Non-classical transcriptional regulation of *HLA-G*: an update

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Abstract

Human leucocyte antigen-G (HLA-G) plays a key role in maternal–foetal tolerance and allotransplantation acceptance and is also implicated in tumour escape from the immune system. The modulation of HLA-G expression can prove to be very important to therapeutic goals in some pregnancy complications, transplantation, cancer and possibly autoimmune diseases. In spite of substantial similarities with classical *HLA*-class I genes, HLA-G is characterized by a restricted tissue-specific expression in non-pathological situations. HLA-G expression is mainly controlled at the transcriptional level by a unique gene promoter when compared with classical *HLA*-class I genes, and at the post-transcriptional level including alternative splicing, mRNA stability, translation and protein transport to the cell surface. We focus on the characteristics of the *HLA-G* gene promoter and the factors which are involved in *HLA-G* transcriptional modulation. They take part in epigenetic mechanisms that control key functions of the *HLA-G* gene in the regulation of immune tolerance.

Keywords: HLA-G • transcription factors • epigenetics • gene regulation

Introduction

Human leucocyte antigen-G (HLA-G) is encoded by the major histocompatibility complex with biological and structural properties associated with a specific function in immune tolerance [1]. HLA-G was first characterized as a protein associated with β_2-microglobulin expressed in the BeWo choriocarcinoma cell line [2] and later as an array of five 37- to 39-kD isoforms in the cytotrophoblasts of placenta [3, 4]. During its 20-year history, HLA-G has been shown to be of crucial importance in the success of implantation and in foetal–maternal symbiosis during human pregnancy [5, 6]. Beyond this perfect example of successful physiological tolerance to semi-allografts, HLA-G has been demonstrated to contribute greatly to the protection of transplanted organs such as heart [7] and kidney/liver [8, 9] allografts against rejection. Evidence has been accumulated showing that HLA-G expression, which is very restricted in non-pathological conditions, may also be a strategy used by malignant tumours [10] and virus [11, 12] to escape the host's immune surveillance. HLA-G exerts these major functions by inhibiting NK and T-lymphocyte-mediated cytotoxicity as well as a proliferative allogenic response [13]. This inhibition is mediated through direct binding to the

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inhibitory receptors ILT-2 (LILRB1/CD85j) [14], ILT-4 (LILRB2/CD85d) [15] and KIR2DL4 (CD158d) [16]. The HLA-G protective effect can occur in the presence of a few HLA-G-expressing cells, by cell-to-cell contact-dependent uptake of HLA-G (trogocytosis) from APC and tumoral cells to T and NK cells, respectively [17, 18]. This process acts through effector cells made to act as suppressor cells locally and temporarily. Finally, ILT2, ILT3, ILT4 and KIR2DL4 expression is up-regulated by HLA-G in antigen-presenting, NK and T cells, suggesting that up-regulation of inhibitory receptors in immune cells might increase their activation thresholds and participate in immune escape mechanisms [19].

The *HLA-G* gene was cloned in 1987 [20] and maps on the short arm of chromosome 6 in the p21.31 region. It presents a gene structure consisting of eight exons and seven introns with approximately 86% similarities with the consensus sequence of the *HLA-A*, *HLA-B* and *HLA-C* genes. Exon 1 encodes the signal peptide, exons 2, 3 and 4 encode the α_1, α_2 and α_3 extracellular domains, respectively, and exon 5 encodes the transmembrane domain. Nonetheless, a stop codon in the second codon of exon 6 results in a shorter cytoplasmic tail region in comparison with classical HLA-class I molecules. A low amount of gene polymorphism is found since only 42 alleles are listed by the WHO Nomenclature Committee for factors of the HLA System (http://www.anthonynolan.org.uk), the *G*010101* group (five alleles) being the predominant one, with frequency varying from 32% to 83% in Japanese, Caucasian and African populations [21]. *HLA-G* alleles are essentially characterized by variations in promoter, 3′UT regions, introns and synonymous substitution in exons, low amino acid changes defining 15 protein variants only [22–25].

An alternative splicing of the primary transcript generates the membrane-bound isoforms HLA-G1 (complete molecule), HLA-G2 (minus exon 3), HLA-G3 (minus exons 3–4), HLA-G4 (minus exon 4) and the soluble isoforms HLA-G5 (soluble HLA-G1 counterpart), HLA-G6 (soluble HLA-G2 counterpart) and HLA-G7 (α_1 domain) (39 to 17 kD) [26–28]. Recent crystallography studies have validated the HLA-G1 heterotrimeric structure with its heavy chain non-covalently associated with β_2m and a nonamer peptide [29]. The loading of high-affinity peptides (KIPAQFYIL) prevents retrieval of the molecule and results in increased cell surface expression of HLA-G1 [30]. Soluble isoforms are encoded by transcripts in which intron 2 (G7) [28] or intron 4 (G5 and G6) [31, 32] are retained and are translated until a premature stop codon that prevents the translation from exon 3 and 5, respectively. Soluble HLA-G1 also may be generated by proteolytic shedding (sHLA-G1) [33], which is likely to be regulated by NF-κB activation [34]. Besides, it has been demonstrated that HLA-G1 and HLA-G5 may also be produced as β_2m-free heavy chains and more importantly as disulphide-bonded homodimers [35–37] and that the ILT-2 and ILT-4 binding sites of HLA-G dimers are more accessible than those of HLA-G monomers [38].

Under non-pathological conditions, HLA-G expression occurs during pregnancy primarily in the pre-implanted embryo [6, 39, 40] and at the maternal–foetal interface on extravillous invasive cytotrophoblasts [3, 4]. A number of other extrafoetal cells also express HLA-G, including amnion epithelial cells [41, 42] and endothelial cells of foetal blood vessels in the placenta [43]. Over the past few years, the HLA-G expression pattern was extended to a few healthy adult tissues in immune privileged sites, namely thymus [44, 45], cornea [46], pancreas [47] and the proximal nail matrix [48]. In addition, HLA-G protein may be produced by human decidual stromal cells [49], monocytes [50], keratinocytes [51] and erythroblasts from primitive to definitive haematopoiesis [52]. In contrast, restrictive expression of HLA-G is abrogated under pathological conditions, with up-regulation observed in grafted organs, inflammatory and autoimmune diseases, and viral aggressions [1]. In particular, more than 1000 malignant lesions have been analysed for the HLA-G expression and definitely demonstrate that HLA-G is switched on in numerous tumours [53]. Interestingly, expression frequency varies according to the tumour type, ranging from less than 30% in tumour lesions such as lung carcinoma [54, 55] and breast carcinoma [56, 57] to at least 80% in tumour lesions such as pancreatic ductal carcinoma, biliary cancer [58] and oesophageal squamous cell carcinoma [59]. Nonetheless, HLA-G1 cell surface expression and HLA-G transcripts may be lost along long-term *in vitro* propagation [60, 61], suggesting that not only HLA-G expression is under the control of genetics but also micro-environmental factors.

