Example 1

Inhibition of the Chemokine Response Prevents Preterm Labor but Not an Invasive Infection in a Nonhuman Primate Model.

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Introduction: Intrauterine infection, which occurs in most early preterm births, triggers an immune response culminating in preterm labor (PTL). Leukocyte activation within the decidua, myometrium and cervix is strongly associated with the inflammatory response and PTL. We hypothesized that inflammation within the decidua is an essential event that enables microbial trafficking across the chorioamniotic membranes and into the amniotic fluid. We hypothesized that administration of a broad-spectrum chemokine inhibitor (BSCI), at the site of bacterial inoculation in the choriodecidual space, could prevent microbial invasion of the amniotic cavity (MIAC) and preterm labor (PTL) in a unique nonhuman primate model.

Methods: Chronically catheterized pigtail macaques at 127 to 138 days gestation received choriodecidual inoculations of either (1) saline (N=7), (2) Group B Streptococcus (GBS; 5x10^8 colony forming units (CFU)/ml; N=5), or (3) BSCI (10 mg/kg pre-treatment and daily intravenous and intra-amniotic daily infusions) + GBS (5x10^8 CFU/ml) (N=4). We serially sampled amniotic fluid in addition to cord blood at delivery to quantitate cytokines (IL1-b,TNFa, IL-6), chemokines (IL-8) and prostaglandins (PGE2, PGF2a) by Luminex and ELISA. Quantitative bacterial cultures were performed at the inoculation site, from amniotic fluid (AF) and multiple fetal tissues. AF cytokines, prostaglandins, and uterine contractility were compared using one-way ANOVA with Bonferroni adjusted pairwise comparisons.

Results: Compared with saline controls, GBS induced PTL in 4 of 5 (80%) animals, but in only 1 of 4 (25%) receiving the BSCI. Despite the relative uterine quiescence in the BSCI group, MIAC and fetal pneumonia occurred in all 4 animals. In accordance with a selective chemokine inhibition, AF IL-8 was significantly lower in the BSCI compared to the GBS group (p=0.03). Surprisingly and despite a clear difference in labor patterns, there was no difference in cytokine or prostaglandin quantities between the BSCI and GBS groups.

Conclusion: Blocking the chemokine response to infection appears to powerfully suppress PTL, but is not sufficient to prevent MIAC and neonatal pneumonia. If tested with antibiotics, a strategy of chemokine inhibition may prolong gestational latency and prevent neonatal infectious morbidity.

Example 2

Novel Non-Classic Progesterone (P4) Receptor PGRMC1 Interactions and Functionality Reveal a Key Role During the Human Decidualization Process.

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Introduction: Progesterone (P4) Receptor Membrane Component 1 (PGRMC1) mediates the antiapoptotic and antimitotic actions of P4 in human granulosa cells. However, its function in the endometrium remains unknown. We previously demonstrated that PGRMC1 is down-regulated in receptive endometria and its overexpression inhibits decidualization. Here, we investigated interactions of PGRMC1 with other proteins and the effect of a PGRMC1 inhibitor (AG205) during decidualization.

Methods: PGRMC1 protein interactions were identified in non-decidualized (ndESC) and decidualized (dESC) endometrial stromal cells through a pulldown assay during a long or short decidualization protocol (P4/E2;8 days or cAMP/MPA;4 days). PGRMC1 sequence was cloned in a pGEX-6P1 vector to express a GST-PGRMC1 fusion protein. Proteins extracted from ndESC and dESC (n=3) were incubated with GST-PGRMC1 or control GST proteins and subsequently identified by mass spectrometry (MS). To better understand the role of PGRMC1 in decidualization, an impermeable P4 (P4-BSA, 1uM) that activates only membrane receptors and the inhibitor AG205 (50 uM)(n=7) were used to block its action. Decidualization was evaluated by analyzing prolactin (PRL) secretion (ELISA) and cytoskeleton morphology (F-actin staining). Global gene expression following AG205 and P4-BSA treatment during a long protocol of decidualization was analyzed by microarray and validated by qPCR (n=4).

Results: Pulldown and MS analysis identified 22 and 25 new significant PGRMC1-interacting proteins in ndESC and dESC, compared to controls ($p<0.01$). Interaction network analysis categorized these proteins mainly into mitochondria and lysosome cellular components, both related to transport activity. Monoamine oxidase B (MAOB) was identified in dESC of both decidualization protocols. The PGRMC1-MAOB interaction was confirmed by immunofluorescence and co-immunoprecipitation in dESC. PRL secretion significantly decreased in the presence of P4-BSA/E2 compared to dESC by P4/E2 ($p<0.05$). Furthermore, PRL levels were significantly increased in the presence of AG205+P4-BSA/E2 compared to ndESC and dESC only with P4-BSA/E2 ($p<0.05$). Finally, microarray analysis showed that AG205 was associated with 91 up- and 147 down-regulated genes compared to ndESC (FDR<0.05). Moreover, biological processes associated with cholesterol/sterol biosynthesis and vesicle-mediated transport were up-regulated.

Conclusion: Novel PGRMC1 protein interactions discovered in ESC and PGRMC1 functional analysis suggest that this protein is implicated in the deep remodeling of ESC during the decidualization process, interacting mainly with proteins involved in intracellular transport and mitochondrial activity.

Example 3

Placenta-Derived Fetal Circulating Factors in Pregnancies Complicated by Maternal Obesity Induce Cardiomyocyte Hypertrophy *In Vitro*.

