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How Micrornas Affect the Expression of Human Leukocyte Antigeng in Pregnancy

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ABSTRACT

The expression of Human Leukocyte Antigen G (HLA-G) on Fetal Extravillous Trophoblast (EVT) cells during pregnancy plays an important role in preventing the fetus from rejection by suppressing the maternal immune system. Decreased expression levels of HLA-G have already shown to be associated with several complications of pregnancy such as pre-eclampsia. However, it remains largely unknown how HLA-G gene expression is regulated with regard to its function and its complications. Polymorphisms and microRNAs affect HLA-G gene expression and the formation of isoforms. Interestingly, three microRNAs, miR-148a, miR-148b and miR-152, downregulate HLA-G expression with functional consequences. Since HLA-G expression levels are reduced in pre-eclampsia without a known cause, we hypothesize that these microRNAs are involved in the development of pre-eclampsia. This review discusses how microRNAs can affect HLA-G gene expression and its functions. Additionally, the role of microRNAs in the development of pre-eclampsia will be reviewed.

Keywords: microRNA, HLA-G, Pre-Eclampsia, miR-148, miR-152

1. INTRODUCTION

In pregnancy, the semi-allogenic fetus expresses both maternal and paternal antigens. As a consequence, the mother induces an immune reaction against the paternal antigens of the fetus. To prevent the fetus from rejection, tolerance of the maternal immune system is induced by fetal trophoblast cells. Fetal Extravillous Trophoblast (EVT) cells lack the expression of classical Major Histocompatibility Complex (MHC) Ia except for a small amount of HLA-C (Ellis *et al*., 1986; King *et al*., 2000). However, EVT cells are able to suppress an immunological reaction by the expression of Human Leukocyte Antigen G (HLA-G) (Chumbley *et al*., 1993; Ellis *et al*., 1986; Kovats *et al*., 1990). In addition to the ability of repressing the maternal immune system, fetal EVT cells are also able to invade the maternal decidua in order to remodel the uterine arteries (Huppertz *et al*., 2012). This transforms the spiral arteries to large capacity tubes which provide the fetus with nutrients, oxygen and it removes carbon dioxide and waste products (Huppertz *et al*., 2012; Kumpel and Manoussaka, 2012).

HLA-G is a non-classical MHC class Ib molecule located on chromosome 6. It consists of 8 exons of which exons 2, 3 and 4 encode for the α1, α2 and α3 regions respectively. The α1 and 2 regions bind peptides in the peptide binding cleft whereas the α3 region is responsible for binding the co-receptors Immunoglobulin-Like Transcript-2 (ILT2) receptor and ILT4 (Clements *et al*., 2005). Moreover, the α3 region of HLA-G has an increased hydrophobicity compared to the classical MHCs. This can possibly enhance the affinity of the ILT receptors to HLA-G (Clements *et al*., 2005).

Seven isoforms of HLA-G can be made via alternative splicing (Ishitani and Geraghty, 1992). In total there are 4 membrane-bound (HLA-G1-G4) and 3 soluble (HLA-G5-G7) HLA-G molecules (Fujii *et al*., 1994; Ishitani and Geraghty, 1992; Kirszenbaum *et al*., 1994; Moreau *et al*., 1995; Paul *et al*., 2000). Depending on the translation of the mRNA, these isoforms can be either membrane-bound or soluble (sHLA-G). Upon translation, exon 5, which encodes for the transmembrane region, will be incorporated and membrane-bound isoforms will be made. However, a

stop codon in intron 4 will block further mRNA translation and prevents incorporation of exon 5 thereby creating soluble isoforms (Fujii *et al*., 1994). In addition, soluble HLA-G5 can also be generated by proteolytic shedding of HLA-G1 via metalloproteinases (Park *et al*., 2004).

In contrast to the other isoforms, both HLA-G1 and HLA-G5 contain β2-Microglobulin (β2M) which is specifically recognized by the ILT2 receptor (Gonen-Gross *et al*., 2005). The inhibitory receptors ILT2, ILT4 and the Killer Immunoglobulin-like Receptor 2DL4 (KIR2DL4) play an important role in the recognition of HLA-G by immune cells (Colonna *et al*., 1998; Navarro *et al*., 1999; Rajagopalan and Long, 1999). HLA-G inhibits Natural Killer (NK) cell-mediated cytolysis, cytotoxic CD8+ T-cells, T-cell alloproliferation, the maturation of dendritic cells and proliferation of T-lymphocytes and NK-cells through the interaction with these receptors (reviewed in Carosella *et al*., 2008).

