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Review

Pregnancy initiation in the rhesus macaque: Towards functional manipulation of the maternal-fetal interface

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Abstract

Nonhuman primates provide an important opportunity to define the mechanisms that contribute to the success of early pregnancy. We have focused for several years now on defining the expression of novel placental major histocompatibility complex (MHC) class I molecules. In parallel, we have used reagents against human immune cell markers to characterize the leukocyte population in the decidua and have demonstrated dynamic changes in these cell populations during the first 5 weeks of gestation. The challenge is to identify the possible role(s) of placental MHC class I in modifying/directing the maternal endometrial or systemic immune system in the post-implantation period. Foremost among the challenges is the difficulty in modifying placental function. In the instance of trophoblast surface proteins, passive immunization studies are feasible, although limitations include the empirical nature of this approach, as well as the inability to modify intracellular function. We have shown that using lentiviral vectors to effect preimplantation gene transfer for transgene expression in the placenta is not only feasible, but of good efficiency. In addition to transgene overexpression, robust approaches for knocking down/knocking out placental gene expression are essential. Recent developments in RNA interference approaches may allow "transient knockout" experiments. While the rhesus monkey has been our model of choice, currently there are limitations in the number of available female rhesus monkeys of reproductive age for research in early pregnancy. It is critical that the technologies for advanced study move forward in other species. The baboon has been used significantly in reproductive tract biology and early pregnancy research and important models have been developed for manipulation of the maternal-fetal interface. Additional characterization of other species, such as the cynomolgus and African green (vervet) monkey is critical. Given the limitations on antigen recognition when using human reagents, we also propose that the development of panels of primate-specific anti-leukocyte antibodies is essential for moving forward nonhuman primate reproductive research.

Background

Early pregnancy represents a fundamentally critical time in the human life cycle. The establishment of human pregnancy is of surprisingly low efficiency, with estimates of pregnancy loss during the first weeks following fertilization as high as 50% [1]. Once established, the success of maternal and fetal health is far from assured, with preeclampsia, fetal growth restriction, prematurity or postmaturity contributing significantly to fetal as well as maternal morbidity and mortality. Finally, even if a pregnancy is

successfully carried to completion, it does not insure postnatal well-being. There is now widespread recognition that a major contributor not only to neonatal health and well-being, but physiology throughout the lifespan is dramatically influenced by the intrauterine environment [2].

There is wide recognition that the placenta has a profound impact in many of these areas of development, however, human pregnancy remains an extremely challenging area for the conduct of clinical research. Invasive studies to determine the influences of placental manipulation are extremely difficult to design because of the special ethical case that a pregnant woman represents, recognizing that interventions which might provide risk to the fetus are not generally acceptable. In addition, it is extraordinarily rare to be able to conduct basic observational or descriptive studies in the earliest stages of human pregnancy (i.e., within several weeks of implantation).

The nonhuman primate represents an important model for understanding basic human biology, and testing therapeutic interventions. A particularly important example of this is in early pregnancy. Nonhuman primates have been shown to be excellent models for studying preimplantation embryo development and postimplantation physiology [3,4]. Indeed, many of the aspects of human preimplantation embryo development, such as the initiation of the expression of chorionic gonadotropin genes, are recapitulated in rhesus monkey embryos similar to human embryos. Careful description of the perimplantation anatomy and development of the placenta and the fetal membranes has been feasible with the nonhuman primate. Finally, experiments to directly intervene in fetal or maternal physiology to understand the outcome in fetal development (e.g., studies in developmental toxicology) are readily accomplished in nonhuman primates. Thus, nonhuman primates provide an important opportunity to define the mechanisms that contribute to the success of early pregnancy. In this report we will review progress in the development of a nonhuman primate model for investigating one area of human pregnancy, the establishment of maternal-fetal immune tolerance. We will describe the approaches we have developed to address the challenges that face investigators in this area, and we will summarize opportunities to address the challenges that remain.

