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ORIGINAL RESEARCH

Galectin-1 influences trophoblast immune evasion and emerges as a predictive factor for the outcome of pregnancy

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ABSTRACT: Galectin-1 (gal-1) is expressed at the feto-maternal interface and plays a role in regulating the maternal immune response against placental alloantigens, contributing to pregnancy maintenance. Both decidua and placenta contribute to gal-1 expression and may be important for the maternal immune regulation. The expression of gal-1 within the placenta is considered relevant to cell-adhesion and invasion of trophoblasts, but the role of gal-1 in the immune evasion machinery exhibited by trophoblast cells remains to be elucidated. In this study, we analyzed gal-1 expression in preimplantation human embryos and first-trimester decidua-placenta specimens and serum gal-1 levels to investigate the physiological role played by this lectin during pregnancy. The effect on human leukocyte antigen G (HLA-G) expression in response to stimulation or silencing of gal-1 was also determined in the human invasive, proliferative extravillous cytotrophoblast 65 (HIPEC65) cell line. Compared with normal pregnant women, circulating gal-1 levels were significantly decreased in patients who subsequently suffered a miscarriage. Human embryos undergoing preimplantation development expressed gal-1 on the trophectoderm and inner cell mass. Furthermore, our *in vitro* experiments showed that exogenous gal-1 positively regulated the membrane-bound HLA-G isoforms (HLA-G1 and G2) in HIPEC65 cells, whereas endogenous gal-1 also induced expression of the soluble isoforms (HLA-G5 and -G6). Our results suggest that gal-1 plays a key role in pregnancy maternal immune regulation by modulating HLA-G expression on trophoblast cells. Circulating gal-1 levels could serve as a predictive factor for pregnancy success in early human gestation.

Key words: gal-1 / HLA-G / human embryo / spontaneous abortion

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Introduction

Galectin-1 (gal-1) is a small β-galactoside-binding protein that belongs to a 15-member protein family expressed by many different cell types. It exerts major roles in the immune system (Perillo *et al.*, 1995; Rubinstein *et al.*, 2004) and is involved in the immune-mediated fetal tolerance during pregnancy, promoting the generation of tolerogenic dendritic cells (DCs), inducing IL-10 expressing T regulatory cells and a Th2 cytokine shift by provoking apoptosis of susceptible Th1 cells (Blois *et al.*, 2007; Kopcow *et al.*, 2008). Gal-1 displays intracellular (i.e. protein–protein interactions) and extracellular (i.e. protein–oligosaccharide interactions) functions (Camby *et al.*, 2006) and constitutes one of the most abundantly expressed galectins in the female reproductive tract. Its expression is under the control of ovarian steroids and this regulation correlates with the timely coordination of embryo implantation in humans and mice (Choe *et al.*, 1997; von Wolff *et al.*, 2005). Moreover, gal-1 is initially synthesized in the trophectoderm of the expanded mouse blastocyst prior to implantation (Poirier *et al.*, 1992). Another important source of gal-1 is the placenta, particularly the cytotrophoblast (CTB) of the mid and distal columns during the first trimester of pregnancy (Vicovac *et al.*, 1998). Putative roles played by gal-1 during placentation include the

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organization of extracellular matrix and the regulation of trophoblast differentiation and cell motility (Vicovac et al., 1998; Jeschke et al., 2010; Kolundzic et al., 2011). However, the role of gal-1 in trophoblast immune evasion mechanism has not been extensively studied yet.

Pregnancy, a highly regulated physiological process, sustains mammalian species survival in this universe. It is an immunological paradox where the fetus expressing paternal antigens is not rejected by the maternal immune system during a successful gestation (Medawar, 1953). Disruption of fetal tolerance mechanisms, which involve pathways regulating innate or adaptive maternal immune responses, may contribute to pregnancy complications (Hunt et al., 2005; Terness et al., 2007). Understanding the mechanisms that direct pregnancy maintenance is an important medical concern in today's society. Reproductive disorders, defined as the biological inability to conceive or to carry a pregnancy to full-term, affect between 3.5 and 26.5% couples with an estimated median of 9% (Boivin et al., 2007; Ombelet et al., 2008). It is also known that ~1% of all women trying to conceive experience recurrent miscarriage (i.e. three previous abortions), the estimate rising to 5% when the condition is defined as two previous miscarriages (Rai and Regan, 2006). A large proportion of these disorders, particularly those previously identified as idiopathic cases, may result from the disruption of the many pathways involved in the establishment of feto-maternal tolerance.

Here, we describe the role of gal-1 in promoting immune evasion mechanism on trophoblast cells. Our data provide evidence that gal-1 regulates placental human leukocyte antigen G (HLA-G) gene expression, highlighting its contribution to the immune evasion machinery by which trophoblast cells influence the maternal immune system during early human pregnancy. To the best of our knowledge, this is the first study demonstrating gal-1 expression in human preimplantation and also on cleavage-stage embryos. Furthermore, we show that circulating gal-1 levels could serve to predict adverse pregnancy outcomes, emerging as a valuable tool for early diagnosis of the risk of miscarriage.

Materials and Methods

Study patients

For analyses of gal-1 level in normal pregnancy, blood samples were collected from healthy pregnant women in the first, second and the third trimesters of pregnancy at their planned visits to the Department of Obstetrics and Gynecology, Umeå University Hospital, and to the Polyclinic Maternity Care Units, for control of pregnancy progression. All the patients involved in this work were properly informed about the purpose of our research and gave their written consent before the sampling. The study was approved by the ethics committee of the Umeå University Hospital. The characteristics of the recruited participants are summarized in Table I.

For measurement of circulating gal-1 levels during the first trimester of normal and pathological pregnancies, samples from a prospective cohort study conducted by the Departments of Internal Medicine, Psychosomatics and Obstetrics at the Charité, University Medicine Berlin, Germany (Arck et al., 2008) were used. Written informed consent was obtained from all the women, and the study was approved by the ethics committee of the Charité. The recruited participants' characteristics are summarized in Table II.

At recruitment, blood samples were taken by venous puncture from all women and delivered to our laboratories within 1–2 h by courier. Serum was harvested from all blood samples upon centrifugation (2000 rpm/20 min) and stored at −80°C until further use.

