Title

Evidence of Cardiac Involvement in the Fetal Inflammatory Response Syndrome: Disruption of Gene Networks Programming Cardiac Development in Nonhuman Primates

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Summary Statement

In a nonhuman primate model of intraamniotic infection, preterm labor and severe fetal inflammation are associated with downregulation of cardiac morphogenesis and vasculogenesis gene networks.

Short Version of the Title

Disruption of fetal cardiac gene networks with fetal infection and inflammation

Abstract

Background: The majority of early preterm births are associated with intraamniotic infection and inflammation, which can lead to systemic inflammation in the fetus. The fetal inflammatory response syndrome describes elevations in the fetal interleukin-6 (IL-6) level, which is a marker for inflammation and fetal organ injury. Understanding the effects of inflammation on fetal cardiac development may lead to insight into the fetal origins of adult cardiovascular disease.

Objective: To determine whether the fetal inflammatory response syndrome is associated with disruptions in gene networks programming fetal cardiac development.

Study Design: We obtained fetal cardiac tissue following necropsy from a well-described pregnant nonhuman primate model (pigtail macaque, *Macaca nemestrina*) of intrauterine infection (N=5) and controls (N=5). Cases with the fetal inflammatory response syndrome (fetal plasma IL-6 >11 pg/ml) were induced by either choriodecidual inoculation of a hypervirulent Group B Streptococcus strain (N=4) or intraamniotic inoculation of *Escherichia coli* (N=1). RNA and protein were extracted from fetal hearts and profiled by microarray and Luminex for cytokine analysis, respectively. Results were validated by quantitative RT-PCR. Statistical and bioinformatics analyses included single gene analysis, Gene Set Analysis, Ingenuity Pathway Analysis, and Wilcoxon rank sum.

Results: Severe fetal inflammation developed in the context of intraamniotic infection and a disseminated bacterial infection in the fetus. IL-6 and IL-8 in fetal cardiac tissues were significantly elevated in fetal inflammatory response syndrome cases versus

controls ($p<0.05$). A total of 609 probe sets were differentially expressed (>1.5-fold change, $p<0.05$) in the fetal heart (ANOVA). Altered expression of select genes was validated by qRT-PCR including several with known functions in cardiac injury, morphogenesis, angiogenesis and tissue remodeling (e.g. ACE2, STEAP4, NPPA and SFRP4; all $p<0.05$). Multiple gene sets and pathways involved in cardiac morphogenesis and vasculogenesis were significantly downregulated by gene set and ingenuity pathway analysis (hallmark TGF beta signaling, cellular morphogenesis during differentiation, morphology of cardiovascular system, all $p<0.05$).

Conclusion: Disruption of gene networks for cardiac morphogenesis and vasculogenesis occurred in the preterm fetal heart of nonhuman primates with preterm labor, intraamniotic infection and severe fetal inflammation. Inflammatory injury to the fetal heart *in utero* may contribute to the development of heart disease later in life. Development of preterm labor therapeutics must also target fetal inflammation to lessen organ injury and potential long-term effects on cardiac function.

Key Words

Animal model, cardiac, chorioamnionitis, development, *Escherichia coli*, E. coli, fetal sepsis, fetal inflammatory response syndrome, fetus, Group B Streptococcus, GBS, heart, intrauterine infection, *Macaca nemestrina*, monkey, morphogenesis, neonate, pigtail macaque, pregnancy, preterm labor, preterm birth, sepsis, vasculogenesis.

Glossary of Terms

ACE2, Angiotensin I converting enzyme 2

ANGPTL7, Angiopoietin-like 7

ANP, atrial natriuretic peptide

cDNA, complementary DNA

CFTR, Cystic fibrosis transmembrane conductance regulator

E. coli, *Escherichia coli*

FAM69C, Family with sequence similarity 9, member c

GBS, Group B Streptococcus

GRIA3, Glutamate Receptor, ionotropic, AMPA3

IRX4, Iroquois homeobox 4

IL-6, interleukin 6

IL-8, interleukin 8

MAL2, Mal T-cell differentiation protein 2

MYL7, Myosin regulatory light chain 2, atrial isoform-like

NPPA, Natriuretic peptide A

qRT-PCR, quantitative reverse transcriptase polymerase chain reaction

SFRP4, Secreted frizzled-related protein 4

STEAP4, STEAP family member 4

Introduction

Infection is associated with the majority of early preterm births, which is a leading cause of neonatal morbidity and mortality.(1) Infection is often subclinical and thought to ascend from the lower genital tract allowing microbes to invade the placenta and amniotic fluid, which can lead to fetal bacteremia and sepsis. The fetal inflammatory response syndrome describes a condition of severe fetal inflammation that often occurs with fetal infection. The fetal inflammatory response syndrome is the counterpart to the adult condition (Systemic Inflammatory Response Syndrome) and is associated with an increased risk for multi-system fetal organ injury.(2-5) Studies have mainly focused on the relationship between the fetal inflammatory response syndrome and injury to the fetal lungs and brain, because they are often imaged and assessed postnatally. Inflammatory injury to other organs, including the fetal heart, has been hypothesized to occur, but is more challenging to demonstrate in human neonates. Although many studies have associated prematurity, low birth weight or fetal growth restriction with cardiovascular risk factors and heart disease later in life, the impact of perinatal infection and inflammation on fetal cardiac development is unknown.(6-16)

