Published in final edited form as:

Am J Reprod Immunol. 2009 June ; 61(6): 453–461. doi:10.1111/j.1600-0897.2009.00706.x.

Suppression of Mamu-AG by RNA interference

Jessica G. Drenzek¹, Jolanta Vidiguriene⁴, Geminis Vidiguris⁴, Richard L. Grendell³, Svetlana V. Dambaeva^{1,2}, Maureen Durning^{1,3}, and Thaddeus G. Golos^{1,2,3}

¹Department of Obstetrics and Gynecology, School of Medicine and Public Health

²Department of Comparative Biosciences, School of Veterinary Medicine

³Department of Wisconsin National Primate Research Center, University of Wisconsin-Madison, Madison WI 53715, USA

⁴Promega Corporation, Madison, WI 53711, USA

Abstract

Problem—The role of placental MHC class I molecules in pregnancy is not well understood. Mamu-AG, the rhesus monkey homology of HLA-G expressed in the human placenta, was targeted for degradation by RNA interference (RNAi), a powerful tool to aid in determining gene function, to determine the effect that this knockdown has on NK cell function.

Method of Study—A series of potential target short hairpin RNA (shRNA) sequences to suppress Mamu-AG expression was screened, which identified an optimal sequence to use in transfection experiments. Knockdown in two different Mamu-AG-expressing cell lines was measured by flow cytometry. Cytotoxicity assays were performed to correlate Mamu-AG expression with NK cell cytotoxicity.

Results—Decreased expression of Mamu-AG by short interfering RNA (siRNA) (70-80%) in cell types tested was associated with increased lysis of Mamu-AG target cells.

Conclusion—Target sequences have been identified that knocked down Mamu-AG expression by RNAi and increased lysis by NK cells. This supports the concept that NK cell receptors recognize this placental nonclassical MHC class I molecule.

Keywords

maternal-fetal immune tolerance; natural killer cells; nonhuman primates

Introduction

The mechanisms by which the semiallogeneic fetus avoids a maternal immune response remain incompletely understood, but it is widely thought that a better understanding of these mechanisms may provide insight into the etiology of early pregnancy loss or abnormal placental growth and development. Nonclassical MHC class I molecules are thought to contribute to the ability of the placenta to evade maternal attack. Mamu-AG is a nonclassical MHC class I molecule expressed in the rhesus monkey placenta thought to be the functional homolog of Human Leukocyte Antigen (HLA)-G in humans.^{1,2} Similarities include limited polymorphism, restricted tissue distribution, alternative splicing of mRNAs, a truncated cytoplasmic domain, a premature stop codon, and synthesis of multiple isoforms in

trophoblasts.^{1,2} Comparable to HLA-G, Mamu-AG is expressed at high levels in the placenta and has restricted low level expression in other tissues.³

In this study we sought to suppress Mamu-AG expression by RNA interference (RNAi) in order to further investigate the role of Mamu-AG. RNAi is a form of sequence-specific posttranscriptional gene silencing in which the introduction of double stranded RNA (dsRNA) into a cell leads to degradation of mRNAs with complementary sequence.^{4,5} The placenta is hypothesized to avoid a maternal immune system attack in part by expressing nonclassical MHC class I molecules that interact with inhibitory receptors on natural killer (NK) cells. The first goal of the following experiments was to identify target sequences that specifically suppress Mamu-AG expression. Two different Mamu-AG-expressing cell lines were transfected with short hairpin RNAs (shRNAs) using a DNA-directed RNAi approach. DNA-directed RNAi is a cloning-based approach that allows direct ligation of hairpin oligonucleotides into a ddRNAi vector.⁶⁻⁸ This method offers the potential of stable, long-term inhibition of gene expression. The second goal was to measure the effect of Mamu-AG suppression with natural killer cell cytotoxicity assays to determine whether cells expressing Mamu-AG were protected from NK cell mediated lysis.

Materials and Methods

Animals

Rhesus monkeys (*Macaca mulatta*) were from the colony maintained at the Wisconsin National Primate Research Center. All surgical procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and under the approval of the University of Wisconsin Graduate School Animal Care and Use Committee.