Collection of human embryos, embryo supernatants and gal-1 labeling

Ethical approval for the use human embryos in this research was obtained from the Human Fertilization and Embryology Authority, UK (HFEA; Project License R0165). Embryos were donated with informed consent by patients treated at the Assisted Reproduction and Gynecology Centre (London, UK). 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. 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 of development after embryo transfer and freezing of any good-quality blastocysts. The cryopreserved embryos donated to this research were thawed using the Vitrolife Thaw kit and allowed to equilibrate in culture for a minimum of 3 h before immunofluorescence studies. The embryos were fixed with 1% paraformaldehyde, then placed in acid Tyrodés solution (Sigma-Aldrich, Germany) to thin the zona pellucida and permeabilized with 0.1% Trixton X-100. The embryos were then washed and blocked with phosphate buffered solution (PBS) supplemented with 2% bovine serum albumin (BSA). All incubation steps were carried out by placing embryos in microdroplets under oil at room temperature (RT). Embryos were incubated with rabbit anti-human gal-1 IgG (Serotec, MorphosysAbD GmbH, Germany) at a dilution of 20 µg/ml for 1 h, washed through sequential droplets of 2% PBS-BSA and incubated with fluorescein isothiocyanate conjugated secondary

Table I Characteristics of the recruited participants included in the first cohort.

Parameters	Non-pregnant (n = 20)	First trimester (n = 30)	Second trimester (n = 20)	Third trimester (n = 20)
Age	28.95 ± 5.97	28.6 ± 3.04	30 ± 4.59	30.15 ± 4.45
GW	—	9–12	18–20	37–41
IUD	5	—	—	—
OC	7	—	—	—
Other methods	8	—	—	—

Exclusion criteria: pregnant women with underlying conditions such as obesity, diabetes mellitus type I or type II, cardiovascular diseases including high blood pressure, autoimmune diseases, hormonal disorders, previous history of recurrent abortions or infertility, chronic diseases, any permanent medication or a smoking habit, pathological pregnancy progression such as an intrauterine growth retardation, pre-eclampsia, intrauterine infections, premature labor, placenta praevia, bleedings and other placental or fetal abnormalities.
GW, gestational age in weeks; IUD, intrauterine device; OC, oral contraception.

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Table II Characteristics of the recruited participants included in the second cohort.

Parameters	Normally progressing pregnancy (*n* = 80)	Subsequent spontaneous abortion (*n* = 55)
Age	29.7 ± 2.80	30.5 ± 3.50
GW	4–12	4–12

Inclusion criteria: week of gestation 4–12, no fertility treatment, no hepatitis B/C or HIV infection; no signs of an imminent miscarriage such as vaginal bleeding, low β-hCG, missing embryonic/fetal heart rate during ultrasound screening. Exclusion criteria for the subsequent spontaneous abortions group: molar pregnancies, abnormal fetal karyotype or infection induced abortion.
GW, gestational age in weeks.

antibody (Sigma-Aldrich). The embryos were then again washed through sequential droplets of PBS and mounted in DABCO (Sigma-Aldrich) and stored at 4°C before analysis. Visualization of immunofluorescence labeling was conducted using a Bio-Rad Radiance 2000 scanning laser confocal microscope.

Purification of CTB and extravillous cytotrophoblastic cells

Placental tissue was obtained from patients undergoing a legal abortion during the first trimester (8–12 weeks of gestation) or at delivery. Informed written consent was obtained from all the patients before their inclusion in the study, for which approval was obtained from the local ethics committee of Geneva University Hospital. Trophoblast cells were isolated as previously described (Bischof *et al.*, 1995). In brief, fresh tissue specimens were isolated and washed several times in sterile Hanks balanced salt solution. Tissue was then enzymatically digested five times for 20 min at 37°C (0.25% trypsin, 0.25 mg/ml Dnase I; Roche, Diagnostics GmbH, USA). After incubation, the trypsin cocktail was neutralized with fetal bovine serum (FBS), and the cells resuspended in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Switzerland). This cell suspension was filtered through a 50-μm mesh laid onto a Percoll gradient (70–5% Percoll diluted with HBSS) and centrifuged for 25 min at 1200 g. The 30–45% percoll layer containing trophoblast cells was collected, the cells washed and resuspended in DMEM (Invitrogen). The cells were then immunopurified with immobilized anti-CD45 antibodies. Ninety five percent of cells were positive for cytokeratin 7 and negative for vimentin.

To obtain extravillous cytotrophoblast (EVCT) cells, the cells were seeded on Petri dishes for 15 min. Supernatants containing EVCT were centrifuged and the cells were resuspended in culture medium and seeded in 6-well plates (4×10^6 cells/well) and in 96-well plates (1×10^5 cells/well). 95% of 24 h cultured cells were positive for cytokeratin 7 and HLA-G and negative for vimentin.

Gal-1 treatment *in vitro*

The human invasive, proliferative extravillous cytotrophoblast (HIPEC) 65 cell line (a generous gift from Prof. D. Evain-Brion, Paris; Pavan *et al.*, 2003) was grown in DMEM high glucose containing 10% FBS (Oxoid AG, Switzerland) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; Invitrogen) at 37°C in a humidified, 5% CO_2 atmosphere. 4×10^5 HIPEC65 cells were seeded in a six-well plate. The following day, cells were serum-starved for 24 h. Cells were then treated with different concentrations (0, 100, 1000 ng/ml) of human gal-1 recombinant (PeproTech Inc., USA) serum-free medium. After a 48-h incubation, RNA was extracted using RNeasy mini kit (Qiagen, Switzerland).

Transfection of HIPEC65 cells with oligonucleotides

Gal-1 expression was reduced by transfection with oligodeoxynucleotides (ODN) directed against gal-1 (Thijssen *et al.*, 2008). For transfection experiments, 3×10^5 HIPEC65 cells were seeded in wells of a six-well plate 24 h prior to transfection without antibiotic treatment. The cells were then treated with 200 pmol of control or gal-1 ODN using 3 μl of oligofectamine in DMEM without antibiotic or FBS. Four hours after incubation, FBS was added to obtain a final concentration of 10% (v:v) FBS. Eight hours after incubation, RNA was extracted using the RNeasy mini kit.

Proliferation assay

Tetrazolium salts (Wst-1) cell proliferation assay was performed as previously described (Ribaux *et al.*, 2012). HIPEC65 cells were seeded at a density of 3×10^4 cells in 96-well plates. Wst-1 cell proliferation assay was used according to the manufacturer's protocol. Absorbance was recorded at 450 nm after 60 min using a 96-well plate reader.