Accumulating evidence in humans and preterm sheep models implicates infection and fetal inflammation in altered fetal cardiac function. Fetal heart rate disturbances (e.g. absence of variability, arrhythmias, cardiac dysfunction) have been associated with chorioamnionitis, an inflammation of the placental membranes often caused by infection.(17-20) In fetuses from pregnancies with preterm premature rupture of membranes, a condition often complicated by microbial invasion of the amniotic cavity,

fetal echocardiography revealed changes in diastolic ventricular function, which may increase cardiac output.(21) In a similar cohort, strain imaging to evaluate right ventricular function found evidence for impairment of systolic and diastolic function and, in cases with funisitis (umbilical cord inflammation), dyskinesia of the right ventricle.(22) These findings are consistent with observations in preterm sheep models of intraamniotic infection (*Candida albicans*) or inflammation (lipopolysaccharide; LPS), in which fetal inflammation was associated with a reduction in mean arterial blood pressure and oxygen saturation, depressed ventricular contractility, diastolic dysfunction and a reduction in cardiomyocyte numbers.(23-25) The mechanism linking inflammation and fetal cardiac injury is unknown and challenging to elucidate in human neonates and sheep models for ethical reasons and the lack of genomic tools, respectively.

Our objective was to identify early biological events in the fetal heart occurring after intrauterine infection and development of fetal inflammatory response syndrome in a nonhuman primate. We hypothesized that development of the fetal inflammatory response syndrome is associated with fetal cardiac inflammation and changes in the gene program responsible for cardiac morphogenesis, analogous to observations that we have made on the effects of intraamniotic inflammation on fetal lung development.(26, 27)

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council and the Weatherall report, “The use of non-human primates in research”. The protocol was approved by the Institutional Animal Care Use Committee of the University of Washington (Permit Number: 4165-01). All surgery was performed under general anesthesia and all efforts were made to minimize discomfort.

Animals and Study Groups

Cases meeting criteria for the fetal inflammatory response syndrome were identified retrospectively in our pregnant nonhuman primate infection program based on an elevated fetal IL-6 level (>11 pg/ml; N=5) and compared with saline controls (N=5). Fetal cardiac microarray analyses were performed on animals inoculated with either: 1) 1-3 X 10^8 CFU of a hyperhemolytic and hypervirulent GBS strain (GBSΔ*covR*; N=4) into the choriodecidual space(28), 2) 5 x 10^4 CFU of *E.coli* RS218 into the amniotic fluid (prototypic strain causing neonatal meningitis; N=1), or 3) saline into the amniotic fluid and choriodecidual space (N=5)(29); citations indicate publications that describe the animal experiments and pregnancy outcomes, but fetal cardiac transcriptomics and IL-1β/IL-6/IL-8 were not previously analyzed or reported. As fetal cardiac tissue from the above saline controls was not saved to allow for protein (cytokine) analysis, an additional four saline controls were performed to enable the comparison of cytokines from fetal cardiac tissues of saline controls with fetal inflammatory response syndrome cases.

In our model, pregnant pigtail macaques were time-mated and fetal age determined using early ultrasound. Temperature in the animal quarters was maintained at 72-82 degrees Fahrenheit. Animals were fed a commercial monkey chow, supplemented daily with fruits and vegetables and drinking water was always available. The animal was first conditioned to a nylon jacket/tether system for several weeks before surgery, which allows free movement within the cage, but protected the catheters. On day 116-125 of pregnancy (term=172 days) catheters were surgically implanted via laparotomy into the maternal femoral artery and vein, amniotic cavity, and choriodecidual interface in the lower uterine segment (between uterine muscle and fetal membranes, external to the amniotic cavity). In the *E. coli* case and saline controls, an additional catheter was implanted into the fetal internal jugular vein. Fetal electrocardiography electrodes and a maternal temperature probe were also implanted. Post-operative analgesia was provided by a 25-microgram fentanyl patch applied the day prior to surgery, in addition to postoperative indomethacin. After 48 hours, the animals appeared to have recovered from surgery based on a return to baseline for activity, appetite, and bowel function.

After surgery, the animal was placed in the jacket and tether with the catheters/electrodes tracked through the tether system. Cefazolin and terbutaline sulfate were administered to reduce postoperative infection risk and uterine activity. Both cefazolin and terbutaline were stopped at least 72 hours before experimental start (~13 half-lives for terbutaline, 40 half-lives for cefazolin, >97% of both drugs eliminated), which represented approximately a 7-10 day period of postoperative terbutaline administration. Cefazolin (1g) was administered intravenously each day in saline controls to minimize the

possibility of a catheter-related infection. Experiments began approximately two weeks after catheterization surgery to allow recovery (~30-31 weeks human gestation).

At our center, term gestation in the non-instrumented pigtail macaque population averages 172 days.

Intraamniotic pressure was continuously recorded, digitized, and analyzed by previously described methods. The integrated area under the intrauterine pressure curve was used as a measure of uterine activity and reported as the hourly contraction area (HCA; mmHg•sec/hr) over 24 hours. Preterm labor was defined as >10,000 mmHg-sec/hr associated with a change in cervical effacement or dilation.