Oligonucleotide design and selection

Two approaches were used to select sequences for antisense RNAs. A series of 25mer antisense oligonucleotides, tiled at one nucleotide intervals, were synthesized complementary to Mamu-AG*02012 mRNA and Mamu-E*05 mRNA by Maskless Array Synthesizer (MAS) technology onto a custom microarray (Roche NimbleGen, Madison, WI). Each oligo synthesized was the complement of the target gene, thereby representing a possible siRNA. Seven copies of each oligonucleotide were randomly placed on the microarray.

Mamu-AG or Mamu-E coding sequence was ligated into a pGEM vector (Promega, Madison, WI), the vector was linearized, and in vitro transcription of RNA from the cDNA templates was performed using T7 RNA polymerase (MEGAscript, Ambion, Austin, TX). The transcription products were purified with the RNeasy kit (Qiagen, Valencia, CA). The in vitro synthesized mRNA was labeled with *Label* IT biotin (Mirus, Madison, WI).

The microarray was prehybridized in a solution composed of 1X MES hybridization buffer containing 0.1 mg/ml herring sperm DNA and 0.5 mg/ml acetylated BSA for 15 min at 45°C, the prehybridization solution was removed, and hybridization solution composed of 10 µg fragmented mRNA, 1X MES hybridization buffer (RocheNimblegen), 1 nM CPK6 Oligo (Roche Nimblegen), 0.1 mg/ml herring sperm DNA, and 0.5 mg/ml acetylated BSA was added. The samples were then hybridized to the microarrays for 16-20 hr at 45°C. Specific hybrids between the target population and the oligonucleotide probes were formed at this time. The next day, the unhybridized probe was washed off and bound probe was stained with Cy-3 streptavidin. Detection of Cy-3 was at 550 nm.

In addition, the Promega siRNA Target Designer Algorithm (www.promega.com/siRNADesigner) was used to predict effective shRNA target

sequences. Selected target sequences were also synthesized into siRNAs (Dharmacon, Lafayette, CO).

Subcloning shRNA sequences

Candidate shRNA oligonucleotides were synthesized that contained additional sequences for ligation into the psiSTRIKE vector (Promega, Madison, WI). The oligonucleotides were synthesized at the University of Wisconsin Biotechnology Center and subcloned into the psiSTRIKE vector (Promega) using the protocol recommended by the manufacturer.

Optimization of shRNA sequences

Mamu-AG cDNA was removed from the pGEM-7zf(+) vector by restriction enzyme digest with *Xho* I and *Hind* III and subcloned into the psiCHECK-2 vector (Promega) digested with *Xho* I and *Pme* I. The psiCHECK-2-Mamu-AG vector was co-transfected with each psiSTRIKE-shRNA vector into BRL cells using FuGENE 6 (Roche, Basel, Switzerland). shRNA against *Renilla* luciferase, nonspecific shRNA, or empty psiSTRIKE vector served as controls. Three thousand BRL cells were plated per well of a 96 well clear bottom black plate (Costar, Corning, NY) in 75 μ l DMEM (Gibco, Carlsbad, CA) supplemented with 10% HI FBS (Atlanta, Lawrenceville, GA) the day before transfection. The FuGENE 6 to DNA ratios were 6:2 and 6:4. For 6:2, 3 μ l FuGENE 6 was diluted into 47 μ l SFM, and 0.5 μ g psiCHECK-2-Mamu-AG and 0.5 μ g psiSTRIKE-shRNA were added. For 6:4, 3 μ l FuGENE 6 was diluted into 47 μ l SFM, and 1 μ g psiCHECK-2-Mamu-AG and 1 μ g psiSTRIKE-shRNA were added. The transfection complex was incubated 15-45 min at room temperature, and 5 μ l of this transfection complex was then added to the appropriate wells in triplicate.

shRNA efficiency was based on the decrease in *Renilla* activity normalized to firefly luciferase activity measured 48 hr post transfection using the Dual-Glo Luciferase Assay System according to the manufacturer's instructions (Promega). The plate was read on the VICTOR₃TM V Multilabel Counter (Model 1420-041, Perkin Elmer, Waltham, MA) at the Keck-UW Comprehensive Cancer Center Small Molecule Screening Facility.

