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The identification of molecular elements regulating the Human Leukocyte Antigen C in human trophoblasts

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Abstract

Limited studies have addressed the importance of Human Leukocyte Antigen C (HLA-C) in the matters of pregnancy but as HLA-C is the only polymorphic HLA expressed on Extravillous trophoblasts (EVT) it actually might be the most important molecule which we should investigate. This thesis focuses on HLA-C expression on EVT and will also discuss HLA-G and HLA-E to a great extent as HLA-G expression is rather EVT specific and HLA-E is able to express HLA-G's leader peptides. The maintenance of fetal-maternal tolerance, while keeping immune responses intact requires a delicate mechanism, which still keeps many scientific questions unanswered.

The first aim of the project was to characterize HLA-C, HLA-E and HLA-G expression in different primary human EVT types and determine the influence of a maternal-fetal HLA-C mismatch and fetal gender on HLA expression levels on these EVT. These observations interlink the HLA expression levels with the invasiveness of various EVT types.

In the second aim we investigated the roles of microRNAs (miRNAs) in regulating HLA expression. miR148a and mir152 are trophoblast specific miRNAs, which are proven to regulate HLA-C and HLA-G surface expression levels on somatic cells and HLA-G transfectants but have not been investigated in EVT. The binding of mature miRNA to its target site lowers the HLA surface expression, while the escape from this posttranscriptional mRNA regulation result in high HLA surface expression levels. We performed knock outs of two miRNA regulators; DiGeorge Syndrome Critical Region 8 (DGCR8) and Argonaute-2 (AGO2), using CRISPR/Cas9, to test whether these proteins are essential for mature miRNA production and therefore HLA regulation.

The obtained data will help to answer crucial questions in the immune paradox of pregnancy and increase the understanding of complex and challenging immunobiology of pregnancy.

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List of Abbreviations

AGO2	Argonaute 2
ATL1	Atlastin-1
APC	Allophycocyanin
ß2-m	ß2-microglobulin
bp	base pair
Cas9	CRISPR associated protein 9
CARD	Caspase activation and recruitment domains
CD	Cluster of differentiation
CEBP	CCAAT-enhancer-binding proteins
cDNA	complementary Deoxyribonucleic acids
CIITA	MHC class II trans-activator
CTB	Cytotrophoblasts
CTL	Cytotoxic T lymphocyte
Cq	quantification cycle
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4',6-diamidino-2-phenylindole
DGCR8	DiGeorge Syndrome Critical Region 8
DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
dNK	Deciducal Natural Killer cells
dNTP	Deoxynucleotide
EBV	Epstein- Barr Virus
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EEO	Electroendoosmosis
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor

eIF	eukaryotic Initiation Factor
EVT	Extravillous trophoblasts
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green Fluorescence Protein
gRNA	guide Ribonucleic acid
HAT	Histone Acetyltransferase
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HPV	Human Papilloma Virus
HSP60	Heat Shock Protein 60
IFN γ	Interferon gamma
IgG	Immunoglobulin G
IL	Interleukin
ILT2	Imunoglobulin-like transcript 2
IRB	Institutional Review Board
IRF-1	Interferon Regulating Factor 1
ISRE	Interferon Stimulated Response Element
kb	kilobases
KIR	Killer cell immunoglobulin-like receptor
KO	knock out
LB	Lysogeny broth
LILRB1	Leukocyte immunoglobulin-like receptor subfamily B member 1
MHC	Major histocompatibility complex
MOPS	3-(N-morpholino)propanesulfonic acid
NCS	New Born Calf Serum

NK	Natural Killer
NLRC5	NOD-like receptor family CARD domain containing 5
NLRP2	NOD Domain, Leucine Rich Repeat and Pyrin Domain Containing 2
No.	Number
NOD	nucleotide-binding oligomerization domain-like receptors
PABP	poly-A-binding protein
PFA	Paraformaldehyde
PBS	Phosphate-buffered saline
PBST	Phosphate buffered saline with Tween-20s
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein Complex
Ran	RAs-related Nuclear protein
Rel.	Relative
RISC	RNA- induced silencing complex
RNA	Ribonucleic acid
ROX	carboxy-X-rhodamine
rpm	revolutions per minute
RT	room temperature
SDS	Sodium dodecyl sulfate
SRY	sex-determining region Y
STBs	syncytiotrophoblasts
TAP	Transporter associated with antigen processing
TBE	Tris/Borate/EDTA
TCR	T-cell receptor
TNF α	Tumor Necrosis Factor alpha
TRBP	TAR RNA-binding protein
UTR	Untranslated region
VT	Villous Trophoblasts

Introduction

The Major Histocompatibility Complex (MHC) molecules are referred to as HLA in humans, meaning Human Leukocyte Antigens. HLA class I molecules are grouped as classical MHC class Ia molecules (HLA-A, HLA-B, HLA-C) and as non-classical MHC class Ib molecules (HLA-E, HLA-F, HLA-G). [2] This thesis is aimed specifically on human HLA-C – a MHC class I gene and will not, to great extent describe class II genes or MHC expression in non-human species.

The history of HLA-C started 10 million years ago on polygenic and polymorphic segment of human chromosome 6, which encodes the histocompatibility antigens. HLA-C is a highly polymorphic heterodimer consisting of heavy alpha chain and ß2- microglobulin chain and displays high variances in-between allotypes. [3] HLA-C was produced by duplication from HLA-B and is only present in chimpanzee, gorilla, bonobo and humans. [4]

HLA-C was discovered by antigen-antibody analysis in the early 70s. [5] Over the next few decades HLA-C has been associated with many diseases, such as viral infections, cancer and many autoimmune diseases. [4] In 1983 the importance of HLA-C in pregnancies and placentation was identified. [6] Soon after, HLA-C was divided into 2 subgroups, dependent on their affinities for Killer cell Immunoglobulin- like receptor (KIR). [7] Less than 10 years ago, the significance of HLA-C in slower Human Immunodeficiency Virus (HIV) progression was demonstrated. [8] These and other important miles stones of the HLA-C and the role of HLA-C in human disease are escribed in Figure 1. However, there are still many questions about the role of HLA-C in human disease to be answered and mechanisms to be explained.

Figure 1 The milestones of HLA-C

YEAR	MILESTONE	REF.
1958	Discovery of 1st MHC "MAC"	[1, 2]
1968	Nomenclature "HLA"	[2, 3]
1970	Evidence of a 3rd sub-locus for HLA-C	[4]
1976	Locus for HLA-C is located on MHC class I chromosome C6 in order HLA-A–HLA-C–HLA-B	[5]
1978	High prevalence of HLA-C (w1 and w2) in spondylarthritisis	[6]
1983	Extravillous trophoblasts are MHC class I positive cells, thus expressing HLA-C Villous trophoblasts are MHC negative cells and do not express HLA-C	[7, 8]
1983	Miscarriage and HLA connected	[9]
1984	Structure of HLA-C locus	[10]
1987	HLA-C shows 91% homology to HLA-B	[11]
1987	Identification of primary structure of HLA-Cw1 and HLA-Cw2	[12]
1988	Nature of polymorphism: 8 HLA-C molecules defined	[13]
1992	HLA-C interacts with NK1 and NK2	[14]
1993	HLA-C is capable of inhibiting NK- mediated cell lysis	[15]
1994	HLA-C associated with psoriasis vulgaris	[16]
1995	HLA-C has been subdivided into 2 groups with distinct affinities for Killer cell immunoglobulin-like 2DL receptors	[17]
1996	HLA-C mRNA was clearly demonstrated in all Extravillous trophoblast samples as well as in JEG-3 and BeWo choriocarcinoma cells	[18]
1997	HLA-C interacts with (uterine) NK cells via Killer cell immunoglobulin-like receptor	[19]
1998	HLA-C (in trophoblast) is resistant to rapid degradation imposed by Human Cytomegalovirus (gene product US2 and US11)	[20]
1999	HLA-C is upregulated by IFNγ	[21]
1999	HIV-1 downregulates HLA-A and HLA-B, while leaving HLA-C unaltered	[22]

2000	Human cytomegalovirus gene products US3 and U6 downregulate HLA-C in trophoblasts	[23]
2000	HLA-C is dependent on CIITA expression	[24]
2000	Definitive evidence that HLA-C, is expressed on the surface of normal trophoblast cells	[21]
2003	HLA-C connected with allographic rejections in liver transplants	[25]
2003	1600 alleles and 1200 proteins have been identified for HLA-C	[26]
2004	Expression of fetal HLA-C and maternal KIR increases risk of pre-eclampsia	[27]
2004	Formation of placenta involves cooperation between KIR and HLA-C	[28]
2005	Lack of the Appropriate Natural Killer Cell Inhibitory Receptors is present in Women with spontaneous abortion	[29]
2009	Fetal maternal HLA-C mismatch is associated with decidual T-cell activation and induction of functional T regulatory cells	[30]
2009	HIV patients highly expressing HLA-C alleles progress more slowly to develop AIDS	[31]
2010	HLA-C expression is not correlated with HLA-G and HLA-E	[32]
2010	NLRC5 is a transcriptional regulator of HLA-A, HLA-B and HLA-C	[33]
2011	Association between HLA-C polymorphism and inflammatory bowel disease	[34]
2011	Trophoblasts express both maternally and paternally inherited HLA-C	[35]
2012	HLA-C- dependent prevention of leukemia	[36]
2014	Cytotoxic T-cell precursor frequency and HLA-C alleles mismatch contribute to prediction of allo-reactivity	[37]
2016	The combination of KIR and HLA-C determines possible pregnancy complications and provides protection against HCMV	[38]
2016	Antibodies against HLA-C play a role in recurrent miscarriages	[39]
2017	NLRP2 is a suppressor of HLA-C expression in trophoblast	[40]
2017	HLA-C express most common cancer mutation KRAS G12D	[41]

Pregnancy paradox

Pregnancy is a very complex, inseparable and important part of life. Surprisingly, only few research groups aim on this essential element. During pregnancy a formation of a new transitory organ occurs. The newly formed placenta serves as an intimate connection between the mother and fetus. [42] Placenta fulfils multiple functions, it provides an interface for gas, nutrient exchange and protection. [43] What's unique, the maternal immune system is capable of fighting pathogens and at the same time it maintains an immune tolerance with the semi- foreign fetus. It has already been 65 years since the first theories about maternal-fetal tolerance were proposed by Peter Medawar. How does it work that the maternal immune system stays alert and battle the foreign invaders while avoiding to reject the fetus? [44] [42]

Specific immune responses are mediated by peptides presented by Major Histocompatibility complexes (MHC), in humans known as Human Leukocyte Antigens (HLA), and their interactions with NK and T-cells. Fetal Extravillous trophoblasts (EVT), the most invasive cell type of the placenta, show an exclusive combination of HLA molecules that contribute to their survival in the maternal tissue. EVT express polymorphic HLA-C and non-polymorphic HLA-E and HLA-G, but lack expression of HLA-A or HLA-B. [45]

The aim of this thesis is to characterize HLA-C, HLA-E and HLA-G expression levels on primary EVT as well as to determine molecular mechanisms of HLA-C, HLA-E and HLA-G regulation in trophoblast cell lines. This is of importance to understand maternal-fetal immune tolerance in healthy pregnancy as well as the contributions to pregnancy complications.

Placenta

Surprisingly, the placenta is not a maternal, but rather a fetal organ. It is formed from fetal cells, especially the extraembryonic fetal membranes: chorion, allantois and amnion. The human viviparous placentation is the most invasive type of placentation. [42] The whole process starts with fertilization, followed by attachment of the blastocyst into maternal endometrium within a week. The attachment causes the change of maternal uterine mucosa into highly specialized tissue supporting the placental invasion, called decidua. High levels of

progesterone during the implantation process are vital for the blastocyst development into fetal precursor and trophoblast cells, which further develop into placenta and fetal membranes over gestation. (Figure 1A.) Placental villi are composed of cytotrophoblasts (CTB) and syncytiotrophoblasts (STB). A stable CTB- STB adhesion is present at the beginning of the gestation, later, a fusion occurs between the STB layer covering the inner CTB layer. The STB layer doesn't undergo division, but CTB supply the villous renewal and constant STB replacement. The CTB-STB cell contact and villous trophoblast- basal membrane interaction overall is mediated by multiple adhesion molecules. The most important attachment is intervened by integrin $\alpha 6\beta 4$, collagen IV, proteoglycans and laminin isoforms. The above-mentioned cell contacts and following signaling events carry out a complex task of maintaining the epithelial integrity, control of cell growth and fusion in the villous interface. [46-50]

At the early stages of development, the placenta is considered as low oxygen environment, what is not beneficial for complete fetal growth, therefore the formation of utero-placental blood circulation is necessary. The CTB lose their attachment to the villous basal membrane, become Extravillous due to multilayering and rupture the uterine tissue and maternal vessels. Next, they replace the endothelium and remove the smooth muscle layer, what results in spiral artery formation. The loss of connection to basal membrane is caused by downregulation of $\alpha 6\beta 4$ integrin and the trophoblast invasion and migration into the endovascular system is mediated by upregulation of L-selectins in decidua basalis. L-selectins are potently expressed also by EVT, what helps the maintenance and establishment of the column architecture, where they are anchored via desmosomes and fibronectin receptor integrin $\alpha 5\beta 1$. By the end of 1st trimester, the intervillous space surrounding the STB is filled with blood, facilitating the nutrient and oxygen interchange. (Figure 1B.) [48, 49, 51]

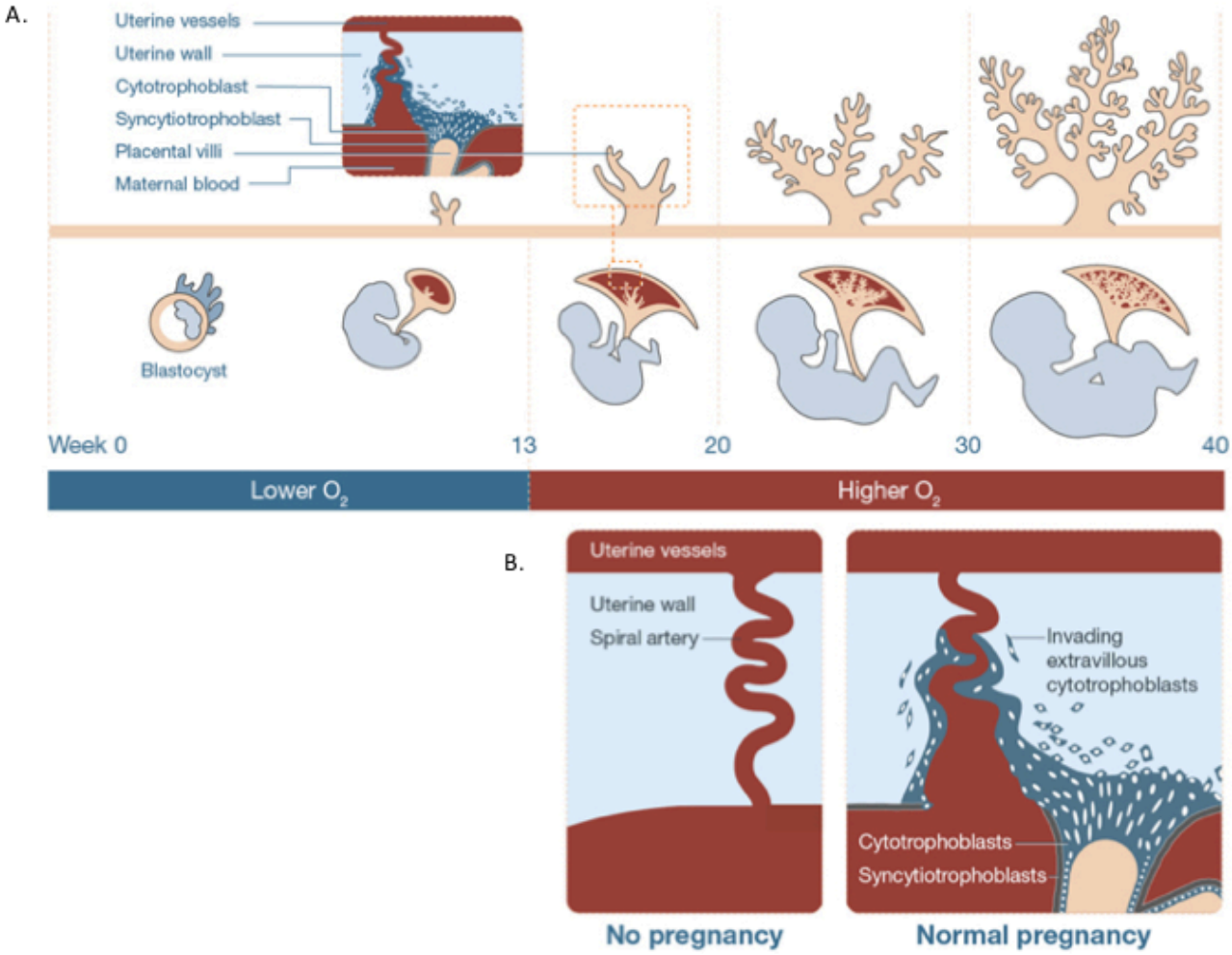


Figure 2. Human placenta formation.
(A.) Schematic illustration of placenta development and the main components involved in human placentation.
(B.) The formation of spiral arteries by Extravillous trophoblasts invasion of uterine endothelium.
Adapted from https://www.nichd.nih.gov

General Immunology and Human Leukocyte Antigens

The immune system compromises of 2 main parts: the innate and adaptive immune system. Innate immunity provides a quick system of defense against the most common pathogens, such as bacteria by recognizing their surface expression patterns. However, there are some pathogens which escape the innate immunity recognition. It activates macrophages, which engulf the bacterium and secrete cytokines or chemokines, which attract neutrophils and monocytes, what triggers inflammation. The inflammation process links the innate and adaptive immunity, as it increases the influx of antigen bearing cells into the lymphoid tissue. Adaptive immunity is slower, but more efficient, plus it provides increased protection when

re-infection with the same pathogen occurs, because of immunological memory. The mechanisms of adaptive immunity resemble the innate immunity at the begining; immature dendritic cells recognize surface expression patterns of pathogens and engulf them. The main function of dendritic cells is an antigen presentation to T-cells and initiation of a pathogen specific lymphocyte response. [52, 53] Pathogen derived antigens are presented to T lymphocytes via polymorphic MHC molecules. We distinguish between two classes of MHC molecules, class I binding CD8+ T- cells, and class II binding CD4+ T-cells.