Histology

After cesarean section, fetal necropsy was performed in all animals and the heart was preserved in RNALater. Histopathologic examination was performed on fetal cardiac samples. A pathologist (AB) blinded to the case/control status examined H&E-stained, full-thickness paraffin sections of the fetal hearts to evaluate inflammation, necrosis, fetal vascular thrombosis, or other histopathological findings.

Cytokine Analysis

Interleukin-6 (IL-6) was assayed from cardiac tissues (nonhuman primate) and umbilical cord plasma (nonhuman primate and human) using Luminex multiplex cytokine kits

(Millipore, Billerica, MA). Fetal cardiac tissue was not available for protein analysis from the *E. coli* infection leaving N=4 (GBSΔcovR) for comparison with controls (N=4).

Bacterial Quantitation

Amniotic fluid (AF) and maternal blood were sampled frequently *i.e.* before (-24 and -0.25 hrs) and after bacterial inoculation (+0.75, +6, +12, +24 hrs and then every 12 hrs until repeat Cesarean section for fetal necropsy). At the time of Cesarean section, we also collected fetal blood, fetal heart tissues and swabs from the fetal lungs, meninges and chorioamnion (placental membranes). For enumeration of GBS and *E. coli* from amniotic fluid or fetal blood (100 µL), serial 10-fold dilutions were plated on TSA (GBS) or Columbia agar with 5% sheep blood (*E. coli*), respectively. Fetal heart tissues from GBS cases were weighed at necropsy, homogenized in sterile PBS and 10-fold serial dilutions were plated on TSA and incubated overnight at 37°C, 5% CO_2 and enumerated the following day as described.(28, 30) Plates were incubated 24 hours at 37°C (GBS) or 72 hours at 35°C (*E. coli*), 5% CO_2 and enumerated. Swabs collected from tissue were aseptically removed from Port-A-Cul vials and streaked to the same media as for blood or fluid. No bacteria were recovered from amniotic fluid, fetal blood or fetal heart tissues from controls.

For cytokine and prostaglandin (PG) analysis, amniotic fluid and blood samples were collected in EDTA tubes. Samples were centrifuged for 5 min. at 1200 rpm immediately after collection and the supernatant was frozen and stored at -80 °C. Prior to freezing, indomethacin (0.3 mM) was added to the samples saved for cytokine and prostaglandin

quantification, respectively. Fetal heart cytokine analysis was performed on tissue homogenates that were diluted 1:1 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1% Triton X-100, supplemented with cOmplete™, Mini, EDTA-free protease inhibitor cocktail (Roche)) and incubated overnight at 4°C. Lysates were then centrifuged at 4000 rpm for 5 min at 4°C, and supernatants stored at −80°C or used immediately for analysis. About 100µl of sample was used in Luminex or ELISA assays as described above.

RNA Extraction and Microarray Processing

To study genetic pathways in *M. nemestrina*, we used the Affymetrix Cynomolgus Array (GeneChip Cynomolgus Gene 1.0 ST, Affymetrix, Santa Clara, CA), which allows interrogation of 40,096 gene-level probe sets based on the *M. fascicularis* genome. *M. nemestrina* and *M. fascicularis* are closely related macaque species and diverged within the last 5-6 million years (31). RNA extraction was performed using miRNeasy mini kits (Qiagen, Valencia, CA) following the manufacturer's established protocol for purification of total RNA from animal tissues. RNA integrity was assessed with an Agilent Bioanalyzer instrument (Agilent, Santa Clara, CA) and was judged by distinct and sharp 18s and 28s ribosomal RNA peaks that were baseline separated. RNA quantity was determined by measuring OD_{260} with a Thermo Scientific NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The NanoDrop instrument was also used to determine purity of RNA samples by measuring $OD_{260/280}$ and $OD_{260/230}$ ratios. Only samples passing this stringent quality control were processed further. Processing of the RNA samples was carried out according to the

Affymetrix GeneChip Whole Transcript Sense Target labeling protocol. Briefly, double-stranded cDNA was synthesized with random hexamers tagged with a T7 promoter sequence. The double-stranded cDNA was subsequently used as a template and amplified by T7 RNA polymerase producing many copies of antisense cRNA. In the second cycle of cDNA synthesis, random hexamers were used to prune reverse transcription of the cRNA from the first cycle to produce single-stranded DNA in the sense orientation. In order to reproducibly fragment the single-stranded DNA and improve the robustness of the assay, dUTP was incorporated in the DNA during the second cycle first-strand reverse transcription reaction. This single-stranded DNA sample was then treated with a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) that specifically recognizes the unnatural dUTP residues and breaks the DNA strand. DNA was labeled by terminal deoxynucleotidyltransferase (TdT) with the Affymetrix® proprietary DNA Labeling Reagent that is covalently linked to biotin. The biotin labeled DNA fragments were hybridized to Affymetrix GeneChip Cynomolgus Gene 1.0 ST arrays, washed, and stained with fluorescent anti streptavidin biotinylated antibody. Following an additional wash step, the arrays were scanned with an Affymetrix GeneChip® 3000 scanner. Image generation and feature extraction was performed using Affymetrix GeneChip Command Console Software.