Transfection of Mamu-AG shRNAs into Mamu-AG expressing BLCL 721.221 cells

721.221 cells stably expressing Mamu-AG were maintained in RPMI 1640 (Gibco) supplemented with 15% HI FBS (Atlanta), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin (Gibco), 1.25 U/ml fungizone (Gibco), and 100 μ g/ml G418 (Gibco).⁹ For transfection, 2×10^6 721.221-Mamu-AG transfectants were centrifuged at 1000 rpm for 10 min and resuspended in 100 μ l Cell Line Nucleofector Kit V buffer (amaxes, Gaithersburg, MD). Two micrograms of plasmid DNA (nonspecific-shRNA/psiSTRIKE-hMGFP, Mamu-AG-shRNA/psiSTRIKE with a puromycin selectable marker, or Mamu-AG-shRNA/psiSTRIKE-hMGFP) or 2 μ g Mamu-AG-siRNA were added to the cells and the suspension was transferred to the nucleofection cuvette. Transfection program A-024 on the nucleofector (amaxes) was used to transfect the cells. The transfected cells were then transferred to a 6 well plate with 3 ml of culture media. Seventy-two hours post transfection, the cells that were transfected with the psiSTRIKE-hMGFP vector were sorted for GFP and then labeled with the anti-Mamu-AG monoclonal antibody 25D310 or the mouse IgG1 isotype-specific control followed by PE anti-mouse IgG1 secondary antibody (BD Pharmingen, San Jose, CA) to determine transfection efficiency and the percent knockdown. Data were collected on a flow cytometer (FACS Calibur, BD Biosciences, San Jose, CA) using Cell Quest Software (BD Biosciences, San Jose, CA), and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Selection of the cells that were transfected with the psiSTRIKE-puro vector was done in culture medium containing 2 μ g/ml puromycin (InvivoGen, San Diego, CA).

Transfection of Mamu-AG shRNAs into first trimester rhesus monkey trophoblasts

Trophoblasts were isolated from a rhesus monkey placenta obtained on day 36 of pregnancy by trypsin/DNase (Sigma) enzymatic digestion and Percoll (Kabi Pharmacia AB, Uppsala, Sweden) gradient as previously described.¹¹ Two million trophoblasts were transfected with 2 µg of siRNA-A, siRNA-B, nonspecific-shRNA in psiSTRIKE-hMGFP, or shRNA-A in psiSTRIKE-hMGFP by nucleofection (amaxa) using program T-023 or U-017. The cells were cultured in DMEM (Gibco) supplemented with 10% HI FBS (Atlanta), HEPES (Sigma, St. Louis, MO), L-glutamine (Gibco), and gentamicin (Sigma). Forty-eight hours post transfection, the transfected trophoblasts were incubated with the 25D3 anti-Mamu-AG monoclonal antibody and analyzed by flow cytometry as described above.

Cytotoxicity Assays

Peripheral blood was collected into heparin tubes from reproductive age female rhesus monkeys. Whole peripheral blood was diluted in three volumes of RPMI 1640 (Gibco) and layered onto a Ficoll-Paque PLUS (Amersham) density gradient. The tubes were centrifuged for 30 min at 2000 rpm with the brake off. The mononuclear cell layer was carefully collected and washed twice with RPMI 1640. The cells were counted and labeled with nonhuman primate anti-CD16 microbeads (Miltenyi Biotec, Auburn, CA) and sorted by MACS on an LS column (Miltenyi Biotec) according to the manufacturer's instructions.

Chromium release cytotoxicity assays were performed by adding $\text{Na}_2^{51}\text{CrO}_4$ (Perkin Elmer, Boston, MA) to $4\text{--}5 \times 10^5$ target cells in 500 µl NK cell medium to achieve a final concentration of 0.1 mCi/ml. NK cell medium is composed of RPMI 1640 (Gibco) supplemented with 10% HI FBS (Atlanta), 2 mM L-glutamine (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco), 0.1 mM sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco), and 55 µM 2-mercaptoethanol (Gibco). The labeled cells were incubated for 1 hr in a CO_2 incubator at 37°C. After the incubation, the labeled cells were washed twice in 10 ml NK cell medium and a viable cell count was performed. The cell concentration was adjusted to 1×10^5 cells/ml in NK cell medium. Fifty microliters of the target cells (total of 5000 cells) were plated into a round-bottom 96 well plate (Nunc) for a total of 18 wells per target (two controls and 4 different ratios in triplicate). One hundred microliters of NK cell medium was added to the control wells for spontaneous release and 100 µl of 1% Triton X-100 (Sigma) in NK cell medium was added to the control wells for maximal release. Four ratios of NK cells were prepared (10:1, 5:1, 2.5:1, and 1.25:1). One hundred microliters of NK cells were added to the targets. The cells were incubated for 4 hr at 37°C with CO_2 . The supernatants were collected with harvesting filters, which were placed inside counter tubes (Biorad, Hercules, CA), and the tubes were placed into a gamma counter (Searle 1185, Chicago, IL). Percent specific lysis = (experimental release-spontaneous release)/(maximal release-spontaneous release) $\times 100$.