MHC class I molecules are encoded on a short arm region of human chromosome 6. (Figure 2.) They are surface glycoproteins expressed on all nucleated cells and consist of non-polymorphic ß2-microglobulin (ß2-m), which has a stabilization function and 3 α domains. The α1 and α2 domain shape the peptide binding groove, while the α3 domain facilitates the connection with CD8+ co-receptor. [54] In humans, 2 groups of MHC class I molecules are identified; highly polymorphic classical MHC Ia (HLA-A, HLA-B, HLA-C) and non-classical MHC Ib (HLA-E, HLA-F, HLA-G) with limited polymorphism. [55]

An immune response directed against antigens of different genetic origin within the same species is called an allogenic response. The most common allogenic response is caused by HLA mismatch and might lead to allograft rejection after transplantation. During pregnancy, the HLA mismatch may be a cause for immunological response and rejection. [25]

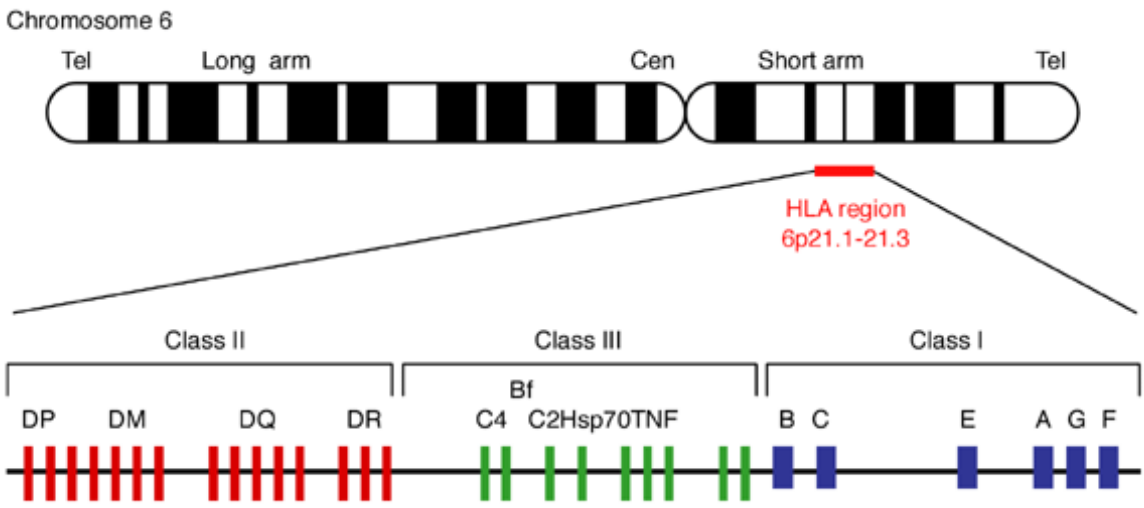


Figure 3. Gene map of the human HLA region.
The figure shows a human chromosome 6, which consists of about 1500 genes and 600 pseudogenes. The HLA region is located on the short arm at 6p21.1-21.3 and among other encodes the classical and non-classical MHC class I molecules. [56, 57]

Role of HLA-C in pregnancy

HLA-C is the only polymorphic MHC molecule expressed by EVT. HLA-C interacts with decidual NK cell (dNK), CD4+ and CD8+ T- cells and mediates two important and contradictory tasks. HLA-C facilitates immune tolerance and protective immunity at the same time.

dNK are the most abundant immune cells in placenta during the 1st trimester, when their population reaches about 70% of total CD45+ lymphocytes. Over gestation, their abundance decreases to about 10-20%, comparable to the population of NK cells in blood (~5-15%). Therefore, it is clear that their presence is essential early in pregnancy, when trophoblast invasion into maternal tissue occurs. [58] dNK interact with HLA-C via Killer cell immunoglobulin-like receptors (KIR) in order to lower their cytotoxicity and induce immune tolerance at the fetal- maternal interface. dNK cytotoxicity can be retrieved upon infection and cytokine secretion to triggered by the immune response to clear pathogens. [59]

CD4+ T-cells do not have a direct recognition mechanism of HLA-C. But antigen processing and antigen presentation in MHC class II expressed by APCs make the recognition of HLA-C by CD4+ T-cells feasible. CD4+ T cells are mostly T helper cells with cytokine production function, what strengthens the effector T cell-, B cell-, NK cell- and macrophage -mediated immune responses and immunization. HLA-C mismatched pregnancies increase the functional T-cell levels (T regulatory cells) in the decidual tissue, what helps the establishment and maintenance of immune tolerance. HLA-C matched pregnancies do not exhibit this phenomenon. [30, 52, 60]

CD8+ T-cells produce a low affinity bond with diverse range of antigens presented by MHC class I molecules via T-cell receptor (TCR). CD8+ T- cells differentiate into Cytotoxic T lymphocytes (CTL) after the antigen presentation in MHC class I. Furtherome, a high HLA-C expression levels or HLA-C mismatch stimulate the immune response by CTLs. At the maternal-fetal interface the CD8+ T-cells provide the protection against viral infection, as well as mediate the alloimmune responses. An elevated CD8+ T- lymphocyte activation causes an influx of CTLs into maternal decidual tissue, what interferes with the established fetal-maternal immune tolerance and impacts the possible pregnancy complications. [30, 61]

Role of HLA-E in pregnancy

HLA-E surface expression on EVT is dependent on nonameric leader peptides derived from HLA class I molecules. Therefore, the HLA-E expression levels are reliant on the leader peptide binding onto its surface mediated by TAPs (Transporter associated with antigen processing). HLA-E binds to CD94/NKG2 receptors and inhibits NK cell cytotoxicity. Remarkably, HLA-G derived leader peptides show high binding affinity with high efficiency of NK cell inhibition. Therefore, HLA-G seems to be a "HLA-E expression booster".

Surprisingly, HLA-E is able to bind and present peptides of non- HLA I origin as well as viral (HIV, influenza, EBV, HCV), mycobacterial or HSP60 proteins can be bound and presented on HLA-E. Human cytomegalovirus (HCMV) is aslo able to directly upregulate HLA-E surface expression.

In addition, HLA-E interacts with special CD8+ T-cells via TCR. These T-cells, known as NK-CTLs, have lysed a target which is susceptible to NK cells and thus express HLA class I- specific inhibitory receptors. The HLA-E – NK-CTL interaction triggers cell lysis and IFNγ production and contributes to immune responses against pathogens. Unfortunately, NK-CTLs have not been confirmed in placental tissues yet. Only the Vδ2 population of T-cells have been identified to recognize HLA-E in decidual tissue. The Vδ2 T-cell population has a high CD94 expression, what resembles the NK cells binding patterns. It is presumed that HLA-E interacts with Vδ2 T-cell subset via both TCR and CD94/NKG2 receptors and assists successful placentation. [62, 63]

Role of HLA-G in pregnancy

HLA-G is a tissue specific, low polymorphic MHC class Ib molecule. It induces immune tolerance at the maternal-fetal interference by inhibition of NK and T- cell cytotoxicity.

HLA-G interacts with dNK cells via KIR2DL4 receptor, which has both inhibitory and stimulatory properties. The HLA-G – KIR2DL4 interaction induces secretion of cytokines (mostly IL6 and IL8) essential for fetal development and maintenance of tolerance. dNK show a tolerogenic behavior towards HLA-G+ EVT, dNK are capable of HLA-G acquisition by trogocytosis, followed by internalization and resulting in reacquisition of HLA-G. Whether the trogocytosis process is receptor specific or receptor independent still has to be investigated.

Ig-like transcript 2 (ILT2, also known as LILRB1) receptor can be expressed by CD4+ T-cells and suppress these ILT2+ T-cells by HLA-G interactions. Additionally, the HLA-G interactions with ILT2 cause a decidual macrophage cytokine responses. These secreted factors have great importance in trophoblast invasion and placenta development. Decidual T-cells are very abundant at term pregnancy, with population of about 50-80% of decidual leukocytes. Most of these T-cells are T regulatory cells which supress immune responses. However, a moderate population of CD8+ T memory cells mediating fetus- and virus- specific immune reaction is also found at the fetal-maternal interphase. [44, 59, 64, 65]

Pregnancy complications

Consistent with section 1.1.2.2, HLA-C interacts with NK cells, the most abundant cells in the decidua, via KIR. We distinguish between two HLA-C allotypes; HLA-C1 and HLA-C2, with different affinities for KIR. HLA-C1 epitope interacts with inhibitory KIR2DL2, KIR2DL3 and activating KIR2DS2 receptors. Epitope HLA-C2 binds to inhibitory KIR2DL1 or activating KIR2DS1 receptors. Besides HLA-C allotypes, we discriminate between two KIR haplotypes. KIR A lacks most of the activating KIR and express mostly inhibitory KIR. KIR B has a varying number of inhibitory and some activating receptors. KIR B haplotypes create an advantage when dealing with infections and reduce the risk of pregnancy complications. [14, 15, 66] KIR2DS1/HLA-C2 interactions enhance viral control via increased degranulation and cytokine secretion in response to virus infected stromal cells.

Placental infections such as Human Papilloma Virus (HPV), Listeria, Herpes Simplex virus and the most common- HCMV, increase the risk of pregnancy complications development. These viral infections induce T-cell activation, what unbalances the immune tolerance at the maternal-fetal interface and prompt pregnancy complications. The viral clearance in immune privileged placenta is essential, but also challenging immune response as an unsuccessful viral clearance may even lead to fetal morbidity. [38, 67]

Since the fetus and the placenta are semi-allogenic tissues they can be recognized as foreign, it would be expected that HLA incompatibility would lead to T-cell upregulation and strong immune reaction and possibly to fetal rejection. Unexpectedly, fetal-maternal histo-incompatibility is necessary for a successful pregnancy. Following this pattern, fetal-maternal

HLA sharing (HLA compatibility) is associated with an increased risk of preeclampsia and recurrent miscarriages. Preeclampsia is still the biggest thread to pregnant women, holding the highest mortality and morbidity rates among all pregnancy complications, whilst recurrent miscarriages affect nearly 2% of pregnant women. Surprisingly, while an induced T- cell activation by HLA-C mismatch doesn't imbalance the maternal-fetal tolerance, a fetal HLA-C KIR epitope – maternal KIR genotype mismatch encourages pregnancy complications. [30, 39, 61, 68]

As stated above, the KIR A allotype increases the risk of pregnancy complications and therefore the risk of preeclampsia. The majority of inhibitory receptors in KIR A allotype cause an inhibition of uterine NK cell, which facilitate the EVT invasion into the uterine mucosa. An inadequate EVT invasion give rise to shallow placentation and insufficient spiral artery formation what continues as placental ischemia and reduces fetal blood supply. Any of these factors significantly decrease the chances of successful and healthy pregnancy. [27, 69]

While most of the focus is put on highly polymorphic HLA-C, HLA-G (and HLA-E expressing the HLA-G leader peptides) with limited polymorphism have an impact on preeclampsia and recurrent spontaneous abortions as the main gatekeeper of fetal-maternal immune tolerance as well. Not only that, HLA-G interacts with KIR2DL4 receptors, whose responses are dependent on the effector cell. Similarly to HLA-C, if the majority of the signals is inhibitory, the inhibition of uterine NK cells is encouraged [70, 71]

Regulation of MHC class I

The MHC class I genes are regulated at transcriptional and post-transcriptional level. The post-transcriptional regulation is mediated by microRNA (miRNA) processing, which will be discussed in the next chapter in detail.

In short, the transcriptional regulation is responsible for constitutive and cytokine- induced MHC class I expression. The MHC enhancosome is located in the promoter region, 250 bp upstream of transcription initiation site. The MHC enhancosome consists of highly conserved cis- regulatory elements; Enhancer A, Interferon Stimulated Response Element (ISRE) and W/S-X-Y element. Enhancer A is a target for NFkB/Rel family transcription factors, whereas W/S-X-Y motif is a target for MHC class I specific trans-activator NLRC5 and MHC class I non-specific trans-activator CIITA. Both trans-activators; NLRC5 and CIITA recruit Histone Acetyltransferases (HATs), what increases the gene expression. [72, 73] However, NLRC5 and CIITA are not expressed in trophoblasts and therefore the MHC class I proteins need a different, trophoblast- specific regulators. Just last year, in 2017, NLRP2, a suppressor of NFkB signaling and HLA-C expression was discovered. NLRP2 is not classified as a transcription factor, because it is lacking nuclear binding domain and localization signals. Despite the HLA-C suppressive nature of NLRP2, NLRP2 is also able to upregulate HLA-C in cytokine-induced manner. [40]

Most of the cell do not express HLA-G and so, the classical MHC I promoter region is not functional alone and needs a tissue specific regulation. HLA-G expression in trophoblasts is influenced by additional cis-regulatory element, located 12 kb upstream of HLA-G coding region. Enhancer L upregulates HLA-G via interactions with CEBP and GATA transcription factors, resulting in chromatin looping. [74]

miRNA regulation

miRNAs have been associated with autoimmune and inflammatory diseases. miRNAs are classified as non-coding RNAs. Their response to extracellular signals is much faster than the response of transcriptional factors, what fine-tunes the gene expression promptly and with high specificity. The miRNA processing pathway starts in the nucleus by RNA polymerase II

transcription or RNA polymerase III splicing, producing long primary miRNAs. The primary RNAs have a stem-loop structure that consists of 5’ cap and 3’ poly-A-tail. Further processing is carried out by RNA polymerase III enzyme Drosha associated with DiGeorge Syndrome Critical Region 8 (DGCR8) cofactor. The products of this processing are about 70 nucleotide long precursor miRNAs. The export of precursor miRNA into the cytoplasm is mediated by Exportin-5 with Ran-GTP bound cofactor. The removal of the loop region is achieved by another RNA polymerase III enzyme Dicer. Dicer produces 22 nucleotide long miRNA:miRNA duplex upon interactions with TAR RNA-binding protein (TRBP) or PACT (a protein activator of interferon-induced protein kinase). Next, the Argonaute family (AGO1-4) facilitates the loading of miRNA duplex onto a RNA- induced silencing complex (RISC) via glycine-tryptophan protein (GW128) interactions. (Figure 3A.) miRNA -induced RISC regulates the gene expression by 2 main modes; mRNA degradation or translational repression. In humans, eukaryotic initiation factor 4F (eIF4F) with 5’ cap and poly-A-binding protein (PABP) help to actively transcribe mRNA via circular structure formation. For the mRNA degradation, the miRNA-induced RICS binds to 3’ UTR of the target mRNA and removes the 5’ cap and 3’ poly-A tail by DCP protein factors and deadenylase complex respectively. By removal of both ends of target mRNA, its stability decreases rapidly and is susceptible for degradation by cytoplasmic 5’-3’ exonucleases. The translational repression happens mostly at the translation initiation level by targeting the 5’ cap. GW182 or AGO competes with eIF4F for 5’ cap binding ending in termination of translation. [75-77]

