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VIP INDUCES THE DECIDUALIZATION PROGRAM AND CONDITIONS THE IMMUNOREGULATION OF THE IMPLANTATION PROCESS

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ABSTRACT

The decidualization process involves phenotype and functional changes on endometrial cells and the modulation of mediators with immunoregulatory properties as the vasoactive intestinal peptide (VIP). We investigate VIP contribution to the decidualization program and to immunoregulation throughout the human embryo implantation process. The decidualization of Human endometrial stromal cell line (HESC) with Medroxyprogesterone-dibutyril-cAMP increased VIP/VPAC-receptors system. In fact, VIP could induce decidualization increasing differentiation markers (IGFBP1, PRL, KLF13/KLF9 ratio, CXCL12, CXCL8 and CCL2) and allowing Blastocyst-like spheroids (BLS) invasion in an *in vitro* model of embryo implantation. Focus on the tolerogenic effects, decidualized cells induced a semi-mature profile on maternal dendritic cells; restrained CD4+ cells recruitment while increased regulatory T-cells recruitment. Interestingly, the human blastocyst conditioned media from developmentally impaired embryos diminished the invasion and T-regulatory cells recruitment in these settings.

These evidences suggest that VIP contributes to the implantation process inducing decidualization, allowing BLS invasion and favoring a tolerogenic micro-environment.

1. INTRODUCTION

Embryo implantation requires a sequence of coordinated events between the endometrial epithelial and stromal cells, the maternal immune system, and the blastocyst (Dey et al., 2004; Gellersen et al., 2007; Stoikos et al., 2008). The endometrium needs to undergo a series of changes that will facilitate blastocyst attachment and invasion. On one hand, progesterone (P4) induces endometrial stromal cell differentiation into epithelioid decidual cells; on the other, decidualized cells will change their secretome associated with the production of immunoregulatory factors which contribute to generate a local immune privileged site for supporting the nidation of a semiallogenic fetus (Dimitriadis et al., 2005; Gellersen and Brosens, 2003; Mesiano et al., 2011).

The importance of the endometrial stromal compartment in regulating early human implantation events is based on the ability of differentiated human endometrial stromal cells (HESC) to respond to embryonic trophoblast signals. Teklenburg et al. demonstrated that decidualized but not undifferentiated HESC selectively recognize the presence of a developmentally impaired embryo and respond by inhibiting the secretion of key implantation mediators and immunomodulators (Teklenburg et al., 2010b).

From the immunological point of view, during the earliest phase of pregnancy, a successful implantation occurs in a regulated pro-inflammatory microenvironment with tissue remodelling and angiogenesis at the maternal-placental interface. The following phase, is characterized by a shift towards a tolerogenic profile that limits the initial pro-inflammatory response and allows the symbiosis between the trophoblast cells and the fetus for fetal growth (Abrahams et al., 2005; Mor and Cardenas, 2010; Weiss et al., 2009). Thus, implantation involves a tight homeostatic control provided by immune cells subpopulations selectively recruited and/or expanded during early stages of gestation, such as the induction of regulatory T lymphocytes (Tregs CD4+CD25+FoxP3+), and tolerogenic dendritic cells (DC) (Gomez-Lopez et al., 2010; Pérez Leirós and Ramhorst, 2013).

Regarding the specialized Treg population, much evidence demonstrated their relevance during pregnancy for preventing a maternal immune response against fetal antigens released by trophoblast or fetal cells at the implantation site and the maternal circulation (Guerin et al., 2009). In fact, Tregs could be induced from CD25- precursors in the peripheral tissue and the draining lymph nodes of the uterus by tolerogenic DC, a subpopulation that could be characterized by their phenotypic and functional profile (Fraccaroli et al., 2009b; Larregina and Falo, 2005; Morelli and Thomson, 2003; Ramhorst et al., 2012; Reis e Sousa, 2006). This subpopulation usually expresses low levels of co-stimualtory molecules such as CD80 and CD86, they are poor producers of IL-12p70 and TNF-α, but high producers of IL-10 and Indolamine 2-3 dioxygenase (Grohmann, 2003; Mellor and Munn, 2004; Morelli and Thomson, 2007).

In this context, the vaosactive intestinal peptide (VIP) is a pleiotropic peptide that promotes anti-inflammatory and tolerogenic profiles through binding to VIP receptors on immune cells. At the early maternal-placental interface, VIP emerges as a key regulator factor with immunomodulatory effects on maternal leukocytes, for example inducing Tregs through a mechanism involving TGF-β1 (Couvineau and Laburthe, 2012; Ekström et al., 1983; Fraccaroli et al., 2009a; Gonzalez-Rey and Delgado, 2007; Leceta et al., 2007).

Regarding VIP contribution to the decidualization program, in previous work we used an *in vitro* model of decidualization, stimulated with P4 and LPS, simulating the inflammatory response during implantation; and we demonstrated that endogenous VIP production contributes to the recruitment of Tregs, differentiated *in vitro*, through a mechanism dependent of the β-chemokine RANTES (CCL5) (Grasso et al., 2014). In fact, since cyclic endometrial decidualization might precondition uterine tissues for a hyperinflammatory response associated with trophoblast invasion, the selectively uterine recruitment of Tregs might control the inflammatory response to sustain cyclic uterine homeostasis (Brosens et al., 2009; Kwak-Kim et al., 2009; Teklenburg et al., 2010a, 2010b; Weiss et al., 2009).

Taking into account that in humans the decidualization of the endometrial stromal cells occurs around 10 days after ovulation, that it depends on elevated levels of cAMP indicating that P4 is not the primary trigger of this differentiation process, and the tight immune control of early implantation, we evaluated the contribution of VIP to the decidualization program and the immunoregulatory process of the human embryo implantation. For that purpose, we developed an *in vitro* implantation model that mimics the human uterine / trophoblast interactions and allows to evidentiate the immunoregulatory effects of the blastocyst conditioned media accordingly with their quality.

2. Materials and Methods

2.1. Cell Lines

Human first trimester trophoblast cell line (Swan-71) and human endometrial stromal cell line (HESC) (a gift by Dr. Gil Mor Medical School, Yale University, USA) were used in these studies. All cells were maintained in DMEM-F12 supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2mM glutamine (Krikun et al., 2004; Straszewski-Chavez et al., 2009). For the different assays, HESC cells were cultured in 24-well plates until they reached 70% confluence.

Decidualization: HESC cells were cultured in 24 wells-plate with DMEM-F12 10% FCS in the presence of VIP (10^{-7}M) or medroxyprogesterone (MPA) (10^{-7}M) and dibutyryl cAMP (db-cAMP) (2,5 10^{-3}M) for 8 days, renewing the stimuli every 48 h and then used in the assays described below. In all cases, cells were washed and the stimuli was removed after differentiation.

Conditioned media (CM): HESC cells were decidualized in the presence of MPA+db-cAMP or VIP, non-decidualized were cultured in DMEM-F12 10% FCS. After the differentiation, the stimuli (VIP and MPA-db-cAMP) were removed and cells cultured overnight in the presence of DMEM-F12 10% FCS, then supernants were collected and maintained at -20°C until use.

2.2. In vitro implantation model

Blastocyst-like spheroids (BLS) generation

Trophoblast cells from a confluent T25 flask of first trimester trophoblast Swan-71 cells were trypsinized and plated into low attachment P60 plates (Corning Incorporated, Corning, NY, USA). Formation of spheroids was monitored, until they reached a compact spherical morphology. Cell viability of the spheroids was evaluated by trypan blue staining (viability >99%).