An alternative non-radioactive cytotoxicity assay method was also performed.¹² Target cells were adjusted to a concentration of 1×10^5 cells/ml in NK cell medium and 50 µl of these cells were aliquoted in triplicate to a clear bottom white 96 well plate (Costar, Corning, NY). NK cells were added to the target cells at ratios of 10:1, 5:1, 2.5:1, and 1.25:1 in NK cell medium in triplicate. The wells for spontaneous release contained no NK cells, but an additional 50 µl of NK cell medium. The cells were incubated for 4 hr at 37°C in CO_2 . The CytoTox-Fluor Cytotoxicity Reagent (Promega) was prepared according to the manufacturer's instructions. Prior to the addition of the reagent, 10 µl of 1.5 mg/ml digitonin (Promega) was added to the maximum release wells. One hundred microliters of the CytoTox-Fluor reagent was added to each well and mixed briefly by orbital shaking. The reaction was incubated for 30 min to 3 hr at 37°C in a CO_2 incubator, and fluorescence was

read on a microplate reader (SpectraMax, Molecular Devices, Sunnyvale, CA). The excitation wavelength was 485 nm, and the emission wavelength was 520 nm.

Results

MHC-E presents antigenic peptides derived from the leader sequences of other MHC class I molecules.^{13,14} It was important to determine regions of Mamu-AG mRNA that were dissimilar to Mamu-E to ensure that a possible target sequence for Mamu-AG mRNA did not also alter Mamu-E mRNA expression. First, microarray analyses were conducted to identify possible target siRNA sequences unique to Mamu-AG. Mamu-AG and Mamu-E mRNAs were hybridized to arrays tiled with 25mers specific to Mamu-AG and the relative intensity of this hybridization is depicted in Fig. 1. Regions of possible siRNA targeting which hybridized with the Mamu-AG mRNA but not with Mamu-E mRNA were between 250-300 nt or 490-570 nt. An alignment of these two sequences indicated that these were possible regions to target with siRNA due to the most nucleotide mismatches (Fig. 2, yellow boxes). Two sequences, shRNA-E and shRNA-F, were designed manually within these regions following general guidelines for shRNA design (avoiding stretches of 4 or more of any nucleotide to avoid premature transcription termination, having a GC content of 30-70%, and starting with a G nucleotide as the preferred initiation nucleotide for the U6 polymerase). Four additional target sequences (shRNA-A, -B, -C, -D) were selected based on the web-based algorithm, and one scrambled sequence (shRNA-G) was also designed (Fig. 2 and 3).

In order to determine which shRNAs initiated effective suppression of Mamu-AG, the hairpins were ligated into the psiSTRIKE vectors and co-transfected with the psiCHECK-2 vector containing Mamu-AG and two luciferase reporter genes. *Renilla* luciferase is fused to Mamu-AG while firefly luciferase serves as an internal control. Effective RNA interference by a specific shRNA directed against Mamu-AG mRNA will lead to degradation of the entire mRNA resulting in decreased *Renilla* luciferase activity. At a ratio of transfection reagent to DNA of 6:4, shRNA-A and shRNA-B were most effective at knocking down expression of Mamu-AG (Fig. 4, black bars). At a ratio of 6:2, shRNA-A and shRNA-D had the greatest effect (Fig. 4, white bars). These experiments demonstrated that shRNA-A and shRNA-B were appropriate shRNA sequences for further studies. Although shRNA-D also appeared to be effective at knocking down Mamu-AG, its mRNA sequence was a perfect match for Mamu-E as well as Mamu-AG, so it was not considered for further study. It is also important to note that shRNA-G is the scrambled sequence of shRNA-A and that this sequence had no effect on Mamu-AG suppression. Suppression of *Renilla* luciferase expression with a previously characterized *Renilla* shRNA served as a positive control.