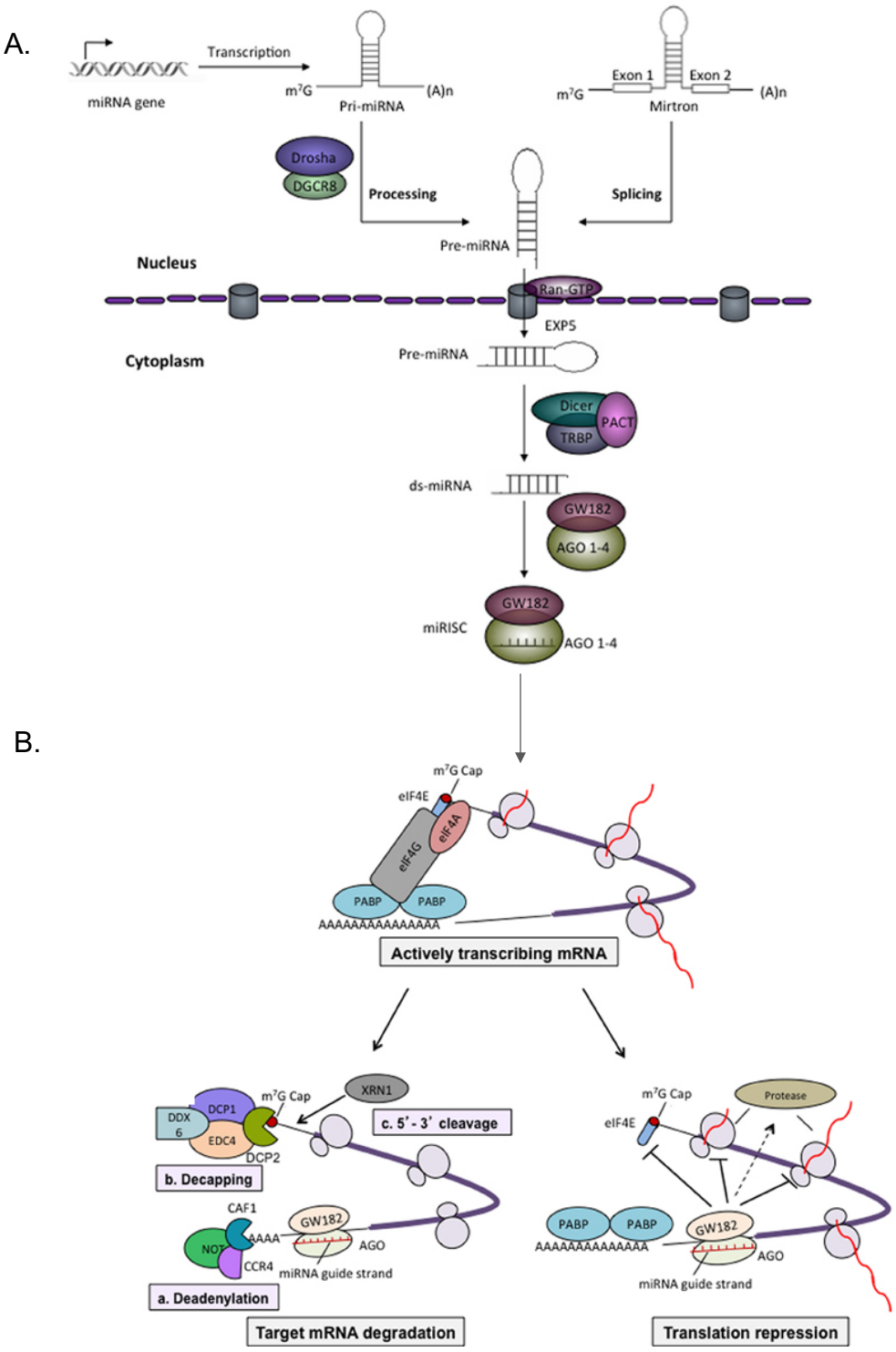


Figure 4. miRNA processing pathway

(A.) miRNA processing is initiated by Polymerase II or III transcription in nucleus, followed by multiple processing step resulting in precursor miRNA formation. The precursor miRNAs are exported out of nucleus by Exportin-5, where the processing steps of dicing, duplexing, unwinding and finally, RICS complex formation continue.

(B.) miRNAs regulate the gene expression by target mRNA degradation or by translational repression Adapted from reference [75].

DiGeorge Syndrome Critical Region 8

DGCR8 is a double stranded RNA binding protein located in the nucleus. It fulfills its microprocessing function at the beginning of the miRNA pathway in association with Drosha enzyme. The RNA binding domains are located at the C-terminal region with Drosha and primary miRNAs binding function, while the WW domain is located at the N-terminus and has an essential role in nuclear localization. [78] DGCR8 recognizes a 10 bp long region of the primary miRNA and mediates the cleavage of the stems near the base of hairpin into precursor miRNA. [77] Surprisingly, an evidence of Drosha- independent processing has also been provided. The Drosha- independent cleavage occurs mostly for intronic miRNAs undergoing splicing. [79]

Argonaute 2

AGO2 belongs to Argonaute family of proteins and plays a role in RNA interference as RISC catalytic component. AGO2 is the only member of the Argonaute protein family in humans which shows an endonuclease activity. AGO2 is maternally expressed and its deficiency has been shown to be lethal in mice due to its essential role in erythrocyte maturation. It is composed of 4 globular and 2 linker domains. Notably, a Dicer- independent miRNA processing has been demonstrated. AGO2 interacts with eiF1A and binds to precursor miRNAs. Functional miRNAs are produced by cleavage of miRNA duplex from the 5' end, followed by urydylation and trimming steps. The result of eiF1A-AGO2 interaction is Dicer-independent miRNA processing pathway along with miRNA guided RNA interference. [80-82]

Hypothesis

In the previous sections, the importance of HLA-C, HLA-E and HLA-G expression on EVT has been shown. The Primary EVT project aims on the characterization of HLA-C, HLA-E and HLA-G expression levels and morphology in different trophoblast cells (1st trimester decidua, and term placenta Decidua Basalis and Chorionic tissue). Next, changes in HLA expression before and after EVT culture on fibronectin, as well as after stimulation with the proinflammatory cytokines IFNγ and TNFα was investigated. Furthermore, Aim 1 determined the influence of

a maternal-fetal HLA-C mismatch and the influence of fetal sex on HLA expression levels on EVT. The characterization of HLA expression was previously conducted using 1st trimester EVT, however no data has so far been published on HLA expression on the term pregnancy EVT in Decidua Basalis and Chorion.

Aim 2 focusses on miRNAs as regulators of HLA expression in EVT. miR148a and miR152 are confirmed regulators of HLA-C and HLA-G expression in trophoblast cell lines. The upregulation of miRNAs causes a decrease in HLA expression levels and vice versa. To test the importance of miRNA processing proteins a knockout of DGCR8 and AGO2 in trophoblast-like choriocarcinoma cell line JEG3 was performed. An increase in HLA expression levels is expected as the formation of mature mRNAs should be repressed. [83-86] This investigation will bring novel insights into the puzzling problem of immune tolerance in pregnancy.

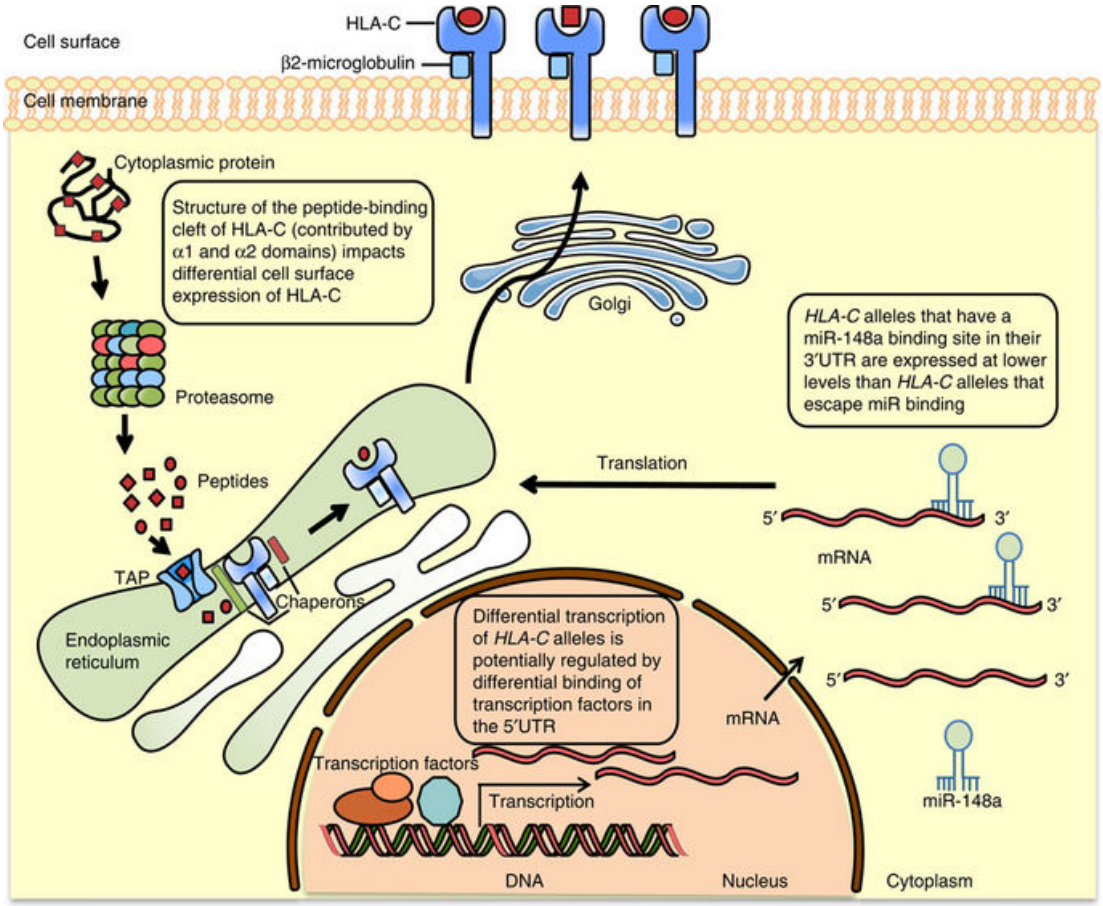


Figure 5. Demonstration of HLA-C protein expression levels regulation.
HLA-C expression is regulated at multiple levels; transcriptional, miRNA processing (post-transcriptional), peptide binding and selectivity. [88]

Materials and Methods

Primary EVT

Isolation of EVT from 1st trimester tissue

Discarded human placental materials (gestational age 6–12 weeks) we obtained from women undergoing elective pregnancy termination in a local reproductive health clinic. All of the human tissue used for this research was deidentified, discarded clinical material. The Committee on the Use of Human Subjects (the Harvard IRB) determined that this use of placental and decidual material is Not Human Subjects Research. The procedure to isolate EVT has recently been described. In short, villous tissue was gently scraped from the basal membrane. Thereafter, the tissue was digested for 8 min at 37°C with a trypsin (0.2%) EDTA (0.02%) solution. Trypsin was quenched with DMEM/F12 medium containing 10% New Born Calf Serum (NCS) and pen/strep (all from Gibco). Cells were filtered over a gauze mesh and were washed once with complete F12 medium and layered on Ficoll (GE Healthcare) for density gradient centrifugation (20 minutes, 2000 rpm). Cells were collected, washed once, and incubated 20 min at 37°C in a tissue culture dish for removal of macrophages. [26]

Isolation of EVT from term placenta tissues

Decidua Basalis and Chorion were obtained from healthy women after uncomplicated pregnancy at term (gestational age > 37 weeks) delivered by elective cesarean section or uncomplicated spontaneous vaginal delivery at Tufts Medical Center. All human tissue used for this research was de-identified, discarded clinical material. The committee on the use of human subjects (Harvard Institutional Review Board) determined that this use of placental and decidual material is not human subject's research. Term placental tissue was collected under a protocol approved by Tufts Health Sciences Institutional Review Board.

Chorionic tissue from term pregnancy was collected by removing the amnion and delicately scraping the decidua parietalis from the chorion. Decidua basalis was macroscopically dissected from the maternal side of the placental disk.

Chorion and decidua basalis tissues were collected and directly placed in complete DMEM/F12 (containing NCS, pen/strep) and PBS respectively. Collected chorion and decidua basalis tissues were washed and digested for 15 minutes at 37°C with 0.25% Trypsin (HyClone™, GE Healthcare)/2% DNAse-I (Sigma). Trypsin was quenched with complete DMEM/F12 medium, plus 0.8% DNAse-I. Cells were filtered through a 100 μm filter and washed with complete DMEM/F12. The undigested tissue was used for a 2nd digestion for 15 minutes at 37°C with 0.25%Trypsin/2% DNAse-I and filtered as described above. Next, cells were layered on Ficoll (GE Healthcare) for density gradient centrifugation (20 minutes, 2000 rpm). Cells were collected and washed once.

Staining and Flow cytometry

Trophoblast isolates were stained for 20 minutes with EGFR1 FITC (Serotec), HLA-G APC, (clone MEM-G/9, Molecular Probes), CD45 PerCP (clone: HI30, BioLegend), HLA-C PE (clone DT9, BD), HLA-E PE (clone 3D12, BioLegend), IgG2b PE (BD) (Supplemental Table 1). The cells were washed with complete DMEM/F12 medium (containing 10% NCS, pen/strep). Flow cytometry was performed at FACS Calibur™ (BD). FACSDiva™ and FlowJO, LCC software was used for analysis.

EVT culture

Trophoblasts were plated on 48-well cell culture plates (Falcon) precoated with fibronectin (100 μL, 20 ng/mL, 45 min, BD), in DMEM/F12 medium (Gibco) supplemented with 10% NCS, pen/strep and glutamine, insulin, transferrin, selenium (Gibco), 5 ng/ml EGF (Peprotech), and 400 units of human gonadotropic hormone (Sigma). Trophoblasts were trypsinized on day 1 (overnight culture) and directly stained for flow cytometric analysis as described above. For stimulation experiments, EVT were stimulated directly after plating with recombinant human TNFα (concentration 5 ng/mL, 20ng/mL) and IFNγ (concentration 20 ng/mL, 100 ng/mL) (both from BioLegend).

Confocal microscopy

Trophoblast were plated on 16 mm coverslip (Ted Pella, Inc.) inside 48- or 24- well cell culture plates (Costar) precoated with fibronectin as described above, in DMEM/F12 medium (Gibco) supplemented with 10% NCS, pen/strep and glutamine, insulin, transferrin, selenium (Gibco), 5 ng/ml EGF (Peprotech), and 400 units of human gonadotropic hormone (Sigma). Trophoblasts were stained either on day 1 (overnight culture) or day 2. Trophoblasts were washed and stained with anti-HLA-G (MEM-G/9) and subsequently with A488 goat-anti mouse secondary antibody. Next the cells were stained with Annexin V A647 (Thermo Fisher) in Annexin V binding buffer (Biolegend) and fixed with 4% PFA (Polysciences, Inc.),) and Rhodamine Phalloidin. At last, the cells were washed and secured onto a coverslip with VECTASHIELD® Hardset Antifade Mounting Medium with DAPI. Acquisition was performed with a Zeiss LSM 700 microscope.