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Introduction: Maternal obesity is associated with cardiac hypertrophy in the fetus and in childhood. MicroRNAs (miRNAs) regulate cardiac hypertrophic signaling and miRNA abundance is often altered in the circulation of adults with cardiac disease. Moreover, plasma miRNAs from pediatric cardiomyopathy patients induce hypertrophy in isolated cardiomyocytes. Maternal obesity alters placental expression of miRNAs, which are known to traffic into the fetal circulation. We hypothesized that RNAs in cord plasma and in the secretome of primary human trophoblast cells (PHTs) isolated from placentas of obese pregnant women induce cardiomyocyte hypertrophy *in vitro*.

Methods: Cord plasma and placental villous tissue were collected from obese pregnant women (body mass index, BMI>30, n=10) and normal controls (BMI<25, n=10) after informed consent. Isolated, syncytialised PHTs were incubated in low serum medium and the conditioned medium was collected after 24 hr. Neonatal rat ventricular myocytes (NRVMs), cultured in serum-free medium, were supplemented (2%) with cord plasma or PHT conditioned medium, from normal or obese pregnancies, or remained untreated. After 72 hr, RNA was extracted from NRVMs and the relative expression of atrial natriuretic factor (*Nppa*), β- and α-myosin heavy chain (*Myh7, Myh6*) determined as markers of pathological hypertrophy. To establish the mechanistic role of circulating RNAs, cord plasma samples were freeze-thawed and RNase treated then supplemented to NRVMs in the presence of liposomal transfection reagent. Data were analyzed by ANOVA with Holm-Sidak post-hoc test.

Results: Treating NRVMs with cord plasma from fetuses of obese, but not normal, women increased *Nppa* expression (+247%, P<0.01) and the ratio *Myh7*:*Myh6* (+121%, P<0.01), compared to untreated NRVMs. Similarly, conditioned medium from PHTs isolated from pregnancies complicated by maternal obesity upregulated the NRVM *Myh7:Myh6* expression ratio (+110%, P<0.05), but not *Nppa* expression, compared to unconditioned medium. RNase-treatment abolished the hypertrophic effect of cord plasma from obese women, such that *Nppa* expression and *Myh7*:*Myh6* ratio were similar in NRVMs treated with lean or obese plasma following RNA digest (interaction obesity x RNase treatment P<0.05).

Conclusion: Circulating factors in umbilical plasma and secreted by cultured PHT cells isolated from pregnancies complicated by maternal obesity induce cardiomyocyte hypertrophy *in vitro*. This effect is in part RNA-mediated. We speculate that changes in the levels of placenta-derived circulating factors, possibly miRNA, underpin fetal cardiac hypertrophy and contribute to the developmental programming of cardiovascular disease in human pregnancies complicated by maternal obesity.

Example 4

ARID1A Loss Results in Non-Receptive Endometrium with FOXA2 Deficiency in Endometriosis-Related Infertility.

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Introduction: Endometriosis is associated with infertility, but the exact pathophysiology of this effect remains unclear. Expression of ARID1A, a SWI/SNF nucleosome remodeling complex protein, is decreased in eutopic endometrium from infertile women with endometriosis. Furthermore, conditional uterine *Arid1a* knockout mice ($Pgr^{cre/+}Arid1a^{f/f}$) are infertile. We hypothesized that ARID1A loss causes endometriosis-related infertility due to a non-receptive endometrium.

Methods: 1) To determine the effect of ARID1A loss on endometriosis development, we surgically induced endometriosis with endometrium of $Pgr^{cre/+}Arid1a^{f/f}$ and control mice. 2) We utilized a non-human primate (baboon) endometriosis model to track ARID1A expression in eutopic endometrium through disease progression. 3) To identify the molecular consequences of ARID1A loss, we performed microarray analysis on uterine tissue from $Pgr^{cre/+}Arid1a^{f/f}$ mice. 4) We analyzed the correlation of ARID1A and FOXA2, a gland-specific transcription factor important for fertility, in endometrial tissue from infertile women with endometriosis and normal controls. 5) We developed $Ltf^{cre/+}Arid1a^{f/f}$ mice to determine the effects of ARID1A loss in the epithelium.

Results: 1) $Pgr^{cre/+}Arid1a^{f/f}$ mice exhibited a significantly increased number of endometriotic lesions after endometriosis induction compared to controls ($p<0.05$). 2) Baboons induced with endometriosis exhibited progressive ARID1A loss measured pre-induction through 15 months post-induction. 3) The microarray analysis identified *Foxa2* and other endometrial gland-related genes to be downregulated at pre-implantation in $Pgr^{cre/+}Arid1a^{f/f}$ mice. Immunohistochemistry also showed remarkable reduction of FOXA2 in $Pgr^{cre/+}Arid1a^{f/f}$ mice at pre-implantation, confirming that FOXA2 expression is dependent on ARID1A. 4) The expression of FOXA2 was significantly lower in eutopic endometrium from women with endometriosis compared to controls ($p<0.001$). ARID1A and FOXA2 protein levels were significantly correlated in a sample set of eutopic endometrium from infertile women with endometriosis and control women based on IHC ($p<0.001$). 5) Similar to $Pgr^{cre/+}Arid1a^{f/f}$ mice, $Ltf^{cre/+}Arid1a^{f/f}$ mice were infertile from implantation and decidualization defects due to non-receptive endometrium.

Conclusion: Endometrial ARID1A loss leads to endometriosis-related infertility by causing endometrial non-receptivity with gland dysfunction as shown in mice, baboons, and samples from infertile women with endometriosis.