Determination of circulating gal-1 levels

Human gal-1 levels were measured with a specific sandwich enzyme-linked-immunosorbent assay (ELISA) protocol, as previously described (Kopcow *et al.*, 2008). Briefly, immunolon 2 ELISA plates (Dynatech Laboratories, USA) were covered with polyclonal anti human gal-1 antibody (2 μg/ml; R&D Systems, USA) and washed with washing buffer (0.5% Tween-20 in PBS). Plates were blocked with 1% BSA in PBS. Individual wells were incubated with serial dilutions of gal-1 or serum samples (diluted 1/20) or human embryo supernatants (diluted 1/5) for 1 h at RT. Wells were washed and incubated with biotinylated polyclonal anti-human gal-1 antibody (0.25 μg/ml in PBS 0.1% BSA; R&D Systems). Plates were washed six times and incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Calbiochem, USA). After eight additional washes, a colorimetric reaction was developed with the 3,3,5,5′-tetramethyl benzidine (TMB) substrate (Pierce Biotechnology, USA). The reaction was stopped by adding one volume of 4 N H_2SO_4. Absorbance at 450 nm was recorded.

Assessment of serum human chorionic gonadotrophin concentrations

Serum samples were tested in competitive ELISA using kits obtained from DRG International (DRG Instruments GmbH, Germany) to quantify human chorionic gonadotrophin (β-hCG) (EIA-4718) according to the manufacturer's recommendations.

HLA-G isoform PCR

To gain insight into which HLA-G isoform mRNAs were present upon gal-1-stimulation or gal-1-ODN treatment on HIPEC-65 cells, HLA-G specific PCR amplifications were performed as previously described by Yao *et al.* (2005) allowing the detection of all alternatively spliced HLA-G mRNAs. The discrimination of the HLA-G products was conducted by ethidium bromide labeling after electrophoresis. All primers were purchased from Tib-Molbiol (Germany).

Cell-ELISA

HIPEC cells were seeded at 3×10^4 cells/well in a 96-well plate. They were serum-starved for 24 h before treatment with various concentrations of recombinant gal-1 for 48 h. The cells were washed, fixed (3%

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paraformaldehyde in PBS, 5 min) and pre-incubated with 3% BSA-PBS for 30 min before incubation with HLA-G antibody (MEM-G/9, Exbio, Germany; diluted 1/100) for 1 h. To remove the unbound antibody, the cells were washed four times in PBS-BSA 3%, and then incubated for 45 min at 4°C with HRP-conjugated goat anti-mouse IgG antibody. After incubation, the cells were washed as described earlier and the TMB substrate (R&D systems) was added. The reaction was stopped by adding 1 M H_2SO_4. Absorbance was read at 450 nm on a microplate reader (Expert plus, Biochrom, Germany).

Immunohistochemistry

Sections of paraffin-embedded tissue ($n = 16$ normal pregnancy and $n = 15$ spontaneous abortion) were cut at 4 μm, deparaffinised, rehydratated and washed in Tris-buffered saline (TBS), followed by blocking of endogenous peroxidase through incubation with 3% H_2O_2 in methanol for 30 min at RT. After incubation with 2% normal serum for 20 min, rabbit anti-human gal-1 IgG (1:500, Serotec, MorphosysAbD GmbH) was incubated overnight (ON) at 4°C. The slides were then washed and incubated with goat anti-rabbit HPR-conjugated secondary Ab (1:200, Jackson ImmunoResearch, Germany) for 1 h at RT and 3,3′-diaminobenzidine chromogen (DAKO, Germany) were used for detection. After washing, nuclei were counterstained with 0.1% Mayer's hematoxylin followed by a standard dehydration procedure and mounting in Vitro-Clud medium (R. Langenbrinck, Germany). For the negative control, the primary antibody was replaced by irrelevant rabbit IgG at an equal concentration.

Immunofluorescence

HIPEC65 cytospins were incubated with peroxidase- avidin- and biotin-blocking solution (Vector, USA), followed by a protein-blocking agent (Immunotech, Germany). The biotinylated mouse IgG1 anti-HLA-G mAb (MEM-G/9, Exbio, AXXORA Deutschland GmbH, Germany) was diluted 1:500 in TBS containing 1% FBS and 0.3% Triton-X100 and incubated ON at 4°C. Control reaction for non-specific binding was carried out by replacing the anti-HLA-G Ab with a mouse IgG1 mAb (BD Biosciences, Germany) at the same concentration as that of the primary antibody. After washing, TRITC-Streptavidin was diluted 1/100 in TBS/1% FBS/0.3% Triton-X100 and incubated for 2 h at 4°C. The nuclei in all the sections were counterstained by incubating for 5 min in 4′,6′-diamidino-2-phenylindole (DAPI) solution, followed by washing and mounting in Shandon Immu-Mount™ (Thermo Scientific, Germany). The sections were analyzed using a confocal laser scanning microscope (cLSM 510, Carl Zeiss, Germany).

Human receptor tyrosine kinases phosphorylation antibody array

Changes in protein phosphorylation upon gal-1- or random- ODN treatment on HIPEC65 cell line were monitored using the human receptor tyrosine kinases (RTKs) phosphorylation antibody array (RayBiotech, Inc., Germany) following manufactures instructions. Briefly, proteins were isolated using lysis buffer containing protease and phosphatase inhibitors cocktail. After the blocking step, 300 μg/ml of proteins were added into each antibody array membrane containing 71 different human RTKs and incubated ON at 4°C. After extensive washing, the membranes were incubated with biotin-conjugated anti-phosphotyrosine antibodies for 2 h at RT with gentle shaking. HRP-conjugated streptavidin was added for 2 h at RT. Phosphorylated RTKs were visualized using enhanced chemiluminescence and exposed to Kodak X-Omat AR film (Sigma-Aldrich).

Immunoblot analysis

Equal amounts of protein from HIPEC65 cell line or human decidua/placenta samples (20 μg) were resolved in polyacrylamide gels under reducing conditions. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences), and equal loading was confirmed by Ponceau S staining (Sigma-Aldrich). Blocked PVDF membranes were incubated with a rabbit anti-human gal-1 IgG (Serotec, MorphosysAbD GmbH) or rabbit anti-β actin (Sigma-Aldrich). Blots were then incubated with peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) and developed using the enhanced chemiluminescence (ECL) detection reagent.

Statistical analysis

All the data are shown as mean and standard error, except where indicated. Results were analyzed with GraphPad Prism 5.0 (GraphPad Software Inc.). All comparisons, except the one in Figs 3A and D and 4 for which we used non-parametric Mann–Whitney *U* test, were performed using analysis of variance (ANOVA) and Tukey's test. Values were considered significantly different when $P < 0.05$.

Results

Gal-1 is up-regulated during normal pregnancy and expressed on human preimplantation embryos

To determine the role played by gal-1 during human pregnancy, we first analyzed circulating gal-1 levels by ELISA in healthy pregnant women during the course of gestation. As depicted in Fig. 1A, we observed a significant increase in gal-1 levels during the first trimester of normal pregnancy compared with the healthy non-pregnant controls ($P < 0.05$). During the second trimester, gal-1 concentrations peaked and remained at similar levels until the end of the third trimester. These data demonstrate that the amount of circulating gal-1 is up-regulated as pregnancy progresses.