HLA-G is one of the major key players in fetal-maternal immune regulation. Expression levels are especially high in the first trimester whereas late term placentas have decreased levels of HLA-G (Yelavarthi *et al*., 1991). Despite the available amount of information, it is still largely unknown how HLA-G expression is regulated in relation to its functions and complications. Nowadays, it is thought that HLA-G can be involved in certain complications during pregnancy such as pre-eclampsia (Hviid, 2006). Additionally, it is seen that alterations in HLA-G expression can have functional consequences.

This review discusses the role of polymorphisms and microRNAs on HLA-G gene expression. Moreover, the review focuses on the effect of microRNAs on HLA-G gene expression. In addition, the possible role of microRNAs in the development of pre-eclampsia will be discussed.

1.1. Polymorphisms and MicroRNAs can Effect HLA-G Gene Expression

1.1.1. Polymorphisms

Unlike the classical MHCs, HLA-G exhibits low polymorphism. HLA-G polymorphisms are present in the coding region of HLA-G, the 5' Upstream Regulatory Region (URR) and the 3' Untranslated Region (UTR). It is thought that polymorphisms can influence HLA-G gene expression because these may affect the expression of certain isoforms and the binding of nuclear factors to regulatory regions. Mechanisms involved in the generation of polymorphisms are point mutations, gene conversions and recombination (Cervera *et al*., 2010).

1.2. Coding Region

To date, 49 HLA-G alleles in the coding region are acknowledged by the WHO Nomenclature Committee for Factors of the HLA System (WHONCFHLAS, 2012). Of these alleles, there are 15 which display HLA-G protein polymorphisms and 2 which are null alleles. Most of the Single Nucleotide Polymorphisms (SNPs) are found in exon 2, 3 and 4 (**Table 1**). Some polymorphisms are able to change the amino acids. For example, in allele HLA-G*01:03 at codon 31 the Adenine (A) can be substituted by a Tyrosine (T) which creates a serine (ser) instead of a threonine (thr). Other substitutions, mostly at the third base of a codon, may be nonsynomous where there is no change of amino acids.

However, not all polymorphisms are substitutions. The HLA-G*01:05N null allele has a deletion of a Cytosine (C) at the last base of codon 129 or the first base of codon 130 in exon 3 (Ober *et al*., 1998; Suarez *et al*., 1997). This causes a frameshift which leads to the formation of a stop codon at codon 189. Hence, the formation of the α2 region will be altered. Consequently, there will be incomplete formation of HLA-G1, -G4 and -G5 but not of the isoforms that do not contain the α2 region (Discorde *et al*., 2005; Ober *et al*., 1998). Remarkably, individuals homozygous for the HLA-G*01:05N mutation are still able to have a term pregnancy without the presence of three different isoforms (Casro *et al*., 2000; Ober *et al*., 1998). Indeed, Discorde and co-workers have confirmed the suggestion that other isoforms can compensate for the loss of HLA-G1,-G4 and-G5 and they have proven to be redundant in homozygous individuals (Discorde *et al*., 2005).

The other null allele, HLA-G*01:13N, is defined by a C to T substitution in the first base of codon 54 (Lajoie *et al*., 2008). This mutation causes a premature stop codon in exon 2, the α1 region. As a consequence, HLA-G will be truncated and probably even nonfunctional. Because of its position in exon 2, the mutation will affect all HLA-G isoforms. Therefore it is not expected to find homozygous individuals for the HLA-G*01:13N allele (Mendes-Junior *et al*., 2010).

Table 1. Overview of polymorphisms in the HLA-G coding region. Data obtained from the (WHONCFHLAS, 2012). Inspiration obtained by (Hviid 2006) and (Moscoso *et al*., 2007). HLA-G alleles are present in the left column and the exons are depicted in the top row. Single nucleotide polymorphisms (SNPs) that alter the amino acids have the name of the new amino acid written below. SNPs without an amino acid written below are silent polymorphisms.