Co-Culture of BLS with HESC cells

HESC cells decidualized in presence of MPA-db-cAMP or VIP andnon-decidualized were grown to confluence in 24 wells-plate (Greiner Bio-One, Kremsmünster, Austria). Swan-71 spheroids were stained with CFSE (CellTrace CFSE Kit, ThermoFisher Scientific, MA, USA) according providers instructions and then (ten spheroids per well) transferred using a transfer pipette and a dissecting microscope. They were co-cultured with confluent HESC and maintained in DMEM supplemented with 10% FBS. All co-cultures were monitored using an Olympus microscope (Olympus Lifesciences, USA) and ImageJ software (NIH, USA). Invasion index was analysed as morphological change and calculated as "1-minor_axis/mayor_axis" of an ellipse surrounding the BLS as shown on figure 3C. For each assay, the average of the invasion index of all the BLS on each well was considered as one single sample and used for the statistical analysis.

2.3. Peripheral Blood Mononuclear Cells (PBMCs)

Blood samples were obtained from fertile women defined as women who had two or more previous normal pregnancies without any miscarriage in their clinical history, non smokers, who were not under pharmacological treatment for at least 10 days before the day of sampling. Blood was obtained by puncture of the forearm vein, and it was drawn directly into heparinized plastic tubes. Studies were approved by the "Academia Nacional de Medicina Review Board" and Ethical Committee. All healthy donors provided written informed consent for sample collection and subsequent analysis. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral

blood by Ficoll-Hypaque PLUS (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), density gradient centrifugation.

2.4. Generation of human DC

After PBMCs isolation, CD14+ cells were separated by performing positive selection with CD14+ micro magnetic beads according to the manufacturer's instructions (Miltenyi Biotec., Bergisch Gladbach, Germany). Cell population purity was checked by FACS analysis using anti-CD14 mAb and was found to be >95%. To obtain DC, monocytes were cultured in RPMI 1640 medium supplemented with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin at 10^6 cells/ml with 20 ng/ml IL-4 and 20 ng/ml GM-CSF for 5 days. The differentiation (over 90%) was evaluated as an increase of CD1a and a decrease of CD14 markers by FACS.

2.5. Real Time PCR

We evaluated decidualization markers PRL, KLF9, KLF13, CCL2, CXCL12, CXCL8 and IGFBP1 expression and VIP/VPAC system expression in HESC cells under non differentiation conditions or decidualized by VIP or MPA-db-cAMP for 8 days. Then, total RNA was isolated following manufacturer recommendations with Trizol reagent (Life Technologies, Grand Island, NY, USA), cDNAs were generated from 1µg of RNA using a MMLV reverse transcriptase, RNAsin RNAse inhibitor and oligo (dT) kit (Clontech; Palo Alto, CA, USA) and stored at -20°C for batch analysis. PCR assay was performed using FastStart SYBR green mastermix (Roche, Penzberg, Germany) following manufacturer's instructions. Primers and melting temperatures are described in Table I. Results were expressed as arbitrary units normalized to GAPDH expression.

2.6. Human Blastocyst conditioned media

All patients were stimulated under ovarian suppression with Gn-RH agonists (Lupron, Abbot Laboratories, Chicago, IL, USA), with rFSH alone Gonal-F, (Ares-Serono Laboratories, Switzerland, actually Merck Serono, Darmstadt, Germany); or Puregon, (Organon NV, Oss, The Netherlands, actually MSD, Kenilworth, NJ, USA) or combined with HMG (Menopur, Ferring Pharmaceuticals, Saint-Prex, Switzerland), or with the same gonadotropins associated with the GnRH Antagonist Cetrorrelix 0.25 (Cetrotide 0.25, Serono Laboratories, Switzerland, actually Merck Serono, Darmstadt, Germany). An initial gonadotropin dose of 225 to 300 IU was maintained for 5 days and adjusted according to ovarian response. A single HCG dose of 10.000 IU (Gonacor 5.000, Ferring Pharmaceuticals, Saint-Prex, Switzerland); or Pregnyl (Organon NV, The Netherlands, actually MSD, Kenilworth, NJ, USA) was administered 34-36 h before oocyte retrieval. From the day after ovarian aspiration until pregnancy was confirmed, 800 mg of intravaginal micronized P4 were administered daily for luteal phase support. Four to five hours after oocyte retrieval, mature oocytes were inseminated (conventional IVF or ICSI was applied according to male evaluation) in drops of 30 µl of GIVF plus medium (Vitrolife AB, Sweden), 37ºC, 6% CO_2, 100% humidity. Fertilization was observed 16-18 h after insemination. Fertilized eggs continued their development in drops of 30 µl of G1 plus medium (Vitrolife, Goteborg, Sweden). On day 3, embryos were transferred individually to drops of 30 µl of G2 plus medium (Vitrolife AB, Sweden) until day 5.

Embryo classification

Embryos were classified following the criteria developed by Gardner and Schoolcraft (1999). Embryo transfer was done with the Frydman Ultra-soft catheter (CCD Laboratoires, Paris, France). After embryo transfer drops of G2 plus were retrieved and allocated individually in vials for further analysis.

2.7. Flow-cytometry analysis

DC were stained with the following mAbs: FITC- or PE-conjugated mAbs anti CD1a, CD83 and CD86 according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). Ten thousand events were acquired in a FACSAria II cytometer and results were analyzed using FlowJo 7.6 Software. Results are expressed as the mean fluorescence intensity (MFI) of CD86 or CD83 normalized to the non-treated control (Media) expressed in arbitrary units (AU).

Intracellular staining for FoxP3 detection

After migration assays, cells were recovered from the lower compartment, washed, surface stained with APC conjugated anti-CD4 mAb (BD Biosciences, CA, USA). Cells were then fixed/permeabilized and intranuclear stained with PE conjugated anti-FoxP3 mAb according manufacturer's instructions (BD Biosciences, CA, USA). Finally, cells were washed with permeabilization buffer and analyzed.

Intracellular staining for VIP detection

HESC cells were decidualized in the presence of MPA -db-cAMP and after 2, 4and 8 days cells were recovered by TrypLe treatment (Gybco, Invitrogen, Argentina). For intracellular VIP detection Stop Golgi was added to the medium in the last 4 h of culture following manufacturer's instructions (Becton Dickinson, San José, CA), to promote intracellular accumulation. Then cells were recovered and, after washing with FACS solution (PBS-2% FBS), cells were incubated with the fixation/permeabilization buffer for 30 min stained with mAb anti-VIP (Abcam, CA). Cells were finally washed with FACS solution and incubated with Alexa 488 conjugated anti-rabbit FITC Ab. Negative controls were performed in the absence of anti-VIP Ab.

Twenty thousand events were acquired in a FACSAria II cytometer and results were analyzed using FlowJo 7.6 Software. Negative control samples were incubated in parallel with an irrelevant, isotype-matched Ab. Results are expressed as the MFI of VIP expression vs non-treated HESC cells (Basal condition) expressed inAU.

2.8. Migration assays

PBMCs were seeded in 5µm-inserts (3 x 10^5 cells/insert) (BD Falcon cell culture inserts), which then were set in a 24-well plate containing the CM from decidualized HESC cells treated with human blastocyst condition media. After 24 h, the cells were recovered from the lower compartment and the frequency of FoxpP3+ cells were quantified by FACS analysis. As a positive control, we used 20% human serum. The results are expressed relative to basal condition for each assay.