Expression of gal-1 at the feto-maternal interface has been established previously (Hirabayashi and Kasai, 1984; Bevan *et al.*, 1994). However, whether gal-1 is expressed in human embryos remains to be elucidated. Therefore, we next used confocal microscopy analysis in order to investigate gal-1 protein expression on human preimplantation embryos. Gal-1 labeling was observed in all embryos analyzed at different stages of development and viability. This included three embryos on Day 3 of development graded as average quality (six- to eight-cell stage)(Fig. 1B left panel), five embryos with arrested development or poor morphology on Day 5, including two embryos that had been diagnosed as chromosomally abnormal by preimplantation genetic diagnosis, and two good-quality blastocysts that had been previously cryopreserved. Strong cytoplasmatic gal-1 labeling was observed on the trophectoderm, the cell layer from which the trophoblast differentiates (Fig. 1B central left panel) and also the inner cell mass that gives rise to the definitive structures of the fetus (Fig. 1B central right panel). Embryos labeled with non-specific anti-rabbit IgG in place of the primary antibody showed only a signal similar to autofluorescence controls (Fig. 1B right panel). These data indicate that under these *in vitro* culture conditions, gal-1 is expressed heterogeneously throughout the embryo at both the cleavage and the blastocyst stages of development. We also determined gal-1 concentrations in the embryo culture supernatants using the ELISA technique.

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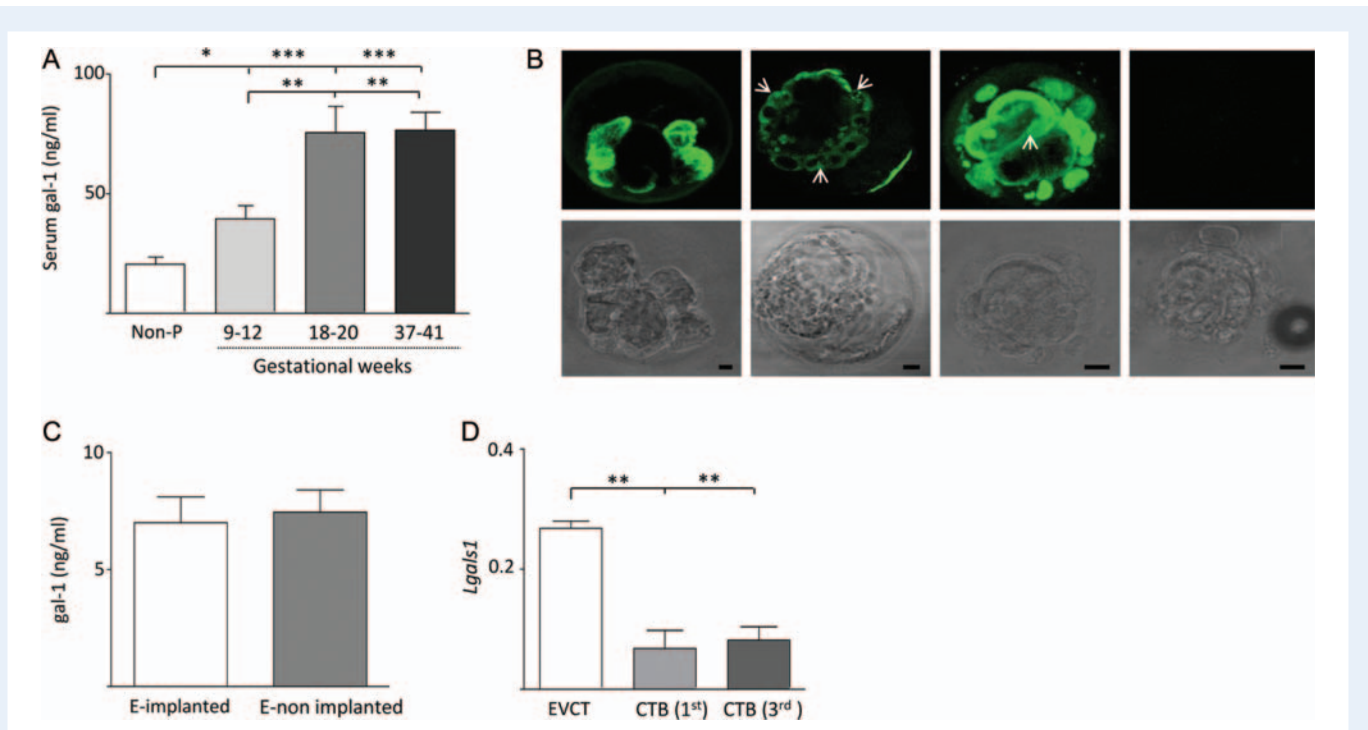

Figure 1 Gal-1 expression during human pregnancy and embryo development. (**A**) Systemic gal-1 levels as measured by ELISA. Gal-1 serum levels from patients with normal pregnancy were increased compared with control non-pregnant (Non-P) patients. Results are presented as mean ± SEM (*, ** and *** denote $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, as analyzed one-way ANOVA with Tukey's post test). (**B**) Confocal microscopy analysis of gal-1 expression in human preimplantation embryos. Top panels; fluorescence projections of Z-stacks and bottom panels; transmission for: (left) cleavage-stage embryo on Day 3 of development, scale bar 10 µm; (central left and right) blastocyst stage embryos on Day 5 of development, arrows denote gal-1 staining on trophectoderm cells (central left) and inner cell mass (central right), scale bar 20 µm. (**C**) Gal-1 levels in human embryo supernatants as measured by ELISA. E = embryo(**D**) Placental gal-1 expression in fresh isolated EVCT and CTBs as analyzed by quantitative PCR. Pronounced gal-1 expression is observed in EVCT compared with CTB (first; 1st and third; 3rd trimester). Results represent the mean and SEM of three independent experiments as analyzed by one-way ANOVA with Tukey's post test, ***$P < 0.001$.

As shown in Fig. 1C, similar levels of gal-1 were found between good-quality embryos that subsequent successfully implanted and those that failed to implant when transferred to the patient.

Although the presence of gal-1 has been documented for different trophoblast types, our next aim was to quantify gal-1 expression on trophoblast cells isolated from fresh first and third trimester placentas, analyzing the EVCT compared with CTBs. Of note, villous CTB stem cells differentiate along two possible pathways: either they fuse to form the overlying syncytiotrophoblast, or in anchoring villi, invasive CTBs break through the syncytiotrophoblast to form extravillous trophoblast columns, those villi that physically attach the placenta to the uterus (Aplin, 1991). As shown in Fig. 1D, EVCT isolated from the first-trimester chorionic villi expressed prominent gal-1 mRNA levels compared with CTB from the first and the third trimesters. No differences were found when comparing the gal-1 mRNA levels between CTB from the first and the third trimesters. These results confirm that gal-1 is highly expressed in EVCT, suggesting a role for this lectin in the physiology of these cells.