On top of the expression regulation of antigen-processing machinery components, post-translational mechanisms such as mRNA stability or protein translation are important for HLA-G expression, in particular during invasiveness of cytotrophoblasts [62]. In this situation, the recent identification of HLA-G-specific miRNAs may be assumed in these mechanisms [24]. On the other hand, a key level of regulation is undoubtedly *HLA-G* gene transcription, since high amounts of *HLA-G* transcripts are observed in cells expressing HLA-G protein, while very low amounts or absence of *HLA-G* transcripts are observed in cells where HLA-G protein is not detected [63–66]. *HLA-G* transcription also exhibits temporal regulation observed during the course of gestation [67], with high levels of *HLA-G* mRNA in first- and second-trimester trophoblasts, whereas a reduced level of *HLA-G* mRNA is observed in term cells [3, 4]. This spatiotemporal pattern of *HLA-G* transcriptional regulation is still partially elucidated and is dependent on transcriptional factors and specific cis-regulatory elements located within a non-classical *HLA-G* gene promoter [68, 69]. We propose herein to present this in detail.

HLA-G gene promoter region: regulatory sites and binding factors

The HLA-G promoter is unique among the *HLA* genes [67] with a divergent proximal region when compared to the other HLA, a trophoblast-specific regulatory element located at −1.2 kb from exon 1 [70] and specific regulatory elements. It is worth noting that in the published data, some findings are less valid then others depending on the method used in evaluating regulatory

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Table 1 Promoter of *HLA-G* gene and associated transcriptional factors

Response element	Location (bp upstream of ATG)	Factors	Methods used for validation	References
CRE/TRE	−1380/−1370; −934; −770	CREB1, ATF-1, c-jun	EMSA, reporter gene, ChIP	[96]
RRE	−1356; −142/−133; −53	RREB-1	EMSA, reporter gene, ChIP	(Flajollet *et al.* unpublished data)
ISRE	−744	IRF-1	EMSA, reporter gene	[100]
HSE	−459/−454	HSF-1	EMSA	[103]
P50, SP1	−187/−171; −166	P50, SP1	EMSA	[72]
X1 box	−124	RFX5	EMSA (negative using ChIP)	[87, 88]
CAAT	−71	CTF		
TCTAAA	−44	TFII		
PRE	−37	PR	EMSA, reporter gene, DNA fragment-binding ELISA	[105]

element location and function. Indeed, the analysis could have been performed *in vitro* (*e.g.* electrophoretic mobility shift assays [EMSAs] and reporter gene assays), *in situ* (*e.g.* chromatin immunoprecipitation [ChIP] and RNA interference) or *in vivo* (*e.g.* transgenic mice), a criterion that should be taken into account in evaluating the impact of the results (Table 1). The *HLA-G* promoter also exhibits a pattern of variations characterized by two divergent lineages, which is consistent with balancing selection. This is probably related to highly regulated expression favouring high- and low-expressing promoters under temporally and/or spatially varying selective pressures [22]. Variations in 3′UTR that could influence RNA stability and/or translation have also been identified [24]. These observations strongly suggest that the *HLA-G* gene polymorphisms should now be considered a very pertinent parameter in the understanding of *HLA-G* gene regulation and more particularly in some *HLA-G*-associated diseases.

The atypical proximal promoter region of the *HLA-G* gene among classical *HLA* class I genes

Classical *HLA*-class I gene promoters contain two main regulatory modules, namely enhancer A/interferon (IFN)-stimulated response element (ISRE) and SXY boxes, located within the 220 bp 5′ of the gene initiation codon (ATG). These two distinct cis-acting elements contribute to the constitutive and inducible level of MHC class I genes, the SXY module being shared by MHC class II genes [71]. The most upstream module contains the enhancer A with κB2 and κB1, two palindromic binding sites for the NF-κB/rel family members and a Sp1 transcription factor site [72, 73]. This module is also composed of a response element localized −180 bp from the ATG (consensus sequence AGTTTCNNTTCT) that may bind factors of the interferon regulatory factor (IRF) family such as IRF-1, IFN consensus sequence binding protein (gene activation), IFN-stimulated gene factor (ISGF)-3 and IRF-2 (repressors) [73]. Additionally, E-box elements can be found in the upstream HLA-class I module and are binding sites for upstream stimulatory factor (USF)-i and USF-2 [73].

The downstream SXY module was first demonstrated to be crucial in the regulation of HLA-class II expression [74]. It comprises the X1, X2 (site α) boxes and Y box (an inverted CCAAT-binding site, also named enhancer B), bound by the multiprotein complex RFX (RFX5, -AP, -ANK/B) [75–78], X2-BP/ATF/cAMP response element-binding (CREB) [79] and NF-Y [80] factors, respectively. All these factors cooperate to allow the formation of a stable multiprotein complex and the binding of the class II transactivator (CIITA), which mediates constitutive and IFN-γ-induced expression of HLA-class I molecules [81–85]. S box function is not fully understood and could possibly play a role in promoter architecture [86].

Like classical *HLA*-class I promoters, *HLA-G* promoter exhibits a CCAAT box and an unusual TATA element, TCTTAA, controlling basal regulation. A transcriptional initiation site located 25 bp downstream of TATA is conserved in *HLA-G*. Nonetheless, a second putative initiation site of *HLA-G* transcription has been reported 51 bp upstream of TATA (CTCACTCCC) (http://www.ncbi.nlm.nih.gov/), but the functionality of both sites has not yet been demonstrated. On the other hand, a modified enhancer A and a deleted ISRE render the *HLA-G* gene promoter unresponsive to NF-κB [72] and IFN-γ [73]. The p50 homeodomain subunit of NF-κB displays a strong binding affinity to the two κB sites *in vitro*, but this subunit without p65/relA does not possess a transactivation function [72]. In addition, the upstream region encompassing the SXY module only contains conserved S and X1 sequences and despite its binding capacity *in vitro*, the intact X1 box is unresponsive to the RFX5 factor *in situ* [87, 88]. The absence of RFX5 binding and the presence of nucleotide

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variations in X2-Y boxes explain the absence of a higher-order complex and the lack of CIITA recruitment at the *HLA-G* promoter *in situ* [88] and in transfection experiments with CIITA expression plasmid alone or in co-transfection experiments with luciferase reporter constructs containing the *HLA-G* promoter fragment [69, 86]. In comparison with non-classical HLA-class I genes, *HLA-E* and *HLA-F*, *HLA-G* promoter, with its unique characteristics, is the odd one out [68] since the HLA-E promoter is induced through the SXY module even if it is not regulated by enhancer A and ISRE, and *HLA-F* promoter exhibits extensive similarities with those of classical HLA-class I genes [69]. Furthermore, a putative negative regulatory element located in exon 1 of HLA class I genes, whose activity can be modulated by hormones [89], is also deleted in the *HLA-G* gene and might, in part, explain why *HLA-G* is expressed in human trophoblasts, whereas HLA class I genes are not.