	EXON 2					EXON 3										EXON 4					EXON 5	
HLA-G alleles	13	27	31	54	57	93	100	104	105	107	110	130	159	169	171	188	189	219	236	258	290	309
HLA-G*01:01:01:01 #HLA-G*01:01	TCC Ser	TAC Tyr	ACG Thr	CAG Gln	CCG Pro --A	CAC His --T	GGC Gly	GGG Gly	TCC Ser	GGA Gly --T	CTC Leu	CTG Leu	TAC Tyr	CAC His	TAC Tyr	CAC His	GTG Val	CGG Arg	GCA Ala	ACG Thr	GGC Gly	AGA Arg --G
HLA-G*01:02				-G- Arg															--C			
HLA-G*01:03			T-- Ser													--T						--G
HLA-G*01:04					--A						A- - Ile											--G
HLA-G*01:05N					--A	--T						xTG del					TGA stop				--T	--G
HLA-G*01:06					--A	--T														-T- Met	--T	--G
HLA-G*01:07	-T- Phe				--A						A-- Ile											--G
HLA-G*01:08					--A	--T												T-- Trp				--G
HLA-G*01:09					--A	--T							C-- His									
HLA-G*01:10			-T- Met																			
HLA-G*01:11			-T- Met		--A						A- - Ile											
HLA-G*01:12		C-- His			--A																	
HLA-G*01:13N				T-- Stop	--A	--T																
HLA-G*01:14					--A		-A- Asp											-A- Gln		-T- Met		
HLA-G*01:15					--A			-T- Val			A- - Ile											
HLA-G*01:16					--A	--T			-G- Cys											-T- Met		
HLA-G*01:17					--A	--T								-G- Asp	C-- His							

In total, there are 28 variants of the HLA-G*01:01 allele which could not be represent all in this table. Therefore the most common polymorphisms are depicted here under HLA-G*01:01.

1.3. Non-Coding Region

The promoter region of HLA-G (5'URR) contains many regulatory elements to provide optimal mRNA synthesis. So far, 29 SNPs are identified within the 5'URR region that may affect binding of nuclear factors to the regulatory elements since the SNPs and regulatory elements are closely situated to each other (reviewed in (Donadi *et al*., 2011)).

Polymorphisms are present in the regions -487 to -475, -754 to -735 and -1201 to -1100 which are associated with the heat shock protein element, interferon-stimulated response element and trophoblast-specific response elements respectively (Pyo *et al*., 2006). Another SNP is the -725 Guanine (G)/C/T polymorphism which is thought to influence NFκβ binding and transcriptional activity (Donadi *et al*., 2011). More importantly, a linkage disequilibrium of the 5'URR with the 3'UTR has been described which can influence the RNA stability of HLA-G (Donadi *et al*., 2011; Rousseau *et al*., 2003).

In addition to the 5'URR there are also polymorphisms associated with the 3'UTR. This region has regulatory sequences including the polyA signal and microRNA binding sites. The most frequent polymorphism of the 3'UTR is the 14bp deletion/insertion in exon 8 (Harrison *et al*., 1993). Interestingly, the insertion of 14 base pairs (bp) at the 3'UTR creates an additional splice event in which the first 92bp of exon 8 are spliced out (Hiby *et al*., 1999). Isoforms with this +14bp insertion have decreased relative expression levels of HLA-G compared to isoforms with a -14bp deletion (Hviid *et al*., 2003). Nonetheless, the removal of these 92bp positively influences the stability of the mRNA. Even though mRNAs with a +14bp polymorphism express low HLA-G levels, they have a better resistance to mRNA degradation (Rousseau *et al*., 2003). Therefore, HLA-G transcripts will be preserved within the cell and more HLA-G can be transcribed. Additionally, the +14bp insertion was also found to be associated with a lower expression of sHLA-G (Chen *et al*., 2008). This indicates that the 14bp deletion/insertion may greatly influence HLA-G gene expression. Furthermore, there are two SNPs located at +3142 and +3187 in the 3'UTR

which may be involved in the regulation of expression levels of HLA-G. The +3142 SNP has a C to G substitution which can influence microRNA targeting (Tan *et al*., 2007). This is further discussed in the section 'MicroRNAs'. Furthermore, the +3187 polymorphism is a G to A mutation which decreases the stability of the mRNA and is associated with pre-eclampsia (Yie *et al*., 2008). In conclusion, the polymorphisms of the coding and non-coding regions discussed here are thought to greatly influence the gene expression of HLA-G. It is seen that some polymorphisms may affect the expression of different HLA-G isoforms or the stability of the mRNA. In addition, some polymorphisms are associated with complications in pregnancy (Hviid, 2006). However, it is still not exactly known what effect these polymorphisms have on the function of HLA-G.