Single gene analysis of microarray data

The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO;

http://www.ncbi.nlm.nih.gov/geo/index.cgi; GSE98459). Analysis of the microarray data focused first on differential expression of single genes, which we refer to as the "ANOVA model." Raw microarray data were pre-processed and analyzed with various Bioconductor packages (http://www.bioconductor.org/)(32). Several quality control steps were carried out to ensure data was of high quality: 1) visual inspection of the GCOS DAT chip images, 2) visual inspection of the chip pseudo-images generated by the Bioconductor *oligo* package, 3) generation and inspection histograms of raw signal intensities, and, 4) generation and comparison of the Relative Log Expression and Normalized Unscaled Standard Errors using the Bioconductor *oligo* package. The data was normalized with the Bioconductor *oligo* package(33) using Robust Multiarray Averaging (RMA).(34) We did not filter out any probesets prior to analysis. From the normalized data, genes with significant evidence for differential expression were identified using the *limma* package in Bioconductor.(35) Changes in expression were inferred using a weighted t-test in conjunction with an empirical Bayes method to moderate the standard errors of the estimated log-fold changes. Array weights were estimated by computing the relative variability of each array, and then using the inverse of the variance to weight each array.(36) This allows smooth adjustment for array quality without having to exclude samples. We selected genes based on an unadjusted p-value < 0.05 and a 1.5-fold difference between groups.

We also fit a conventional linear model using IL-6 concentrations (log base 2) as a marker for infection severity, which we refer to as the "IL-6 model." In this model, we excluded two saline controls for which we did not have an IL-6 level. We selected genes

with an unadjusted p-value < 0.05 and an absolute slope > 0.07. This slope can be interpreted as a 5% change in expression for every doubling of IL-6 concentration. Given that the range of observed IL-6 concentrations is > 14 logs (e.g., more than 14 doublings in concentration), this represents an approximate 1.6-fold change in expression, at minimum, over the entire range of observed IL-6 concentrations.

Gene Set Analysis

Next, the data was analyzed using gene set tests to investigate categories of genes, using the *romer* function from the Bioconductor *limma* package. The *romer* function is intended to perform a competitive test against a battery of gene sets, assessing the statistical significance of pre-defined gene sets/pathways as a whole rather than of single genes. This method facilitates identification of modest but concordant changes in expression of a set of genes that may be missed by single gene analysis. Gene set testing considers all the genes in the experiment and allows for the identification of gene sets that are more highly ranked, as a set, than would be expected under the null hypothesis. The p-values are based on random rotations of the residuals (9,999 random rotations), which is similar to conventional permutation tests, but permits unlimited numbers of permutations to be tested. In addition, the *romer* function works with any linear model, not just ANOVA; we used the linear regression function against IL-6 concentration for gene set testing and Ingenuity Pathway Analysis (see below). In the IL-6 model, we excluded two controls for which fetal plasma was not obtained and the IL-6 level was unknown (Saline 1 and 3, Table 1). We used the Gene Ontology and

Hallmark gene set collections from the Broad Institute (http://software.broadinstitute.org/gsea/msigdb)(37).

To determine overlap in gene expression between our data and the fetal blood transcriptome associated with the fetal inflammatory response syndrome(38), we created a self-contained gene set using 36 of the 41 validated genes from the fetal blood transcriptome study that could be mapped to our nonhuman primate array. Using the roast function from the Bioconductor *limma* package, we tested if the 36 genes in the set were, on average, differentially expressed.

Ingenuity Pathway Analysis

The Core analysis feature of the Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com) was used to discover pathways and transcriptional networks in the gene expression microarray data from the IL-6 model. The Functional Analysis feature identified the biological functions and/or diseases that were most significant to the data set. Given that the range of observed IL-6 concentrations is > 14 logs, this represents an approximate 1.6-fold change in expression, at minimum, over the entire range of observed IL-6 concentrations. Ingenuity Pathway Analysis uses a right-tailed Fisher's exact test to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity

Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Knowledge Base, but are represented as a single node in the network. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with labels that describe the nature of the relationship between the nodes (see figure legends for details). Ingenuity Pathway Analysis also allows prediction of the activation or inhibition of transcription factors involved in the gene expression patterns seen in our study.

Validation of cDNA microarray by qRT-PCR

We used qRT-PCR to validate expression changes of genes of interest that had been identified by microarray analysis. Briefly, reverse transcription was performed according to the manufacturer's established protocol using total RNA and the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA.). For gene expression measurements, 2 µL of cDNA was included in a PCR (12 µL final volume) that also consisted of the ABI TaqMan® Gene Expression Assays mix and the TaqMan Gene Expression Master Mix according to the manufacturer's protocol (Applied Biosystems, ThermoFisher Scientific, Foster City, CA). Amplification and detection of PCR amplicons were performed with the ABI PRISM 7900 system (Applied Biosystems Inc., Foster City, CA) with the following PCR profile: 1 cycle of 95°C for 10 min, 40 cycles of 95°C for 30 s, and 60°C for 1 min. GAPDH amplification plots derived from serial dilutions of an established reference sample were used to create a linear regression formula in order to calculate expression levels, and β-actin gene expression levels were utilized as an

internal control to normalize the data.