Finally, Monarch 1, a factor belonging to the CATERPILLER gene family, which is expressed primarily by myeloid-monocytic cells, is the only factor found to enhance both classical HLA-class I and *HLA-G* genes. More particularly, Monarch-1 was demonstrated to enhance the *HLA-B* promoter but the regulatory target elements have not been identified to date [90].

Alternative regulatory elements within the *HLA-G* gene promoter

To investigate alternative transactivation pathways to the conserved MHC class I regulatory routes, one of the strategies commonly used was to perform transient transfections with luciferase reporter constructs containing *HLA-G* promoter fragments of different lengths. This identified a negative regulatory sequence within the *HLA-G* promoter fragment extending 450 bp from the ATG and a slightly induced activity of intron 2-containing construct in JEG-3 choriocarcinoma cells [91]. Other major strategies were the use of *HLA-G* transgenic mice into which *HLA-G* transgenes of different lengths were introduced, *in silico* analysis of the *HLA-G* gene promoter sequence, and the identification of the target sites of specific modulatory agents present in the placental or tumoral micro-environment. From these analyses emerged regions identified as putative key components of the *HLA-G* gene regulation. However, precise target sites and/or transcription factors have not always been clarified, as is the case for IFN-γ, leukaemia inhibitory factor (LIF) and hypoxia.

The locus control region

A positive regulatory region of the *HLA-G* gene was found that contained in a 244-bp HindIII/EcoR1 fragment located -1.2 kb from the ATG, using *HLA-G* transgenic mice [70, 92]. This fragment was demonstrated to be critical for spatio-temporal expression of HLA-G mRNAs by using a *HLA-G* transgene including the entire coding region, 1 kb of the 3′ flanking region and 1.2 or 1.4 kb of the 5′ flanking region (5.7- and 6.0-kb fragment, respectively). Indeed, the presence of the 244-bp HindIII/EcoR1 region was associated with a tissue-specific pattern of HLA-G expression in spongiotrophoblasts and mesenchymal cells in placenta and in thymus. HLA-G transcription obtained with the 6.0-kb fragment parallels that seen in human extraembryonic tissues during the course of gestation, reaching the highest levels of expression in trophoblast at day 12.5 and then decreasing progressively before parturition. The distal 244-bp fragment that is required for tissue-specific expression of HLA-G presents a similarity in function with a locus control region (LCR). In agreement with this, sequence analysis of the regulatory fragment has revealed similarities to important elements for the activity of the H3S region of the β-globin LCR [93] such as the TATA symmetrically surrounded by GGGTGG and the putative AP1-binding site [94]. Moreover, DNA binding assays with nuclear extracts from HLA-G$^+$ and HLA-G$^-$ cells revealed the formation of several complexes in this region. Some of them are specific of HLA-G expression status, whereas others are shared complexes [65, 95]. One of these shared complexes has been identified in an independent study by Van den Elsen's group and corresponds to the DNA binding of ATF1/CREB1/c-jun on the CRE/TRE (cyclic AMP-response element/TPA-response element) located at position $-1380/-1370$ in the putative HLA-G LCR [96]. ChIP assay demonstrated the *in situ* binding of CREB-1 and c-jun to this region [96].

cAMP response element/TPA response element

The computer-assisted search for alternative putative regulatory elements in the promoter had led Van den Elsen's group to localize two additional functional CRE/TRE elements dispersed through the promoter region at positions -934 and -770 from the ATG [96]. EMSAs demonstrated that CRE/TRE$_{-934}$ and CRE/TRE$_{-770}$ bind CREB1 and ATF1/CREB1 factors, respectively. Promoter activity assays and mutagenesis studies revealed the crucial role of the three CREs for the basal level of *HLA-G* promoter activity and its transactivation, with the most important contribution of CRE$_{-1380/-1370}$ within putative LCR. In accordance with this, transient transfection of the CREB repressor ICER (inducing cAMP early repressor) inhibits the CREB-induced transactivation of the *HLA-G* gene's 1438-bp promoter, while enhanced transactivation occurs with the co-activators CBP/P300 (CREB binding Protein). Notably, CREB, CBP/P300 and *HLA-G* are co-expressed in extravillous cytotrophoblasts. Nonetheless, CREB association to the *HLA-G* gene promoter *in situ* was also observed in HLA-G$^-$ cell lines, which strongly suggests that tissue-specific expression of HLA-G involves additional regulation mechanisms, including epigenetics.

Interferon-stimulated response element

The cascade of events initiated by IFNs involves the activation of JAK/STAT transduction pathways and the transactivation of the gene promoter with the ISRE and IFN-γ activation site (GAS). Despite a non-conserved HLA class I ISRE in the proximal promoter of HLA-G, several investigations have revealed that the *HLA-G* gene is responsive to up-regulation following treatment with IFN-α, IFN-β and IFN-γ [97, 98]. Enhancement of steady-state levels of *HLA-G* mRNA upon IFN treatments was observed in

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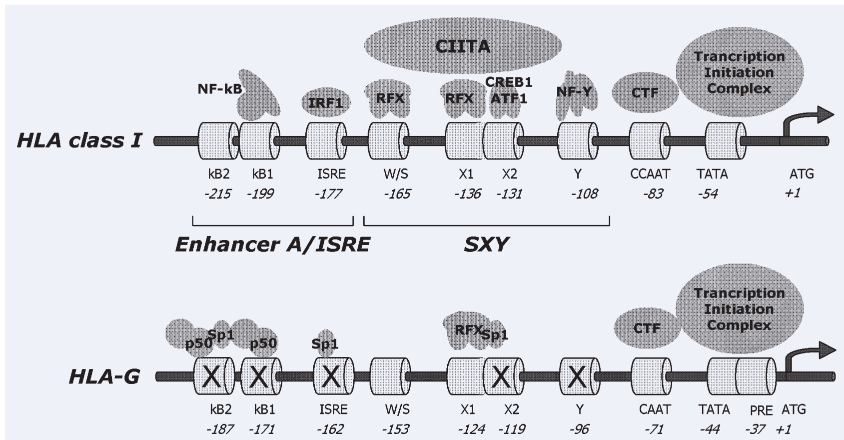

