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The role of methylation, DNA polymorphisms and microRNAs on *HLA-G* expression in human embryonic stem cells

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ABSTRACT

The human leukocyte antigen *(HLA)-G* gene seems to play a pivotal role in maternal tolerance to the fetus. Little is known about *HLA-G* expression and its molecular control during *in vivo* human embryogenesis. Human embryonic stem cells (hESC) provide an interesting *in vitro* model to study early human development. Different studies reported discrepant findings on whether *HLA-G* mRNA and protein are present or absent in hESC. Several lines of evidence indicate that promoter CpG methylation and 3′ untranslated region (3′UTR) polymorphisms may influence *HLA-G* expression.

We investigated how *HLA-G* expression is linked to the patterns of promoter methylation and explored the role of the 3′UTR polymorphic sites and their binding microRNAs on the post-transcriptional regulation of *HLA-G* in eight hESC lines.

We showed that, while the gross expression levels of *HLA-G* are controlled by promoter methylation, the genetic constitution of the *HLA-G* 3′UTR, more specifically the 14bp insertion in combination with the +3187A/A and +3142G/G SNP, plays a major role in *HLA-G* mRNA regulation in hESC.

Our findings provide a solid first step towards future work using hESC as tools for the study of early human developmental processes in normal and pregnancy-related disorders such as preeclampsia.

1. Introduction

Human leukocyte antigen G (HLA-G) belongs to the non-classical HLA class I antigens and is a tolerogenic molecule that acts on cells of both innate and adaptive immunity. Besides its immunosuppressive function in transplantation, autoimmunity and tumour progression, HLA-G expression is associated with implantation and protection of the semi-allogeneic fetus from the maternal immune system (Carosella et al., 2008; Rebmann et al., 2007; Hunt et al., 2005). Trophoblast cells, which preferentially express HLA-G as well as small amounts of HLA-C, -E and -F, play a major role in tolerance, implantation and vascular remodelling (Berger et al., 2010; Ishitani et al., 2003). These cells originate from the trophectoderm (TE) cells at the blastocyst stage of embryo development, prior to implantation. Despite the essential role in implantation that has been attributed to the soluble form of HLA-G (Jurisicova et al., 1996a,b; Yao et al., 2005; Shaikly et al., 2008; Fuzzi et al., 2002), little is known about *HLA-G* gene expression and its molecular control during *in vivo* human embryogenesis. The knowledge on HLA-G and its role in implantation is largely based on *in vitro* co-culture studies using decidua and trophoblast tissue (Teklenburg and Macklon, 2009; Helige et al., 2008; Moser et al., 2010). Although these studies provide very valuable information, the degree to which they can be extrapolated to the human *in vivo* situation is limited.

In previous work, we showed that both the TE and inner cell mass (ICM) cells of human full blastocysts display HLA-G on the surface membrane (Verloes et al., 2011). Interestingly, we found that during blastocyst expansion, HLA-G was down-regulated in the inner ICM cells (the precursors of the epiblast or EPI) but not in the outer ICM cells (the precursors of the hypoblast). This is in line with the fact that the HLA-G5 protein is consistently found in the mesoderm of the yolk

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sac, in the first hematopoietic cells of the erythroid lineage as well as in endothelial cells of developing blood vessels of the embryo/fetus, all structures originating from the hypoblast (Menier et al., 2004).

As mentioned above, research on the mechanisms of control of gene-expression during human embryogenesis is limited. The main reasons for this are legal and ethical issues on one hand and the scarcity of human preimplantation material for research purposes on the other hand. Conversely, human embryonic stem cells (hESC), which are derived from blastocyst-stage ICM cells, are an interesting alternative research model for early human embryogenesis (Dvash and Benvenisty, 2004; Friedrich Ben-Nun and Benvenisty, 2006; Rubin, 2008).

During our work on the expression of HLA-G in human pre-implantation embryos, we detected expression of HLA-G both at the mRNA and protein levels in three in-house derived hESC lines (Verloes et al., 2011). Intriguingly, different studies by other groups reported discrepant findings on whether HLA-G mRNA and protein are present (Verloes et al., 2011; Grinnemo et al., 2006; Marchand et al., 2011; Hanna et al., 2010) or absent in hESC (Drukker et al., 2002; Suarez-Alvarez et al., 2010; Zhu et al., 2012; Telugu et al., 2013; Li et al., 2013). These differences might be due to the significant genetic and epigenetic diversity that is found amongst different hESC lines. In turn, this diversity may be linked to the genetic and epigenetic status of the embryo that was used to derive the line or may be variation acquired during derivation and *in vitro* culture (Richards et al., 2004; Abeyta et al., 2004). Another likely alternative is that the differences reflect the endogenous mechanisms of gene expression control. In this context, hESC provide an interesting opportunity to investigate these mechanisms in cells closely related to those found in early human embryos.

HLA-G, encoded by the major histocompatibility complex, is located on chromosome 6p21.3 and is composed of eight exons and seven introns with a stop codon at exon 6. The gene exhibits a 5′-upstream regulatory region (5′URR)/promoter extending at least 1.4 kb from the ATG initiation codon and a 3′ untranslated region (3′UTR) (Solier et al., 2001; Moreau et al., 2009). At the transcriptional level, *HLA-G* expression is mainly controlled by its promoter.