HLA-C typing

DNA extraction was performed with E.Z.N.A.® Tissue DNA Kit (Omega bio-tek) following the manual's instructions. DNA concentration was quantified by NanoDrop™ (Thermofisher). HLA typing was performed by the American Red Cross tissue typing facility, Dedham, Massachusetts.

Y chromosome screening

PCR reaction was preformed using a master mix containing 0.1 μL Phusion® High Fidelity DNA Polymerase, 4 μL GC buffer, 1 μL dNTP (all from BioLabs), 1 μL forward primer (the primer sequences can be found in Supplemental Table 4.), 1 μL reverse primer, 60-80 ng of DNA and dH_2O for final volume of 20 μL. The following amplification conditions were used:

Table 1 PCR conditions for Y chromosome screening

Incubate at 95°C for 3 minutes
Incubate at 95°C for 30 seconds.
Incubate at 68°C for 30 seconds.
Incubate at 72°C for 30 seconds.
Cycle to step 2 for 36 more times.
Incubate at 72°C for 5 minutes.
Incubate at 4°C forever.

Genome editing

Designing and cloning of CRISPR gRNA

The PCGT2 vector (B2M Bulldozer, #84381, Addgene) was digested with BbsI restriction enzyme (BioLabs). [55]

Oligonucleotides were designed as follows:

- Sense: 5'-CACC(G)-20nt
- Antisense: (C)-20nt-CAAA-5'

The oligonucleotides (10 μM stock) were annealed using a master mix containing 2 μL sense, 2 μL antisense, 20 μL 10x annealing buffer (NEB Buffer 3) (BioLabs) and 176 μL of H_2O for 2 minutes at 95°C.

Furthermore, the oligonucleotides were phosphorylated using a master mix containing 5.8 μL of annealed oligonucleotides, 0.6 μL T4 PNK buffer 10x/T4 ligase buffer, 0.6 μL T4 PNK (both from BioLabs). The following conditions were used:

- 37°C for 30 minutes incubation
- 50°C for 20 minutes inactivation

The annealed and phosphorylated oligonucleotides were ligated using a master mix containing 7μL of annealed phosphorylated oligonucleotides, 1 μL of digested PCGT2 vector, 1 μL T4 10x buffer, 1 μL T4 DNA ligase (BioLabs) for an hour at room temperature.

Transformation in Escherichia coli (E. coli) competent cells

50 μL of E. coli competent cells with 10 μL of ligation product were incubated on ice for 20°C. Next a 42°C heat shock in shaking water bath for 1 min was performed, followed by further

incubation on ice for 5 minutes. 500 μL of LB medium was added to the mixture and 100 μL were plated onto LB plate with Ampicillin. The plates were incubated overnight at 37°C. Furthermore, single colonies were picked and incubated in LB medium (with 50 μg/mL ampicillin supplementation) overnight at 37°C in shaking incubator.

Small and Large scale DNA isolation (Mini and Maxi Prep)

Small scale DNA isolation was performed with QIAprep Spin Miniprep Kit (QIAGEN) and Large scale DNA isolation was performed with Qiagen HiSpeed Plasmid Midi Kit (QIAGEN) following the manual's instructions. DNA concentration was quantified by NanoDrop™.

Maintenance/ Propagation of JEG3 cells

JEG3 cells were cultured in RPMI medium (Gibco) containing 10% Fetal Bovine Serum (FBS, Atlanta Biologicals) and pen/strep (Gibco). JEG3 cells were passaged at 80-90% confluency approximately every 4 days in 1:6 ratio with 0.25% Trypsin-EDTA (Gibco) for 3 minutes and thereafter incubated at 37°C and 5% CO_2 concentration.

Transfection of JEG3

JEG3 cells were split in 1:6 ratio into 10 cm dishes. At 80% confluency 2.5 μg of CRISPR gRNA, 5 μg of Cas9 plasmid, 300 μL of Opti-MEM (Gibco) and 30 μL of FuGENE® (Promega) were mixed and incubated at room temperature for 20 minutes. Afterwards, the mixture was added to cells dropwise.

DNA extraction

DNA extraction was performed with E.Z.N.A.® Tissue DNA Kit (Omega bio-tek) following the manual's instructions. DNA concentration was quantified by NanoDrop™

PCR – screening

PCR reaction was preformed using a master mix containing 0.1 μL Phusion® High Fidelity DNA Polymerase, 4 μL GC buffer, 1 μL dNTP (all from BioLabs), 1 μL forward primer, 1 μL reverse primer (the primer sequences can be found in Supplemental Table 4.), 60-80 ng of DNA and

dH_2O for final volume of 20 μL. Different cycling conditions were used for individual miRNA regulators.

Table 2 PCR conditions for AGO2 and DGCR8

AGO2	DGCR8
Incubate at 95°C for 2 minutes	Incubate at 95°C for 3 minutes
Incubate at 95°C for 15 seconds.	Incubate at 95°C for 30 seconds.
Incubate at 67.8°C for 15 seconds.	Incubate at 68.7°C for 30 seconds.
Incubate at 72°C for 15 seconds.	Incubate at 72°C for 30 seconds.
Cycle to step 2 for 36 more times.	Cycle to step 2 for 36 more times.
Incubate at 72°C for 2 minutes	Incubate at 72°C for 5 minutes.
Incubate at 4°C forever.	Incubate at 4°C forever.

Gel electrophoresis – genotyping

The PCR reactions were analyzed with 2% agarose gel. The SeaKem® low EEO agarose powder (lonza) was dissolved in TBE (Biorad) buffer with 0.00003% GelRed™ (Invitrogen). For the size determination 100 bp DNA ladder (BioLabs). Gel electrophoresis was carried out with Gel Running Station filled with TBE buffer (both Biorad). AlphaImager HP geldocumentation system (Protein Simple) was used for analysis.

Gel extraction and sequencing

Gel extraction was performed with QIAquick Gel Elution Kit (Qiagen) following the manual's instructions. DNA concentration was quantified by NanoDrop™. The extracted DNA was submitted for sequencing to https://www.genewiz.com, following the webside's instructions.

Staining and Flow cytometry of JEG3 cells and clones

JEG3 were trypsinsed with 0.25% Trypsin-EDTA (Gibco) and stained with HLA- G APC, (clone MEM-G/9, Molecular Probes), HLA-C PE (clone DT9, BD), HLA-E PE (clone 3D12, BioLegend), IgG2b PE (BD) (Supplemental Table 1). Flow cytometry was performed at FACS Calibur™ (BD). FACSDiva™ and FlowJO, LLC software was used for analysis.

Western Blot

AGO2 and DGCR8 clones were lysed using 1x Cell Lysis Buffer (Cell Signaling Technology) with Protease Inhibitor Cocktail (10 μL/mL, Sigma). 3x Reduing Blue SDS Loading buffer with 1/10 volume of 30x Dithiothreitol (both Cell Sigaling Techlogies) was added, and the sample was boiled for 10 minutes at 95°C. The samples were loaded into Bolt™ 4-12% Bis-Tris Plus gel (Thermo Fisher Scientific) in NuPAGE® MOPS SDS Running Buffer (Life Technologies) and gels were run 80 minutes at 150V. Next, the samples were bloted onto iBlot® Gel Transfer Stacks Nitrocellulose using iBlot™ device (both Thermo Fisher Scientific) for 7 minutes at 20V. The nitrocellulose membrane was blocked for 1-2 hours in 5% milk/ PBST and then incubated with primary antibody in 1% milk/ PBST overnight. Thereafter, the membrane was washed and stained with secondary antibody in 1% milk/ PBST for 1-2 hours at RT. Nitrocellulose membrane were washed 3-times and developed by using 1:1 Immobilon™ Western (Millipore) and X-OMAT (evolve). (Supplemental Table 3). The results were quantified relative to HSP70 expression with PictureJ (NIH).

RNA isolation, cDNA synthesis and quantitative real-time qPCR

RNA isolation was performed with RNeasy Mini Kit (QIAGEN) following the manual's instructions (Appendix C). RNA concentration was quantified by NanoDrop™.

cDNA synthesis was performed with qScript cDNA synthesis kit (QuantaBio) for mRNA qPCR and TaqMan® Advanced miRNA Assays (Thermo FIsher) for miRNA qPCR following manual's instructions.

mRNA qPCR was performed with qScript™ One-Step SYBR® Green qRT-PCR Kit (QuantaBio) and miRNA qPCR was performed with TaqMan® Advanced miRNA Assays (Thermo FIsher) following manual's instructions. (The primer sequences can be found in Supplemental Table 4.) Quantitative PCR was run on ViiA 7 (Thermofisher) and the following conditions were used:

Table 3 qPCR conditions

	mRNA qPCR (SYBRgreen)			miRNA qPCR (TaqMAN)		
Step	Temperature	Time	Cycles	Temperature	Time	Cycles
Enzyme activation	95°C	5 minutes	1	95°C	20 seconds	1
Denature	95°C	10 seconds	40	95°C	1 second	40
Anneal/Extend	60°C	30 seconds		60°C	20 seconds	

Relative expression was determined with Excel (Microsoft).

Statistical analysis

All data were analyzed by GraphPad Prism software. All data are depicted as median values with interquartile range. To determine differences among more than 2 unpaired groups, a non-parametric Kruskal-Wallis test with Dunn's multiple comparison post-test was performed. To determine differences among more than 2 paired groups, a non-parametric 2-way ANOVA with Tukey's multiple comparisons test was performed. P-values <0.05 were considered to denote significant differences. * $p<0.05$; ** $p<0.01$; *** $p<0.005$.

Results

Primary EVT

HLA expression profiles of three types of freshly isolated primary human EVT.

The EVT were isolated according to the protocols described in the Material and Methods, section 2.1.1 and 2.1.2, which were developed at Strominger Lab by Tamara Tilburgs, PhD. For the term placenta, a lot of tissue was left after the 1st digestion procedure and therefore the residual tissue was digested again using the same conditions. However, no significant differences in HLA-C, HLA-E or HLA-G expression levels were observed upon different digestions.

The trophoblasts were stained directly after isolation and the HLA-C, HLA-E and HLA-G expression within HLA-G+ EVT was determined for each sample (see Supplemental Figure 1 for the gating strategy). It was crucial to determine the relative expression (e.g. Rel. HLA-C) as the isotype control differs for the 1st trimester and term placenta. The expression of a certain HLA molecule was normalized against the isotype control (IgG) to obtain the Relative expression, which allows comparison and the performance of statistical analysis.

The HLA-C and HLA-E expression was highest in the 1st trimester EVT.

HLA-G expression levels were significantly increased compared to HLA-C and HLA-E expression on all three EVT types. The Decidua Basalis EVT have the highest expression levels of HLA-G, which is responsible for induced immune tolerance. Remarkably, Decidua Basalis EVT showed the greatest variation in the expression of all 3 examined HLA molecules between different samples, suggesting that variation in patient demographics and/or clinical variables possibly contribute to modification of HLA expression patterns in this tissue. [3]

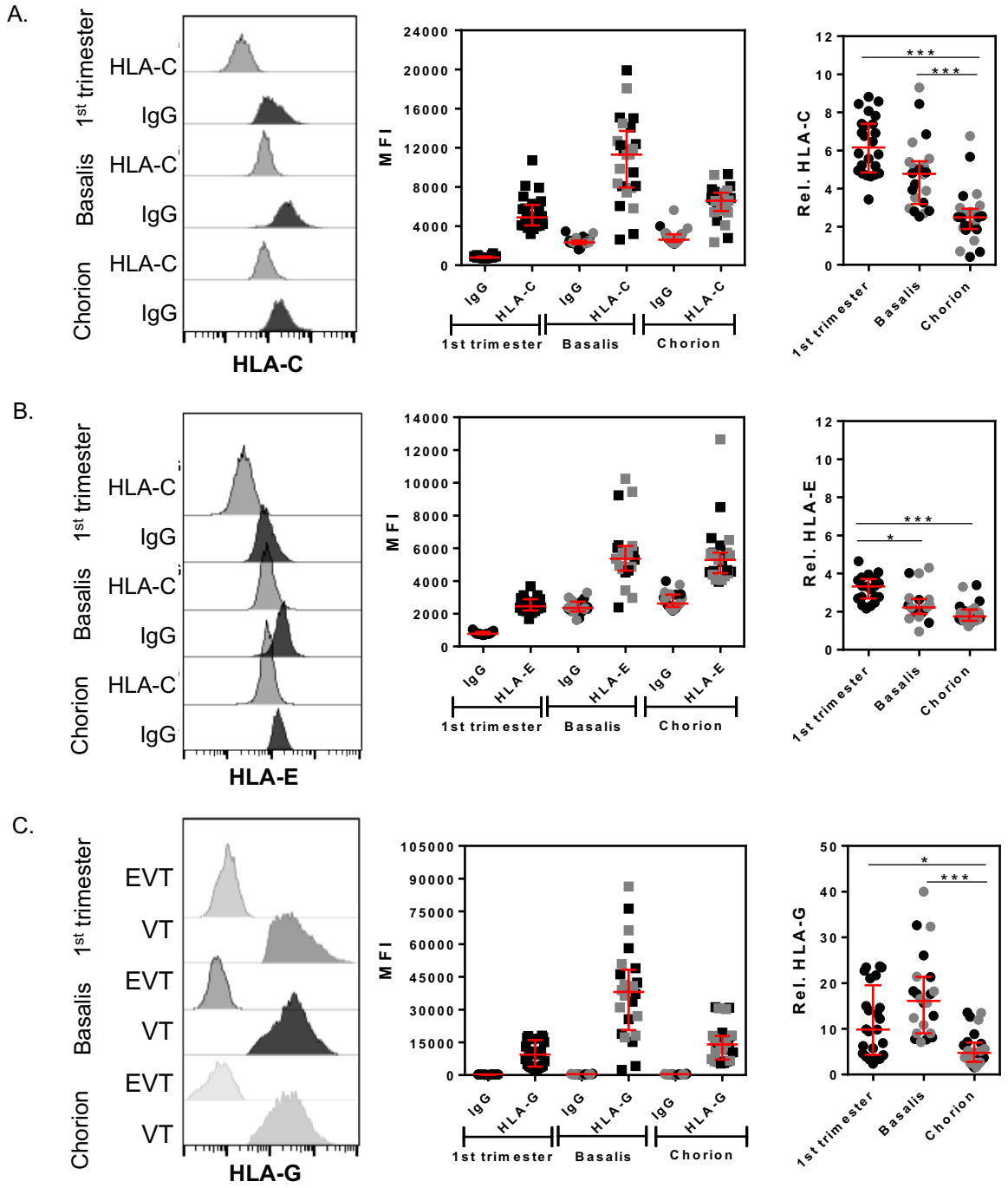


Figure 6. 1st trimester EVT has highest HLA-C and HLA-E expression, while Decidua Basalis EVT has the highest HLA-G expression levels.

The histograms show the HLA expression within CD45- gate and the subsequent selection of HLA-G+ cells in freshly isolated 1st trimester, Decidua Basalis and Chorion EVT. The relative HLA expression in HLA-G+ EVT is normalized against the isotype control (IgG).

Representative FACS plots depict the Mean Fluorescence Intensity (MFI) and Relative expression of (A.) HLA-C (DT9), (B.) HLA-E (3D12) and (C.) HLA-G (MEM-G/9) in 1st trimester, Decidua Basalis and Chorion HLA-G+ EVT. Black data points represent the 1st digestion and grey data points represent the 2nd digestion of the same tissue.