Fig. 1 HLA-class I and *HLA-G* gene promoter: cis-regulatory sequences and their interacting factors. Boxes with X within HLA-G promoter indicate that mutations prevent binding of classical HLA-class I transacting factors.

several cell types such as trophoblast cell lines [97], blood cells (monocytes and macrophage cell lines) [50] and glioblastoma cell lines [99]. In particular, IFN-β enhances the levels of *HLA-G* transcripts in trophoblast explants, amnion and thymus-derived epithelial cells [100]. However, it is a general rule that a basal *HLA-G* transcriptional level is required for IFN-induced up-regulation of *HLA-G* mRNAs [97] and then up-regulation of HLA-G cell surface expression.

Computer-assisted searches within the *HLA-G* promoter sequence led our group to identify an ISRE motif, which is highly homologous to the consensus ISRE. It is located at position −744 bp upstream of ATG, beside a GAS-like element (−734) previously shown to be unable to interact with a GAS-binding complex [98]. We demonstrated that the HLA-G ISRE is a binding site for IFN-response factor-1 (IRF-1) and transactivates HLA-G expression following IFN-β treatment [100]. Despite a weak induction in JEG-3 cells [96, 100], the activity of *HLA-G* promoter was clearly significant in the thymic epithelial cell LT-TEC2 [100].

Although IRF-1 binds to HLA-G ISRE, no transactivation effect in response to IFN-γ was observed in luciferase assays using the 1.4-kb *HLA-G* promoter [91, 96]. Besides, the use of a model system consisting of mouse fibroblasts transfected with a 6.0-kb fragment containing the whole *HLA-G* gene demonstrated the presence of elements that respond to IFN-γ [101]. Consequently, other regulatory pathways or IFN-γ responsive elements should be located outside the 1.4-kb promoter region with the *HLA-G* gene and/or the 3′UT region.

Heat shock element

Stress-induced proteins have been implicated in balancing immune responses during various diseases [102]. This prompted us to evaluate the effect of stress on *HLA-G* gene expression in the M8 (melanoma) and T98G (glioblastoma) HLA-G$^-$ cell lines using heat shock at 42°C or arsenite treatment for 2 hrs. Stress induced an increase in the level of *HLA-G* mRNA with a specificity compared to other HLA class I transcripts. Interestingly, HLA-G6 transcript was induced prior to the other *HLA-G* transcripts, suggesting tight control of *HLA-G* alternative splicing. The study also identified a heat shock element (HSE) within the *HLA-G* promoter at position −459/−454 that is defined as a repetition of the pentanucleotide NGAAN arranged in alternating orientation. The HSE seems to be functional since it binds heat shock factor-1 (HSF-1) *in vitro* by EMSAs [103]. Nonetheless, additional functional analysis using a reporter gene under the control of *HLA-G* promoter with wild-type or mutated HSE would be necessary to improve *HLA-G* HSE functionality.

Progesterone response element

Progesterone is an essential steroid to maintain pregnancy and has been suggested to be an important immune modulator during this time. That is why Librach's group investigated the potential effects of progesterone on *HLA-G* gene expression, revealing that at 10, 100 and 1000 ng/ml progesterone enhanced *HLA-G* mRNA expression in JEG-3 cells by 2.36-, 10.53-, and 17.58-fold, respectively, as compared to controls [104]. More recently, this group demonstrated that the *HLA-G* gene promoter is up-regulated by progesterone through a specific binding site for the progesterone receptor (PR) complex [105]. The identified progesterone response element (PRE)-like sequence is a 15-bp non-classical consensus core sequence that has 60% homology to the wild-type mouse mammary virus (MMTV) PRE and a weaker affinity for PR complexes than MMTV-PRE, probably because of variations in the fixed half site of steroid hormone response elements. This PRE is located −37 bp from the ATG and overlaps the HLA-G TATA box, but the authors do not exclude the presence of other PREs in the *HLA-G* promoter region. Indeed the chloramphenicol acetyltransferase reporter gene assay was not performed with the scrambled HLA-G PRE site and although the authors stipulated that PRE is specific of the *HLA-G* promoter, very similar sequences can be found at the same location in the HLA-class I promoter.

Leukaemia inhibitory factor target site

LIF is a pleiotropic cytokine that is expressed at the maternal–foetal interface and plays an essential part in embryo implantation

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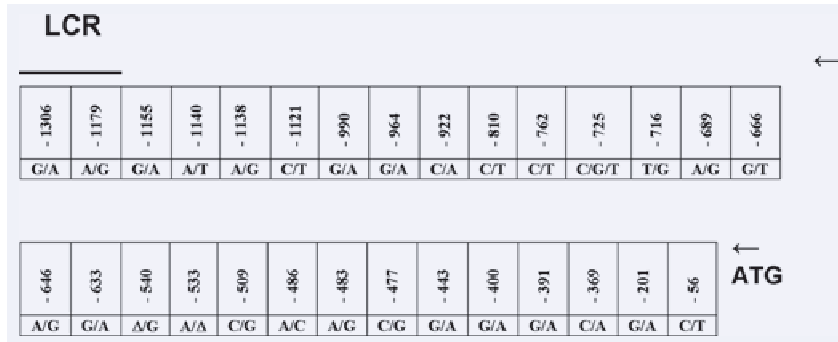

Fig. 2 SNPs along the 1.4 kb of the *HLA-G* gene promoter sequence. ATG: initiation codon; LCR: locus control region; Δ: deletion.

and in mediating interactions between maternal decidual leucocytes and trophoblasts [106, 107]. Upon 72 hrs stimulation with LIF, an up to 3.6-fold elevation of HLA-G mRNA has been demonstrated with JEG-3 choriocarcinoma cells. Luciferase reporter gene assays demonstrated that the stimulation of transcription was driven by a 890-bp promoter fragment of the 5′ *HLA-G* gene flanking region [108]. Nonetheless, precise target site(s) have not yet been identified. Moreover, endoplasmic reticulum aminopeptidase-1 (ERAP1) is also induced by LIF and plays a role in presenting antigenic peptides to HLA-G and then mediates HLA-G cell surface expression [109].