1.4. MicroRNAs

MicroRNAs are small non-coding RNA molecules of about 22 nucleotides which can negatively regulate gene expression (Bartel, 2004). Binding of miRNAs to the 3'UTR of a gene can result in the suppression of translation or in the degradation of mRNA. Recently, it has been suggested that microRNAs (miRNAs) may play a role in the regulation of HLA-G gene expression (Veit and Chies, 2009). Additionally, there is a C to G polymorphism at position +3142 which has been suggested to influence miRNA targeting to the 3'UTR region of HLA-G (Tan *et al*., 2007). Moreover, three miRNAs have been found that can bind in the 3'UTR end of the HLA-G gene and can inhibit its gene expression. These are: miR-148a, miR-148b and miR-152 (Tan *et al*., 2007). It has been shown that both miR-148a and miR-152 can downregulate the expression of HLA-G and reduce the binding of the inhibitory receptor ILT2 to HLA-G cells (Manaster *et al*., 2012; Zhu *et al*., 2010). As a consequence, NK cell-mediated cytotoxicity is increased. Further, it is predicted via in silico analysis that more miRNAs can bind to other SNPs in the 3'UTR, indicating that other miRNAs might be possible regulators of HLA-G as well (Castelli *et al*., 2009). These studies provide evidence that miRNAs can be important regulators of HLA-G gene expression in addition to the earlier described polymorphisms.

1.5. miR-148a, miR-148b and miR-152 Expression

MicroRNAs bind to a seed region, which is suggested to be important in target recognition. This region consist of 2-7 nucleotides that are perfectly complementary to that of the mRNA. The seed region of miR-148a and miR-148b is the same and the predicted binding energies are similar (Manaster *et al*., 2012; Tan *et al*., 2007). Therefore it is thought that these two miRNAs have similar activity. Furthermore, in all three miRNA (miR-148a, miR-148b and miR-152) seed regions, the +3142 C to G SNP is located. This SNP was suggested to influence HLA-G expression since the binding energies of the C variant were lower than the G variant (Tan *et al*., 2007). Though binding of miRNAs to the G variant is more stable and expresses lower HLA-G expression. Additionally, the +3142G genotype of the mother showed to be protective against the development of asthma in their children (Tan *et al*., 2007). Contrarily, Manaster and co-workers showed that the +3142 polymorphism does not influence the in vitro miRNA targeting of HLA-G (Manaster *et al*., 2012). Both alleles (+3142C or G) are evenly distributed throughout the population (TIHC, 2003). Thus in order to investigate if the +3142C to G polymorphism influences the expression of HLA-G and thereby also the clinical outcome of a pregnancy, the genotype of normal and complicated pregnancies have to be determined.

All three suggested miRNAs are able to bind to their target site within the 3'UTR of HLA-G (Manaster *et al*., 2012; Tan *et al*., 2007; Zhu *et al*., 2010). In order to bind to HLA-G, microRNAs must be expressed in EVT cells. Zhu and co-workers showed that low levels of miR-152 are expressed in the HLA-G high JEG3 cell line (Zhu *et al*., 2010). Since JEG3 is a human placenta choriocarcinoma cell line that is used to study EVT cells in vitro it can be assumed that results will resemble in vivo situations. In addition, the expression levels of miR-148a and miR-148b were tested in both primary Cytotrophoblast Cells (CTB) and JEG3 cells (Manaster *et al*., 2012; Tan *et al*., 2007). Both cell types showed low expression levels of miR-148a and miR-148b. Although the in vitro expression of these miRNAs is low, it is likely that they can still be produced in EVT cells. Moreover, it is expected to find low microRNA expression in uncomplicated pregnancies. In fact, the expression of microRNAs should be low during pregnancy because they are likely to negatively regulate HLA-G expression and thus lead to complications. Furthermore, Dicer, a protein involved in the miRNA processing machinery, was found to be present in villous and extravillous trophoblast cells, whereas it was absent in syncytiotrophoblast cells (Forbes *et al*., 2012). All together this is an indication that miR-148a, miR-148b and miR-152 can be expressed and produced in EVT cells.