Case study in nonhuman primate pregnancy research – development of a primate model for maternal-fetal immune tolerance

Advances in the 20th century in our understanding of immune mechanisms of transplant rejection revealed that mammalian pregnancy poses a novel challenge to fetal survival. The tolerance of the paternal component of the fetal genome, particularly the polymorphic MHC class I genes, has intrigued reproductive biologists for half a century. This question is all the more intriguing given the realization that the solutions that have evolved across different mammalian orders is diverse in the extreme (Table 1). For example, mice have been reported to have no trophoblast MHC expression, wheras, horses normally

Table 1: Comparison of nonprimate and primate placental MHC biology

bovine:
• trophoblasts lack MHC class I expression [5]
• Trophoblasts from cloned fetuses express MHC class I, cow has altered endometrial immune cells [5]
horse:
• trophoblasts express classical MHC class I [6]
• mare raises an alloresponse to paternal MHC [7]
mice:
• no trophoblast MHC class I [8]
• pregnancy is successful despite transgenic MHC expression (or β2 m knockout) [8, 9]
• disruption of T cell or complement regulation detrimental to pregnancy success [10, 11]
nonhuman primate:
• lack of functional HLA-G locus [28]
• nonclassical MHC class I expression: Mamu-AG [29], Mamu-E [30]
• novel MHC class I expression, Mamu-I [31]
human:
• extravillous trophoblast HLA-G expression [12, 13]
• abundant placental nonclassical MHC expression (HLA-E, HLA-F) [15,22]
• placental HLA-C expression in some cytotrophoblasts [14]
HLA-G homologs have not been identified in nonprimate species

express paternal and maternal MHC class I in the endometrial cups and raise an antipaternal MHC response in the mare.

Perhaps the most significant attention has been allotted to human placental MHC, given the broad palette of reagents and information on human MHC expression. While human trophoblasts do not express MHC Class II molecules, human trophoblasts express a novel pattern of primarily nonclassical MHC class I (HLA-E, -F and G) as well as limited HLA-C expression [12-15]. In particular, since HLA-G expression is largely restricted to placental trophoblasts, it has received significant investigation by reproductive biologists.

In vitro studies with human trophoblasts and immune cells have suggested that there are specific receptors for HLA-G on various maternal leukocyte compartments. These compartments include the novel population of uterine natural killer cells also known previously as decidual large granular lymphocytes [16], as well as endometrial macrophages and possibly dendritic cells [17-19]. The identity of this latter cell type remains controversial [18,19]. Previously it had been hypothesized that trophoblast nonclassical MHC class I molecules served to avoid the problems that would be imposed by expression of paternal MHC on trophoblasts exposed directly to maternal immune cells. In the absence of any MHC class I, NK cells would be activated by lack of self to mount an immune response to the placenta. Indeed, NK cell inhibitory receptors (KIRs) are expressed on uterine NK cells and may function as HLA-G receptors, however this remains a matter of contention [20,21]. It is clear that HLA-E receptors are widely expressed in NK cells, including those in the decidua [22]. In contrast, monocyte-derived cells express a related family of Leukocyte Ig-like Receptors (LILRs), and two of these (LILRB1/ILT2, LILRB2/ILT4/LIP2) are putative HLA-G receptors [23,24].

Regardless of the receptor identity, the effects of placental MHC molecules may not simply be to down-regulate antitrophoblast immune responses. Although information in humans or primates is limited, in mice there is substantial literature that describes an important role of uterine leukocytes in pregnancy success despite the lack of MHC expression on mouse trophoblasts. Mice with components of the nonspecific immune system deleted by homologous recombination have defects in decidual differentiation and vascular development [25] indicating that pregnancy success may rely on the presence and function of nonspecific immune cells, i.e., NK cells. With regard to human placental MHC, much interest has also arisen centered on not only local immunity, but the possible effects of HLA-G in the peripheral circulation on extrauterine tolerance, not only in the setting of pregnancy [26], but as well in cancer and transplantation medicine [27]. Thus understanding the biological function of nonclassical MHC expression has significance beyond the setting of pregnancy.

Despite this substantial interest, well over a decade of research into the expression of HLA-G and its effects on the maternal immune system has failed to precisely identify a significant function for the molecule. Much less even still is known about the relative contributions the other placental MHC class I molecules make to maternal tolerance of pregnancy. For this reason, it was necessary to understand whether an animal model may serve in functional studies of maternal-fetal immune tolerance.