Next, it was important to determine if these shRNA sequences can suppress Mamu-AG in Mamu-AG expressing cells. Human BLCL 721.221 cells do not endogenously express MHC class I molecules, with the exception of HLA-E. 721.221-Mamu-AG stable transfectants were transfected with the psiSTRIKE-hMGFP vector containing either shRNA-A, shRNA-B, or a nonspecific sequence. Seventy-two hours post transfection, the cells were sorted by FACS for GFP to enrich for transfected cells, and stained with Mamu-AG-specific 25D3. Relative to nonspecific shRNA (Fig. 5A), there was 81% suppression with shRNA-A (Fig. 5B) and 80% suppression with shRNA-B (Fig. 5C).

Mamu-AG expression was next evaluated in freshly isolated day 36 rhesus monkey trophoblasts that were transfected by nucleofection on the same day as they were isolated. The psiSTRIKE-hMGFP vector was used, but the cells were not sorted for GFP. The transfected cells were analyzed by flow cytometry for Mamu-AG with 25D3 forty-eight hours post transfection. With direct transfection of siRNAs, relative to nontransfected

trophoblasts (Fig. 6A), siRNA-A caused 70% suppression using program T-023 (Fig. 6B) and 78% suppression using program U-017 (Fig. 6C). When compared with the nonspecific shRNA (Fig. 6D), Mamu-AG expression was suppressed by shRNA-A by 72% (Fig. 6E).

We evaluated the effect that Mamu-AG knockdown had on interactions with putative target cells by determining the cytotoxicity of peripheral NK cells against Mamu-AG-suppressed cells. 721.221- Mamu-AG transfectants and Mamu-AG-shRNA-A stable transfectants were used as targets in cytotoxicity assays with NK cells. At ratios of 5:1 and 2.5:1, there was significantly higher cytotoxicity of Mamu-AG shRNA transfectants by NK cells (Fig. 7). It was a possibility that HLA-E expression may also be altered by RNAi directed against Mamu-AG since HLA-E transports the leader peptide of transfected MHC class I molecules to the cell surface and is expressed by 721.221 cells. However, expression of HLA-E by the 721.221-Mamu-AG transfectants was so low at the onset that its role in the cytotoxicity assays was negligible (data not shown).

Discussion

Potential target sequences for suppressing Mamu-AG by RNAi were identified by two predictive approaches. Our rationale for the array-based approach was that by probing with intact mRNA, we would be able to exclude siRNA candidates which would be unable to hybridize under intra-cellular conditions due to RNA secondary structure. However, the web-based algorithm was more effective in identifying target sequences than hybridizing Mamu-AG and Mamu-E mRNAs to a Mamu-AG tiled microarray. The optimal target sequence was effective in two different Mamu-AG expressing cell types, BLCL 721.221- Mamu-AG transfectants and primary first trimester rhesus monkey trophoblasts. Mamu-AG expression was suppressed up to 81% in the 721.221-Mamu-AG transfectants and up to 78% in the primary trophoblasts. In addition, the cytotoxicity assays demonstrated for the first time that rhesus monkey NK cells are able to recognize Mamu-AG, as evidenced by inhibition of lysis of Mamu-AG transfectants. The cytotoxicity assays also revealed that even the reduced Mamu-AG expression in the face of suppression by RNAi was sufficient to confer a protective effect against NK cell mediated lysis.