1st trimester EVT, but not term placenta EVT increase HLA-C expression on culture on fibronectin

Next, the expression of chosen HLA-C, HLA-E and HLA-G after overnight culture (~24 hours) on Fibronectin was investigated. A 24- hour incubation time was chosen, because of a short MHC class I half-life. The median half-life of MHC class I proteins is approximately 21 hours. [56]

Upon culture on fibronectin HLA-C expression increased by about a ~1.5 fold (Figure 6A.), whereas HLA-G increased by ~4 fold (Figure 6C.) on the 1st trimester EVT confirming previous studies [26]. HLA-E expression increased about ~1.5 fold in the 1st trimester EVT, however similar increase in HLA-E expression was observed for both term EVT types and therefore it is not considered to be significant. (Figure 6B.) Interestingly, both term pregnancy EVT from Decidua Basalis and Chorion did not increase HLA-C, HLA-E or HLA-G expression upon culture on fibronectin. The observed HLA-C and HLA-G increase may be important in the 1st trimester EVT invasion and establishment of fetal-maternal immune tolerance, but not in term EVT when the invasion has been completed and tolerance established. [57]

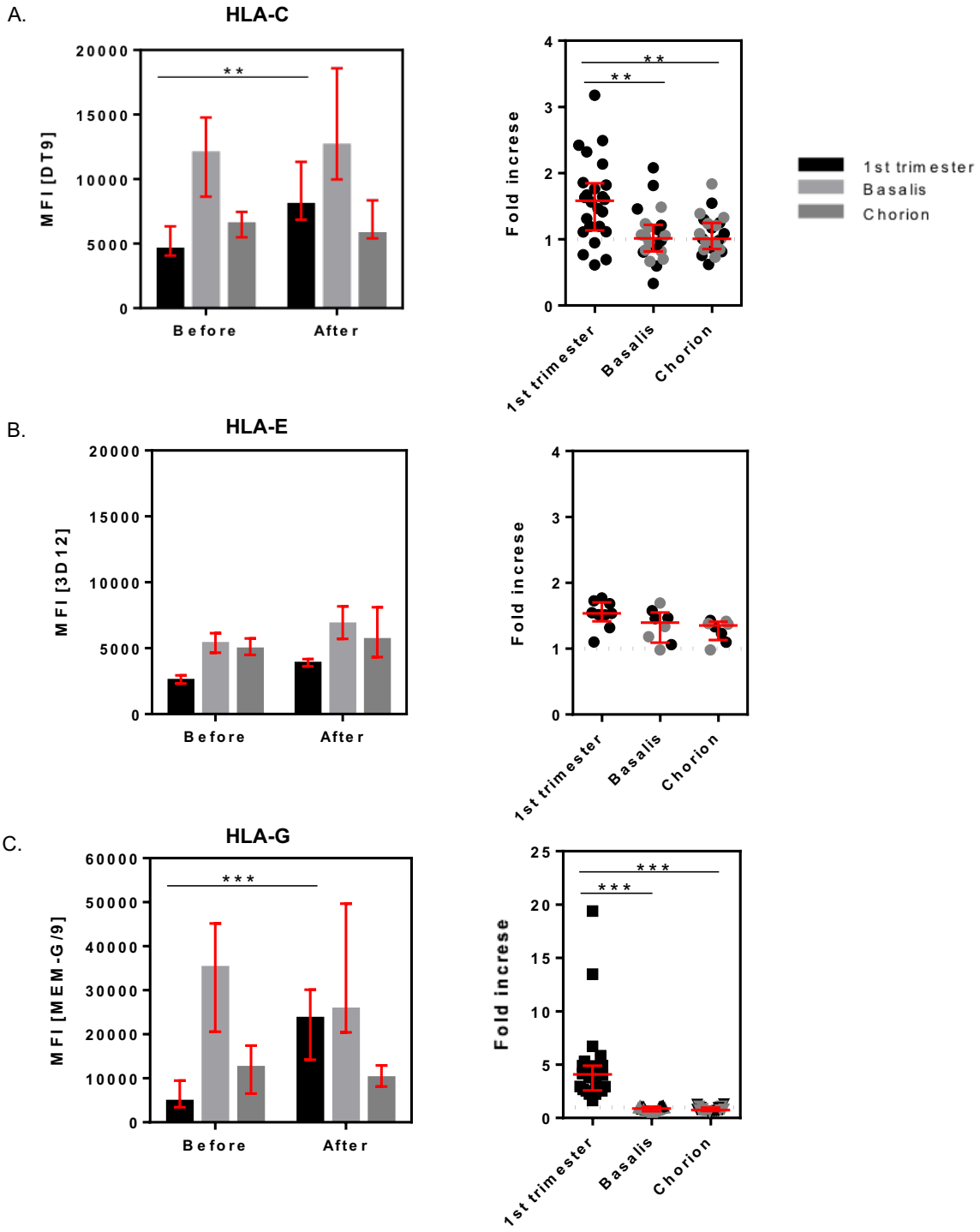


Figure 7 Culture on fibronectin increases HLA-C and HLA-G on 1st trimester EVT but not on EVT at term pregnancy.

Graph depict the Mean Fluorescence intensity (MFI) and fold increase after overnight EVT culture on Fibronectin of (A.) HLA-C (DT9), (B.) HLA-E (3D12) and (C.) HLA-G (MEM-G/9) expression levels in 1st trimester, Decidua Basalis and Chorion HLA-G+ EVT. In fold increase graphs, the black data points represent the 1st digestion and grey data points represent the 2nd digestion of the same tissue. In MFI graphs the black color represents 1st trimester, light grey Decidua Basalis and dark grey Chorion EVT.

HLA-C (but not HLA-G) is upregulated by IFN**γ** only in the 1st trimester EVT

Next, we investigated the influence of cytokine stimulation on HLA expression on the three types of EVT. IFN**γ** significantly increased expression of HLA-C by ~2 fold on the 1st trimester EVT, confirming previous studies. [58]

Surprisingly, the upregulation of HLA-C by IFN**γ** wasn't observed in term pregnancy EVT isolated from Basalis and Chorion. Moreover, TNFα didn't upregulate HLA-C, nor HLA-G in any EVT type.

Previous investigations have shown that proinflammatory cytokines, such as IFN **γ**, have no effect on HLA-G levels in the 1st trimester EVT. [58] Although in Figure 7B. a significant upregulation of HLA-G has been observed, we assume, that it is so small it is probably not relevant.

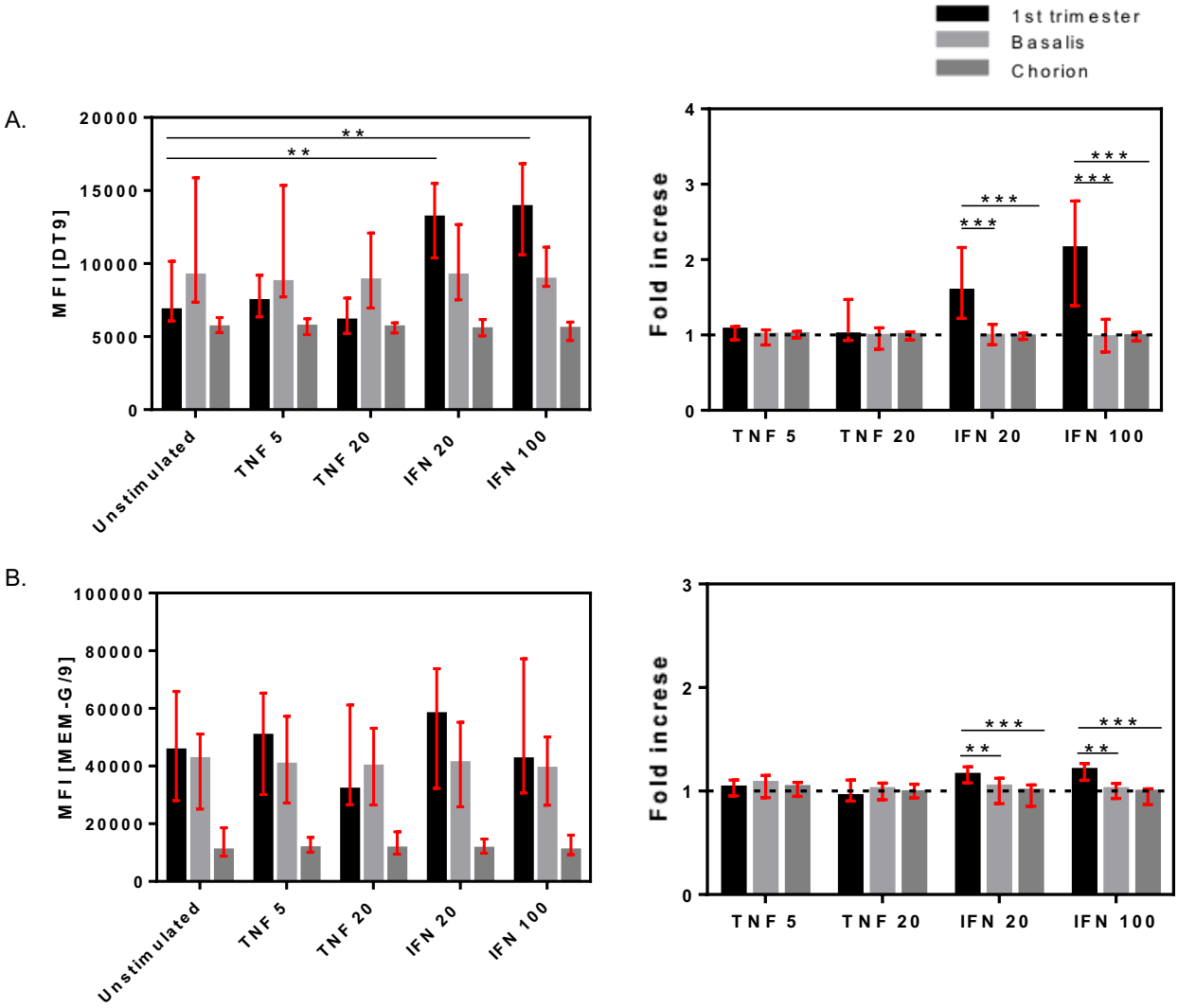


Figure 8. HLA-C (but not HLA-G) is upregulated by proinflammatory cytokine IFNγ only in the 1st trimester EVT.
The graphs depict the Mean Fluorescence intensity (MFI) and fold increase after TNFα and IFNγ stimulations of (A.) HLA-C (DT9) and (B.) HLA-G (MEM-G/9) expression levels in 1st trimester, Decidua Basalis and Chorion HLA-G+ EVT.
The cytokines were used at following concentrations; TNFα - 5 ng/mL (TNF5) or 20 ng/mL (TNF20) and IFN γ - 20 ng/mL (IFN20) or 100 ng/mL (IFN100). Black color represents the 1st trimester, light grey the Decidua Basalis and dark grey the Chorion HLA-G+ EVT.

Fetal- maternal HLA-C match or mismatch doesn't influence the HLA expression levels

To determine the possible differences in HLA expression between HLA-C- matched and HLA-C- mismatched mother and fetus pairs, DNA was isolated from villi and decidual tissues and sent for HLA-C typing at the Red Cross tissue typing facility.

No significant differences in HLA-C, HLA-E and HLA-G expression levels on EVT between the HLA-C matched and HLA-C mismatched fetal and maternal pairs were observed on all three EVT types. (Figure 8.) However, due to a failure to analyze many maternal DNA samples possible due to contamination with fetal DNA, the analysis lacked statistical power and larger sample size is required to draw conclusions.

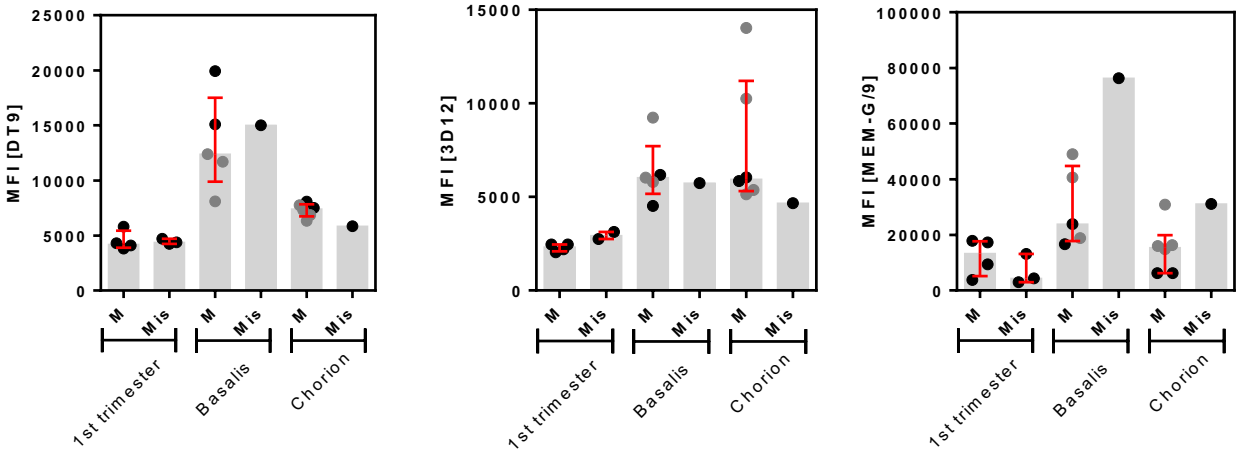


Figure 9. HLA-C matched, and HLA-C mismatched pregnancies do not affect HLA expression levels in any EVT type.
The representative FACS plots show the Mean Fluorescence Intensity (MFI) of HLA-C (DT9), HLA-E (3D12) and HLA-G (MEM- G/9) in 1st trimester, Decidua Basalis and Chorion HLA-G+ EVT, dependent on HLA-C match (M) /mismatch (Mis) in between fetal and maternal HLA-C allotypes. Grey data points represent placenta that had significant inflammation that could be identified macroscopically.

Fetal gender influences the HLA-C expression levels in the 1st trimester and HLA-G expression levels at term pregnancy

To investigate if fetal gender affects the expression of HLA on EVT, DNA was isolated from villous tissues (of fetal origin) from all samples used in Figure 5. The Y chromosome screen was performed with PCR using sex determining region Y (SRY) primer pair. Surprisingly, EVT isolated from placental tissues from a pregnancy with male fetuses expressed significantly higher HLA-G in the term EVT (in both Decidua Basalis and Chorion EVT), whereas female fetuses express slightly higher, and significant different HLA-C levels at 1st trimester pregnancy.

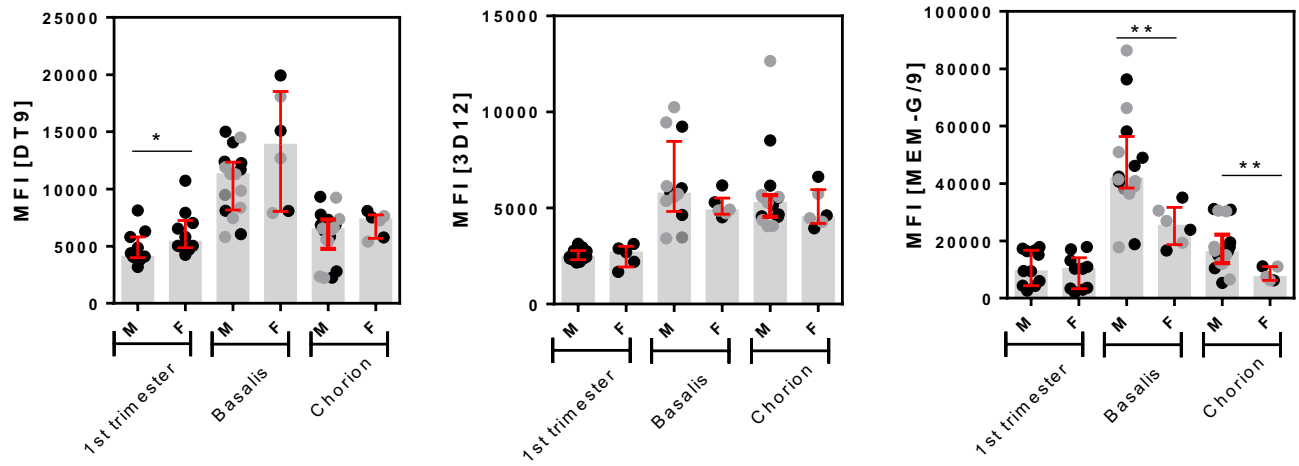


Figure 10. Male fetuses express significantly higher HLA-G at term pregnancy.
From left to right; the Mean Fluorescence Intensity (MFI) of HLA-C (DT9), HLA-E (3D12) and HLA-G (MEM- G/9) in 1st trimester, Decidua Basalis and Chorion HLA-G+ EVT, dependent on the sex of the fetus (M- male, F- female). The black data points represent the 1st digestion and grey data points represent the 2nd digestion of the same tissue.

The invasiveness of EVT types is correlated with their morphology.