Exogenous and endogenous gal-1 regulates the tolerogenic molecule HLA-G on EVCT

Gal-1 exhibits immunosuppressive properties at the maternal interface in early human pregnancy, in particular the ability to induce apoptosis on T cells (Kopcow *et al.*, 2008). However, the relevance of gal-1 to trophoblast immune evasion mechanisms remains unrevealed. In order to comprehend its importance, we focused our attention on HLA-G expression by trophoblasts, which is normally associated with placentation and protection of the allogeneic fetus from the maternal immune system (Moreau *et al.*, 1998).

First, we postulated that gal-1 may function as a paracrine signal affecting EVCT behavior. Therefore, a set of experiments was conducted with a well-characterized extravillous human trophoblastic cell line (HIPEC65) (King *et al.*, 2000; Pavan *et al.*, 2003) in order to determine whether the increased availability of gal-1 positively correlated with HLA-G expression. Treatment of HIPEC65 cells with human recombinant gal-1 (hrgal-1) induced a significant increase in HLA-G expression, as analyzed by the cell ELISA method (Fig. 2A). In addition, we analyzed the pattern of HLA-G isoform expression using PCR. Interestingly, only HLA-G1 and -G2 isoforms were up-regulated upon exogenous gal-1 stimulation (Fig. 2B). Furthermore, treatment of HIPEC65 cells with recombinant gal-1 at different concentrations did not result in promotion of cell proliferation compared with the control HIPEC65 cells (Fig. 2C).

Knowing that EVCT express gal-1, the second hypothesis dealt with the autocrine role of gal-1 regarding the modulation of HLA-G expression. Thus, we aimed to knockdown endogenous gal-1 expression by using a published gal-1 specific antisense ODN (Thijssen *et al.*, 2006)

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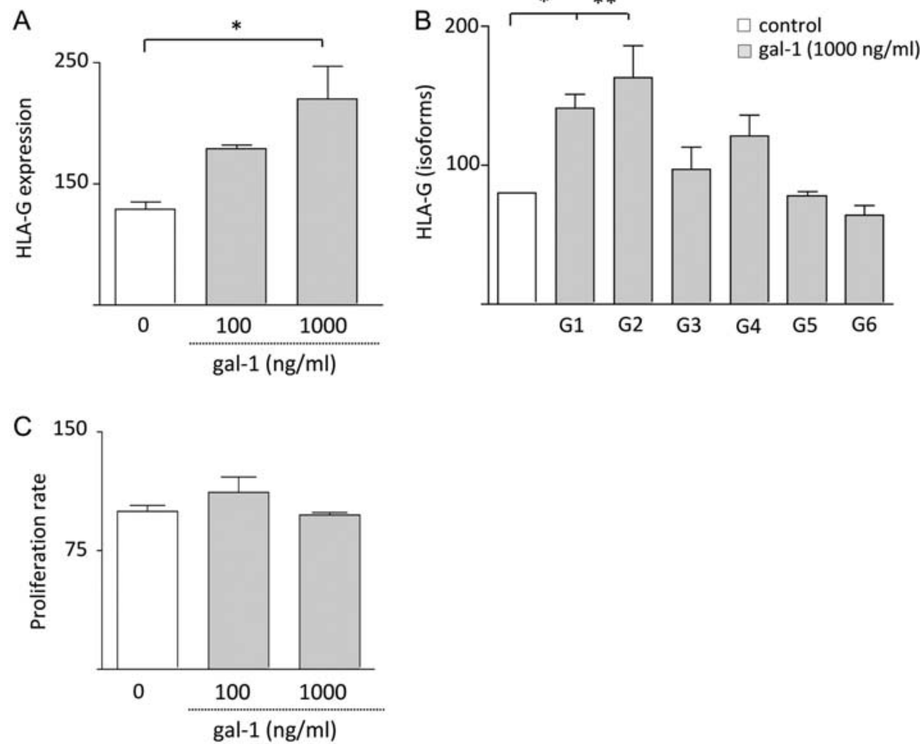

Figure 2 Exogenous gal-1 stimulation regulates HLA-G expression on the HIPEC65 cell line. (**A**) HIPEC65 cells were incubated with increasing concentrations of hrgal-1 for 48 h as described in Materials and Methods. HLA-G expression was quantified by cell-ELISA in HIPEC65 cells treated with hrgal-1 for 48 h. Data show the HLA-G expression on HIPEC65 cell relative to non-treated controls. (**B**) Analysis of HLA-G isoforms upon gal-1 stimulation on HIPEC65 cell line analyzed by PCR. (**C**) Proliferation rate is shown for HIPEC65 cells treated for 48 h with increasing concentration of hrgal-1. In all figures, results are represented as mean ± SD of three independent experiments. * and ** denote $P < 0.05$ and $P < 0.01$, respectively, as analyzed by the Tukey's test.

and investigate HLA-G expression in HIPEC65 cells. As shown in Fig. 3A, reduction of gal-1 mRNA and protein expression by HIPEC65 could be observed upon gal-1 ODN treatment, whereas a random ODN had no effect. Next, we determined whether HLA-G expression is regulated by the availability of endogenous gal-1 in HIPEC65 cells. Therefore, cytospins from gal-1 ODN treated HIPEC65 cells were stained with the MEM-G/9 mAb that specifically recognizes the HLA-G isotype HLA-G1 and its soluble counterpart -G5. Confocal microscopy revealed that knockdown of endogenous gal-1 down-regulated HLA-G expression by HIPEC65 cells (Fig. 3B), suggesting that gal-1 is involved in the modulation of trophoblast maternal immune evasion mechanisms. As shown in Fig. 3C, HLA-G1, -G2, -G5 and -G6 isoforms were down-regulated in response to gal-1 knockdown treatment in HIPEC65 cells. In contrast, HLA-G3 and -G4 did not show an altered expression pattern in comparison with HIPEC65 cells treated with the random ODN (Fig. 3C). Although gal-1 ODN treatment did not significantly modify the cell growth kinetics of HIPEC65 cells compared with a random ODN (Fig. 3D), we observed a reduction of src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites (SRMS) and TXK tyrosine kinase (TXK), two non-receptor tyrosine kinases [protein tyrosine kinase (PTK)] that belong to the proto-oncogene tyrosine protein kinase sarc (Src) and Tec protein tyrosine kinase (Tec) family, respectively (Fig. 3E), suggesting that intracellular gal-1 is able to modulate tyrosine phosphorylation in trophoblast cells, while their cell growth kinetics remains unaltered (Hunter, 1998).