Ras response elements

To further identify factors involved in the regulation of *HLA-G* gene expression, our group recently developed a specific proteomic approach to characterize proteins differentially bound to the proximal and distal *HLA-G* gene promoter. Biotinylated, double-stranded *HLA-G* promoter fragments were incubated with nuclear protein extracts of HLA-G$^+$ and HLA-G$^-$ cells for transcriptional activity and isolated with streptavidin-coated magnetic beads. This DNA-affinity strategy was followed by a 2D separation and the proteins of interest were analysed using mass spectrometry. This allowed us to identify the zinc finger protein Ras responsive element binding 1 (RREB-1) [110] that is capable of binding three Ras response elements (RREs) along the *HLA-G* gene promoter.

We demonstrated that RREB-1 is involved in the repression of *HLA-G* transcriptional activity, acting through the recruitment of factors such as histone deacetylase 1 (HDAC1) and C-terminal binding protein (CtBP) [111] implicated in chromatin remodelling (Flajollet *et al.*, unpublished data).

Sequence polymorphism within the *HLA-G* gene promoter and the 3′UT region

Evidence has been accumulated showing that the *HLA-G* gene polymorphism is involved in the regulation of the *HLA-G* gene transcriptional activity. In particular, some *HLA-G* allelic variants are associated with differences in the pattern of *HLA-G* mRNA isoforms and *HLA-G* mRNA levels [112, 113]. On the one hand, polymorphism in the 3′UT region such as the absence or presence of 14 bp of ‘exon 8’ has been studied more extensively. The presence of the 14 bp is associated with low levels of mRNA expression [113, 114] and mediates or is involved in the out-splicing of the first 92 b of exon 8 [115]. These transcripts were shown to be more stable than the complete RNA [116]. Moreover, a C/G single nucleotide polymorphism (SNP) at +3142 bp in the *HLA-G* mRNA has recently been demonstrated to influence the targeting of three microRNAs [24]. On the other hand, an effort has been made to extend the analysis of *HLA-G* gene variations to the promoter region. To date, 29 SNPs have been identified [22, 23, 117] within this region (Fig. 2) and we cannot exclude that in some cases polymorphism on the promoter may be in linkage disequilibrium with 3′UT variants and that some of them could influence alternative splicing [118]. Interestingly, many of the polymorphisms either coincide with or are closed to the known regulatory elements and thus may affect the binding of the corresponding regulatory factors (Fig. 2).

Modulation of *HLA-G* transcription by micro-environmental factors with unidentified target sites

The restricted expression of *HLA-G* in physiological conditions and its up-regulation in pathological situations reveals a significant correlation of *HLA-G* transcription with biochemical environment [49]. This is strongly supported by the fact that *HLA-G* expression and transcription may be specifically down-regulated or even lost during long-term culture of biopsy-derived cancer cells [60, 61].

Variations of environmental factors such as growth factor, cytokines, hormones, physical conditions and stress dramatically occur during pregnancy as well as during inflammation, viral infection and cancer. Interestingly, numerous micro-environmental factors and molecular circuits are shared by placental and pathological situations such as cancer [119]; some of them have been demonstrated to be involved in *HLA-G* gene transcription. However, with the exception of IFNs and progesterone, the mechanisms by which most of these key regulatory molecules exert control on the *HLA-G* gene transcription, either directly on the *HLA-G* promoter or not, need further exploration.

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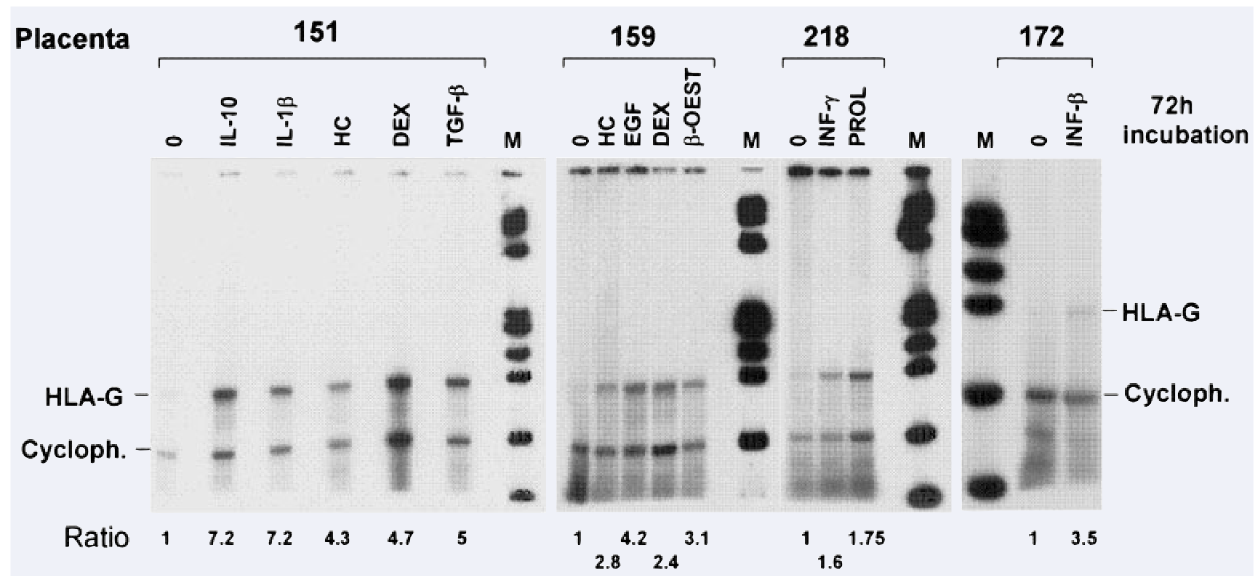

Fig. 3 RNAse protection assay analysis of *HLA-G* mRNA levels in four trophoblast organ explants (namely 151, 159, 218, 172) of first-trimester gestation incubated 72 hrs without (0) or with micro-environmental factors (IL-10: 2 ng/ml; IL-1β: 17 pg/ml; HC: 10^{-8} mol/l hydrocortisone; DEX: 10^{-7} mol/l dexamethasone; TGF-β: 10 ng/ml; EGF: 20 ng/ml; β-OEST: 10^{-7} mol/l β-oestradiol; IFN-γ: 100 U/ml; PROL: 10^{-10} mol/L prolactine; IFN-β: 1000 U/ml). The HLA-G template used for the riboprobe was obtained by PCR amplification of *HLA-G* genomic fragment located in the 3′UT region as previously described [124]. Ratio indicates the values obtained for HLA-G signals normalized to the constitutively expressed cyclophilin (Cycloph.) signal compared with untreated trophoblasts (assigned as value of 1). M: molecular weight size marker.