To determine the cell morphology of the three different types of EVT, the cells were fixed and stained according to the protocol described in Material and Method section 2.1.5. Each EVT type displayed a distinct and characteristic morphology. The cell shape, number and/or length of projections suggest the invasive character and function of certain EVT type.

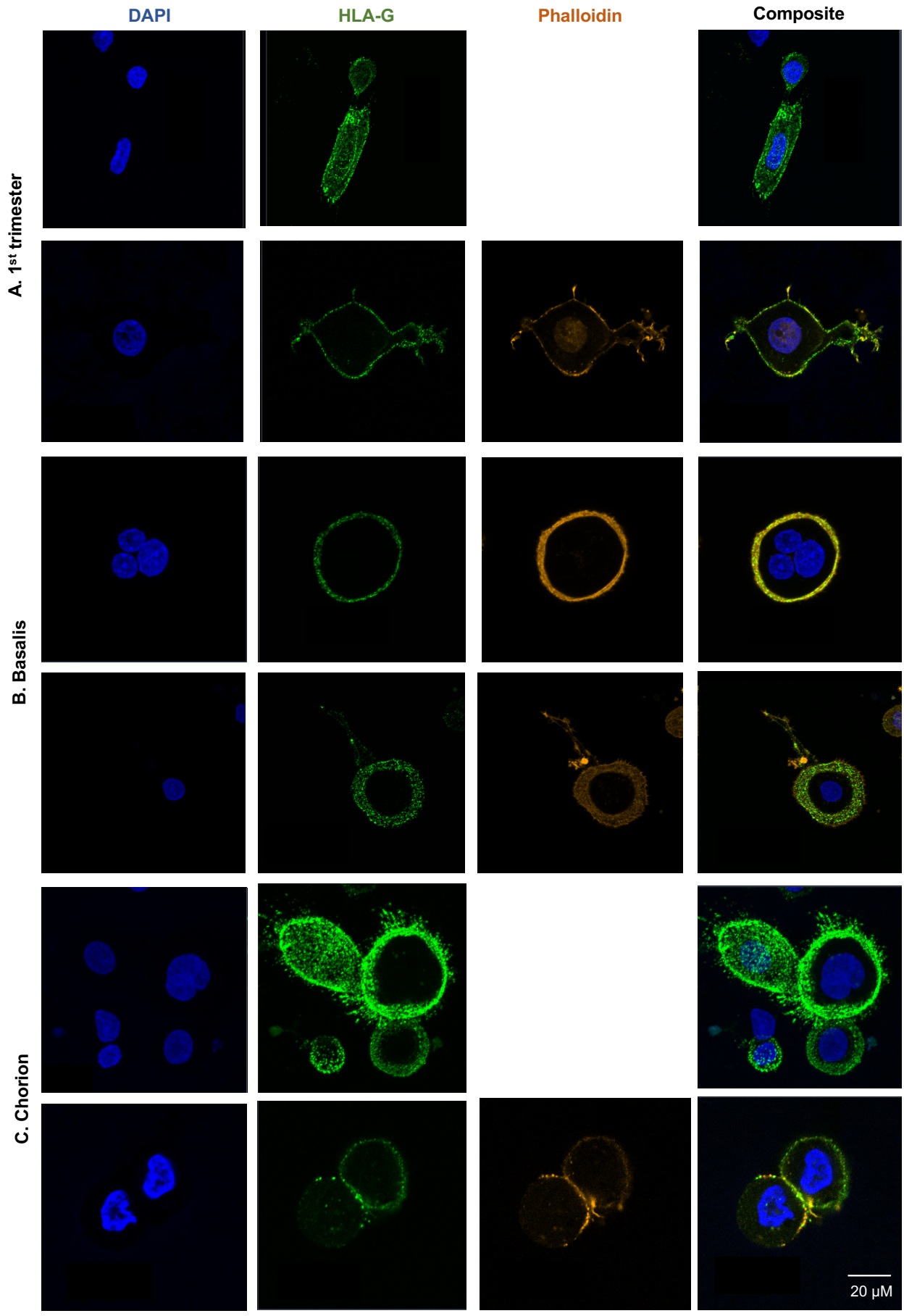
In Figure 10A. we observe an elongated cell morphology with many projections, that is consistent with the superior invasive nature of 1st trimester EVT. [59] The projections haven't been classified yet, but we hypothesize that these projections might be nanotubes. [60]

Basalis EVT (Figure 10B.) are characterized as less invasive trophoblasts (than 1st trimester EVT) and are mostly large multi-nucleated cells with or without projections. In Figure 10D. a giant multinucleated cell (18 nuclei) was found in Decidua Basalis EVT.

Last, in Figure 10C. Chorion EVT morphology differs from round single- to multi- nucleated cells with few short projections, what demonstrate that Chorion EVT are the least invasive trophoblast type.

In conclusion, elongated cells with many projections and connections can be classified as 1st trimester EVT, the most invasive EVT type among the three examined EVTs. Large multinucleated cells with or without projections can be identified as Decidua Basalis EVT, whereas large single and multi- nucleated cells with few and short projection are categorized as least invasive Chorion EVT.

However, more investigation and quantification of EVT cell morphology is needed. For instance, quantification experiments including the characterization of cell size and shape, percentage of cells with short and/or long projections, number of nuclei per cell etc. Nevertheless, some preliminary observations can be stated.


DAPI
HLA-G
Phalloidin
Composite
A. 1st trimester
B. Basalis
C. Chorion
20 µM

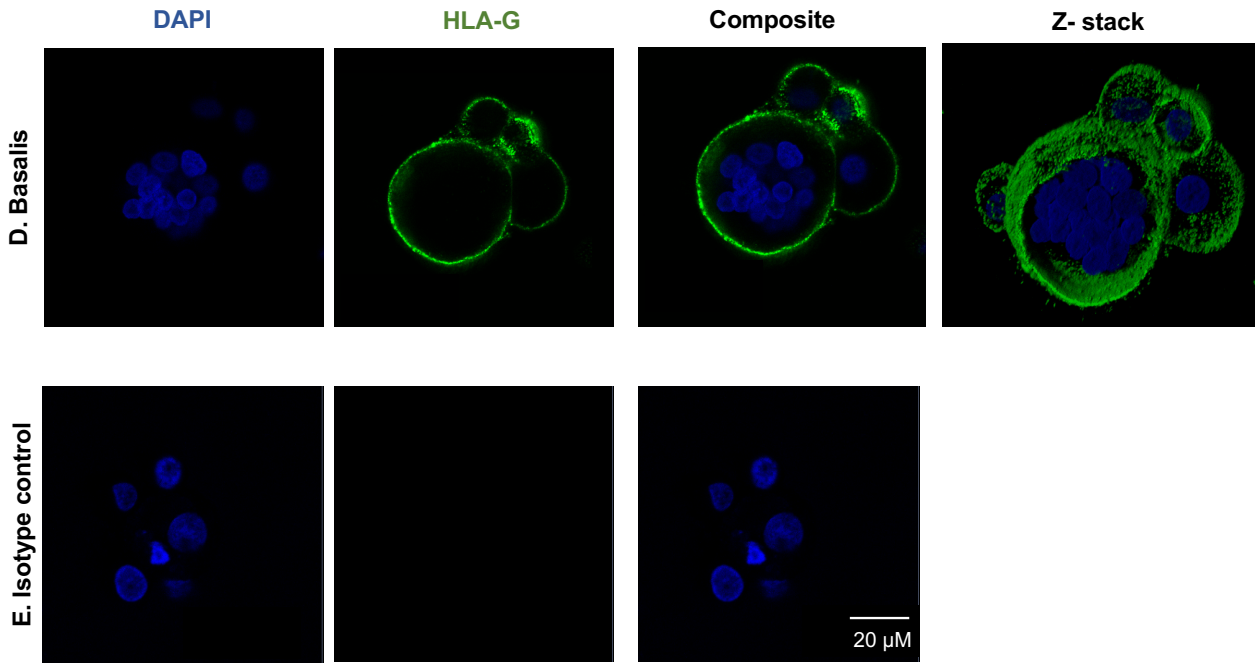


Figure 11. 1st trimester, Decidua Basalis and Chorion HLA-G+ EVT display different morphologies dependent on their function in invasion and immune tolerance maintenance.
Confocal microscopy of (A.) 1st trimester, (B. and D.) Decidua Basalis and (C.) Chorion HLA-G+ EVT, showing different cell morphologies in the matter on nuclei number and projection structure. The panel depicts DAPI, HLA-G, Phalloidin, composite and Z stack images.
(E.) shows Isotype control stain IgG+ GAM-A488 performed on the 1st trimester HLA-G+ EVT in order to confirm HLA-G specific binding.

Genome editing

DGCR8/AGO2 Knock Out

To test our hypothesis, whether the KO of miRNA regulators (DGCR8 and AGO2) alters the HLA-C, HLA-E or HLA-G expression levels in the trophoblast-like choriocarcinoma cell line JEG3, we utilized a CRISPR/Cas9 genome editing strategy. The target of the KO was located on Exon1 of DGCR8 and AGO2 in JEG3. A schematic representation of the targeting strategy can be seen in the Supplemental Figure 3A. Four different guide RNAs (gRNA) for each gene (Supplemental Table 3.) were designed, cloned and tested to obtain the best cutting efficiency. Next, JEG3 were transfected with CRISPR gRNAs and Cas9_GFP. The transfection efficiency was checked 24 hours post-transfection using a fluorescence microscope. The non-transfected cells (negative control) showed no GFP expression, while the transfected cells expressed GFP. The FACS sort for GFP positive cells was carried out 48 hours post- transfection. The GFP positive

sorted cells were re-plated in low density, to allow colonies derived from a single parent cells to grow. Subsequently, the colonies were picked into 96-well plate and used for large scale PCR screening followed by genotype confirmation using qPCR, Western Blot and DNA Sequencing (Supplemental Figure 3.).

We were able to establish 4 DGCR8 KO clones confirmed by sequencing but surprisingly not by Western Blot and qPCR. The DNA sequencing showed a 133 bp deletion (deletion with frameshift) for all 4 DGCR8 KO clones. However, the DGCR8 protein still appear to be present in the Western Blot analysis (data not shown). Nor the qPCR data confirmed the DGCR8 KO, as a lower delta CT value of DGCR8 mRNA suggests more transcripts to be present. (Figure 11A.). The miRNA qPCR data showed no difference between the miR148a, miR150 and miR152 content in DGCR8 KO clones and also no difference inbetween the examined genotypes. (Figure 12.) Despite the fact, we decided to continue the project and new qPCR primer pairs are currently being tested.

Moreover, 10 AGO2 KO clones confirmed by Sequencing, Western Blot and qPCR were established. The length of deletions varied from 11bp to 80 bp and only the clones containing a frameshift deletion were considered to be AGO2 KO clones. Western Blot data showed clear KOs with no protein being present (Supplemental Figure 3E.), as well as the elevated delta CT value of AGO2 mRNA implicate the KO phenotype (Figure 11B.). Similarly, to DGCR8 KO clones, the miRNA qPCR data didn't show a difference in miRNA content for AGO2 KO clones. (Figure 12.)

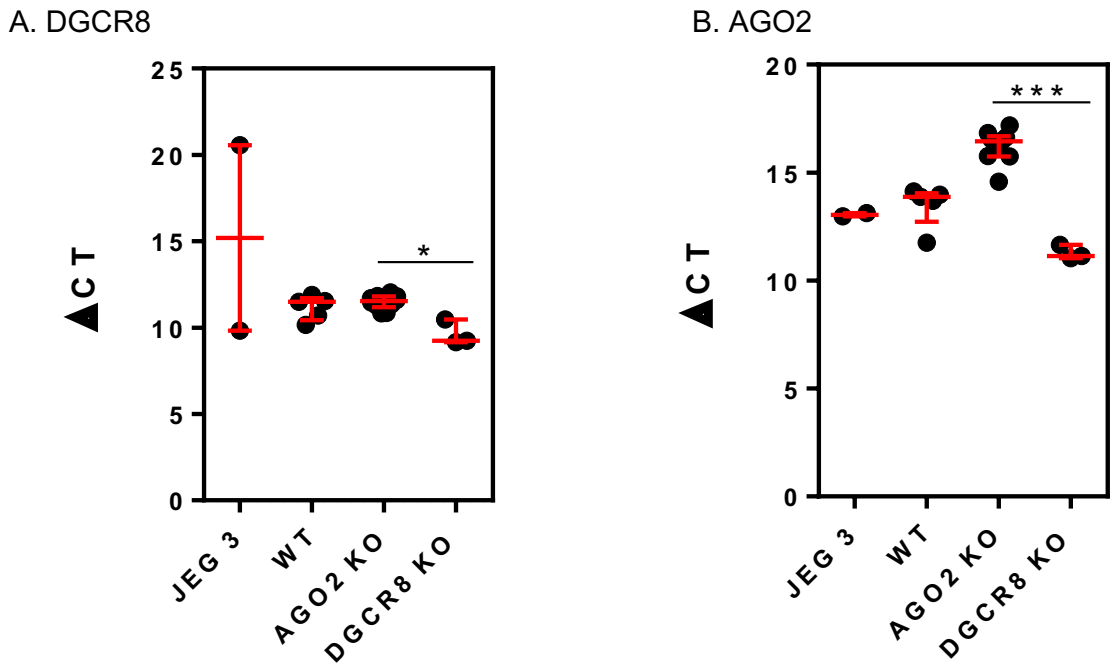


Figure 12. DGCR8 mRNA delta CT values of DGCR8 KO clones are lower than AGO2 KO clones, while AGO2 mRNA levels of AGO2 KO are the highest among examined genotypes.

The graphs depict the (A.) delta CT values of DGCR8 and (B.) AGO2 mRNA levels in parental JEG3, WT, AGO2 and DGCR8 KO clones.

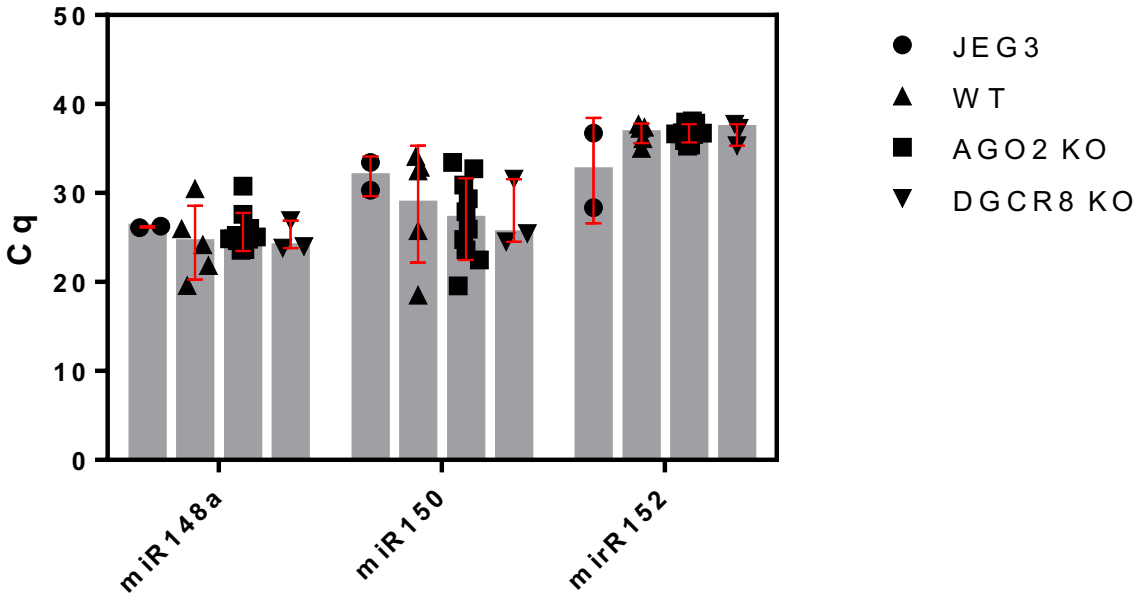


Figure 13. No change in miR148a, miR150 and miR152 content between JEG3, WT, AGO2 and DGCR8 KO clones.