Statistical Analysis

Statistical analyses related to the transcriptomics data are detailed above in their respective sections. We used a t-test for the analysis of Real-Time PCR data. A Mann-Whitney test was also performed to test comparisons between cases and controls for cytokine quantities.

Results

Preterm Labor and Fetal Cardiac Infection in a Nonhuman Primate Model of Severe Fetal Inflammation

To understand biological events induced by inflammation in the fetal heart, we used a unique, chronically catheterized, nonhuman primate model (pigtail macaque, *Macaca nemestrina*) of infection-induced preterm labor. From our pregnancy infection program, we retrospectively identified five cases with severe fetal inflammation consistent with the fetal inflammatory response syndrome (N=4 GBS and N=1 *E. coli*; fetal plasma IL-6: 157 – 29,294 pg/mL) and three saline controls (Table 1). In all fetal inflammatory response syndrome cases, there was acute and severe chorioamnionitis. The E. coli case was delivered in preterm labor 2.5 days after inoculation. Three of the four GBS cases were delivered at the time of preterm labor (0.3, 1.0 and 2.0 days after inoculation). One of the four GBS cases was delivered 2.0 days after inoculation without preterm labor in an effort to avoid stillbirth due to a marked change in color and turbidity of the amniotic fluid indicating heavy bacterial growth.(28) All controls were delivered in

the absence of preterm labor at 7 days post-inoculation to obtain gestational age matched tissues.(26)

In four of five cases, the fetal inflammatory response syndrome accompanied a disseminated bacterial infection into the fetal blood and/or organs. Bacteria were cultured and quantified from fetal heart tissues in all four fetal inflammatory response syndrome cases induced by GBS inoculation (Fig. 1A). Although fetal heart tissue was not specifically tested for bacteria from the *E. coli* case, 10^7 CFU were recovered from both fetal lungs and blood indicating a severe fetal infection. In fetal cardiac tissues, levels of IL-6 and IL-8 were significantly higher in fetal inflammatory response syndrome cases than in controls (Fig. 1A, p=0.03). IL-1β and TNF-α levels were not significantly different, but approached statistical significance for TNF-α (Fig. 1A, p=0.06). Blinded evaluation of fetal cardiac tissues did not reveal significant changes in gross pathology (Table S1). In the case with the highest fetal plasma IL-6 levels (induced by *E. coli*), there were areas with cytoplasmic pallor, but otherwise neutrophilic infiltrates or significant inflammation was not observed (Fig. 1B-C).

Fetal Cardiac Single Gene Analysis and Quantitative RT-PCR (qRT-PCR) Validation

To identify changes in the fetal cardiac gene program, we performed a single gene analysis of microarray results by two methods: 1) ANOVA (case-control comparison) and 2) linear regression correlating fetal plasma IL-6 to gene expression regardless of experimental group (IL-6 model). In the ANOVA analysis, 609 probe sets were differentially expressed >1.5 fold ($p<0.05$) between fetal inflammatory response

384 syndrome cases and controls. According to the IL-6 model, 1162 probe sets were
385 significantly differentially expressed ($p<0.05$). Genes significantly downregulated in both
386 types of analyses included *SFRP4*, *NPPA*, *PLA2G7*, *GRIA3*, *MTHFR*, *EPHA4*,
387 *ANGPTL7* (ANOVA: $\log_2$ fold changes range from -2.7 to -1.3, $p<0.05$; IL-6 model:
388 $p<0.02$; Table 2, Fig. S1). In both analyses, genes significantly upregulated included
389 *S100A8*, *FAM69C*, *PLA2G2*, *CFTR*, *STEAP4*, and *IRX4* (case-control: $\log_2$ fold
390 changes range from 1.2 to 2.6, $p<0.05$; IL-6 model: $p<0.02$; Table 2). Of the 609
391 significant genes from the ANOVA analysis, 452 were also significant in the IL-6 model
392 (with consistent direction of change). The probability that this would occur by chance,
393 under a hypergeometric null distribution, corresponds to a p-value less than 1×10^{-16}.
394 To validate our microarray data, we performed quantitative RT-PCR on 10 genes of
395 interest. Nine of ten genes were significantly differentially expressed between fetal
396 inflammatory response syndrome cases and controls: *SFRP4*, *NPPA*, *MAL2*,
397 *ANGPTL7*, *FAM69C*, *ACE2*, *CFTR*, *STEAP4* and *IRX4* ($p<0.05$, Fig. 1D).
398
399 *Gene Set and Ingenuity Pathway Analysis*
400 To explore relationships among differentially expressed genes, we identified gene sets
401 and pathways with concordant changes in expression by performing Gene Set Analysis
402 for data obtained with the IL-6 model. Multiple gene sets related to morphogenesis were
403 significantly downregulated with increasing IL-6 level including cellular morphogenesis
404 during differentiation ($p=0.008$, Fig. 1E), neuron differentiation ($p=0.008$), axonogenesis
405 ($p=0.008$), and regulation of DNA replication ($p=0.03$; Table 3). To place the findings of
406 disruptions in gene networks for cellular morphogenesis within a visual construct, the