Taken together, these data suggest that both extracellular and cytoplasmic gal-1 participate during the reprogramming of local maternal immune modulation by regulating HLA-G expression on EVCT.

Gal-1 and adverse pregnancy outcomes

There is growing evidence suggesting that adverse pregnancy outcome (e.g. spontaneous abortion) could be associated with abnormal immunologic interactions at the feto-maternal interface. Indeed, local gal-1 expression decreased during early pregnancy loss in mice and humans, a finding that is consistent with a role for this lectin in the establishment of maternal tolerance (Liu et al., 2006; Blois et al., 2007). This line of evidence, together with the lack of effective tools for the early diagnosis of pregnancy disorders (e.g. spontaneous abortion) encouraged us to investigate the possible link between circulating gal-1 levels and pregnancy outcomes. As depicted in Fig. 4A, serum gal-1 levels were significantly reduced in the first-trimester pregnant women who subsequently suffered from spontaneous abortion compared with the women who had a successful pregnancy. However, the β-hCG values of the pregnant women who subsequently suffered

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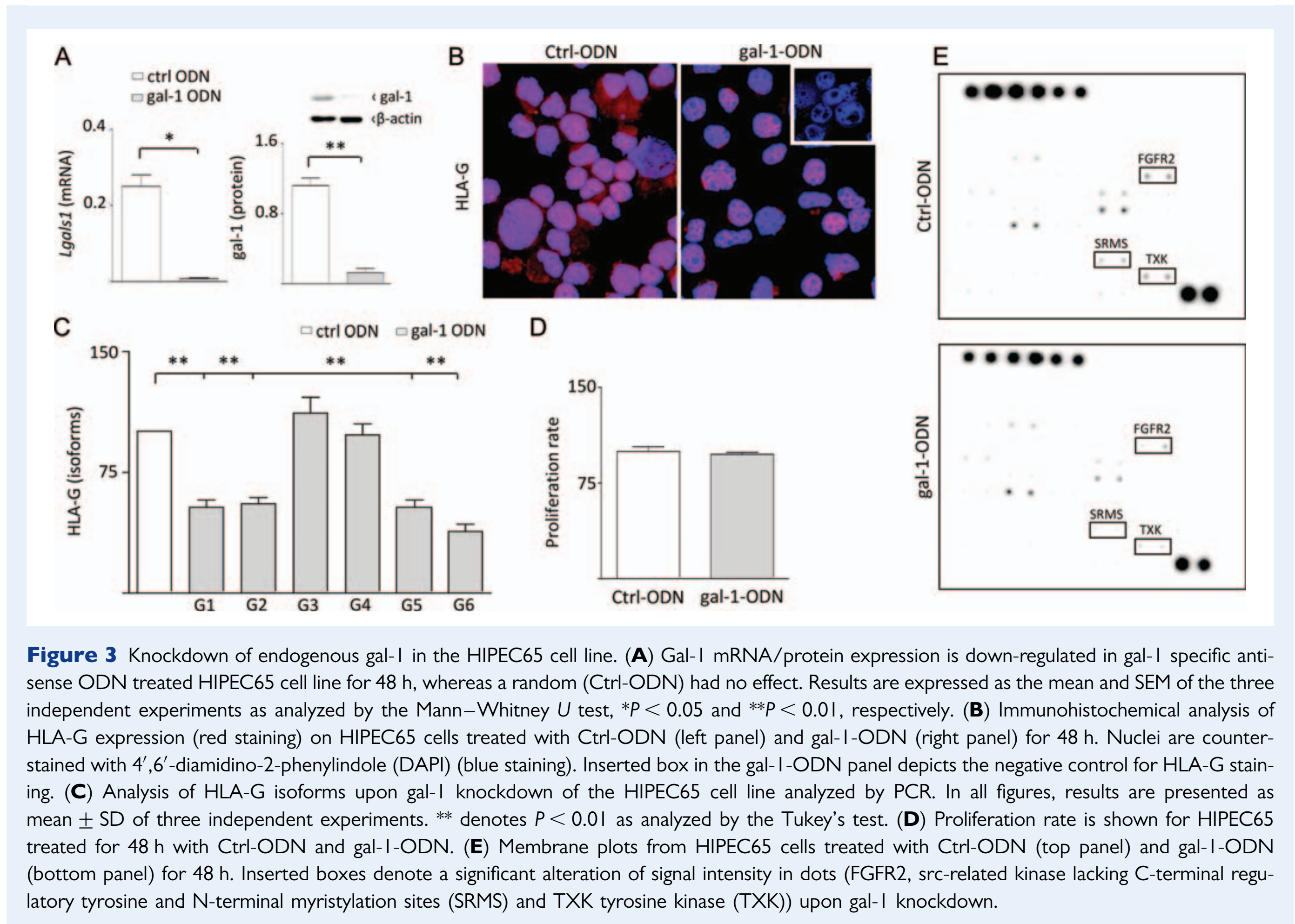

Figure 3 Knockdown of endogenous gal-1 in the HIPEC65 cell line. (**A**) Gal-1 mRNA/protein expression is down-regulated in gal-1 specific antisense ODN treated HIPEC65 cell line for 48 h, whereas a random (Ctrl-ODN) had no effect. Results are expressed as the mean and SEM of the three independent experiments as analyzed by the Mann–Whitney *U* test, $*P < 0.05$ and $**P < 0.01$, respectively. (**B**) Immunohistochemical analysis of HLA-G expression (red staining) on HIPEC65 cells treated with Ctrl-ODN (left panel) and gal-1-ODN (right panel) for 48 h. Nuclei are counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (blue staining). Inserted box in the gal-1-ODN panel depicts the negative control for HLA-G staining. (**C**) Analysis of HLA-G isoforms upon gal-1 knockdown of the HIPEC65 cell line analyzed by PCR. In all figures, results are presented as mean ± SD of three independent experiments. ** denotes $P < 0.01$ as analyzed by the Tukey's test. (**D**) Proliferation rate is shown for HIPEC65 treated for 48 h with Ctrl-ODN and gal-1-ODN. (**E**) Membrane plots from HIPEC65 cells treated with Ctrl-ODN (top panel) and gal-1-ODN (bottom panel) for 48 h. Inserted boxes denote a significant alteration of signal intensity in dots (FGFR2, src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristylation sites (SRMS) and TXK tyrosine kinase (TXK)) upon gal-1 knockdown.

from spontaneous abortion were within the normal range (Fig. 4B). Our next aim was to extend the investigation of gal-1 expression to decidual-placental tissues from spontaneous abortion and normal pregnancy during the first trimester. Using Western blot, we observed that expression of gal-1 is decreased in spontaneous abortion samples compared with normal pregnancy (Fig. 4C). We next analyzed gal-1 distribution in the normal pregnancy decidual-placental specimens. The immunostaining of gal-1 was observed mainly in decidua and villous CTB. Analysis of the staining intensity of localized gal-1 protein in the decidua and CTB of first-trimester normal pregnancy and spontaneous abortion showed significant differences between both groups ($P > 0.05$) (Fig. 4D). These data suggest that circulating gal-1 levels could predict the risk of spontaneous abortion early in human pregnancy.

