–7.7</td>
<td>2/3</td>
<td>1/4</td>
</tr>
<tr>
<td>Patient 7</td>
<td>–20</td>
<td>3/3</td>
<td>1/3</td>
</tr>
<tr>
<td>Patient 8</td>
<td>–3.5</td>
<td>1/3</td>
<td>0/2</td>
</tr>
<tr>
<td>Patient 9</td>
<td>7</td>
<td>2/5</td>
<td>0/4</td>
</tr>
<tr>
<td>Patient 10</td>
<td>Not available</td>
<td>Not available</td>
<td>3/6</td>
</tr>
<tr>
<td>Patient 11</td>
<td>19.7</td>
<td>1/1</td>
<td>0/4</td>
</tr>
<tr>
<td>Patient 12</td>
<td>–4.2</td>
<td>3/5</td>
<td>0/4</td>
</tr>
</tbody>
</table>

1. The mean difference of the detected value above or below the mean plasma sHLA-G of the group.
2. The number of samples in which sHLA-G was detected over total number of follicular fluid samples tested.
3. The number of samples in which sHLA-G was detected over total number of embryo supernatant samples available for testing where samples from: a, days 1–2; b, days 2–3; c, days 3–4; and d, days 4–5 of development.
4. Number of embryos with HLA-G-positive staining over total number of embryos stained; IHC, immunohistochemistry.

### FIGURE 3. Wide-field fluorescence images and single particle analysis.

A. Pattern of diffraction limited spots obtained on a control polylysine-coated glass slide loaded with MEM-G/9-PhyE probe only. B. Typical diffraction limited spots obtained following incubation of MEM-G/9-PhyE with JEG-3 cells. C. Histogram showing the statistical analysis of the total number of diffraction limited spots and their signal intensity as counts (cts) on a polylysine-coated glass as in image in A. D and E. Representative histograms showing the statistical analysis of the total number of diffraction limited spots and their signal intensity on two separate JEG-3 cell images.
selection of embryos for transfer using sHLA-G screening in early development stages may result in similar pregnancy rates to those achieved through extended culture and blastocyst transfer. This would reduce the risks in IVF treatment associated with extended culture of embryos in vitro and the cost of testing would be offset against that of extended culture.

The small sample volume of embryo culture medium available for testing in this study was not compatible for use with the automated ELISA analyzer, which requires a minimum sample volume of 200 µl for sample aspiration. Thus, the samples were loaded manually then the remaining ELISA steps were conducted using an automated setup. It was found that using this semiautomated method, sHLA-G testing could be implemented within IVF practice, and also that the results of sHLA-G expression by the embryo would be available the same day as testing to assist with embryo selection at the time of transfer.

sHLA-G expression in embryos undergoing preimplantation genetic diagnosis has not been previously reported. We observed no significant differences in sHLA-G expression between embryos diagnosed as chromosomally normal or abnormal. This is consistent with the findings of Desai et al. (16), who noted that some embryos diagnosed as chromosomally abnormal by PGD had the capacity to secrete sHLA-G. We conclude that sHLA-G could be used in conjunction with current morphological parameters to identify embryo implantation potential. However, this study has shown that the presence of sHLA-G is not indicative of chromosomal normality.

Noci et al. (14) reported that only sHLA-G-positive embryos lead to pregnancy. Sher et al. (11) and Desai et al. (16) reported significantly higher pregnancy rates when sHLA-G-positive embryos were transferred but also reported a pregnancy rate of 25 and 36%, respectively, with sHLA-G-negative embryos. In the 26 patients included in this study, embryos that were sHLA-G-positive during their development showed a higher rate of implantation, but sHLA-G-negative embryos also resulted in pregnancies. Varied limits of the sensitivity of the ELISA methods and protocols used in different studies could account for different values and correlations of sHLA-G expression and implantation. Some embryos may be sHLA-G positive but at levels not detectable by the ELISA and so counted as sHLA-G negative. In addition varying culture environments and embryo manipulation protocols may affect sHLA-G expression. Ovarian stimulation, immune therapy, and clinical technique are variables that affect pregnancy rates in IVF treatment. Our results indicate that although sHLA-G secretion by the embryo may not be a prerequisite for pregnancy, sHLA-G testing could assist in embryo selection at the time of transfer to the uterus and improve IVF implantation rates. This in turn would make the option of single embryo transfer increasingly cost effective and acceptable to patients, thus reducing the risks associated with multiple pregnancies in assisted conception.

This study has confirmed the expression of sHLA-G in cleavage stage embryos during days 1 and 2 of human development. In mice we have previously detected MHC class I transcripts at the one-cell stage of preimplantation development (34). However, in humans, activation of the embryonic genome is thought to occur between days 2 and 3 of development after the second cleavage division (35). The possibility that maternal protein expression of HLA-G may be a carryover to the oocyte and be implicated in HLA-G expression of the embryo has also been considered. Rizzo et al. (25) measured sHLA-G in follicular fluid and found that this correlated with sHLA-G measured in the corresponding embryo supernatant. In contrast to this study, Lédée et al. (26) detected sHLA-G in 96% of follicular fluids but found this was not correlated to sHLA-G in the corresponding embryo supernatants or related to implantation. We were able to detect sHLA-G in approximately half of the follicular fluids tested, but in only approximately one-quarter of embryo culture supernatants. Although we only reported sHLA-G in the follicular fluid samples from 11 patients here, additional experiments also showed that <50% of follicular fluids tested positive for sHLA-G (N. Fernández and Valerie R. Shaikly, unpublished data).