2.9. VIP determination

VIP secretion was quantified in supernatants obtained from HESC cells and HESC decidualizedcells after 24 h of culture, with VIP EIA Kit (Peninsula Laboratories-Bachem, San Carlos, CA, USA). Briefly, 25 µl of antiserum and 50 µl of standard or sample were incubated in 96-well immunoplates for 1 h at room temperature. Then, 25 µl of biotinylated (Bt)-tracer was added and incubated for 2 h. After five washings with EIA Buffer, 100 µl of Streptavidin-HRP were added and incubated at room temperature for 1 h. After washed with EIA Buffer, TMB solution and 2N HCl were sequentially added for color development. Absorbance was determined in iMarkTM Absorbance Microplate Reader (Bio-Rad, Hercules, CA, USA) at 595 nm and 450 nm for the blue and yellow products. Results were expressed in pg/ml.

2.10. IL-10 secretion

Dendritic cells were cultured in the absence or presence of CM obtained from HESC cells (basal condition), after VIP-decidualization (VIP) or MPA-db-cAMP-decidualization (Dec) protocol during 24 h, then supernatants were collected and IL-10 was quantified by ELISA (BD Biosciences, USA) performed according to the manufacturer's protocols.

2.11. Statistical analysis

The significance of the results was analyzed by the Student´s t-test and ANOVA with Tukey's post test for parametric analysis of HESC cell line-samples. Mann-Whitney U-test was used for the analysis of non-

parametric samples from maternal PBMCs. We used the GraphPad Prism6 software (GraphPad, San Diego, CA) and a value of *p<0.05 was considered significant.

3. Results

3.1. Decidualization modulates VIP expression and VIP induces decidualization markers

Taking into account that VIP and its receptor subtype VPAC1 is expressed by human stromal cells, we first evaluated if the decidualization modulates the VIP/VPAC system. As depicted in Figure 1A and B, HESC decidualized by MPA-db-cAMP significantly increased VIP expression and secretion. In fact, we performed a time-course analysis for VIP production by HESC cells decidualized in the presence of MPA-db-cAMP and after 2, 4 and 8 days VIP intracellular production were quantified by FACS analysis. As depicted Figure 1C, endogenous VIP production significantly increases after 2 days of differentiation, supporting the hypothesis that VIP might contribute to the decidualization program from earliest stages.

VPAC1 expression was not significantly modulated (Figure 1D) and VPAC2 was not detected under these conditions. Decidualization also decreased DPP4 (CD26), an intrinsic membrane glycoprotein and a serine exopeptidase expressed in glandular and endometrial stromal cells able to cleave VIP, suggesting that VIP may have an increased halflife (Figure 1E) (Ou et al., 2013; Tan et al., 2014).

Since the decidualization process is accompanied by an increase in VIP production and VPACs are coupled to Gs/AMPc/PKA signaling, we evaluated VIP effect as an early factor able to trigger decidualization. For that purpose HESC cells were treated with VIP or with MPA-db-cAMP for 8 days. Then, the expression of different decidualization markers was evaluated by realtime PCR. Particularly, we evaluate the classical markers of differentiation as IGFBP-1, prolactin (PRL), and the ratio between the transcription factors KLF9 and KL13. KLF9 is highly expressed on endometrial stromal cells where acts as a co-activator of P4 receptor B (PGRB) during initial differentiation and once the differentiation process begins, KLF9 expression starts diminishing and KLF13 expression increases through the induction of BMP-2 (bone morphogenetic protein 2). In this stage, KLF13

replaces KLF9 as PGR-B co-activator allowing endometrial cells to respond to P4(Pabona et al., 2010).

As shown in Figure 2, VIP significantly increased the expression of characteristic decidualization markers such as IGFBP1 (A) and PRL (B), transcription factors KLF13/KLF9 ratio (C). In fact, the decidualization program also increases the expression of several chemokines and chemokine receptors as the SDF-1 (CXCL12), IL-8 (CXCL8), and MCP-1 (CCL2)(Ren et al., 2012). Also CXCL12 and CXCL8 contribute to selective recruitment of Tregs toward decidualized cells, while CCL2 recruits monocytes and DC (Ramhorst et al., 2016). Therefore, we also evaluate the modulation of the main chemokines associated with receptive endometrium in HESC cells treated with VIP or with MPA-db-cAMP. As depicted Figure 1D-F, both decidualization treatments significantly increase CXCL12, CXCL8, and CCL2 expression in comparison with HESC non-decidualized suggesting VIP might induce different decidualization markers.

3.2. VIP treatment favors trophoblast cell invasion in an *in vitro* model of embryo implantation

Since VIP is able to induce decidualization markers, we next evaluated a functional study if cells decidualized by VIP are able to allow trophoblast adhesion. To answer this question we developed an *in vitro* model of human embryo implantation. Swan-71 cells, a human first trimester cell line were cultured on non-adherent plates for 24 to 48h to form blastocyst-like spheroids (BLS). The BLS were morphologically selected, tagged with CFSE and seeded over confluent monolayers of HESC cells with different decidualization treatments (non-decidualized, decidualized by VIP or by MPA-db-cAMP). All co-cultures and each BLS were monitored by fluorescence microscopy and the invasion index obtained as described in the M&M section. Pictures in Figure 3A and C show that those cells decidualized by VIP allowed BLS invasion as HESC cells decidualized with MPA-db-cAMP, and displayed a peak after 48 h of co-cultured. Figure 3B shows representative microphotographs of BLS tagged with CFSE invading the HESC monolayers under different treatments and how the invasion index was quantified. The present results suggest that VIP not only induces

decidualization markers in endometrial stromal cells, but also displayed functional activity of the embryo implantation in *in vitro* model.

3.3. Decidualized cells condition the microenvironment inducing a tolerogenic profile

Another crucial step during the early implantation period is the generation of a tolerogenic microenvironment to sustain embryo nidation in a homeostatic context. We focused on the main leukocytes populations able to induce a maternal tolerogenic response, dendritic cells (DC) and Tregs. Therefore, we evaluated if the decidualized cells were able to induce tolerogenic or semi-mature DC and if they were able to recruit Tregs.

First, DC cells were differentiated from maternal CD14+ cells by magnetic beads as described in the M&M section, and then were cultured in the absence or presence of CM obtained from HESC cells after the decidualization protocol with VIP or MPA-db-cAMP. As Figure 4A shows, CM from decidualized HESC cells under both protocols did not induce CD86 expression, a co-stimulatory molecule, nor CD83, a maturation marker on DC. Figure 4A right panel shows representative dotplots with the frequency of CD86+CD83+ DC treated with CM from HESC before and after decidualization (with VIP or MPA-db-cAMP). Furthermore, DC increase IL-10 production in the presence of CM from decidualized HESC cells in comparison with non-decidualized cells (Figure 4B), suggesting that DC remain in a semi-mature or tolerogenic state by factors produced by decidualized cells with VIP or MPA-db-cAMP.

Since Tregs is an essential population for pregnancy maintenance, and they are selectively recruited during the peri-implantation period as demonstrated in murine models, here we evaluated the recruitment of maternal Tregs toward CM from decidualized cells. Maternal PBMCs were seeded onto the upper compartment of a transwell system and T lymphocyte migration was quantified by FACS analysis. We observed a significantly decrease in the recruitment of CD4+ cells toward CM from decidualized cells, in the presence of VIP and MPA-db-cAMP, in comparison with those non-decidualized (Figure 4C). However, when we specifically evaluated the frequency of

CD4+FoxP3+ cells, we observed a significant increase of recruited Tregs toward decidualized cells treated with MPA-db-cAMP (Figure 4D). In fact, this result agrees with the increased expression of CXCL12 and CCL2 associated with the selective recruitment of Treg toward the uterus (see Figure 2).