The graph depicts the miRNAs (miR148a, miR150 and miR152) expression levels in parental JEG3, WT, AGO2 and DGCR8 KO clones respectively. Circular data points show JEG3, triangular the wild-types, square AGO2 KO clones and reverse triangular DGCR8 KO clones.

Constitutive HLA-C, HLA-E and HLA-G expression

After the establishment of DGCR8 and AGO2 KO cell lines, FACS (Figure 13. left) and qPCR (Figure 13. right) for HLA-C, HLA-E and HLA-G was performed to determine the constitutive expression of these HLA molecules.

JEG3 express higher levels of HLA molecules than WT (Figure 13.), however we can only speculate about the cause of this phenomenon. We suppose there might be a genotypic drift due to high number of passages or the transfection procedure. These discrepancies might suggest some sort of contamination or truncated proteins as well. [61]

The variation in HLA expression levels between all clones is high, especially for HLA-C and AGO2 KO clones, one of the reasons might be a different length of deletion and therefore a different frameshift. Notwithstanding, this doesn't explain the variation in WT expression levels. HLA-E expression levels didn't show variation (Figure 13B.)

DGCR8 KO clones have lower HLA-G expression than AGO2 KO clones (Figure 13C.), but this data is only supported by the FACS and not by the qPCR results. Consistent with the data discussed in the previous section, and the hypothesis stated in section 1.3, the KO of AGO2 protein caused an increase in HLA-G expression levels via miRNA regulation.

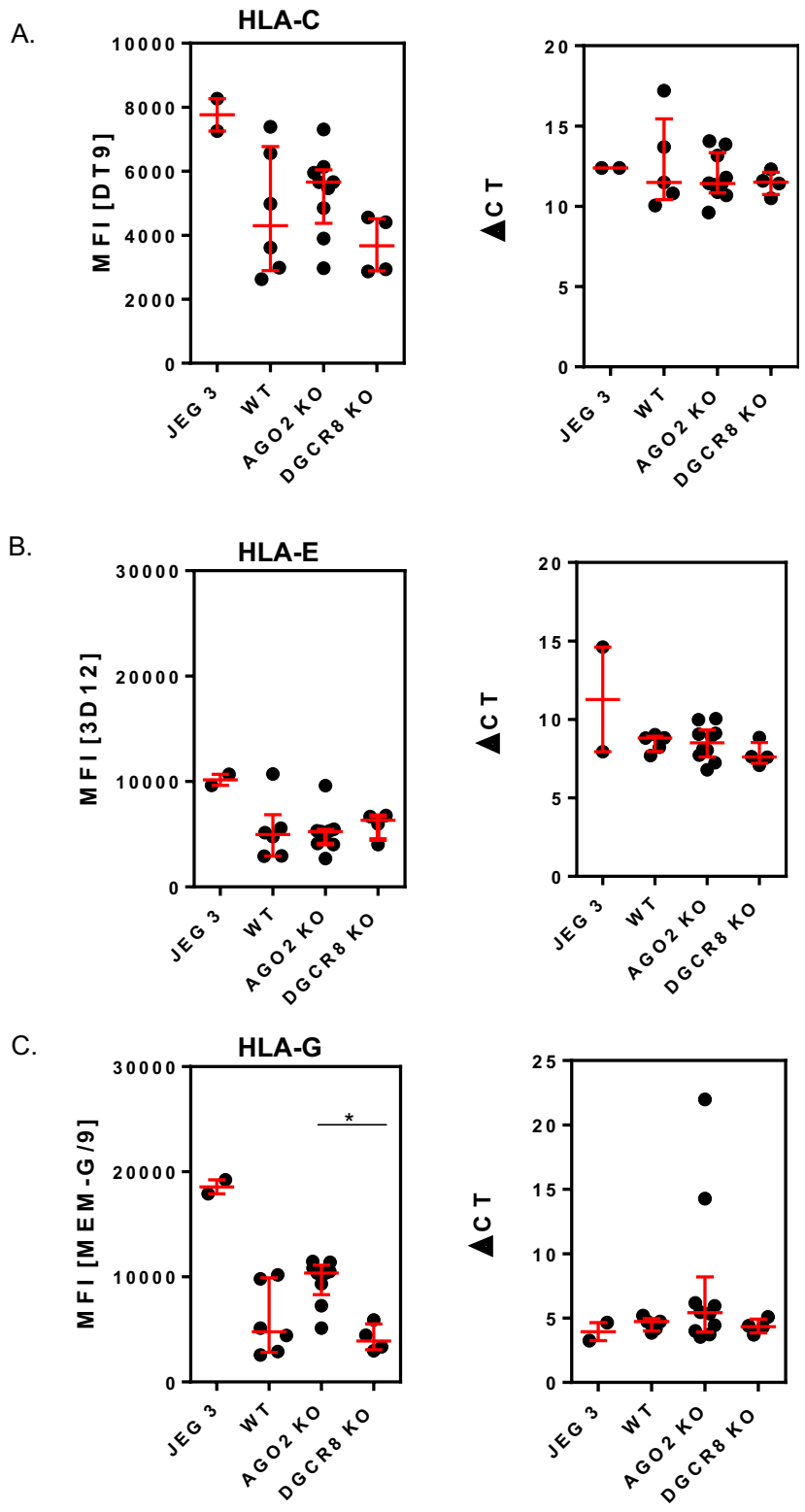


Figure 14. DGCR8 KO clones have lower HLA-G expression than AGO2 KO clones.
The representative FACS plots and graphs depicting mRNA levels of constitutive HLA-C (DT9), HLA-E (3D12) and HLA-G (MEM- G/9) expression in parental JEG3, WT, AGO2 and DGCR8 KO clones.

Induced HLA-C, HLA-E and HLA-G expression

Next, we determined HLA expression on all clones before and after stimulation witg TNF**α** and IFN **γ**. The HLA-C expression levels were upregulated by IFN **γ** only in JEG3, WT and AGO2 KO clones, but not in the DGCR8 KO clones. The upregulation of HLA-C was significantly higher for JEG3 and WT, ~5 fold, while about ~3 fold upregulation can be observed for AGO2 KO clones (Figure 14A.).

IFN **γ** upregulates HLA-G only in WT (~3 fold), AGO2 KO clones (nearly 2 fold) and DGCR8 KO clones (~3 fold) but not in JEG3. (Figure 14C.) It has been previously investigated that the expression of HLA-G (in placenta) is not altered by IFN **γ**. [62] And thus, it seems legitimate to state that a genotypic drift has occurred in the transfected clones.

In resemblance to primary EVT stimulation results, no effect of TNF**α** on any investigated HLA molecule was observed.

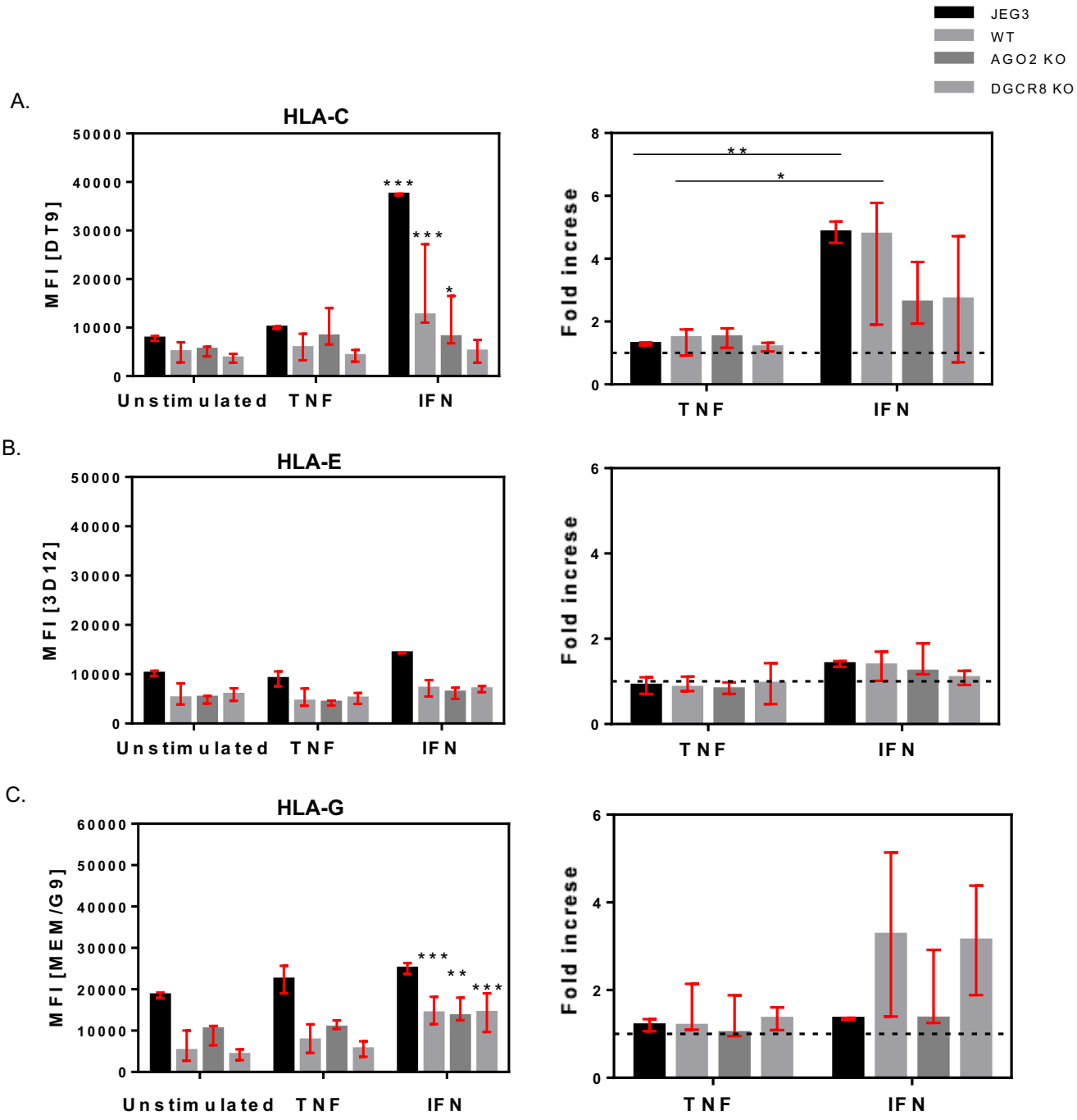


Figure 15. HLA-C expression is upregulated by IFNγ only in parental JEG3 and WT clones, while HLA-G expression is upregulated by IFNγ in WT and both KO lines.
The graphs depict the Mean Fluorescence intensity (MFI) and fold increase after TNFα and IFNγ stimulations of (A.) HLA-C (DT9) and (B.) HLA-G (MEM-G/9) induced expression levels in parental JEG3, WT, AGO2 and DGCR8 KO clones.
TNFα was used at 20 ng/mL concentration and IFN γ at 100 ng/mL. The significance is expressed with respect to unstimulated samples.

Discussion

Important mechanisms establishing the fetal-maternal immune tolerance during pregnancy are majorly mediated by HLA-C, HLA-E and HLA-G surface expression levels on EVT and the HLA interactions with immune cells such as NK and T cells. [63]

The obtained results demonstrate that different EVT type express different levels of HLA molecules, have different morphology and react to the environment differently. 1st trimester EVT have the highest HLA-C and HLA-E expression levels, while Decidua Basalis EVT have the highest HLA-G expression. The interactions with extracellular fibronectin upregulated HLA-C and HLA-G in the 1st trimester EVT. The upregulation is beneficial for cell adhesion interactions during the placenta formation and establishment of maternal-fetal tolerance and therefore was not detected for term EVT, where the tolerance has been already established. [64]

IFNγ upregulated HLA-C in the 1st trimester tissue, but not in term EVT. The cytokine upregulation mechanism is given by the MHC class I enhancosome structure, especially the ISRE element, which binds the regulatory transcription factor IRF-1. [39] Furthermore, the trophoblast specific transcriptional regulator NLRP2 also influences cytokine induced expression of HLA-C. [40] IFNγ is a class II interferon, which is able to of direct viral inhibition and has profound immuno- stimulatory and modulatory effects. The IFNγ production is mainly mediated by NK cells, which are abundant just during the 1st trimester pregnancy and their population decreases over gestation. Additionally, HLA-C molecules are the main ligands for KIR receptors on NK cells. [65, 66] The non-responsiveness of HLA-G to IFNγ has been previously described due to IFNγ- inducible gene expression silencing. [58] In contrast, TNFα as an endogenous pyrogen, which induces fever and inflammation, didn't cause a HLA-C (HLA-E or HLA-G) upregulation. The main immune cells responsible for production are macrophages, which interact with MHC class II and not with MHC class I molecules. [67]

Interestingly, fetal gender correlates with HLA-G expression levels. EVT from pregnancies with male fetuses have about 2-fold higher HLA-G expression levels in both, Decidua Basalis and Chorion, term EVT, than with female fetuses. To begin with, male fetuses tend to be larger and heavier at term and are associated with longer pregnancies. At term pregnancy, the

testosterone levels in maternal periphery are the highest (about 830 pg/mL for male fetuses pregnancies). Even increased soluble HLA-G levels in amniotic fluid are related to male fetuses. [68, 69]

Additional regulatory mechanisms for male fetuses may be necessary to allow immune tolerance for Y-chromosome related proteins that the maternal immune system can recognize as foreign. However, the immune responses and immunization of male fetuses is smaller than of female fetuses. As a result of increased immune responses of female fetuses, they are more susceptible to develop autoimmune diseases. Some of the reasons, explaining the greater immune responses are higher progesterone levels which reduce NK cell cytotoxicity and overall higher T cell population. These factors may explain increased HLA-C expression levels in 1st trimester EVT isolated from pregnancies with female fetuses in matters of establishing the tolerance. Moreover, placentas from pregnancies with female fetuses show higher expression of immune autosomal genes, such as Janus Kinase1, Clusterin or Interleukin-2 receptor subunit beta. [70, 71].