The question of why sHLA-G levels in blood plasma do not appear to correlate with those levels found in the follicular fluid is unclear. Proteins in human follicular fluid originate from blood plasma through crossing of the follicular/blood barrier or are synthesized directly by metabolism of follicular granulosa cells. In this study, we report levels of sHLA-G in follicular fluid almost 10 times lower than in the maternal blood plasma. Schweigert et al. (36), using a proteomic approach, showed qualitative and quantitative differences in some plasma and follicular fluid proteins. It was suggested that a selective transport process of filtration across the blood follicle barrier may account for this observation. Rebbmann et al. (37) also reported significantly less sHLA-G in maternal samples of amniotic fluid and cord blood than in blood plasma. Thus, it could be hypothesized that the blood follicular fluid barrier may act to limit the sHLA-G in the follicle. As oocyte retrieval is performed by vaginal aspiration under ultrasound guidance, follicular fluids are usually aspirated with some blood contamination. Levels of sHLA-G in circulating blood can be high and a small amount of blood in the follicular fluid could allow it to be counted as sHLA-G positive. Therefore, differences in the reported sHLA-G detection in follicular fluid samples could be due to blood contamination or varying sensitivity of ELISA protocols used.

In the patient group tested in this study, sHLA-G detection in embryo culture supernatants did not appear to show a relationship with maternal variables, sHLA-G detected in blood plasma, follicular fluid, or HLA-G protein in the patients embryos. Unfortunately, laboratory and clinical practice did not allow for traceability of individual oocytes from follicle to embryo. Rizzo et al. (25) identified granulosa cells as the producer of sHLA-G5 and polymorphonuclear cells as the source of sHLA-G1 in follicular fluid. Our results also show that HLA-G protein is present in a subpopulation of granulosa cells but using MEM-G/9, which is specific for HLA-G1, shed sHLA-G1, and sHLA-G5, the isoform could not be identified. The sHLA-G detected in follicular fluid was of a similar concentration and range to that detected in embryo supernatants, which may be of physiological relevance. It would be interesting to investigate fluid from the fallopian tubes after ovulation; the detection of sHLA-G in such samples would indicate further importance of this molecule in preimplantation development.

To gain further insight into the role of HLA-G in early development, we used confocal microscopy to identify HLA-G protein in preimplantation embryos. Previously, Jurisicova et al. (23) reported MHC class I labeling in 6 of 13 oocytes and in 2 of 5 blastocysts incubated with the monoclonal class I Ab W6/32. Using the murine mAb 1B8, raised against the α polypeptide chain of HLA-G, this group reported HLA-G staining in 2, 4, and 16 cell embryos and 7 of 9 blastocysts. HLA-G expression was localized in the trophectoderm and never observed in the inner cell mass. However, Desoye et al. (38) and Roberts et al. (39) failed to detect MHC class I protein using W6/32 in 3 embryos and 3 blastocysts, respectively. Yao et al. (24) used the HLA-G-specific Ab MEM-G/9, the same mAb used in this study; labeling was found in 15 of 20 embryos at various cleavage stages and in all 8 morulae and blastocyst examined. However, mRNA for sHLA-G5 was not observed by these authors in cleavage-stage embryos and in only 20% of blastocysts. mRNA for HLA-G1, which is also detected by MEM-G/9, was only observed in 80% of blastocysts and 20% of cleavage stage embryos. Thus, there appears to be a mismatch.

The Journal of Immunology
between observed expression of HLA-G mRNA and HLA-G protein during preimplantation development. Although cleavage-stage embryos were not available for this study, HLA-G protein was observed in 58% of embryos that had arrested at varying cleavage stages before day 6 and in 77% of day 6 blastocysts. Thus, our findings support that of Yao et al. (24) in that we detected HLA-G in cleavage-stage embryos and in a higher percentage of blastocysts. Many of the embryos used in this study were considered to be of poor quality as they were not suitable for treatment or cryopreservation on day 6 of development. This may explain why we report HLA-G protein in a lower percentage of embryos to that of Yao et al. (24), where good quality embryos that had been previously cryopreserved were investigated. In this study, HLA-G protein was observed not only in good quality embryos thawed after cryopreservation, but also in embryos that arrested, poor quality blastocysts, and embryos diagnosed as chromosomally abnormal that were not considered suitable for IVF treatment.