Discussion

Owing to its functional polyvalence with a wide range of biological activities, gal-1 is proposed as one of the master regulators of immune tolerance (Almkvist and Karlsson, 2004). Human pregnancy implies maternal immune system regulation, which prevents rejection of the fetus despite expressing paternal antigens. The expression of gal-1 significantly increases in the late secretory phase endometrium and shows a specific pattern of expression within the decidua and placenta (Maquoi *et al.*, 1997; Vicovac *et al.*, 1998; von Wolff *et al.*, 2005). In this report, we clearly show that circulating gal-1 levels significantly increase during the first trimester of normal pregnancy, peak during the second trimester and remain similar until the end of the third trimester. The increase in peripheral gal-1 level coincides with the period of establishment of the placenta during the first trimester, suggesting that trophoblast cells could be one of the sources of the circulating lectin. Indeed, we describe for the first time to our knowledge, the presence of gal-1 on the trophoblast cells of human blastocysts prior to implantation. This could imply that gal-1 is a key protein for trophoblast differentiation in the first trimester of human pregnancy, a possibility that awaits further investigation. In this context, gal-1 is expressed in the CTB of middle and distal cell columns differentiating toward fully invasive trophoblast (Vicovac *et al.*, 1998) and has been recently reported as a component of the human trophoblast invasion machinery. Thus, while gal-1 silencing has a negative effect on trophoblast cell invasion capacity, exogenous gal-1 stimulation has been shown to enhance this process (Kolundzic *et al.*, 2011). This observation is in agreement with the identification of gal-1 expression as a signature of cell invasiveness when comparing highly and poorly invasive mammary carcinoma cell lines (Harvey *et al.*, 2001). In this study, we also confirm that CTBs expressed gal-1 during the first trimester of normal pregnancy, while syncytiotrophoblasts did not show immunoreactivity against gal-1. This result suggests that CTB differentiation into syncytiotrophoblast

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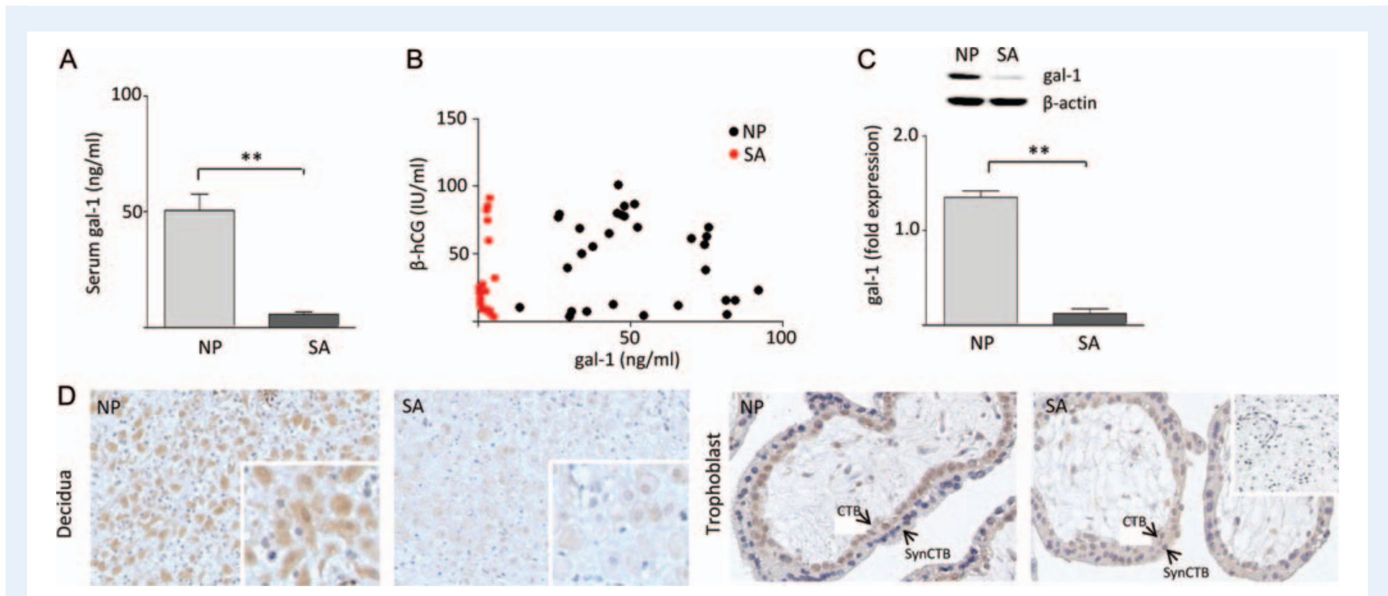

Figure 4 Systemic and local gal-1 levels during pathological human pregnancy. (**A**) Gal-1 serum levels from control patients were increased compared with pregnant women who subsequently had a miscarriage. (**B**) β-hCG and gal-1 levels measured by ELISA in serum between 4 and 12 gestational weeks. While pregnant women who subsequently had a miscarriage shown β-hCG levels within the normal range, peripheral levels of gal-1 were significantly decreased compared with control patients. (**C**) Gal-1 expression in crysections from normal and spontaneous abortion patients during the first trimester as analyzed by Western blot. (**D**) Immunohistochemical study of gal-1 in paraffin-embedded decidua-placental secions. Prominent gal-1 staining (dark brown) was detected on the decidual stroma and only in CTB cells during early normal pregnancy compared with pregnant women with miscarriage (right panel, magnification ×10). (Bottom inserted boxes; magnification ×20) Arrows denote CTB and SynCTB localization. Inserted box in trophoblast SA panel shows negative control for gal-1 staining. NP, normal pregnancy; SA, spontaneous. In all figures, results are presented as mean ± SD of three independent experiments. ** denotes $P < 0.01$ as analyzed by the Mann–Whitney U test.

leads to the loss of gal-1 expression. In this regard, it has been reported that gal-1 stimulates the fusion of human villous trophoblast and Bewo cells, inducing the formation of syncytium *in vitro* (Fischer *et al.*, 2010). However, gal-1 expression is retained and even up-regulated on EVCT, which are also derived from CTB cells. This could be explained by the evidence that two distinct populations of CTB are committed to the differentiation of syncytiotrophoblast and EVCT (James *et al.*, 2005). It remains to be investigated to what extent gal-1 drives trophoblast differentiation during early gestation and contributes to placentation, which is essential for the development of the embryo and the success of pregnancy.