3.4. Human Blastocyst conditioned media effect on an *in vitro* implantation model

A key point of the decidualization process is that human decidualized cells acquire the ability to sense embryo quality, and then change their secretome contributing to the implantation process or not, according to embryo quality(Brosens et al., 2015). Here we evaluated if decidualized cells by VIP or MPA-db-cAMP display this ability using the *in vitro* implantation model. For that purpose we evaluated the invasion index of BLS in the presence of human blastocyst condition media (bCM). The bCM were obtained from women that received conventional fertilization treatments as IVF or ICSI and was applied according to male evaluation, as described in the M&M section. Mature oocytes were inseminated and fertilized eggs continued their development in drops individually until day 5. The embryos were classified following the criteria developed by Gardner and Schoolcraft (Gardner et al., 2000).

Then, HESC cells decidualized by VIP or MPA-db-cAMP were co-cultured with BLS in the absence or presence of bCM from developmentally competent or impaired blastocysts and the invasion index was quantified after 48 h. As shows Figure 5A, bCM from developmentally impaired (I) human blastocyst decreased the invasion on HESC cells decidualized by MPA-db-cAMP in comparison with bCM from competent (C) blastocyst. The present results suggest that blastocyst also modulates the implantation process through the production of soluble factors and decidualized HESC cells might sense embryo quality.

To evaluate another mechanism of decidualized cells that may respond to blastocyst quality, and since Tregs were specifically recruited toward decidualized cells, we evaluated whether the bCM modulate CD4+FoxP3+ cells recruitment toward decidualized cells under both treatments. As

previously described, maternal PBMCs were seeded on the upper compartment of a transwell system and CD4+FoxP3+ migration was quantified by FACS analysis. CD4+FoxP3+ cells were selectively recruited toward CM from HESC decidualized by MPA-db-cAMP in the presence of competent (C) bCM while this effect was not observed in the presence of the bCM from blastocysts with impaired (I) competence (Figure 5B).

4. Discussion

The decidualization program involves many regulatory molecules that generate a network to control implantation processes such as trophoblast adhesion, invasion and the maternal immune regulation (Dimitriadis et al., 2010; Fraccaroli et al., 2011; Terness et al., 2007; Yoshinaga, 2010).

After the endometrium undergoes a decidual response, the integrity of the tissue becomes dependent upon continuous P4 signaling, however, the mediators that might contribute to trigger decicualization are still an open question(Brosens et al., 1999).

Here, we show that VIP might be one of the first mediators produced by HESC cells upon decidualization that through its interaction with the VPAC1 receptor triggers cAMP signaling to induce the decidualization program. The results presented in this study also demonstrated that VIP induces decidualization associated with the expression of decidualization markers. Furthermore, from the functional point of view, it allows the BLS invasion in an *in vitro* model of human embryo implantation. VIP also displays an immunomodulatory role in this process, since VIP decidualized cells were able to control the immune microenvironment by conditioning DC to a tolerogenic profile similar to the positive control of differentiation. Even, our results suggest that CMfrom VIP-decidualized are able to induce a tolerogenic profile on DC and considering that decidualized cells secrete several immunomodulators, we cannot suggest that only endogenous VIP production could be responsible for the modulation of DC profile.

Regarding T cells recruitment, we observed a significant decrease in CD4+ cells recruitment toward CM from decidualized cells (by VIP or MPA-db-cAMP). In this sense, it was reported in mice that effector T cells cannot accumulate within the decidua and the impaired accumulation might be attributable to the epigenetic silencing of key T cell-attracting inflammatory chemokine genes in decidual stromal cells (Nancy et al., 2012).

Through interactions of chemokines and their receptors, the maternal–fetal interface recruits immunoregulatory Treg cells, helping to form an immune tolerant microenvironment (Ramhorst et al. 2016). Lin et al. have demonstrated that CXCL12 produced by trophoblast enhances exogenous CD4+CD25+ T-cell migration and prevents embryo loss in non-obese diabetic mice (Lin et al., 2009).In this sense, also decidual cells produce various types of chemokines, such as CCL2, CXCL8, CX3CL1, CXCL10 and CXCL12, at significant levels (revised by Du et al. 2014). Paticularly, throught the interaction between CXCR4–CXCL12 interaction and CCR2-CCL2 decidual cells allow the recruitment of Tregs (Du et al. 2014).

The present work also shows for the first time a selective recruitment of maternal Tregs toward decidualized cells by MPA-db-cAMP, suggesting that decidual stromal cells have the ability to restrain the attraction of CD4+ cells, potentially Th1 or T cytotoxic profiles, while recruit Tregs as a strategy to prevent potential tissue damage. Recently, Teles et al. using 2-photon imaging demonstrated that FoxP3+ cells accumulated in the mouse uterus during the receptive phase of the estrus cycle and the *in vivo* depletion of Tregs in two FoxP3.DTR-based models prior to pairing, drastically impaired implantation and resulted in infiltration of activated T effector cells as well as in uterine inflammation and fibrosis in both allogeneic and syngeneic mating combinations (Teles et al., 2013).

Pregnancy depends on intimate interactions between a developmentally competent embryo and a receptive endometrium. Human implantation sites are for ethical reasons inaccessible *in vivo*, therefore, the understanding of early pregnancy events is based on knockout studies in mice and on gene expression studies in the human endometrium, aimed at identifying those factors that underpin

the transient receptive phenotype. In the present study we collected conditioned medium of individually cultured human embryos and then characterized the maternal response *in vitro* using an human implantation model able to test soluble factors produced by low-quality human embryos and embryos of proven developmental competence. We observed that bCM from impaired embryos decreased the invasion index, therefore decidualized cells in this model would also have the ability to select embryo quality. In fact, a very interesting point is that bCM from developmentally impaired blastocyst also decreased the recruitment of Tregs toward decidualized cells, which are involved in the control of the maternal immune response towards a tolerogenic profile as discussed above. Therefore, our results suggest that developmentally impaired human blastocyst rather than being biologically silent or inert, they could secrete factors, that impair the modulation of the maternal response required for the decidual transformation of the endometrium. Even though this effect was not observed in those cells decidualized by VIP, it is important to highlight that the treatment with MPA-dbcAMP strongly induces decidualization. Though VIP was able to significantly induce decidualization markers and chemokine expression, increase spheroid invasion index and induce tolerogenic DC, these results don't reach the same levels as we observed on HESC differentiated using the standard decidualization protocol (MPA-db-cAMP). These results suggest that VIP could be one factor that contribute to initiate the decidualization program, however others could be involve to complete the differentiation program. This is also supported by the kinetic of VIP production, since VIP production significantly increased during all the standard differentiation protocol with a peak production at day 2.

In this sense, Teklenburg et al. characterized key soluble factors involved in crosstalk between endometrial stromal cells and an interacting embryo; and they demonstrated that endometrial stromal cells have the ability to selectively recognize and respond to the presence of a developmentally impaired embryo but only upon differentiation into decidual cells(Teklenburg et al., 2010b). Later, Brosens et al. showed that signals emanating from developmentally competent embryos triggered short oscillatory Ca^{2+} fluxes, while developmentally impaired blastocysts

prolonged Ca^{2+} response able to induce an endoplasmic stress response in human decidual cells, contributing to an active selection of human embryos at implantation (Brosens et al., 2015).