Cytokines, growth factors and hormones

Placenta is the main HLA-G-producing site that releases and/or is in contact with a variety of cytokines, both anti-inflammatory (IL-10, IL-4, IL-5, IL-6) and pro-inflammatory (tumor necrosis factor [TNF]-α, IL-1β, IL-2), and transforming growth factors (TGF-β), granulocyte macrophage colony stimulating factor [GM-CSF], granulocyte colony stimulating factor [G-CSF], colony-stimulating factor [CSF-1], LIF and epidermal growth factor [EGF]). Autocrine and paracrine mechanisms occur through specific cytokine receptors. Cytokines are crucial for successful embryo implantation and contributes to the maternal metabolic changes necessary to accommodate the increased energy needs of the foetus. Pregnancy is associated with dynamic changes in cytokine levels and ratios and is mainly characterized by an increase in the concentration of cytokines in the second half of pregnancy, with many changes orchestrated around IL-12 [120, 121]. Pregnancy also affects hormones in the body, mostly because of the effects of hormones produced by the placenta. In particular, human chorionic gonadotrophin, produced by the developing placenta, stimulates the ovaries to produce the oestrogen and progesterone needed to sustain pregnancy. By the fourth month of pregnancy, the placenta takes over from the ovaries as the main producer of oestrogen and progesterone [122]. These hormones are involved in womb changes to make room for the growing baby. Other hormones come into play that help the womb to contract during and after labour (oxytocin) as well as stimulate the production and release of breast milk (prolactin).

Our group suggested that these molecules might influence *HLA-G* gene expression, so we investigated the effect of a part of the network of cytokines and hormones involved in the placental or shared tumoral micro-environment on the *HLA-G* transcription of cultured trophoblast explants (Fig. 3). As a result, we observed that the amounts of *HLA-G* transcripts increased approximately 7.2-fold following treatment with IL-10 [123] and IL-1β, and from approximately 1.6- to fivefold with glucocorticoid hormones [124], TGF-β, EGF, β-oestradiol, prolactin and IFNs in comparison with *HLA-G* transcript levels in untreated trophoblasts. Besides, IL-10, a major suppressor of the immune response and inflammation [125], was demonstrated to increase HLA-G mRNA and cell surface protein expression by monocytes [123] and renal cell carcinoma (RCC) cell lines [126], *HLA-G* mRNA and soluble HLA-G protein by mononuclear cells from patients suffering from non-Hodgkin lymphomas (T-NHL) [127], acute myeloblastic leukaemia (AML) and acute lymphoblastic leukaemia (B-ALL), and HLA-G protein cell surface expression by decidual stromal cells [49] and the FON melanoma cell line [60]. Notably, reduced placental IL-10 production occurs in human pathological pregnancies such as in pre-eclampsia [128] in which a defect in HLA-G transcription is observed [114, 129]. Likewise, IL-10 production in cancer is associated with *HLA-G* expression [130] and the IL-10 homolog produced by cytomegalovirus (CMV) can up-regulate HLA-G protein expression at the monocyte cell surface [131]. On the other hand, GM-CSF treatment combined with IFN-γ and/or IL-2 has been shown to enhance both *HLA-G* mRNA and soluble

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HLA-G protein in mononuclear cells from patients suffering from T-NHL [127], AML and T-ALL [132]. Moreover, cell surface expression on the U937 monohistiocyte cell line was demonstrated, but the effect on the *HLA-G* transcription was not investigated [133, 134] (Table 2).

Yet, although preliminary data show a clear potential effect of cytokines, growth factors, and hormone on the modulation of *HLA-G* transcription, some of them require additional investigation and it is likely that a combination of agents might be an interesting and pertinent avenue of research. Moreover, the molecular mechanisms responsible for enhancing *HLA-G* transcription by almost all the modulators described, either alone or in combination, remain to be elucidated. In particular, they could act through promoters of transacting factors instead of direct interaction with the *HLA-G* gene promoter and they also might be involved in RNA stability. Whatever the mechanisms at work, the efficiency of IL-10 or IFN treatment requires basal transcriptional activity to enhance *HLA-G* gene activity. A hypoxic environment is one possible candidate factor to reverse *HLA-G* gene repression.

Hypoxia

Maintenance of oxygen (O_2) homeostasis is critical for the maintenance of life. Hypoxia induces a series of adaptive physiological responses observed in biological processes such as maternal-foetal interactions and cancer, since it is associated with cell proliferation. Hypoxia followed by normoxia regulates the depth of the trophoblastic invasion and the vascular remodelling of the uterine tissues [135]. Tumoral hypoxia (up to 50–60% of solid tumours) arises as a result of an imbalance between the supply and consumption of O_2 [136]. In response to hypoxic conditions and to restore pO_2 homeostasis, cells instantaneously express a key factor, the hypoxia-inducible factor (HIF), which activates transcription of over 70 genes controlling glycolysis, glucose transport, cell survival and death, cell adhesion, angiogenesis and erythropoiesis [137, 138]. HIF is a heterodimer composed of constitutively expressed HIF-1β and inducibly expressed HIF-1α subunits. Under normoxic conditions (21% O_2), HIF-1α is hydroxylated and targeted by the van Hippel–Lindau tumour-suppressor protein, which upon synthesis causes its rapid degradation by the ubiquitin-26S proteasome pathways. With a low O_2 level ($<$6%) hydroxylation in HIF-1α is inhibited, resulting in a translocation of the subunit into the nucleus, a dimerization with HIF-1β, and binding to the hypoxia responsive element (HRE) (RCGTG consensus sequence) on the promoter of target genes [139]. Besides, HIF is able to interact with the transcriptional co-activators CBP/P300, SRC1, TIF2, which potentiate transactivation [137, 140].

Stress conditions such as heat shock or arsenite treatment were demonstrated to induce *HLA-G* gene transcription in HLA-G$^-$ cells. The effect of hypoxic stress on the *HLA-G* transcription was first assessed by Ferrone's group using the iron chelator desferrioxamine (DFX) to stabilize HIF-1α in HLA-G$^-$ melanoma cells. They found that DFX treatment induces *HLA-G* gene transcription in seven of 13 melanoma cell lines. The effect is dose- and time-dependent and approximatively 16-fold lower than the level of constitutive mRNA in the JEG-3 choriocarcinoma cell line [141]. Our group confirmed these results with the M8 melanoma cell line, thus supporting hypoxia as a candidate micro-environmental factor to reverse *HLA-G* gene repression [142]. The effect on the *HLA-G* mRNA up-regulation was further observed with undifferentiated cytotrophoblasts isolated from first-trimester placenta cultured with different concentrations (20%, 8%, 2%) of O_2 [143] and with Raji (Burkitt's B lymphoma) cells [144]. The computer search analysis of the *HLA-G* gene promoter sequence identified a putative consensus HRE located -243 bp upstream of the ATG. Whether this HRE is functional is still unknown [141].