Ingenuity Pathway Core Analysis feature was used to map functional networks of relevant genes. We also used the data obtained with the IL-6 model as input for the Ingenuity Pathway Analysis. Multiple pathways differentially regulated within the Ingenuity Pathway Analysis “Diseases and Biological Functions” category were related to cardiac morphogenesis and vasculogenesis including morphology of the cardiovascular system (Fig. 2), cellular movement and migration of cells, growth of smooth muscle, morphology of vessel, growth of muscle tissue, and vasculogenesis and migration of endothelial cells (Table 3; p value range: $4 \times 10^{-8} – 7 \times 10^{-21}$). Ingenuity Pathway Analysis also has the capability to predict activation states of transcriptional regulators based on the activation or suppression of downstream genes. The top transcription factors predicted to be associated with changes in gene expression were *TNF* (tumor necrosis factor), *TGFB1* (transforming growth factor, beta 1), and *IL-1B* (IL-1 beta; p value range: $2 \times 10^{-10} – 2 \times 10^{-14}$). These data suggest that multiple pathways related to fetal cardiac morphogenesis may be impacted by the development of the fetal inflammatory response syndrome and/or fetal bacteremia.

The human blood transcriptome associated with the fetal inflammatory response syndrome has been described by Madsen-Bouterse et al(38) and provides an opportunity to directly compare gene expression in different fetal compartments between human and nonhuman primate fetuses. Thirty-six of the 41 validated genes reported by Madsen-Bouterse et al could be mapped to our nonhuman primate array. A self-contained gene set test was then used to determine if any of the 36 genes identified in the human fetal blood transcriptome were differentially expressed in the nonhuman

primate fetal cardiac tissues. We observed that 33% of these genes were upregulated in our ANOVA model (p=0.04) and 44% were upregulated in the IL-6 model (p=0.008). Overall, there is significant overlap between the transcriptomes derived from human blood and nonhuman primate cardiac tissues in the setting of severe fetal inflammation.

Comment

Principal Findings of the Study

Our study is the first to demonstrate that fetal infection and inflammation was associated with changes in the cardiac developmental gene program involving a disruption in gene networks for morphogenesis and vasculogenesis in a nonhuman primate model of infection-associated preterm labor (conceptual model, Fig. 3). Principal findings of the study are**:** 1) pro-inflammatory cytokines (IL-6, IL-8) are elevated in the fetal myocardium without significant evidence of histopathologic inflammation during an early stage of the infection and inflammatory response; 2) a consistent set of genes is differentially regulated with fetal infection and fetal inflammatory response syndrome by either case-control analysis or correlation with IL-6 levels (e.g. *NPPA*); and 3) genes differentially expressed in the nonhuman primate fetal heart are involved in cardiac morphogenesis and vasculogenesis.

Results in the Context of What is Known

Strong epidemiological evidence implicates a fetal origin for adult heart disease and related risk factors.(6-16) Our results of perturbations in the fetal cardiac developmental gene program in cases with preterm labor, infection and the fetal inflammatory response

syndrome provide a link between preterm birth and adult cardiovascular disease. During pregnancy, diastolic dysfunction has been reported in human fetuses after preterm premature rupture of membranes, which is a condition typically complicated by an intraamniotic infection.(21) In preterm sheep models, amniotic fluid inoculation of either lipopolysaccharide or *Candida albicans* was associated with fetal cardiac dysfunction (diastolic dysfunction, reductions in ventricular contractility and cardiomyocyte density, hypertrophy of mature cardiomyocytes).(23, 24) Our work extends these observations in pregnancy by associating intraamniotic infection with the production of pro-inflammatory cytokine mediators in the fetal heart, severe fetal inflammation and perturbations in the preterm cardiac developmental gene program.

The long-term effects of infectious and/or inflammatory injury on the developing fetal heart are unknown, but the idea that adverse events in fetal life might predispose towards adult disease is consistent with a body of literature often referred to as the "Barker hypothesis".(39) David Barker, a British epidemiologist, proposed a causal association between intrauterine growth retardation, low birth weight and preterm birth with the development of cardiovascular disease, hypertension and diabetes in middle age based on a cohort in the United Kingdom.(40-42) Subsequent epidemiological studies supported a relationship between preterm birth and increased blood pressure,(7, 43-52) a major risk factor for coronary heart disease and stroke. A recent large epidemiologic study using a Swedish birth cohort found a 17-fold increased risk of heart failure in the first year of life after preterm birth (95% CI: 7.96 - 36.3), which provides even stronger evidence for an ominous effect of preterm birth on cardiac

development.(16) A series of physiologic and imaging studies in 102 adults born preterm revealed significant changes in their myocardial structure and function compared to controls.(53-55) Experiments in animal models and observations from human neonates born preterm also demonstrate altered cardiac structure (cardiomyocyte and cardiac hypertrophy) following preterm birth(56, 57) or neonatal exposure to a hyperoxic environment.(58, 59) Our data suggests that preterm birth complicated by severe fetal inflammation is more likely to be associated with a long-term risk of heart disease for surviving neonates.