The initiation of the decidualization process depends on one hand, on an elevated and sustained intracellular cAMP concentrations in stromal cells, and on the other, on the simultaneous downregulation of inhibition of the conversion cAMP stimulation back to AMP (Bartsch et al., 2004; Brosens et al., 1999). Here we show that VIP might be one of the earliest factors that active adenylate cyclase through their receptors and trigger the decidualization process. In this sense, an inhibitor of the phosphodiesterases was effective in elevating intracellular cAMP concentrations to achieve maximal *in vitro* decidualization, as determined by measurement of the expression of the decidual marker genes (PRL and IGFBP-1)(Bartsch et al., 2004).

Approximately half of all human embryo implantations result in failed pregnancy. Although several factors may contribute to this problem, many cases of implantation failure are attributed to poor uterine receptivity. Indeed, implantation remains as the rate limiting step for the success of *in vitro* fertilization. Previously, we observed a lower frequency of infiltrated CD4+VIP+ cells in endometrium from patients with recurrent spontaneous abortions (RSA) in comparison with fertile women; supporting the idea that a lower frequency of VIP-producers endometrial T cells might pre condition RSA patients to an imbalance of the immune response (Fraccaroli et al., 2012). In fact, failure of the endometrium to express an adequate decidual phenotype disables natural embryo selection upon implantation and causes recurrent *in vitro* implantation failures (Salker et al., 2010).

Even though the research in the past few years provided a better understanding of the trophoblast-decidua-blastocyst crosstalk, it raised a number of unanswered questions regarding for example, the earliest triggers of the decidualization program, the nature of the embryonic signals able to modulate the decidual secretome and the intercellular connections between decidualized cells program. While it seems reasonable to assume that many of these implantation factors will be evolutionary conserved, especially in mammals where pregnancy depends on invasion of maternal tissues, it should also be acknowledged that there are important interspecies differences, highlighting

the versatility and utility of the humans *in vitro* implantation models. Finally, understanding the immunoregulation at implantation would have major implications for patients with reproductive failures, especially for those with *in vitro* fertilization implantation failures, so further clinical studies are required to determine the immune mechanisms underlying impaired decidualization and embryo recognition.

5. Authors' contributions: EG developed the *in vitro* model of implantation, VIP decidualization protocols and *in vitro* assays with bCM. EG, ES, LG and LF carried out all the experiments with decidualized HESC, qPCRs data analysis and interpretation. DP performed the migration of T cells assays. SG and GS performed dendritic cells differentiation. GM, MI and C.R. recruited the patients that participate in this study and obtained the conditioned media of blastocysts. CPL and RR designed the whole study, discussed the results and prepared the manuscript. All authors read and approved the final manuscript.

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7. Declaration of interest: Authors fully declare any financial or other potential conflict of interest.

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9. Figure Legends

Figure 1: Decidualization modulates VIP expression and secretion on HESC

HESC cell line was decidualized by MPA-db--cAMP (Dec) and **A)** VIP expression were evaluated after 8 days of differentiation by real time PCR. **B)** At the same time supernatants were collected and VIP release was quantified by EIA kit. Results are expressed as mean pg/ml ± S.E.M. of at least 3 independent experiments (*p<0.05, Student t-Test). **C)** Endogenous VIP intracellular production was quantified by immunofluorescence staining and FACS analysis. HESC cells were cultured in the presence of MPA-db-cAMP during 2, 4 and 8days, then cells were permeabilized and stained with primary anti-VIP Ab, followed by Alexa 488 conjugated anti-rabbit Ab. Negative controls were performed in the absence of anti-VIP Ab. Results are expressed as the mean fluorescence intensity (MFI) of VIP expression normalized to HESC non-decidualized cells (Basal) (*p<0.05, ANOVA Tukey's post test). **D)** VPAC1 and **E)** DPP4(CD26) expression were evaluated by real time PCR. Results for qPCR are expressed as gene expression relative to GAPDH expression $2^{-\Delta Ct}$±S.E.M. of at least 4 independent experiments (*p<0.05, Student t-Test).

Figure 2: VIP induces decidualization markers. HESC cells were cultured in 24 wells-plate with DMEM-F12 10% FCS in the absence/presence of VIP (10^{-7}M) or MPA (10^{-7}M) and db-cAMP ($2,5\ 10^{-3}$M) for 8 days, renewing the stimuli every 48 h. Then the expression of **A)** IGFBP1, **B)** PRL, **C)** KLF13/KLF9 ratio, **D)** CXCL12 **E)** CXCL8 and **F)** CCL2 were evaluated by real time PCR. The expression was normalized by GAPDH. (* p<0.05 Anova Tukey post-test. Bars: Mean± SEM from 5 independent experiments).

Figure 3: VIP treatment favors trophoblast cell invasion in an *in vitro* model of embryo implantation. **A)** Swan-71 cells were cultured on non-adherent plates for 24 to 48 h to form the blastocyst-like spheres (BLS). The BLS were morphologically selected, tagged with CFSE and seeded over non decidualized, VIP-decidualized or MPA-db-cAMP-decidualized HESC cells monolayer. After the differentiation, the stimuli (VIP or MPA-dbcAMP) were removed and cells were washed with fresh media before the transfer of the BLS. There is no VIP nor MPA-db-cAMP added during BLS invasion specifically to avoid any effect on the Swan-71 spheroids. **B)** The BLS invasion was followed during 24, 48 and 96 h by fluorescence microscopy and morphologically analyzed (* p<0,05 ANOVA Tukey's post-test. Bars: Mean± SEM from 6 independent experiments). **C)** Figure shows representative microscopy pictures of BLS tagged with CFSE invading the HESC monolayers under different treatment. Invasion index was analysed as morphological change and calculated as "1-minor_axis/mayor_axis" of an ellipse surrounding the BLS as shown on the figure. For each assay, the average of the invasion index of all the BLS on each well was considered as one single sample and used for the statistical analysis.

Figure 4. Decidualized cells condition the microenvironment inducing a tolerogenic profile.

DC differentiated from maternal monocytes were cultured in the absence or presence of CM obtained from HESC cells (Basal), after VIP-decidualization (VIP) or MPA-db-cAMP-decidualization (Dec) protocol during 24 h. Then cells and supernatants were collected and the expression of CD86 andCD83 were evaluated by FACS analysis and IL-10 secretion by ELISA. **A)** Results are expressed as the MFI of CD86 and CD83 normalized to the non-treated control (Media) expressed in arbitrary units (AU). The right panel shows representative dot plot with the % of CD83+ DC, CD86+ DC and CD83+CD86+ DC under the different treatments. Result is representative from 4 independent experiments with 4 different fertile-PBMCs samples. Negative control samples were incubated in parallel with an irrelevant, isotype-matched Ab. **B)** IL-10 secretion, results are expressed as the mean pg/ml ± S.E.M. of at least 4 independent experiments (*p<0.05 Mann-Whitney test). **C-D)** For the migration assays, the maternal PBMCs were seeded in 5µm-inserts, in the upper compartment,