The origin of miR-148a, miR-148b and miR-152 can be either canonical or intronic. In the canonical pathway the miRNA is transcribed from its own promoter and forms a hairpin structure after which it is processed in the nucleus and cytoplasm (Miyoshi *et al*., 2010). A minority of microRNAs are intronic (mirtrons). Mirtrons are found in the introns of pre-mRNAs and are not transcribed from their own promoter but instead expressed from their host transcript (Bartel, 2004). No co-expression of miR-148a is expected to be found since there is no specific gene located near this miRNA. On the contrary, miR-148b and miR-152 are located within the coatomer protein complex subunit zeta 1 (COPZ1) and COPZ2 respectively. miR-152 is located within an intron of COPZ2 and therefore it can be thought that this miRNA can be simultaneously expressed with the COPZ2 transcript. Indeed, it was found that the expression of these two transcripts are similar in human endometrial cancer cell lines (Tsuruta *et al*., 2011). Therefore it can be suggested that miR-152 is regulated via the intronic pathway. In contrast, miR-148b had no coherent expression with its host gene COPZ1 (Liang *et al*., 2007). This indicates that miR-148b, just like miR-148a, is most likely regulated via the canonical pathway.

Several articles already showed that the in vitro binding of miR-148a, miR-148b and miR-152 to the 3'UTR of HLA-G resulted in a decreased expression of HLA-G protein. However, the mechanism of this inhibition is still an issue of discussion. Manaster and coworkers show that mRNA degradation is involved, whereas Zhu and coworkers show inhibition of translation is the responsible mechanism. It is possible that miRNA mediated repression of HLA-G expression functions via both mechanisms. The miRNA will act via mRNA cleavage or inhibition of translation depending on the amount of complementary sequence of the mRNA (Bartel, 2004).

Conclusively, it is plausible that miR-148a, miR-148b and miR-152 influence HLA-G expression. The microRNAs are likely to be expressed and processed in EVT cells. Genotypes of normal and complicated pregnancies have to be determined to examine if the +3142C to G polymorphism can additionally influence HLA-G expression. More research is needed to investigate which mechanism is involved in HLA-G repression. In addition, it is important to examine when during pregnancy these miRNAs are expressed to identify the impact that miRNAs can have on gene expression and consequently on pregnancy.

1.6. Functional Consequences of HLA-G Targeting by microRNAs

Binding of miR-148a, miR-148b and miR-152 to HLA-G resulted in a decreased (s)HLA-G expression. Since the 3'UTR is present in all HLA-G isoforms, it is likely that miRNAs can regulate the expression of all HLA-G isoforms. This altered HLA-G expression can have functional complications. As described earlier, repression of HLA-G by miRNAs diminished the interaction with ILT2 and enhanced NK cell-mediated killing (Manaster *et al*., 2012). Another important function of HLA-G in protecting the fetus besides inhibition of NK-cell mediated cell death/apoptosis, is the inhibition of Cytotoxic Lymphocyte (CTL) responses. HLA-G is able to suppress the allocytotoxic T lymphocyte responses against paternal antigens of the fetus in vitro (Kapasi *et al*., 2000). This induces an protective Th2 response in pregnancy, in which IL-10 is upregulated and the expression levels of TNF-α and IFN-γ are reduced. However, it was found that lower levels of HLA-G were unable to suppress CTLs to the same extent as normal HLA-G levels and thereby it promotes the Th1 response. This can have serious consequences for pregnancy. Therefore it is essential to examine if microRNAs can be involved in the inhibition of CTL suppression via the reduced expression of HLA-G. Furthermore, it would be interesting to investigate if other functions of HLA-G are impaired as well because of the induction of microRNAs.