Several mechanisms have been proposed as responsible for maternal immune tolerance against placental alloantigens. In this context, one of the most relevant strategies for immune evasion relies on the unique pattern of expression of histocompatibility antigens displayed by trophoblast cells, of which human EVCT express only the non-classical major histocompatibility complex type one molecule, HLA-G, generating the necessary maternal tolerance by inhibiting cytotoxic T lymphocytes responses and natural killer (NK) cell activity (Kapasi *et al.*, 2000; Contini *et al.*, 2003; Hunt *et al.*, 2005). Cytokines such as interferons (IFN-alpha, IFN-beta, IFN-gamma) (Lefebvre *et al.*, 2001), tumor necrosis factor-alpha (Yang *et al.*, 1995), IL-10 (Moreau *et al.*, 1999), leukemia inhibitory factor (Bamberger *et al.*, 2000) and hormones (e.g. progesterone and glucocorticoids) (Moreau *et al.*, 2001; Yie *et al.*, 2006a) are capable of enhancing steady-state levels of HLA-G on trophoblast cells. Here, we provide evidence that gal-1, a lectin with a potent role in modulating immune tolerance during mammalian pregnancy, has a stimulatory effect on HLA-G gene expression. Most intriguing is the finding that exogenous gal-1 regulated membrane-bound forms (HLA-G1 and -G2), whereas endogenous gal-1 in addition modulated the expression of soluble variants (sHLA-G5 and -G6) on the human EVCT derived cell line. This result is in agreement with several studies showing the capacity of gal-1 to exhibit extracellular and intracellular functions (Fischer *et al.*, 2005; Camby *et al.*, 2006). It is attractive to speculate that the gal-1-HLA-G axis on EVCT would be relevant to the migration toward the maternal vasculature, since HLA-G is expressed only by CTBs that invade the uterus (McMaster *et al.*, 1995). However, sHLA-G (e.g. HLA-G5) has been proposed as a negative regulator of temporal and spatial growth factor-induced trophoblast invasion (McCormick *et al.*, 2009). In this context, the autocrine effect of gal-1 on soluble forms of HLA-G could represent a key mechanism by which trophoblasts self-regulate invasion once they reach the maternal vasculature. Interestingly, for sHLA-G, a possible role in vascular remodeling has already been identified (Fons *et al.*, 2006; Le Bouteiller *et al.*, 2007). Furthermore, the finding that both gal-1 and HLA-G are regulated by hypoxia in tumor cells (Mouillot *et al.*, 2007; Zhao *et al.*, 2010) could be relevant to trophoblast, since during early pregnancy trophoblast invasion is executed under hypoxic conditions.

We also observed that blocking endogenous gal-1 negatively affected the phosphorylation of SRMS and TXK, two non-RTKs (PTK) that belong to the Src and Tec family, respectively. Signaling pathways induced upon activation of PTKs comprise the ras-mitogen activated protein kinase (Lowenstein *et al.*, 1992) and the subsequent translocation to the nucleus to phosphorylate specific transcription factors, such

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as Sp1, E2F, Elk-1 and AP-1 (Seger and Krebs, 1995). Interestingly, previous studies have reported that gal-1 binds ras (Paz et al., 2001; Belanis et al., 2008) and increases Sp1 transactivation and DNA binding due to the reduced threonine phosphorylation of Sp1 (Fischer et al., 2005). Since the HLA-G promoter region contains three ras response elements (Flajollet et al., 2009) where Sp1 binds (Donadi et al., 2011), the negative regulation of HLA-G expression observed in this study may result from reduced PTK phosphorylation upon gal-1 silencing. However, it must also be noted that a progesterone receptor response element is located along the HLA-G gene promoter (Yie et al., 2006b), and thus the cross talk between gal-1 and progesterone could be relevant to the regulation of HLA-G expression during early pregnancy. Such interactions between gal-1 and progesterone seem to be important for supporting pregnancy maintenance in mice (Blois et al., 2007; Hirota et al., 2012).

Several reports have highlighted the importance of gal-1 during gestation (Blois et al., 2007; Kopcow et al., 2008). Gal-1 is up-regulated within the decidua (on stromal cells and NK cells) and can induce tolerogenic DCs, emerging as a key modulator of T cell functions (Blois et al., 2007; Kopcow et al., 2008; Than et al., 2008). In addition, gal-1 has been found to induce trophoblast invasion and fusion during placentation, and may thus be important for the regulation of trophoblast differentiation (Fischer et al., 2010; Kolundzic et al., 2011). Together with its influence on the immune evasion machinery in trophoblast cells by regulating HLA-G expression described here, these data strongly suggest that gal-1 plays a central role in the maintenance of pregnancy. Our study further provides the first evidence regarding the importance of gal-1 as a predictive factor of pregnancy success in humans, given that circulating gal-1 levels were found to be diminished in healthy pregnant women who subsequently suffered from spontaneous abortion, when the β-hCG values were still within the normal range. A previous study has shown a down-regulation of gal-1 expression in placental villous tissues from early pregnancy loss patients (Liu et al., 2006), whereas our results also showed low expression of gal-1 in spontaneous abortion specimens in villous CTB and also in stromal decidual cells. Thus, circulating levels of gal-1 appear to correlate positively with local levels of expression, further enhancing its predictive value as a marker for spontaneous abortion. Given its remarkable functional plasticity, ability to modulate cell growth and differentiation processes, angiogenesis and immune responses, gal-1 is most likely to play a central role in the complex signaling network involved in pregnancy maintenance, with potential implications for the diagnosis and treatment of reproductive disorders.

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Authors' roles

S.M.B. and M.C. conceived and designed the research; I.T.-G, N.F., G.B., V.S., M.C. and S.M.B. performed the research; O.N., M.S., L.K., B.F.K. and L.M.-N. contributed essential reagents; I.T.-G., V.S., M.C. and S.M.B. analyzed the data; G.B., M.C. and L.M.-N. gave input on writing the manuscript; S.M.B. wrote the paper.

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Conflict of interest

None declared.

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