Next to the significant evidence that CpG methylation of the *HLA-G* promoter is important for the transcriptional activity of the gene (Moreau et al., 2003; Mouillot et al., 2005), *HLA-G* expression is also post-transcriptionally regulated by the 3′UTR. Several polymorphic sites in the 3′UTR have been described to modulate the expression of the *HLA-G* transcripts and proteins (Rousseau et al., 2003) and alternative splicing (Moreau et al., 2003; Mouillot et al., 2005; Auboeuf et al., 2002). First, there is a 14bp insertion/deletion (INDEL) containing an AUUUG sequence. It has been reported to influence the mRNA stability and splicing pattern (Rousseau et al., 2003) and has been associated with a lower level of *HLA-G* mRNA (Hviid et al., 2003). Next, the single nucleotide polymorphism (SNP) +3187A/G appears to mediate mRNA stability and degradation (Yie et al., 2008). The presence of an A(denine) at the +3187 position has been shown to lead to a decreased *HLA-G* mRNA expression (Yie et al., 2008). Furthermore, there is the +3142C/G SNP, which influences the binding affinity for specific microRNAs (miRNAs). The presence of G(uanine) at position +3142 increases the affinity for miR-148a, -148b and -152, downregulating *HLA-G* expression by mRNA degradation (Tan et al., 2007; Castelli et al., 2009).

In hESC, despite the variation of HLA-G expression observed by the different studies, only one study reported on the promoter CpG methylation status in a mRNA negative line, the Sheffield-1 (Shef-1) line. This line displayed a partially methylated CpG island around its transcriptional start site with exception of four fully methylated CpG sites (region -211 to -272) suggesting that this methylation pattern was responsible for the lack of *HLA-G* mRNA expression (Suarez-Alvarez et al., 2010). However, no promoter methylation data are available for HLA-G positive lines, and the role of the polymorphisms in the 3′UTR has not been previously explored in hESC.

The aim of this study was to identify the control mechanisms of *HLA-G* gene expression in hESC. For this, we evaluated how *HLA-G* expression is linked to the patterns of promoter CpG methylation and explored the role of the 3′UTR polymorphic sites and their binding miRNAs on the post-transcriptional regulation of *HLA-G*.

2. Material and methods

2.1. Cell cultures

HESC lines were derived and characterized in our laboratory as previously described (Mateizel et al., 2006, 2010, 2012). The lines are registered at the European Stem Cell Registry (www.hescreg.eu). The cells were maintained in 20% O_2, 5% CO_2 at 37 °C in standard hESC medium: KnockOut™-DMEM (Life Technologies, Ghent, Belgium) containing 20% KO-SR (KnockOut™ SR, Serum Replacement for ES cells; Life Technologies), 2 mM Glutamine (Life Technologies), 1% non-essential amino acids (Life Technologies), 1 mM β-mercapto-ethanol (Sigma-Aldrich, St. Louis, MO, USA), 6 ng/ml bFGF (Life Technologies) and 1% Pen/Strep (Life Technologies)] on mytomycin-inactivated CF1 mouse embryonic fibroblasts (MEF) and were passaged by mechanical dissociation.

To study the effect of different passaging methods, VUB01 and VUB04_CF were split in two sublines. One subline was passaged mechanically and the other enzymatically with 200 U/ml of collagenase IV (Life Technologies). Cell pellets were collected using a non-enzymatic cell dissociation solution (Sigma-Aldrich) to exclude contamination of differentiated cells and MEFs.

The human *HLA-G*-expressing choriocarcinoma cell line JEG-3 (American Type Culture Collection), used as a positive control, and the M8 melanoma cell line transfected with a pcDNA3.1 hygromycin vector, used as a negative control, were cultured as previously reported (Verloes et al., 2011).

HESC lines (VUB01, VUB02 and VUB04_CF) were cultured for 4 or 5 days and then treated with the demethylating agent 5-Aza-2′-deoxycytidine (5-Aza-dC) (Sigma-Aldrich) for 48 h at a final concentration of 10 μM. Untreated cells, grown in parallel, were used as controls.

2.2. DNA and RNA extraction and cDNA synthesis

Genomic DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Germany). Total RNA was extracted using the RNeasy Mini kit (Qiagen) and a DNAse treatment (RNase-Free DNase Set; Qiagen) was performed on all the samples. The isolated RNA was reverse-transcribed using the First-strand cDNA Synthesis Kit (GE Healthcare, UK) with the *Not*I-d(T) 18 primer.

2.3. Bisulphite sequencing

DNA bisulphite treatment was performed with the EZ DNA Methylation-GoldTM kit (Zymo Research, Germany) and the DNA was immediately amplified using the primers F: 5′-TGGGTTAAGATTT AGGGAGATA-3′ and R: 5′-TAACTTCTCTAAAAACCTATCACCTAA-3′ (Suarez-Alvarez et al., 2010). PCR products were cloned using the TOPO TA Cloning kit (Life Technologies). After transformation, single colonies were purified based on blue/white selection. The length of the insert of the colonies was determined by PCR with the M13 primers on lysed bacteria, followed by fragment analysis on a 1.5% agarose gel. PCR products of the correct length were then sequenced using the M13 Forward and M13 Reverse primers with the Big Dye Terminator kit on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Nieuwkerk, The Netherlands). As a control for completeness of bisulphite modification, the conversion of cytosine residues outside CpGs was analysed in each sequence. In general, cytosines outside CpGs are unmethylated and should therefore be converted by bisulphite treatment. Only sequences with >96% bisulphite conversion of cytosine residues outside CpGs were included in the results to exclude false results due to incomplete DNA modification by bisulphite.