As for the effect of hypoxia on HLA-G cell surface expression, results differ depending on the cell type or culture conditions. It is likely that post-transcriptional mechanisms may be involved since the lack of HLA-G translation can be observed in cells with hypoxia-induced mRNA [141, 142]. On top of that, the down-regulation of HLA-G expression is reported in cells expressing HLA-G at the cell surface following exposure to low O_2 concentration [145] or DFX [142].

Chromatin remodelling at the *HLA-G* gene locus

The epigenetic control of gene promoters is a critical mechanism in transcriptional regulation since it determines the accessibility and recruitment of regulatory factors to the DNA. Epigenetic modifications involve DNA methylation and histone tail modifications such as acetylation, phosphorylation, methylation, ubiquitylation and sumoylation [146]. Epigenetic processes control implantation, placentation, organ formation and foetal growth. In particular, there is a stepwise decline in DNA methylation from fertilization until the morula stage [147] and this correlates with the activation of *HLA-G* gene transcription [39, 40]. Alteration in epigenetics may contribute to pathological situations such as pre-eclampsia [148] and are widely recognized as contributing to tumorigenesis [149].

Evidence for HLA-G silencing by a DNA methylation process was reported first by Le Bouteiller's group in the HLA-G$^-$ choriocarcinoma JAR cell line [150, 151]. The use of demethylating agents such as 5-azacytidine and 5-aza-2′-deoxycitidine (5-Aza-dC) further demonstrated that the repression of *HLA-G* gene activity in cultured cell lines of various origins is reversed by demethylating treatment [144, 150, 152–155] and is maintained at least 5 days [153]. This treatment may also enhance steady state levels of *HLA-G* mRNA [60, 153] and it directly induces HLA-G protein expression in JAR, Raji (Burkitt's B lymphoma, LCL721.221 (lymphoblastoid B cell) [152], OCM-1A (melanoma) [154] and RCC cells [155] as well as human leukaemia cell lines [156]. The HLA-G inhibitory process seems to be independent of the expression of other HLA-class I and HLA-class II, which are detected differentially according to the cell line. By direct sequencing of bisulphite-treated DNA, one study found no correlation

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Table 2 *HLA-G* transcriptional effectors and effect on HLA-G expression

Effector molecules	Modulation of ***HLA-G*** **gene expression**				References
	mRNA	**Protein**			
		Flow cytometry	**Western blot**	**ELISA/ELIspot**	
Growth factors/cytokines					
EGF	↑ . Trophoblasts	nd	nd	nd	Present paper
GM-CSF + IFN-γ	↑/= . Mononuclear cells from T-NHL, AML,T-ALL	↑ . U937 (histiocytic lymphoma)	↑ . Mononuclear cells from T-NHL	↑/= . Mononuclear cells[m] from AML, B-ALL, T-ALL, T-NHL = . PBMC	[127] [132–134]
IL-1 β	↑ . Trophoblasts	nd	nd	nd	Present paper
IL-2 + IFN-γ	↑ . Mononuclear cells from T-NHL	↑ . U937	nd	↑ . Mononuclear cells from T-NHL	[127] [133]
IL-2 + IFN-γ + GM-CSF	↑ . Mononuclear cells from T-NHL	nd	↑ . Mononuclear cells from T-NHL	↑ Mononuclear cells[m] from B-ALL, T-NHL = . PBMC	[127]
IL-10	↑ . Trophoblasts . Monocytes ↑/= . Mononuclear cells from AML, B-ALL, T-NHL . RCC cell lines	↑ . Monocytes . Decidual stromal cells ↑/= . RCC cell lines = . FON (melanoma)	↑ . Mononuclear cells from T-NHL . Decidual stromal cells	↑ . JEG-3 ↑/= . Mononuclear cells from AML, T-NHL = . PBMC	[49] [60] [123] [126, 127] [132] [162]
IFN-α	↑ . JEG-3 . U937 . Blood monocytes = . JAR	↑ . U937 .THP-1 (acute monocytic leukaemia) = . JEG-3	nd	↑ . JEG-3 . Serum of treated patient with melanoma	[50] [162, 163]
IFN-β	↑ . JEG-3 . U937 . Blood monocytes .Thymic epithelial cells . Amniotic epithelial cells = . JAR	↑ .Thymic epithelial cells . Amniotic epithelial cells ↑ . U937 . THP-1 = . JEG-3	nd	↑ . JEG-3	[50] [99] [162]

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Table 2 Continued

Effector molecules	Modulation of *HLA-G* gene expression				References
	mRNA	Protein			
		Flow cytometry	Western blot	ELISA/ELIspot	
IFN-γ	↑ . JEG-3 . U937 . Blood monocytes . THP-1 . RCC cell lines . Glioblastoma cell lines = . JAR	↑ . U937 . Blood monocytes . THP-1 . HL-60 (acute promyelocytic leukaemia) . Decidual stromal cells . FON (melanoma) . RCC cell lines = . JEG-3	↑ . Decidual stromal cells	↑ . JEG-3 = . PBMC[m] . Mononuclear cells[m] from AML, B-ALL, T-ALL	[49, 50] [60] [123] [126] [162] [164]
LIF	↑ . JEG-3	↑ . JEG-3 = . FON	nd	↑ . JEG-3	[60] [108] [109]
TGF-β	↑ . Trophoblasts	↓ . FON	nd	nd	Present paper [60]
TNF-α/PMA	= . JEG-3	↓ . JEG-3 . FON . M8-HLA-G1 (Melanoma transfectant)	↑ . JEG-3 . FON . M8-HLA-G1	↑ . M8-HLA-G1	[34]
Hormones					
β-oestradiol	↑ . Trophoblasts	nd	nd	nd	
Progesterone	↑ . JEG-3	nd	↑ . JEG-3 .Cytotrophoblasts	↑ . JEG-3 . Trophoblasts	[104, 105] Present paper
Progesterone +cAMP	nd	↑ . Decidual stromal cells	↑ . Decidual stromal cells	nd	[49]
Glucocorticoids	↑ . Trophoblasts	nd	nd	nd	Present paper
Prolactin	↑ . Trophoblasts	nd	nd	nd	Present paper
Stress					
Arsenite	↑ . M8 [a] (melanoma) . T98G [a] (glioblastoma)	= . M8 . T98G	nd	nd	[103]