We found HLA-G detection to be strong on the trophectoderm of blastocysts, trophectoderm hatching from the zona pellucida, and trophectoderm projections. In previous studies, it has been shown that trophectoderm projections are associated with hatching of the embryos from the zona pellucida, blastocyst movements, and initial contact with the maternal epithelia (40). Our findings reinforce the concept that HLA-G could be implicated in initial attachment and implantation of the embryo in the maternal uterus.

SPIF allows visualization of individual molecules at the cell surface within a spatial resolution of ~25 nm (31). This technique permits the determination of positional information of molecules and the single-diffraction limited spot intensity determines the number of molecules. The large three-dimensional nature of embryo blastomers prevents a clear plane of focus on the cell surface. However, SPIF analysis of the JEG-3 cell line was conducted to serve as a model of HLA-G spatial and temporal expression. Similarly to confocal microscopy analysis of HLA-G expression in embryos, results showed that HLA-G expression was not uniform in the population of cells analyzed; some showed high density, and evidence of HLA-G self-associations and clustering were also observed. Some cells show lower expression where particles were mostly monomers but dimer associations and higher order oligomeric clusters were also observed. This observation is in line with studies performed by Boyson et al. (5) who demonstrated that HLA-G forms disulfide-linked dimers on the cell surface of an HLA-G transfected cell line. In addition Clements et al. (41) reported on the crystal structure of HLA-G, which suggested a head-to-tail mode of dimerization. The interaction of HLA-G monomers and dimers and their oligomeric state warrants further investigation and may be of significance in the function of HLA-G in early embryonic development.

This study has shown that some preimplantation embryos express sHLA-G as part of their secretome. The expression of sHLA-G is associated with successful development of the embryo to the blastocyst stages in vitro and successful implantation of the embryo after transfer to the uterus. Thus, those embryos expressing sHLA-G appear to have a selective advantage against those not expressing sHLA-G.

One approach to understand the role of HLA-G in reproduction is to relate the physiological role of HLA-G in adults with its expression during embryogenesis and pregnancy. In adults, HLA-G is associated with Ag-presenting functions, although it binds a limited repertoire of peptides. HLA-G also serves as a ligand for specific NK and IgG receptors. HLA-G is also associated with allograft acceptance and masking of tumors or diseased cells from immune attack (4). The exact role for expression of HLA-G in early development remains unclear. sHLA-G expression in early cleavage embryos may act as a modulator of activity of target cells through interaction with killer inhibitory receptors in uterine NK cells, CD8+ T cells, macrophages, and dendritic cells, all subsets known to be present in the uterus (42). sHLA-G appears to be produced by the embryo in small quantities compared with levels in the mother’s plasma; however, the small quantity of sHLA-G in embryos may be sufficient to sensitize uterine cells. In contrast, the fact that sHLA-G is expressed in vitro, in the absence of target cells suggests that HLA-G may have a role per se on the embryos as a selective marker of key developmental stages. Indeed, HLA-G may be part of a stage-specific Ag synthesis favoring cleavage, differentiation and cell division, similar to the role of differentiation markers in lymphoid development (43).

It has also been considered that HLA-G is the human homolog of the preimplantation ped gene, performing a similar role in embryogenesis (44). In mice, the product designated Ped belongs to the MHC class Ib gene family; it encodes for the protein H-2 class I Qa-2 molecule, which when expressed results in faster embryo cleavage rates and confers reproductive advantage (45). The H-2Qa molecule is anchored to the cell membrane by a phospholipids tail and thus it could form associations with other MHC class I molecules for signaling (46). One possibility, which cannot be ruled out at present it that the observed HLA-G expression may be the result of factors encoded by genes in linkage disequilibrium with HLA-G, which confer a beneficial effect for implantation. These genes may also promote cell division or be involved in MHC-mediated transmembrane signaling pathways favoring cell division.

In this study, HLA-G protein was identified on the hatching trophectoderm, cells of the blastocysts that come directly into contact with uterine epithelium at implantation. It could be postulated that HLA-G may provide the blastocyst with local immunoprotection from uterine NK cells either directly or through cleavage from the trophectoderm cell membrane. Alternatively, HLA-G may have nonimmune functions such as promoting cell adhesion and control of trophoblast invasion. sHLA-G and HLA-G protein expression was identified in chromosomally abnormal embryos, embryos with arrested development, and was not found to be a prerequisite for implantation. Taken together, these findings indicate that although sHLA-G testing could be used as an additional tool to assist with embryo selection, it should be used in conjunction with already established methods of embryo selection and screening in IVF protocols. Further interlaboratory studies are required to fully understand the nature of sHLA-G expression in relation to other proteins, cytokines, and metabolic factors known to influence preimplantation development.

Acknowledgments
We thank the staff at the Assisted Reproductive Gynaecology Centre for all their help and assistance in the course of this study. We thank the staff at HCA Laboratories (London) for their advice and access to the Triturus ELISA analyzer. We also thank Dr. Richard Jurd (University of Essex, Essex, U.K.) for reading the manuscript and the anonymous reviewers for the helpful feedback on this article.

Disclosures
The authors have no financial conflict of interest.

References