Pro-inflammatory response in the fetal heart without evidence of histopathologic inflammation in the acute stages of fetal infection and the fetal inflammatory response syndrome

Neutrophils can injure cardiomyocytes through respiratory burst and oxidative injury(60), but neutrophilic infiltration was minimal or absent in the heart tissues in our study suggesting that either bacteria or cytokines played a greater role in perturbing fetal cardiac developmental gene networks. Although it is well established that bacterial cell wall components (e.g. lipopolysaccharide) and inflammatory cytokines can induce direct cardiodepressive effects on adults with sepsis, evidence is only beginning to accumulate that the fetal heart is similarly vulnerable to infectious and inflammatory injury.(61-64) GBS does not produce LPS, but the GBS hemolytic toxin overexpressed by the strain in this study [GBSΔ*covR*,(28)] has been shown to injure cardiomyocytes *in vitro* and induce a rapid loss of function and viability.(65) Polymicrobial infections are common in cases of early pretem birth and there is evidence that cell wall fragments

from gram-positive and gram-negative bacteria (e.g. peptidoglycan and lipopolysaccharide) can also synergize to induce release of cytokines and injure organs.(66) The latency between bacterial inoculation and delivery was fairly short in our study (maximum 2.5 days); therefore, fetuses exposed to infection for a longer time *in utero* may have a greater degree of leukocytic infiltration in their cardiac tissues, more complex mechanisms of cardiac injury and changes in the expression of their developmental gene networks.

Differential gene expression in the fetal heart with infection and the fetal inflammatory response syndrome

A consistent set of genes was differentially regulated in association with the diagnosis of the fetal inflammatory response syndrome or rising IL-6 levels and included *SFRP4*, *NPPA*, *MAL2*, *ANGPTL7*, *FAM69C*, *ACE2*, CFTR, *STEAP4* and *IRX4*. *MYH6* was also significantly downregulated when correlated with IL-6 levels. Of these genes, *NPPA* and *MYH6* have known roles in cardiac morphogenesis and gene mutations have been linked to development of arrhythmias and cardiomyopathy in the adult.(67, 68) *NPPA* encodes atrial natriuretic peptide (ANP), which is an early and specific marker for functional myocardium of the embryonic heart; expression of *NPPA* is essential for formation and expansion of the cardiac chambers.(69, 70) During fetal life, there is a dynamic and patterned expression of *NPPA* throughout the heart and ANP suppresses proliferation of cardiomyocytes near term in response to growth-promoting stimuli.(71, 72) A premature downregulation of NPPA may restrict growth potential of the fetal heart. *ACE2* expression is thought to have a protective role for the heart in pathologic settings

and downregulation of *ACE2* may also be harmful for development.(73) Several single genes with altered expression in our study have known functions in cardiac development or are implicated in heart disease or cardiac injury repair.

Downregulation of cardiac morphogenesis and vasculogenesis gene networks

Multiple gene sets, biological pathways and transcription factors related to morphogenesis and vasculogenesis (cellular movement, TGF-β signaling, epithelial-mesenchymal transition, cellular morphogenesis during differentiation, morphology of the cardiovascular system, and morphology of blood vessel) were disrupted in the context of fetal infection and inflammation. By Gene Set Analysis and Ingenuity Pathway Analysis, multiple pathways related to cardiac morphogenesis and vasculogenesis were differentially regulated in cases with fetal infection and the fetal inflammatory response syndrome suggesting multiple mechanisms and pathways for injury. Although the atria and ventricles have formed by week 10, the fetal heart continues to develop and grow until birth(71); disruptions in cardiomyocyte growth patterns imposed by an infection in the late second or third trimester may restrict growth potential and lead to compensatory changes such as cardiomyocyte hypertrophy. Downregulation of vasculogenesis during fetal life is particularly important as coronary vessels continue to develop *de novo* in postnatal life, which is necessary to augment growth of the coronary vasculature.(74) There was also significant overlap between the our data in nonhuman primate cardiac tissues and the human blood transcriptome from fetuses with the fetal inflammatory response syndrome (38); as leukocytes in fetal cardiac tissues were minimal or absent, we conclude that an overlap in gene expression

between our studies reflects a shared biological response across preterm cardiomyocytes and leukocytes to systemic infection and inflammation. Notably, a similar transcriptomic profile of downregulation in morphogenesis pathways was observed in our prior studies of gene expression in the nonhuman primate fetal lung in response to intraamniotic inflammation with a lesser degree of fetal inflammation.(26, 75)

Clinical Implications

The question of whether infection and fetal inflammation disrupts fetal heart development is of critical importance, because an intraamniotic infection occurs in approximately half of pregnancies with preterm premature rupture of membranes (pPROM).(76-78) Cardiac dysfunction has also been described in fetuses with pPROM.(21, 22) Further, an elevated plasma IL-6 level, diagnostic of the fetal inflammatory response syndrome, is associated with neonatal hypotension, which can be the result of low cardiac output and altered myocardial contractility.(79, 80) Our study provides critical evidence that infection and severe inflammation in utero disrupts the developmental genetic program of the fetal heart *before* birth. The extent to which an interrupted genetic program for organ development can be restored post-birth is unknown, but a number of interventions in the neonatal intensive care unit are also associated with an inflammatory stress (e.g. mechanical ventilation, supplemental oxygen).(81-83) Overall, this study suggests that in addition to risks of neonatal mortality, neurologic and pulmonary morbidity, some preterm infants have altered cardiac development beginning with inflammatory injury *in utero*.