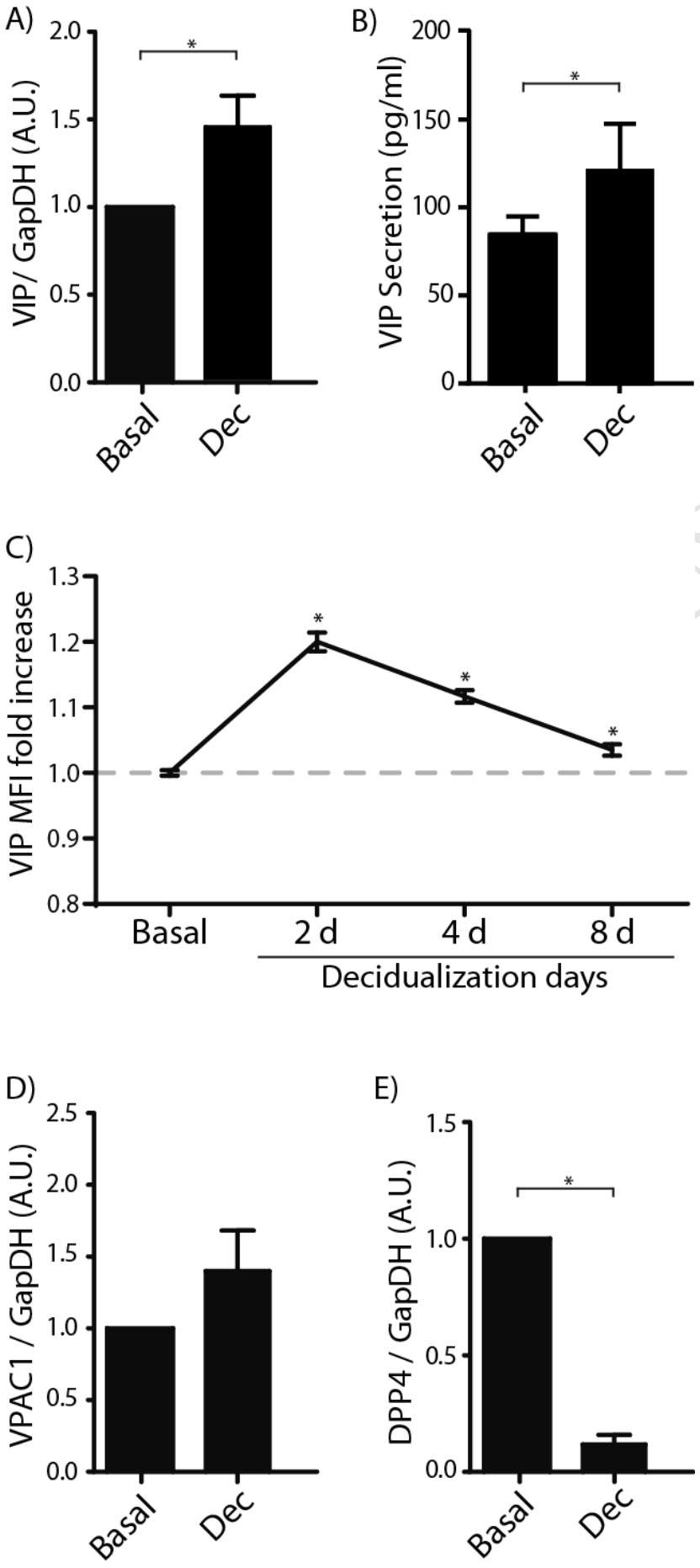
and CM from HESC cells under different treatment in the lower compartment. The migration of CD4+ and CD4+FoxP3+ cells was evaluated by FACS analysis. **C)** The results were analysed into the electronically gated CD4+ cell population and **D)** CD4+FoxP3+ cells from 3 independent experiments using different fertile-PBMCs samples (*p<0.05 Bars: Mean± SEM, Mann Whitney test).

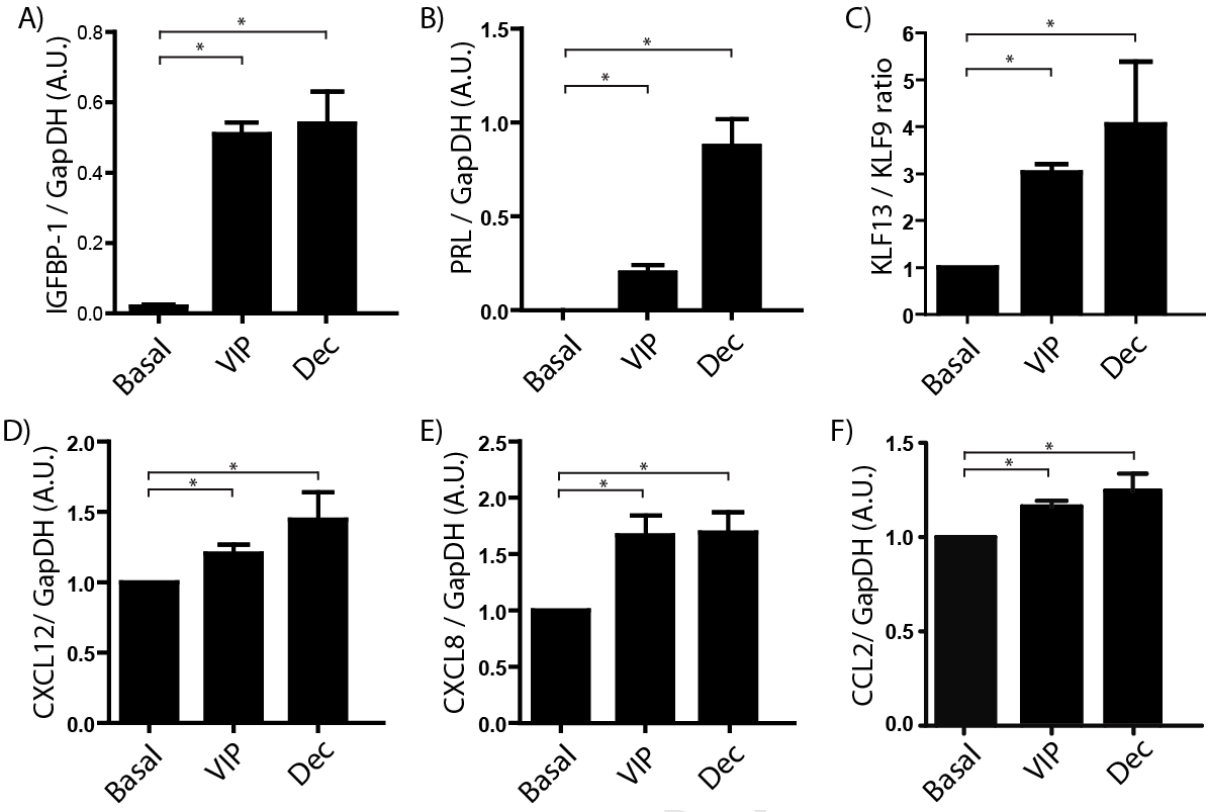
Figure 5: Human Blastocyst conditioned media effect on an *in vitro* implantation model