The novel presence of HLA-G during pregnancy remains a provocative phenomenon. Since the expression of HLA-G is most prominent in the placenta during pregnancy, it is hypothesized to contribute significantly to maternal-fetal immune tolerance. Previous studies have been performed that examined the effects of knockdown of HLA-G in human trophoblasts.15 JEG-3 cells, a human choriocarcinoma cell line that is likely derived from extravillous trophoblasts and expresses HLA-G, were transfected with siRNA for HLA-G or a scrambled sequence.15 Three doses of nucleotides were used (200 nM, 100 nM, and 50 nM) and transfected cells were analyzed by Western blot. The highest dose almost completely reduced HLA-G, the next dose reduced expression by 50%, and there was no suppression at the lowest dose. It was suggested that residual siRNA-resistant HLA-G expression may be due to the long turnover time of HLA-G on the surface of these cells.15

The rhesus monkey is an excellent model to study maternal-fetal immune tolerance since the hemochorial villous rhesus monkey placenta is developmentally and structurally similar to the human placenta. The pattern of Mamu-AG in the rhesus placenta does differ somewhat from that in the human, in that in the definitive placenta, Mamu-AG is expressed in villous syncytiotrophoblasts, whereas cell-surface HLA-G expression is restricted to the extravillous trophoblasts in the human placenta. This raises the possibility that trophoblasts interact with maternal peripheral blood lymphocytes in addition to the interaction of invasive extravillous trophoblasts with decidual lymphocytes.2 Although, the NK cell receptors for Mamu-AG have not been identified, our results demonstrated that Mamu-AG

expression on 721.221 cells suppresses lysis by rhesus peripheral blood NK cells. It has been shown that homologs of putative receptors for HLA-G, KIR2DL4, LILRB1, and LILRB2, are also expressed in the rhesus monkey.^{16–18} The rhesus KIR2DL4 homolog has a cytoplasmic tail which differs from human KIR2DL4.¹⁹ It is a possibility that the inhibitory function of LILRB1 and LILRB2 may counter the activating signal of KIR2DL4 on uterine NK cells.¹⁶ Therefore, it is possible that when Mamu-AG expression is suppressed by RNAi, a similar counterbalance may occur and rhesus monkey KIRs become activating. In addition, MHC-E must also be considered since it is known that the CD94/NKG2 heterodimer recognizes HLA-E in humans, and we have shown that rhesus monkeys express CD94 and NKG2 receptors.^{19–21} Therefore, it is also possible that upregulation of HLA-E on 721.221 cells is also protecting cells from lysis. Further experiments directly investigating the effect of RNAi on Mamu-E expression are necessary.

In addition to gaining insight into the role of nonclassical MHC class I molecules during pregnancy through their RNAi-mediated suppression, the suppression of MHC molecules could aid in the tolerance of allografts. A major obstacle in the development of tissues for regenerative medicine is HLA incompatibility.²² If donor cells can undergo MHC suppression via RNAi then the possibility of allograft acceptance could be enhanced, and the risk of rejection could be reduced.^{22–24} In conclusion, RNAi is an effective mechanism for investigating gene function. By utilizing this technique, the function of nonclassical MHC class I molecules during pregnancy and their interactions with their putative receptors could be clarified.

Acknowledgments

We thank Andrew Niles from Promega and Igor Slukvin from the W. N. P. R. C. for assistance with the cytotoxicity assays, Noel Peters from the Keck U. W. C. C. Small Molecule Screening Facility for help with the luciferase assay, the W. N. P. R. C. Animal Care Staff, Veterinary Staff, and Assay Services, Judith Peterson for help with manuscript preparation, and Robert DeMars and David Watkins for the MHC transfectant stable cell lines. This research was supported by NIH grants R01 HD34215 and R21 AI076734 to T.G.G. and T32 HD041921 to J.G.D. This research was also supported by NIH grant P51 RR000167 to the W. N. P. R. C. and was conducted in part at a facility constructed with support from Research Facilities Improvement Program grant numbers RR15459-01 and RR020141-01. This publication's contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

References

1. Langat DK, Hunt JS. Do nonhuman primates comprise appropriate experimental models for studying the function of human leukocyte antigen-G? *Biol Reprod.* 2002; 67:1367–1374. [PubMed: 12390864]
2. Slukvin II, Boyson JE, Watkins DI, Golos TG. The rhesus monkey analogue of human lymphocyte antigen-G is expressed primarily in villous syncytiotrophoblasts. *Biol Reprod.* 1998; 58:728–738. [PubMed: 9510960]
3. Slukvin II, Watkins DI, Golos TG. Tissue distribution of the mRNA for a rhesus monkey major histocompatibility class Ib molecule, Mamu-AG. *Tissue Antigens.* 1999; 53:282–291. [PubMed: 10203022]
4. Ramaswamy G, Slack FJ. siRNA. A guide for RNA silencing. *Chem Biol.* 2002; 9:1053–1055. [PubMed: 12401489]
5. Elbashir SM, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.* 2001; 15:188–200. [PubMed: 11157775]
6. Leung RK, Whittaker PA. RNA interference: from gene silencing to gene-specific therapeutics. *Pharmacol Ther.* 2005; 107:222–239. [PubMed: 15908010]
7. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev.* 2002; 16:948–958. [PubMed: 11959843]