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Table 2 Continued

Effector molecules	Modulation of *HLA-G* gene expression				References
	mRNA	Protein			
		Flow cytometry	Western blot	ELISA/ELIspot	
Heat shock	↑ . M8 [a] . T98G [a] . JAR . Raji	= . M8 . T98G	nd	nd	[103] [141] [144]
Hypoxia/DFX/CoCl2	↑ . Extravillous cytotrophoblasts ↑ . Melanoma cells (M8 [a], 1074mel [a]) . Raji [a] (Burkitt's B lymphoma) = . JAR	↓ . HTR-8/SVneo on matrigel (first-trimester cytotrophoblast) . FON . JEG-3	↓ . HTR-8/SVneo on matrigel	nd	[142] [144, 145] [165]
Epigenetic treatments					
5-azacytidine/5-aza-2′deoxycytidine	↑ . JAR [a] . FON . Melanoma cell lines (OCM-1a[a], M8 [a], FON) . Glioblastoma (U87MG, LN-229, LN-428) . B,T and myelo-monocytic Leukaemia cell lines. . BG-1 [a] (ovarian cancer cells) ↑/= . RCC [a] cell lines ↑ . Tera-2 (lung embryonic carcinoma)	↑ . JAR . FON . Raji . LCL721.221 (lym-phoblastoid B cells) = . NKL (NK cell leukaemia) . KG1a (acute myel-ogenous leukaemia) . M8	↑ . JAR . Raji . LCL721.221 = . NKL . KG1a . M8	↑/= . RCC cell lines	[60] [126] [144] [150] [152–154] [156] [158]
NaBu/TSA/VA	↑ . M8 . JAR . Raji	nd	nd	nd	[144] [152]

ALL: acute lymphoblastic leukaemia; AML: acute myeloblastic leukaemia; DFX: desferrioxamine; NHL: Non-Hodgkin lymphoma; PBMC: peripheral blood mononuclear cells and VA: valproic acid.
[a]: Raise HLA-G gene repression.
[m]: Mean of several experiments.
↑: up-regulation (↑ : low).
↓: down-regulation (↓ : low).
=: no effect.
↑/=: up-regulation or no effect.
↓/=: down-regulation or no effect.
nd: not determined.

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between *HLA-G* gene transcriptional activity and methylation of 63 CpG islands at the *HLA-G* locus in blood cells expressing ($CD2^+$ lymphocytes) or not expressing ($CD34^+$ haematopoietic cells) *HLA-G* mRNA [157]. Nonetheless, recent data focussing on the promoter region covering 450 bp 5′ from the ATG (19 CpG sites) strongly argue for a cis-acting CpG methylation associated with *HLA-G* gene silencing [153, 154, 158]. Moreover, the analysis of chromatin remodelling at the *HLA-G* locus performed with histone deacetylase inhibitors (HDAC), trichostatin A (TSA) and sodium butyrate (NaBu) treatments, demonstrated the *HLA-G* gene activation in M8 (melanoma), JAR and Raji cells, despite a lower mRNA level in comparison with 5-Aza-dC treatment [144, 152]. To back this up, the analysis of H3 and H4 histone acetylation by ChIP of the proximal and distal *HLA-G* gene promoter showed the presence of hyperacetylated histones in $HLA\text{-}G^+$ cells (FON and JEG-3 cells), while hypoacetylated histones were predominant in $HLA\text{-}G^-$ cells (M8 and JAR cells) [153]. Thus, the acetylation of lysine residues of H3 and H4 renders the chromatin in a permissive state, arguing in favour of *HLA-G* gene expression.

Concluding remarks

The present data highlight the complexity of the regulation of non-classical *HLA-G* transcriptional activity, which is likely to be associated with the tight control of HLA-G function participating in immune tolerance. Several regulatory elements have now been identified, but some of them need further functional validation using reporter genes and interference RNA tools. The demonstration of their presence or absence *in situ* using ChIP assays is also required since the chromatin environment is crucial for binding and function. Cytokines and micro-environmental agents act on *HLA-G* gene expression in a cell-specific manner and may have pleiotropic activities. From this point of view, the medical relevance of the current findings on the HLA-G regulation should be carefully evaluated. In this regard, the development of animal models would make it possible to validate *in vivo* both the concept that HLA-G is a key component in immunoregulation and the molecular mechanisms modulating its expression. Moreover, an emerging point of view is that the impact of *HLA-G* gene polymorphism and temporal data on regulatory processes are needed to understand modifications at the *HLA-G* locus, particularly epigenetic changes following micro-environmental signals during placentation and various pathological situations.

Considering all the regulatory mechanisms known to date, they support a coherent regulatory model of *HLA-G* gene expression based on those previously proposed by Ferrone's group [159]: HLA-G is generally not expressed under non-pathological conditions *in vivo*, probably because the promoter is inactivated by DNA methylation and at least histone hypoacetylation. During *in vivo* proliferative processes, cells will be exposed to stress (*e.g.* hypoxia) and undergo epigenetic changes such as DNA demethylation and histone acetylation, leading to opened chromatin and accessibility to transcription factors. It is likely that sequence variations at specific transcription factor target sites influence the level of response. Upon gene activation, cytokines and hormones of the micro-environment will enhance the amounts of *HLA-G* transcripts and then protein expression. The micro-environment could also induce antigen-processing machinery components, thus contributing to the transport and stabilization of HLA-G molecules at the cell surface. TNF-α may enhance intracytoplasmic HLA-G cell content and may enhance HLA-G1 proteolytic shedding following NF-κB activation. Upon adaptation to tissue culture *in vitro*, cells may not be exposed to the same stressful conditions and *HLA-G* transcription may be changed. Thus long-term growth *in vitro* may subsequently lead to methylation and hypoacetylation of the *HLA-G* promoter and silence the gene.

Finally, the fact that demethylation treatment may activate *HLA-G* receptor genes [160, 161] should be considered in cancer therapy using treatments blocking HDAC and/or reversing DNA methylation to enhance tumour suppressor genes. These treatments might favour the enhancement of both *HLA-G* at the cell surface of tumoral cells, and KIR protein expression at the cell surface of tumour infiltrating lymphocytes might thus favour tumour escape. Therefore, in addition to the crucial need to better understand HLA-G function, extensive studies on the control of *HLA-G* gene expression are fundamental to developing non-deleterious therapeutic strategies.

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