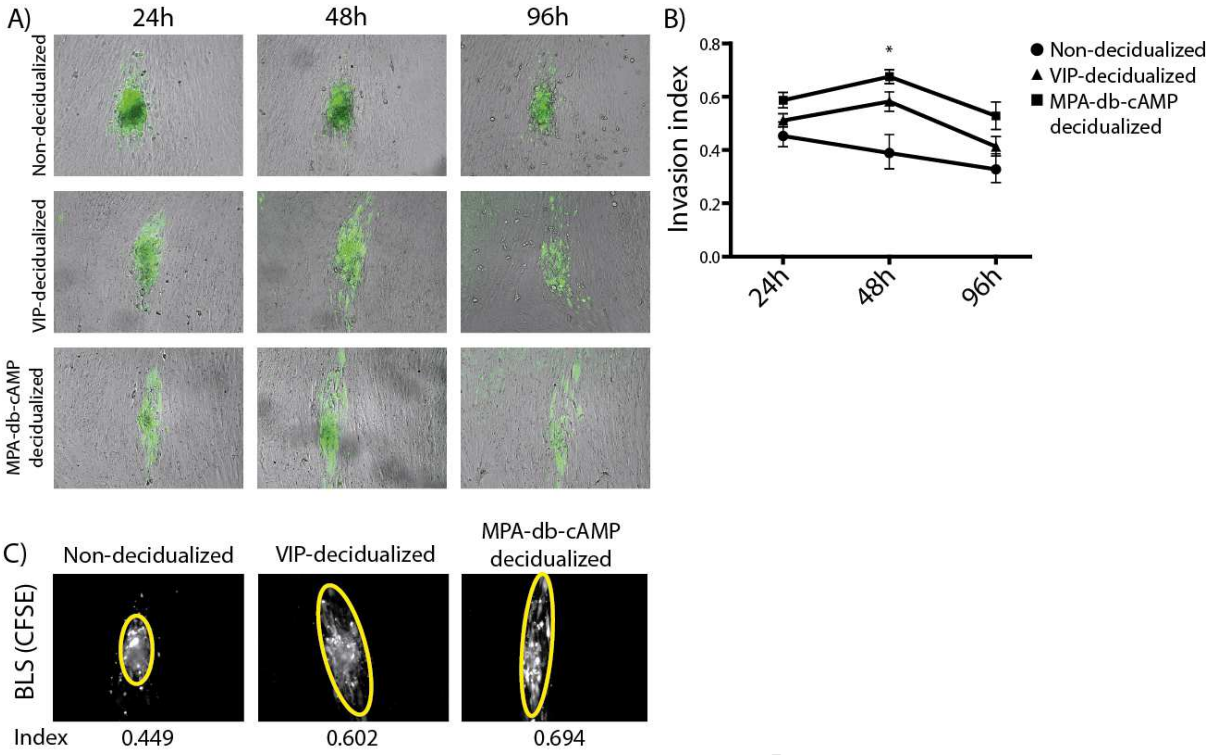
A) After VIP-decidualization (VIP) or MPA-db-cAMP-decidualization (Dec) protocol was completed, differentiation stimuli was removed and BLS were seeded in the presence of bCM developmentally competent (C) or impaired (I) or G2 plus medium as negative control (-) and the invasion index was quantified after 48 h (*p<0,05 ANOVA Tukey's post-test. Bars: Mean± SEM from 4 independent experiments). The lower panel shows representative microphotographs. **B)** Maternal PBMCs were seeded in the upper compartment and CM obtained from HESC cells under different stimuli in the lower compartment. The migration was evaluated in the presence of bCM developmentally competent (C) or impaired (I) by FACS analysis. The results are expressed as the frequency CD4+FoxP3+ cells from 3 independent experiments using different maternal PBMCs (Mann Whitney test *p<0.05). The right panel shows representative dot plot with the % of CD4+FoxP3+ in the presence of CM from Dec + bCM from developmentally competent (Dec+C) or impaired (Dec+I) blastocysts. Result is representative from 4 independent experiments used a pool of bCM from 3 blastocyst (with the same quality score).