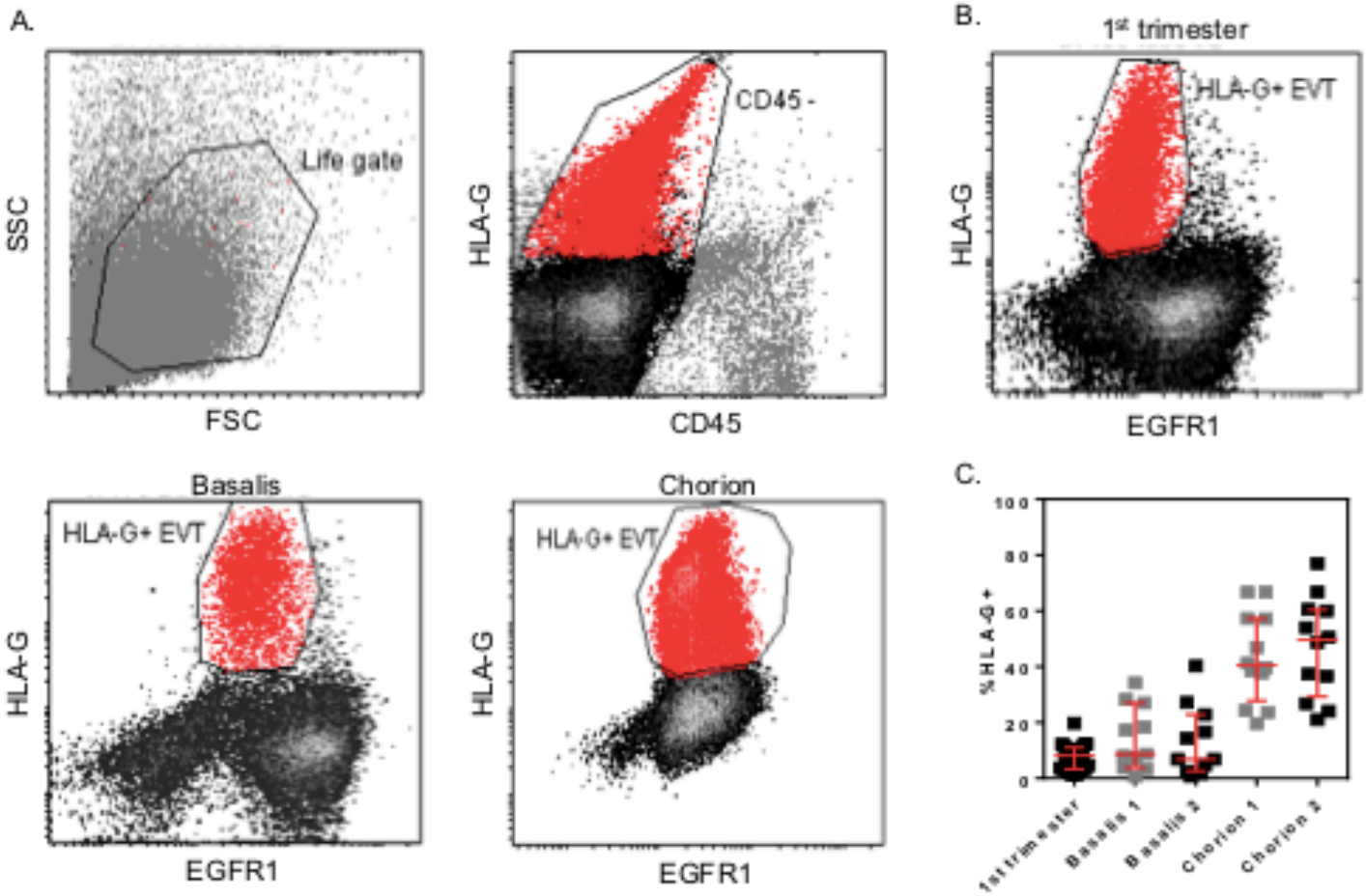
The HLA class I expression is regulated at transcriptional and post-transcriptional level, but it is also regulated by the stability of the HLA protein. The post-transcriptional regulators of HLA expression are miRNAs. The production of mature miRNAs is dependent on micro processing events mediated by regulators such as DGCR8 and AGO2. [43]

We were able to generate 10 AGO2 and 4 DGCR8 KO lines and determine their constitutive and induced HLA cell surface expression, but the acquired data, do not fully support the hypothesis stated in section 1.3. The KO of DGCR8 and AGO2 proteins didn't decrease the miRNA content and nor increase the HLA expression levels. Despite the confirmation of protein knock outs by sequencing, the knock out wasn't confirmed by qPCR and for DGCR8 even western blot didn't show conclusive results. Therefore, we speculate that the proteins may be still present at very low levels or a truncated version of protein is present. Still, it is not clear, whether they are functional or not. A possible interpretation is that a truncated, but still functional DGCR8 protein was produced due to exon skipping or an alternative use of a start codon. Another theory includes compensating mechanisms and other regulators

overtaking the AGO2 function. One of the candidates is Dicer, which can interact with AGO2. However, Dicer independent miRNA processing was also reported. [47]

Unfortunately, I was not able to investigate more and finish the project due to time limitations, but I believe that Strominger Lab members will keep going and finish the project soon.

Annex



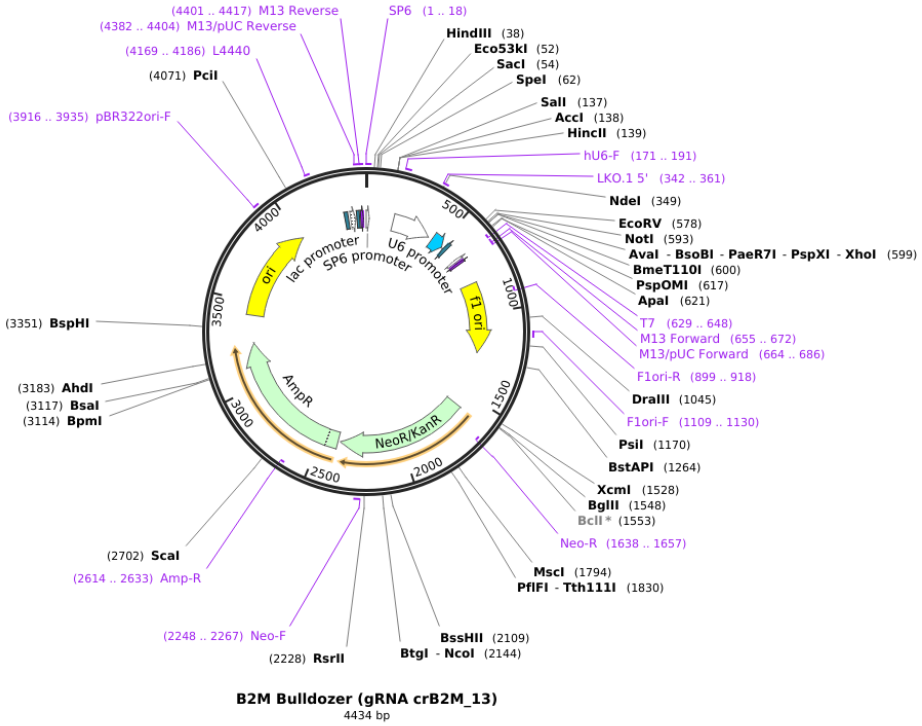
Supplemental Figure 1. Gating strategy used for FACS
(A.) A representative dot plot of total acquired events directly after trophoblast isolation, live cells were gated out and the selection was used to gate CD45- population.
(B.) The dot plots show CD45- cells within live gate and were used to gate out HLA-G+ EVT for each trophoblast type; 1st trimester, Decidua Basalis and Chorion respectively. The gated HLA-G+ EVT were used for the determination of HLA-C, -E and –G expression levels.
(C.) Shows the purity of HLA-G+ trophoblast isolates within CD45- gate for 1st trimester, Decidua Basalis (1st and 2nd digestion) and Chorion (1st and 2nd digestion) EVT. Chorion shows the greatest EVT purity with 40-50% HLA-G+ EVT in the CD45- gated population.

Supplemental Table 1. List of antibodies used for flow cytometry

Antibody	Species	Company	Catalog No.	Dilution
Anti-human EGF Receptor FITC	Rat	Serotec	MCA1784F	1:50
Anti-human mAb HLA-G APC, clone MEM-G/9	Mouse	Molecular Probes	A15708	1:50
Anti-human CD45 PerCP, clone HI30	Mouse	Biolegend	304026	1:100
Anti-human HLA-C PE, clone DT9	Mouse	BD	566372	1:50
Anti-human HLA-E PE, clone 3D12	Mouse	Biolegend	342604	1:100
Isotype control IgG2b PE	Mouse	BD	556437	1:50

Supplemental Table 2. List of antibodies and dyes used for confocal microscopy

Primary Antibody	Species	Company	Catalog No.	Dilution
Anti-HLA-G, clone MEM-G/9	Mouse	Abcam	Ab7758	1:50
Purified anti-mouse IgG$_1$, clone A85-1	Mouse	BD Biosciences	553440	1:50
Secondary Antibody	**Species**	**Company**	**Catalog No.**	**Dilution**
A488 Goat anti-mouse IgG	Mouse	Invitrogen	A11029	1:100
Dye	**Company**	**Catalog No.**	**Dilution**	
Rhodamine Phalloidin	Invitrogen	R415	1:200	
Annexin V, A647 conjugate	Thermo Fisher	A23204	1:10	
Mounting Medium	**Counterstain**	**Company**	**Catalog No.**	**Drop volume**
VECTASHIELD® HardSet Antifade Mounting Medium	DAPI	Vectro Laboratories	H-1500	10 μL



Supplemental Figure 2. B2M Bulldozer (gRNA crB2M_13)
PCGT2 vector (Addgene plasmid #84381) was used for CRISPR gRNA cloning. [27]

Supplemental Table 3. The list of gRNA used.
This table contains all oligonucleotide sequences that were used to clone the respective CRISPR gRNAs into PCGT2 vector. The gRNA sequence is in uppercase and the vector backbone is in lower case.

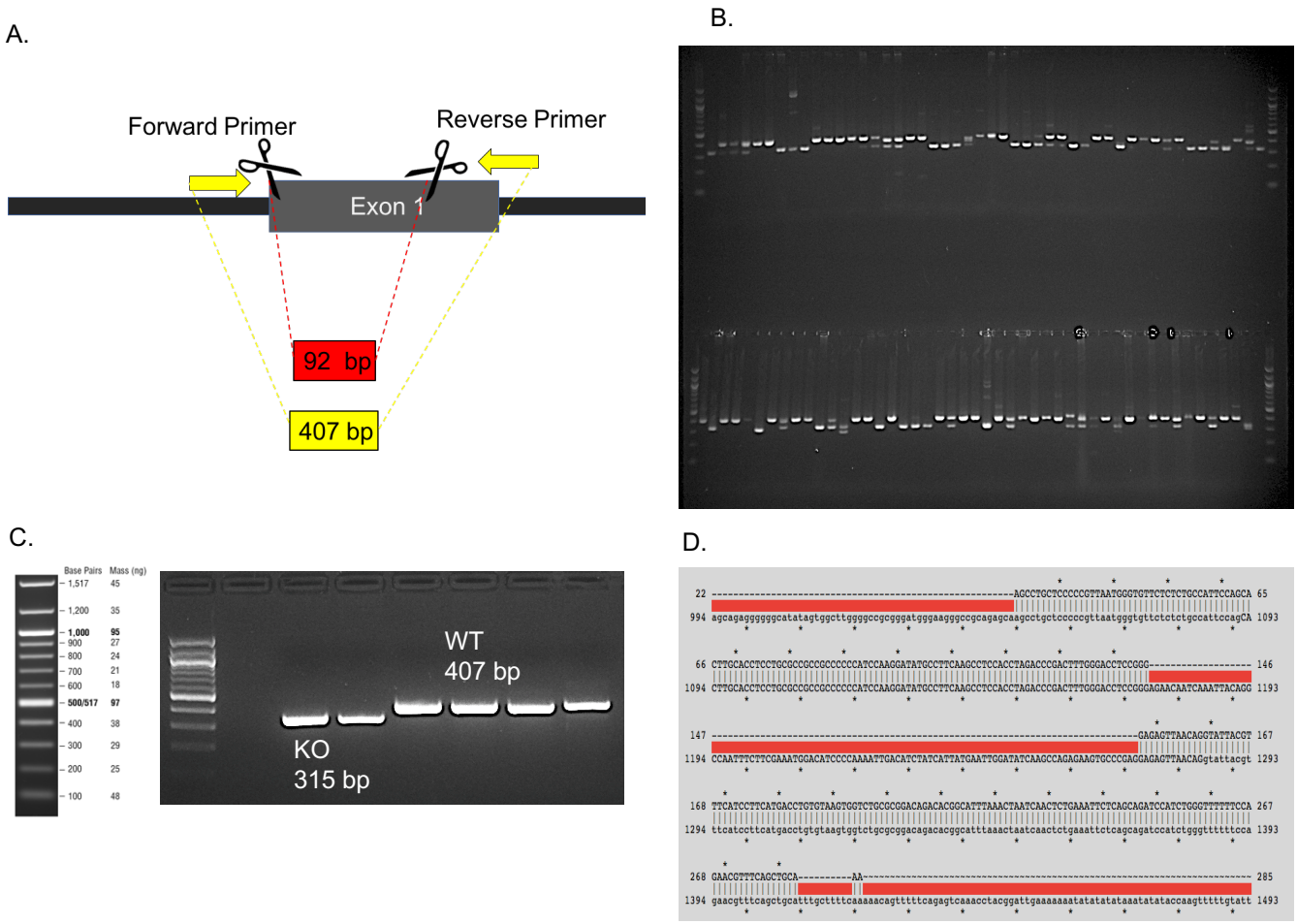
Name	Gene	Direction	Sequence 5'to 3'
AC1	*AGO2*	Forward	caccgCTCCACCTAGACCCGACTTT
AC1	*AGO2*	Reverse	aaacAAAGTCGGGTCTAGGTGGAGC
AC2	*AGO2*	Forward	caccgTCAAGCCAGAGAAGTGCCCG
AC2	*AGO2*	Reverse	aaacCGGGCACTTCTCTGGCTTGAC
AC3	*AGO2*	Forward	caccgCGGGAGAACAATCAAATTAC
AC3	*AGO2*	Reverse	aaacGTAATTTGATTGTTCTCCCGC
AC4	*AGO2*	Forward	caccgAGTGCCCGAGGAGAGTTAAC
AC4	*AGO2*	Reverse	aaacGTTAACTCTCCTCGGGCACTC
DG1	*DGCR8*	Forward	caccgTGTGAGGGCTTGTAAAACTC
DG1	*DGCR8*	Reverse	aaacGAGTTTTACAAGCCCTCACAC
DG2	*DGCR8*	Forward	caccGCAAACGTCCAGTGGTGCAG
DG2	*DGCR8*	Reverse	aaacCTGCACCACTGGACGTTTGC
DG3	*DGCR8*	Forward	caccgAGTCTTAAGCGCTTTTAATA
DG3	*DGCR8*	Reverse	aaacTATTAAAAGCGCTTAAGACTC
DG4	*DGCR8*	Forward	caccGGTGGTGGAGACTGCTCACG
DG4	*DGCR8*	Reverse	aaacCGTGAGCAGTCTCCACCACC

Supplemental Table 4. List of primers used for PCR and qPCR
This table consists of all primer sequences to amplify AGO2, DGCR8, in both PCR and qPCR, and all investigated HLAs in qPCR.

Gene	Direction	Sequence 5'to 3'	Application
Primary EVT			
ATL1	Forward	GCCACAGTATTTGCCCTTAGC	PCR
ATL1	Reverse	AATTCGTATGGGAAACTCCAGTC	PCR
SRY	Forward	AGAGAATCCCAGAATGCGAAC	PCR
SRY	Reverse	CTTCCGACGAGGTCGATACTT	PCR
Genome editing			
AGO2	Forward	GGGCATATAGTGGCTTGGGG	PCR
AGO2	Reverse	TGCAGCTGAAACGTTCTGGA	PCR
AGO2	Forward	GCACCGGCAGGAGATCATAC	qPCR
AGO2	Reverse	AGACACCGTCGCGGTAGAA	qPCR
DGCR8	Forward	GTCTCAGCGGACTTGTGCAT	PCR
DGCR8	Reverse	TACTCCTGCAGCTCTCGGTA	PCR
DGCR8	Forward	GCAGAGGTAATGGACGTTGG	qPCR
DGCR8	Reverse	AGAGAAGCTCCGTAGAAGTTGAA	qPCR
GAPDH	Forward	GAAGGTGAAGGTCGGAGT	qPCR
GAPDH	Reverse	GAAGATGGTGATGGGATTTC	qPCR
HLA-C	Forward	GTGTCCACCGTGACCCCTGTC	qPCR
HLA-C	Reverse	ATTCAGGTTCTTAACTTCAT	qPCR
HLA-E	Forward	GCACAGATTTTCCGAGTGAAT	qPCR
HLA-E	Reverse	CAGCCATGCATCCACTGC	qPCR
HLA-G	Forward	GCTGCCCTGTGTGGGACTGAGTG	qPCR
HLA-G	Reverse	ACGGAGACATCCCAGCCCCTTT	qPCR

Supplemental Table 5. List of antibodies used for western blot.
This table compiles all primary and secondary antibodies used to detect AGO2 and DGCR8, as a loading control, expression in wildtype and knockout cells.

Primary Antibody	Species	Company	Catalog No.	Dilution
Anti- Argonaute-2 antibody	Rabbit	Abcam	ab186733	1:1000
DGCR8 Polyclonal Antibody	Rabbit	Thermo Fisher	PA5-41899	1:1000
Purified anti-HSP70 (HSPA8), clone 9/2	Mouse	BioLegend	682102	1:500
Secondary Antibody	**Species**	**Company**	**Catalog No.**	**Dilution**
ECL™ Anti-mouse IgG, Horseradish Peroxidase linked	Sheep	GE Healthcare	NA931V	1:5000
ECL™ Anti-rabbit IgG, Horseradish Peroxidase linked	Donkey	GE Healthcare	NA934V	1:2000



Supplemental Figure 3. Schematic representation of confirmation of genotype and establishment of clones for AGO2.

(A.) CRISPR/Cas9 strategy

(B.-C.) PCR screen of AGO2, with guide efficiency of approximately 30%.

(D.) Sequencing was performed by genewiz. A deletion of 79 bp is shown and therefore a KO clones is present.

(E.) Confirmation of genotype by western blot.

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