An in silico screening of miRNAs that are potentially involved in the regulation of HLA-G revealed that miR-19a had the exact same binding energy as miR-148a, miR-148b and miR-152 and also has a target site which included the +3142 polymorphism (Castelli *et al*., 2009). This indicates that miR-19a can have an important function in the regulation of HLA-G as well. Recently, miR-19a expression was found to be upregulated by shear stress in human umbilical cord endothelial cells (Qin *et al*., 2010). The expression of this miRNA inhibited the cell cycle of endothelial cells. It would be interesting to test if miR-19a can also have a potential role in the regulation of HLA-G in EVT cells. This can be done by verifying miR-19a binding to HLA-G and measuring the activity of HLA-G in the presence of miR-19a. Additionally, it is interesting to investigate if miR-19a could be associated to cell cycle processes in EVT cells as well. It might be that miR-19a is involved in vascular remodeling.

Several functions of HLA-G can be diminished upon low expression of HLA-G. However, the cause of the reduced HLA-G expression is still unknown and it is possible that microRNAs are involved. The NK cell-mediated cell death has shown to be enhanced through inhibition of HLA-G by miRNAs. Here, we suggest that CTL mediated cell death could also be regulated via the same mechanism. Additionally, miR-19a would be a good candidate for further investigation of its involvement in HLA-G expression and of its functions.

1.7. The Involvement of microRNAs in Pre-Eclampsia

Reduced expression of HLA-G by the negative regulation of miRNAs may influence the amount of sHLA-G and membrane-bound HLA-G during pregnancy. It is possible that miRNAs indirectly can cause complications in pregnancy, such as pre-eclampsia. Naturally, this will depend on several factors such as the gestational age of the fetus, which miRNAs are expressed and when during pregnancy these miRNAs are expressed.

Pre-eclampsia is a pathological condition that can occur from 20 weeks of gestation onwards in which maternal blood pressure rises and oedema and proteinuria develop (Duley, 2009). This condition occurs in 5-8% of all pregnancies and can lead to eclampsia. Both pre-eclampsia and eclampsia are nowadays still a major cause of maternal and perinatal mortality and morbidity. Currently, there are a few interventions to prevent pre-eclampsia but there is no treatment except for the termination of pregnancy or delivery (WHO, 2012).

During pregnancy invasive EVT cells are able to remodel the uterine arteries in order to have a high flow and low resistance blood supply to the fetus (Huppertz *et al*., 2012). It has been found that in pre-eclampsia EVT cells start to differentiate but cannot complete differentiation. Therefore EVTs are less able to invade the uterine arteries and as a consequence, the arteries will have a low flow and high resistance (Lim *et al*., 1997). This inadequate blood supply to the fetus can eventually result in poor growth or prematurity. Although the exact cause of pre-eclampsia is not determined yet, there are indications that many factors are involved. One of these factors is the genetic background of HLA-G expression.

In a pre-eclamptic pregnancy, the expression of HLA-G is much lower compared to control pregnancies at both mRNA and protein level (Hara *et al*., 1996; Lim *et al*., 1997; O'Brien *et al*., 2001; Yie *et al*., 2004; Zhu *et al*., 2012). The exact cause of this reduction in HLA-G level has yet to be determined. Considering that miRNAs negatively regulate HLA-G expression, it can be hypothesized that miRNAs are involved in the development of pre-eclampsia. To test this hypothesis, sHLA-G and miR-148a, miR-148b and miR-152 levels need to be tested from the blood of normal and pre-eclamptic pregancies.

There are already some indications that certain microRNAs are involved. Several studies aimed to identify miRNAs involved in pre-eclampsia by comparing miRNA expression between normal and pre-eclamptic placentas (Mayor-Lynn *et al*., 2011; Pineles *et al*., 2007; Zhu *et al*., 2009). Each study found various miRNAs that were either up- or downregulated in pre-eclamptic placentas. Zhu and coworkers were able to identify almost a two-fold increase of miR-152 expression in pre-eclamptic placentas (Zhu *et al*., 2009). This is in line with the suggestion that miR-152 could be involved in the development of pre-eclampsia by the downregulation of HLA-G. However, they also found a decreased expression of miR-19a. Although this conflicts with our hypothesis that we discussed earlier, it does acknowledge the suggestion that miR-19a can be involved in vascular remodeling. Additionally, other microRNAs with other targets than HLA-G are found to be involved in the modulation of angiogenesis in Human Umbilical Vein Endothelial Cells (HUVEC) (Poliseno *et al*., 2006). This implies that more miRNAs might play a role in the vascular organization in pre-eclampsia.