We have defined the MHC class I expression within the rhesus monkey placenta. Intriguingly, the rhesus monkey does not express a functional HLA-G ortholog [28], but expresses at a high level a locus actually derived from rhesus MHC *A* [29]. Nonetheless, this locus (which we designated *Mamu-AG*) retains novel characteristics associated with *HLA-G*, including a shortened cytoplasmic domain, novel mRNA splicing, expression of glycosylation isoforms, and predominant protein expression within the placenta. The rhesus placenta also expresses the mRNAs for *Mamu-E* [30] and a novel locus in the rhesus designated *Mamu-I* [31]. The protein expression of these molecules has not been fully explored. Of particular interest is that we have shown that within a week of implantation, Mamu-AG protein expression is readily detectable within the invasive trophoblasts penetrating the uterine decidual stroma, as well as endovascular trophoblasts within maternal decidual blood vessels [32]. It is critical to note that this observation is unlikely to have been made in human pregnancy, due to the virtual unavailability of 1 week implantation sites for such analysis. Our studies with Mamu-AG, as well as others with HLA-G, have clearly shown the importance of highly specific antibodies because of the likely translational control of MHC class I expression. Although *Mamu-E* and *-I* mRNAs are found in the placenta, confirmation of their protein expression awaits the characterization of antibodies specifically recognizing these rhesus proteins.

Decidual immune cells

The maternal side of the primate maternal-fetal interface is equally fascinating. Primate pregnancy is characterized by the appearance of a unique population of resident tissue leukocytes. A majority of these cells are large granular natural killer (NK) cells, termed uterine NK (uNK) cells. The phenotype of these cells in both human and nonhuman primate pregnancy is distinct from peripheral blood NK cells, notably with high levels of expression of CD56 (NCAM-1) [33]. It remains unclear whether these cells are resident cells expanded by the initiation of pregnancy, if

they arise due to trafficking and differentiation of peripheral blood NK cells to the decidua, or if both mechanisms are at play. In addition to these NK cells, there is a modulation and modification of decidual macrophages in the first months of human and monkey pregnancy. While it has been recognized that macrophages are present in substantial numbers in the first trimester human decidua, we have recently undertaken to delineate this population in the earliest stages of rhesus monkey pregnancy, within 1 to 3 weeks postimplantation. Unexpectedly, there were distinct differences in the distribution of macrophages around the invading interstitial and endovascular trophoblasts [34]. Whereas NK cells were relatively uniformly distributed throughout the decidua and did not give the distinct impression of selective localization, macrophages were in many cases clearly preferentially located in close proximity to the trophoblasts. This is in keeping with recent reports of expression of chemokines and macrophage attractant proteins to the human implantation site [35], although macrophage distribution is not as dramatic as in these early pregnancy images. It would appear that macrophage-trophoblast interactions may very well be one of the earliest placental-immune interactions.

Developing approaches for placental manipulation – ART in support of primate transgenesis

The expression of placental MHC and the phenotype and appearance of nonhuman primate decidual leukocytes is supportive of the relevance of this animal model to human pregnancy. In reality it becomes clear that it is very difficult to conduct invasive studies to disrupt placental function. The problem is that classical ablation-replacement experiments such as those to investigate the hypothalamic-pituitary-ovarian reproductive axis are not relevant in a pregnant monkey. The potential opportunities seem limited to passive immunization, which can be fruitful but has significant drawbacks, including the empirical nature of such experiments, and perhaps treatment with soluble placental MHC class I to determine effects on systemic immunity.

For a solution to this dilemma, we considered the origin of the placenta. Since the placental trophoblasts arise from the trophectoderm of the preimplantation embryo, we selected IVF-produced or *in vivo* recovered embryos as a target for placental modification, via placental transgenesis. Although the methods of transgenesis are well-established in mice and clearly feasible in other species, the relative scarcity of rhesus embryos as well as the logistical difficulties of synchronization and surrogate embryo recipient identification compelled us to consider ways to improve the efficiency of gene transfer. We elected to adapt the tools of the gene therapy researcher to obtain high efficiency transfer to embryos. We considered and conducted studies with plasmid-based EBV vectors [36], adeno-associated virus type 2 [37], which had low efficiency of gene transfer in rhesus embryos (Wolfgang and Golos, unpublished observations), and ultimately settled on lentiviral-based vectors [38]. Using a green fluorescent protein (GFP) transgene, we found relatively reliable GFP expression within several days of gene transfer (Fig. 1). We conducted 24 transcervical nonsurgical transfers of GFP-transduced IVF or *in vivo* recovered embryos (Table 2). An important facet of these studies is that the animal care staff working with these animals had years of experience with their reproductive cycles and patterns, including previous pregnancies. Interestingly, the transfer of GFP-transduced embryos suggested a broader window of implantation to that previously determined [39] for "wild-type" embryos. It may be that gene transfer alters embryonic development such that asynchrony between embryo and recipient is not as detrimental as in transfer of unmodified embryos.