8. Sui G, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci U S A*. 2002; 99:5515–5520. [PubMed: 11960009]
9. Boyson JE, Iwanaga KK, Golos TG, Watkins DI. Identification of a novel MHC class I gene, Mamu-AG, expressed in the placenta of a primate with an inactivated G locus. *J Immunol*. 1997; 159:3311–3321. [PubMed: 9317129]
10. Slukvin II, Lunn DP, Watkins DI, Golos TG. Placental expression of the nonclassical MHC class I molecule Mamu-AG at implantation in the rhesus monkey. *Proc Natl Acad Sci U S A*. 2000; 97:9104–9109. [PubMed: 10922066]
11. Golos TG, Handrow RR, Durning M, Fisher JM, Rilling JK. Regulation of chorionic gonadotropin-alpha and chorionic somatomammotropin messenger ribonucleic acid expression by 8-bromo-adenosine 3',5'-monophosphate and dexamethasone in cultured rhesus monkey syncytiotrophoblasts. *Endocrinology*. 1992; 131:89–100. [PubMed: 1612035]
12. Niles AL, Moravec RA, Hesselberth PE, Scurria MA, Daily WJ, Riss TL. A homogeneous assay to measure live and dead cells in the same sample by detecting different protease markers. *Anal Biochem*. 2007; 366:197–2006. [PubMed: 17512890]
13. Huddleston H, Schust DJ. Immune interactions at the maternal-fetal interface: a focus on antigen presentation. *Am J Reprod Immunol*. 2004; 51:283–289. [PubMed: 15212681]
14. Braud VM, Allan DS, Wilson D, McMichael AJ. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr Biol*. 1998; 8:1–10. [PubMed: 9427624]
15. Wengenmayer T, Poehlmann TG, Markert UR. Inhibition of HLA-G production in JEG-3 choriocarcinoma cells by RNA interference. *Am J Reprod Immunol*. 2004; 51:189–191. [PubMed: 15209386]
16. Yan WH, Lin A, Chen BG, Zhou MY, Dai MZ, Chen XJ, Gan LH, Zhu M, Shi WW, Li BL. Possible roles of KIR2DL4 expression on uNK cells in human pregnancy. *Am J Reprod Immunol*. 2007; 57:233–242. [PubMed: 17362384]
17. Apps R, Gardner L, Sharkey A, Holmes N, Moffett A. A homodimeric complex of HLA-G on normal trophoblast cells modulates antigen-presenting cells via LILRB1. *Eur J Immunol*. 2007; 37:1924–1937. [PubMed: 17549736]
18. Slukvin II, Grendell RL, Rao DS, Hughes AL, Golos TG. Cloning of rhesus monkey LILRs. *Tissue Antigens*. 2006; 67:331–337. [PubMed: 16634871]
19. LaBonte ML, Hershberger KL, Korber B, Letvin NL. The KIR and CD94/NKG2 families of molecules in the rhesus monkey. *Immunol Rev*. 2001; 183:25–40. [PubMed: 11782245]
20. Kelley J, Walter L, Trowsdale J. Comparative genomics of major histocompatibility complexes. *Immunogenetics*. 2005; 56:683–695. [PubMed: 15605248]
21. Mavilio D, Benjamin J, Kim D, Lombardo G, Daucher M, Kinter A, Nies-Kraske E, Marcenaro E, Moretta A, Fauci AS. Identification of NKG2A and NKp80 as specific natural killer cell markers in rhesus and pigtailed monkeys. *Blood*. 2005; 106:1718–1725. [PubMed: 15899917]
22. Figueiredo C, Seltsam A, Blasczyk R. Class-, gene-, and group-specific HLA silencing by lentiviral shRNA delivery. *J Mol Med*. 2006; 84:425–437. [PubMed: 16520945]
23. Gonzalez S, Castanotto D, Li H, Olivares S, Jensen MC, Forman SJ, Rossi JJ, Cooper LJ. Amplification of RNAi--targeting HLA mRNAs. *Mol Ther*. 2005; 11:811–818. [PubMed: 15851019]
24. Haga K, Lemp NA, Logg CR, Nagashima J, Faure-Kumar E, Gomez GG, Kruse CA, Mendez R, Stripecke R, Kasahara N, Ciciarelli JC. Permanent, lowered HLA class I expression using lentivirus vectors with shRNA constructs: Averting cytotoxicity by alloreactive T lymphocytes. *Transplant Proc*. 2006; 38:3184–3188. [PubMed: 17175217]