One specific microRNA found in all three studies is miR-210 (Mayor-Lynn *et al*., 2011; Pineles *et al*., 2007; Zhu *et al*., 2009). In two studies, miR-210 was found to be increased by a three-fold in (severe) pre-eclampsia, while Lynn and coworkers found a small downregulation. In another study of miR-210 it was found that this microRNA is overexpressed in hypoxic conditions and causes a reduction in the formation of capillary-like structures (Fasanaro *et al*., 2008). Furthermore, it decreases endothelial cell migration and survival. Since hypoxia is an important hallmark of pre-eclampsia, this can indicate that miR-210 is involved in the development of pre-eclampsia.

Pre-eclampsia is caused by the underdevelopment of the uterine arteries and it can be thought that both a

change in NK cell-mediated killing and the enhanced CTL responses can be responsible for this complication. Kaposi and coworkers already suggested that CTL responses may influence the development of pre-eclampsia. In pre-eclamptic pregnancies there is a Th1 response whereas normal pregnancies are characterized by a Th2 response. The Th1 response, which results from the enhanced allocytotoxic responses, is an indication that this function of HLA-G might be involved in the development of pre-eclampsia.

With regard to the suggestion that miRNAs are involved in the development of pre-eclampsia and the in vitro results that show an increase in NK cell-mediated cell death in the presence of miRNAs, it can be hypothesized that there will be a difference in NK cell-mediated cell death between normal and pre-eclamptic placentas. At the moment, there is no consensus yet about the NK levels in normal versus pre-eclamptic placentas. Borzychowski and coworkers found a significant increase in the NK1/NK2 ratios in pre-eclamptic pregnancy versus normal pregnancy (Borzychowski *et al*., 2005). On the other hand, Sánchez-Rodríguez and coworkers found no differences in NK cells from pre-eclamptic and normal pregnancies (Sanchez-Rodriguez *et al*., 2011). More research has to be done to identify the proportion and activity of NK cells in pre-eclampsia. In order to investigate if miRNAs are involved, the activity and amount of NK cells in pre-eclamptic and normal placentas should be tested together with the levels of miRNA expression in the same samples.

To summarize, microRNAs can be potentially involved in the aberrant gene expression of HLA-G in pre-eclampsia. We hypothesize that miR-148a, miR-148b and miR-152 can be associated with pre-eclampsia via the downregulation of HLA-G. We also suggest that both NK cell-mediated cell death and the activation of CTL responses, by the reduced expression of HLA-G via miRNA binding, can cause the development of pre-eclampsia.

2. CONCLUSION

The HLA-G gene expression of fetal EVT cells can be affected by both polymorphisms and microRNAs. Until recently, there was not much focus on the involvement of microRNAs in pregnancy. However, last years it becomes apparent that the placenta is an important site for miRNA expression and that they might be important regulators in placental development. Recently, miR-148a, miR-148b and miR-152 have found to bind the 3'UTR of HLA-G and reduce the expression of HLA-G. Reduced expression levels of HLA-G have shown to have functional effects. Further research should focus on the consequences of this microRNA dependent HLA-G suppression. It was already shown that NK cell-mediated killing is enhanced. Nevertheless, it can be thought that other functions of HLA-G, for example CTL mediated cell death, are altered. In addition, more microRNAs, such as miR-19a, can be tested for their involvement in HLA-G expression and vascular remodeling.

Reduced expression levels of HLA-G were observed in pregnancies with pre-eclampsia and (recurrent) spontaneous abortions. This indicates that HLA-G is an important factor in the development of pregnancy disorders. Pre-eclampsia is characterized by the reduction of HLA-G and the induction of a Th1 response. Here, we hypothesize that miRNAs may be one of many factors in involved in the development of pre-eclampsia since miRNAs have proven to reduce the HLA-G expression. Examination of the role of miR-148a, miR-148b and miR-152 and other miRNAs in HLA-G expression and in pathological conditions of pregnancy are needed to confirm this suggestion. This information will eventually create a better understanding of the tightly regulated mechanisms during pregnancy.

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