At the time of delivery, we noted excellent transgene expression in placentas from all offspring, including a lost twin pregnancy [38]. However, evidence for transgene expression in cord blood cells or skin cells of the offspring was lacking [38], and subsequent follow-up studies have likewise failed to detect extraplacental GFP protein expression in PBLs (Garthwaite et al, unpublished observations). Site-of-integration effects are well-known in transgenesis, but it seems surprising that of three offspring there would be a lack of transgene expression and further studies of these individuals are ongoing. Significantly, placental transgene expression was tolerated by the maternal immune system, even in the face of a robust immune response by the dam [38]. Additional study of the dams is also warranted and this is an advantage of the nonhuman primate model, in which we can study subsequent pregnancies potentially over many years.

Conclusions – limitations, challenges and future opportunities in primate maternal-fetal medicine research

There are opportunities as well as challenges in manipulating the placental phenotype in the primate. Whereas overexpression of a selected gene can be of value, of perhaps greater broad importance will be the development of gene-knockdown approaches. Standard gene deletion by homologous recombination will require advances in embryonic stem cell technologies with rhesus monkey ES cells, but a viable alternative is the use of RNAi technology within the lentiviral system [40]. Other areas of interest are listed in Table 3.

Table 4 lists additional important priority areas in pregnancy research with the nonhuman primate model. One of the critical limitations in the use of the rhesus monkey