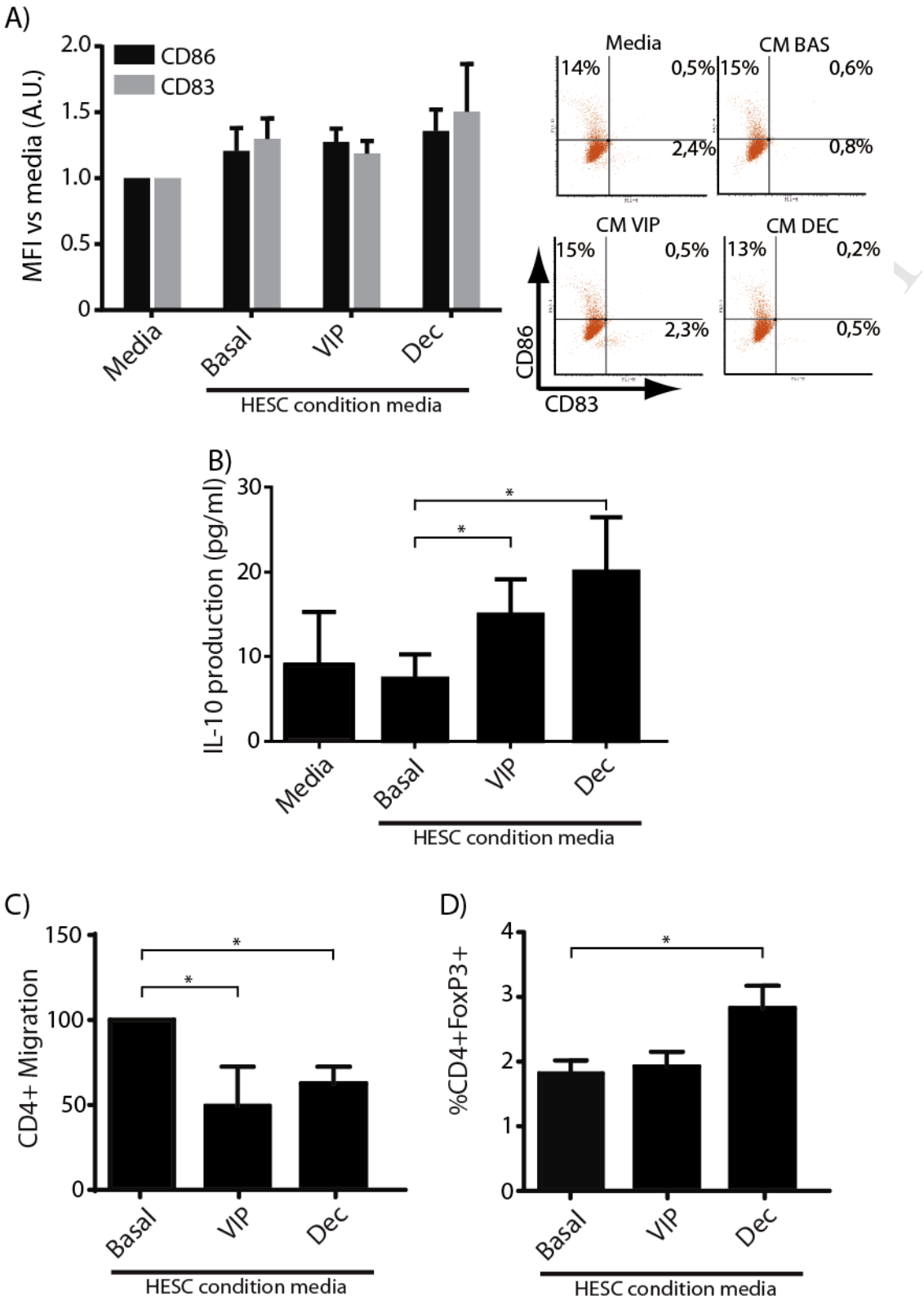
Table I

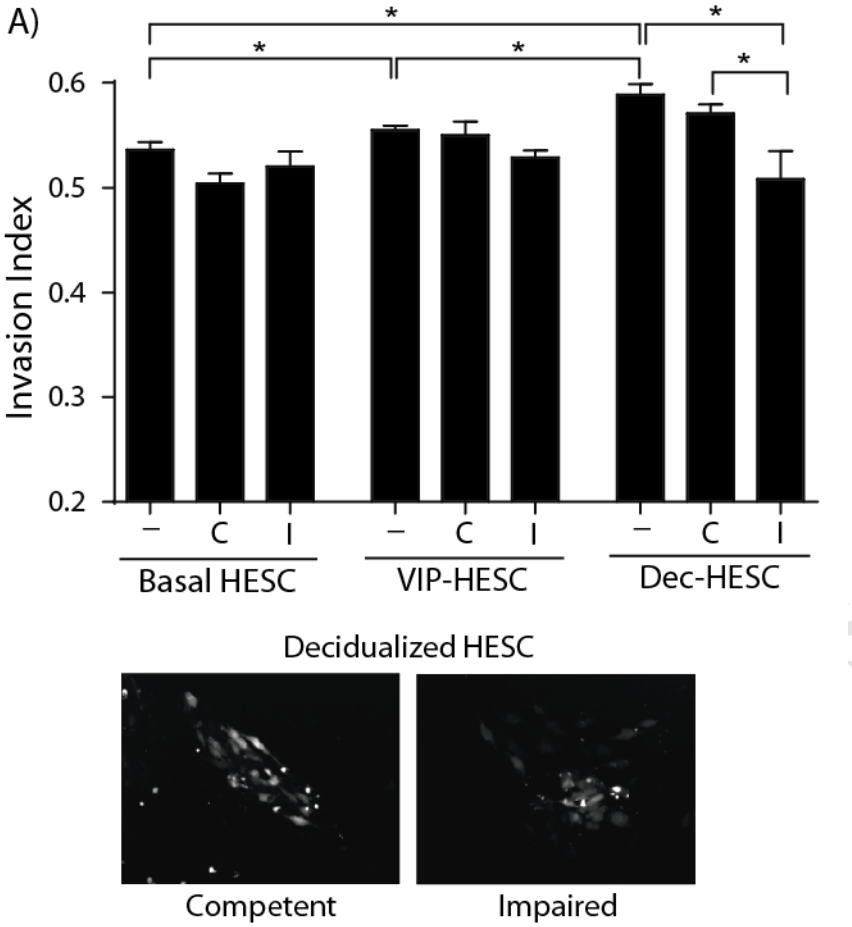
Gene		Primer sequence (5' - 3')	Tm (C)	Prod. length (bp)
PRL	F	CCACTACATCCATAACCTCTCCTC	63	134
	R	GGCTTGCTCCTTGTCTTCGG		
VIP	F	CAGTAACAGCCAACCCTTAGCC	60	196
	R	TGAGAAGAGTCAGGAGCACAAGG		
KLF13	F	TTCGGTGGTTCCTTGGTGACTGG	61	169
	R	TGGACCCTTGGATTCTGCCTTGG		
IGFBP1	F	GAGCACGGAGATAACTGAGGAG	58	192
	R	TTGGCTAAACTCTCTACGACTCTG		
VPAC1	F	CCCCTGGGTCAGTCTGGTG	58	100
	R	GAGACCTAGCATTCGCTGGTG		
VPAC2	F	CCAGATGTCGGCGGCAACG	56	196
	R	GCTGATGGGAAACACGGCAAAC		
CXCL12	F	TGCCCTTCAGATTGTAGC	60	186
	R	CGTCTTTGCCCTTTCATC		
CXCL8	F	CCAACACAGAAATTATTGTAAAGC	62	163
	R	CACTGGCATCTTCACTGATTC		
CCL2	F	CAGCAGCAAGTGTCCCAAAG	64	149
	R	GAGTGAGTGTTCAAGTCTTCGG		
KLF9	F	CCCATCTCAAAGCCCATTACAG	60	151
	R	CACAGCGGACAGCGGAAC		
GAPDH	F	TGATGACATCAAGAAGGTGGTGAAG	64	240
	R	TCCTTGGAGGCCATGTAGGCCAT		

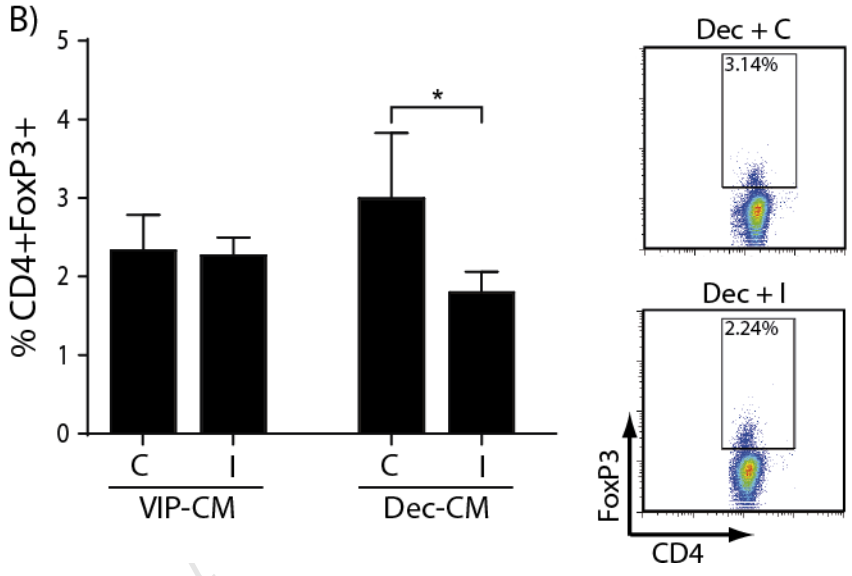

A)
VIP/ GapDH (A.U.)
2.0
1.5
1.0
0.5
0.0
*
Basal
Dec
B)
VIP Secretion (pg/ml)
200
150
100
50
0
*
Basal
Dec
C)
VIP MFI fold increase
1.3
1.2
1.1
1.0
0.9
0.8
*
*
*
Basal
2 d
4 d
8 d
Decidualization days
D)
VPAC1 / GapDH (A.U.)
2.5
2.0
1.5
1.0
0.5
0.0
Basal
Dec
E)
DPP4 / GapDH (A.U.)
1.5
1.0
0.5
0.0
*
Basal
Dec






A)
MFI vs media (A.U.)
2.0
1.5
1.0
0.5
0
CD86
CD83
Media
Basal
VIP
Dec
HESC condition media
Media
CM BAS
CM VIP
CM DEC
14%
0,5%
2,4%
15%
0,6%
0,8%
15%
0,5%
2,3%
13%
0,2%
0,5%
CD86
CD83
B)
IL-10 production (pg/ml)
30
20
10
0
*
*
Media
Basal
VIP
Dec
HESC condition media
C)
CD4+ Migration
150
100
50
0
*
*
Basal
VIP
Dec
HESC condition media
D)
%CD4+FoxP3+
4
3
2
1
0
*
Basal
VIP
Dec
HESC condition media


A)
Invasion Index
0.6
0.5
0.4
0.3
0.2
*
*
*
*
*
–
C
I
–
C
I
–
C
I
Basal HESC
VIP-HESC
Dec-HESC
Decidualized HESC
Competent
Impaired


B)
% CD4+FoxP3+
5
4
3
2
1
0
*
C
I
C
I
VIP-CM
Dec-CM
Dec + C
3.14%
Dec + I
2.24%
FoxP3
CD4

Highlights

- VIP induced decidualization in HESC cells along with increased decidualization markers.

- Blastocyst-like spheroids were able to invade HESC decidualized with VIP or MPA+dbcAMP.

- Decidualized HESC conditioned media induced a semi-mature profile on denditic cells.

- Decidualized HESC conditioned media restrained CD4+ cells recruitment while increased CD4+Foxp3+ recruitment.

- of human blastocyst conditioned media from developmentally impaired embryos diminished the invasion and Treg recruitment in these settings.