Fig. 1. Mamu-AG and Mamu-E mRNA hybridized to a microarray spotted with Mamu-AG 25mer oligonucleotides. Possible siRNA sequences for Mamu-AG are located where the relative intensity of hybridization to the microarray is the strongest for Mamu-AG (blue) while there is very low intensity for Mamu-E (pink).

Fig. 2. Sequence alignment of Mamu-AG and Mamu-E. The regions in yellow indicate where the hybridization signal was maximally different between the Mamu-AG and Mamu-E and indicate possible regions to target with siRNA. Target siRNA sequences are underlined in red. Target sequences A-D were based on the web-based algorithm, and sequences E and F were based on the microarray data.

shRNA-A: 5'-GAAGAGCTCAGATAGATAA-3'
shRNA-B: 5'-GGATGGAGAGGAACAAACT-3'
shRNA-C: 5'-GGGAGACACAGAACATGAA-3'
shRNA-D: 5'-GGAGAGGAGCAGAGATACA-3'
shRNA-E: 5'-GAACATGAAGACCGCGACA-3'
shRNA-F: 5'-GAACACCCAGCGCAAGTGG-3'
shRNA-G: 5'-GAGGAGAAAGATACATTAC-3'

Fig. 3.

Oligonucleotide Mamu-AG sequences that were subcloned into the psiSTRIKE vector. Sequence G is the scrambled sequence of A.

Fig. 4.

Target site selection of the Mamu-AG gene. BRL cells were co-transfected with the psiCHECK-2 vector expressing Mamu-AG mRNA and with psiSTRIKE vectors expressing Mamu-AG shRNA target sequences or controls. The ratio of transfection reagent to DNA was either 6:4 or 6:2. Knockdown was determined by luciferase assay and expressed as *Renilla* activity normalized to firefly luciferase. Results are presented as the mean of three wells + SEM.

Fig. 5.

Flow cytometric analysis of 721.221-Mamu-AG transfectants treated with shRNA. The psiSTRIKE-hMGFP vectors containing shRNA or siRNA targeting Mamu-AG were transfected by nucleofection. Seventy-two hours post transfection, the shRNA transfected cells were sorted by FACS for GFP and then stained for Mamu-AG. A: nonspecific shRNA transfectants, B: shRNA-A transfectants, and C: shRNA-B transfectants. The mean fluorescent intensities (MFI) are included on each dot plot and used to calculate percent knockdown.

Fig. 6.

Flow cytometric analysis of day 36 rhesus monkey trophoblasts transfected with shRNA or siRNA for Mamu-AG. Cells were stained for Mamu-AG 48 hr post transfection. A: nontransfected trophoblasts stained with anti-Mamu-AG 25D3 mAb, B: trophoblasts transfected with siRNA-A using program T-023, C: trophoblasts transfected with siRNA-A using program U-017, D: trophoblasts transfected with nonspecific shRNA, and E: trophoblasts transfected with shRNA-A. The mean fluorescent intensities (MFI) are included on each dot plot and used to calculate percent knockdown.

Fig. 7. NK cell-mediated cytotoxicity against 721.221-Mamu-AG transfectants and Mamu-AG shRNA transfectants. NK cells from five different monkeys were incubated with 721.221 cells transfected with Mamu-AG or Mamu-AG expressing cells transfected with vector expressing shRNA-A. There were no obvious differences between the two cytotoxicity assay methods used so the data was combined. The data are presented as the mean $\pm$ SEM. (*) represents significant difference ($P < 0.05$) determined by Student's t test.