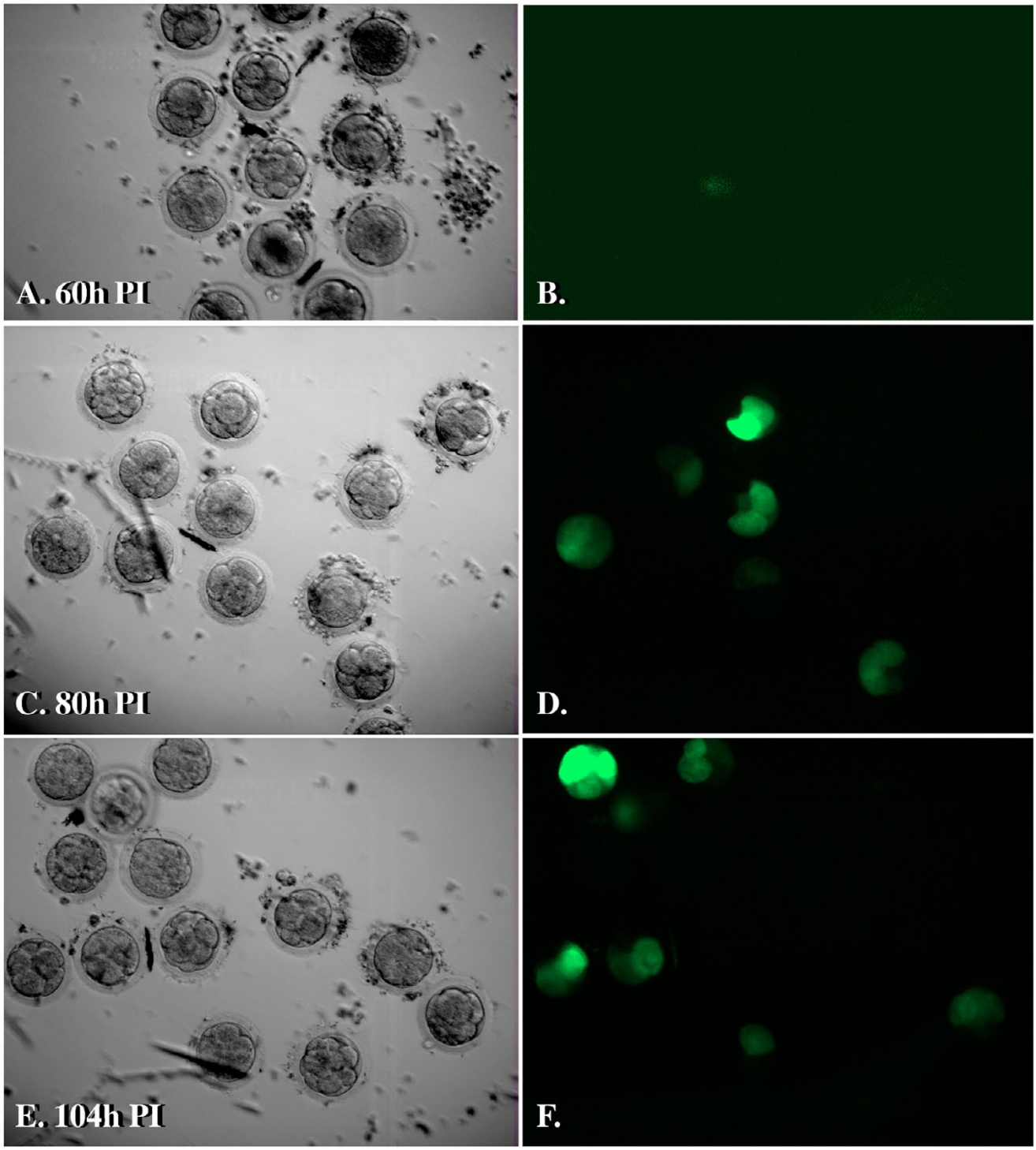

Figure 1
Brightfield (left) and fluorescent (right) images of lentivirus-transduced rhesus monkey preimplantation embryos. IVF-derived embryos were injected with lentivirus stock with GFP expression under the control of the EF-1α promoter. Injections were done at the 8–16 cell stage (60 hrs postinsemination (PI), Panel A) and embryos were imaged at 10–12 hour intervals thereafter. Most embryos were at the blastocyst stage within 4 days (panels I, J).

Table 2: Outcome of transfer of embryos injected with lentiviral vector

Total Transfers	Recipient Day Post LH	Outcome
2	3	No pregnancy initiation
7	4	1 live/1 stillborn twin, 1 blighted ovum
8	5	4 blighted ova
3	6	1 live birth
3	7	1 live birth, 1 blighted ovum
1	8	1 early twin loss
24		3 live births, 8 lost pregnancies

Table 3: Challenges in manipulation of the placental phenotype

- Targeted delivery of vector to the placenta: promoter and receptor-based approaches
- Implementation of gene knock-down technology
- Regulated expression would be of significant utility
- Useful approaches will include minimally invasive monitoring of transgene expression during pregnancy
- Improvement in vector application (replacement vs. insertion)
- Alignment of enthusiasm with reality of resource availability = prioritization of research goals

Table 4: Opportunities and areas of growth in nonhuman primate pregnancy research

- Basic reproductive biology of additional primate species
- Reagents for reliable assisted reproductive technologies
- Forward implementation of improved embryo transfer and reconstitution technology
- Availability of nonhuman primate-specific antibodies
- Continued focus on cooperative training and collaborations among primate researchers

is simply the availability of sufficient numbers of animals to conduct embryo transfers with modified embryos. A substantial pool of animals is needed in the absence of reliable synchronization of recipient cycles, and this will remain a limitation when these methods become available, given the long (165 day) gestation period of this species. Dramatically expanding the rhesus monkey population at the National Primate Research Centers is one critical need.

The development of basic knowledge in the reproductive biology and physiology of pregnancy in additional species with close similarities to human pregnancy is also important. The baboon (Papio anubis) already is an excellent model in early pregnancy research [41]. The cynomolgus monkey (Macaca fasciculata) and African green (vervet) monkey (Circopithecus aethiopes) have attractive characteristics of size, cost, and in the vervet, a relatively straight cervix to facilitate embryo transfer. The stimulation of nonhuman primates with recombinant human gonadotropins remains the state of the art, and current efforts to develop recombinant homologous nonhuman primate hormones is critical to the long-term success of primate transgenesis.

Finally, with regards to modification of the decidual immune cells, fewer options are available. The study of the reproductive immunology of these species suffers from laborious and expensive testing of anti-human antibodies to define appropriate reagents for identifying antigens on nonhuman primate leukocytes. The development of panels of primate-specific antibodies will not only improve detection and phenotyping of monkey leukocytes, but may facilitate the development of passive immunization or immunodepletion strategies for probing the function of reproductive tract immune cells in pregnancy success. When these approaches and resources become available, investigators will have a dramatically enhanced ability to bring molecular embryology, microarray phenotyping, stem cell biology, and homologous recombination into the arsenal of research in primate reproduction.

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