Research Implications

A nonhuman primate model of infection-associated preterm labor affords a rare opportunity to investigate the fetal origins of abnormal cardiac development that can lead to adult heart disease. Many important questions remain as to how this risk may be amplified by common neonatal interventions and whether anti-inflammatory therapeutics administered in utero or postnatally might lessen this risk. In our study, fetuses were exposed to intraamniotic infection and had severe systemic inflammation, but whether a disruption of cardiac development occurs in preterm labor cases with a lesser degree of fetal inflammation is unknown. Our experiments were not originally designed to investigate heart function and additional work is needed to correlate changes in fetal cardiac transcriptomics with heart function and anatomical changes in the myocardium and vascularity. Whether therapeutic inhibition of inflammation in preterm infants may reduce the long-term risks of heart disease is unknown, but a critical question for prevention of life course morbidity. Therapeutics targeting inflammation in pre-clinical studies of preterm labor should be studied for their effect on the transcriptomic profile of fetal organs to determine if disruptions in developmental gene networks can be ameliorated in the fetal heart.

Strengths and Weaknesses

The strength of our study is in the novel finding of a disruption in morphogenesis gene networks in the fetal heart following intraamniotic infection and development of the fetal inflammatory response syndrome. Furthermore, the nonhuman primate shares many key features with human pregnancy (e.g. placentation, hormonal onset of labor), which

592 differ in other animal models of preterm birth (e.g. murine, sheep). The main study
593 limitation is the modest sample size, which is necessary for ethical reasons,
594 conservation of nonhuman primates and expense of the studies. Notably, our
595 nonhuman primate model is consistent with the effects of a disseminated bacterial
596 infection and severe systemic fetal inflammation; whether or not our results are
597 applicable to inflammation in the absence of bacteremia or a lesser magnitude of the
598 fetal inflammatory response syndrome is unknown.(84) Another important feature of our
599 model is the short time course from bacterial inoculation to delivery and fetal necropsy
600 (typically 2 days); histopathologic inflammation or overt signs of cardiac injury in the
601 fetal heart may not have had sufficient time to develop. Our results are best applied to a
602 severe infectious/inflammatory insult to the fetus in the late second or early third
603 trimester (~28 weeks) as the influence of infection on fetal development is likely
604 gestational age dependent.(85) The combination of cases using two different microbes
605 (GBS and *E. coli*) as inciting agents of the fetal inflammatory response syndrome was
606 necessary to establish sufficient power for the analysis; however, this mix of microbes is
607 also typical of human case series of preterm labor and the fetal inflammatory response
608 syndrome. Finally, we acknowledge that the effect of systemic fetal inflammation and
609 preterm birth on gene expression in the fetal heart may involve multiple factors beyond
610 simply the infectious/inflammatory component that may also function to increase the risk
611 of cardiovascular disease in adulthood such as a reduction in fetal systolic blood
612 pressure due to sepsis(86); oxidative stress(87); nutritional support such as lipid
613 infusions(88); and formula feeding.(89)
614

Conclusion

Our study offers new insight into the effect of fetal infection and inflammation on the dynamic regulation of genetic pathways programming fetal cardiac morphogenesis and vasculogenesis. This data provides a framework by which to understand how early biological changes impart a risk for cardiovascular disease later in life. Rapid changes in the fetal cardiac genetic program after bacterial inoculation suggest that inflammatory injury to the fetal heart occurs quickly after a virulent bacterial strain invades the amniotic cavity. An important question is whether an intervention combining antibiotic and anti-inflammatory therapeutics can ameliorate fetal organ inflammatory injury and restore a normal developmental genetic program in the fetal heart.

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Data Availability

The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/index.cgi; GSE98459) and will be made publicly available upon manuscript acceptance.

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Table 1. Fetal Cytokines, Pregnancy Outcomes and Amniotic Fluid Cultures

Group	Fetal Plasma Peak (pg/ml)		Chorio-amnionitis	Preterm Labor	Peak AF Cultures (CFU)
	IL-6	IL-8			
E. coli 1	29,293.7	2,273.1	Acute Severe	YES	1.4×10^7
GBS 1	2,152.6	3,265.6	Acute Severe	NO	1.0×10^7
GBS 2	1,015.5	1,634.1	Acute Severe	YES	9.4×10^7
GBS 3	254.4	6,307.5	Acute Severe	YES	2.5×10^7
GBS 4	156.8	3,341.7	Acute Severe	YES	2.9×10^7
Saline 1	*	*	NO	NO	No growth
Saline 2	2.0	523.4	NO	NO	No growth
Saline 3	*	*	NO	NO	No growth
Saline 4	0.9	182.3	NO	NO	No growth
Saline 5	2.3	223.0	NO	NO	No growth

Table presents the mean (SEM) for amniotic fluid and fetal plasma peak cytokines. Histopathologic evidence of inflammation in the placental membranes (chorioamnionitis) was diagnosed using Redline criteria.(90)

*In two saline controls, fetal plasma was not obtained.

With the exception of E. coli 1, remaining data in this table has been previously published(26-28) and is presented to provide context for the fetal cardiac transcriptomics and cytokine data.

Table 2. Select differentially expressed probe sets in the fetal heart of animals developing the fetal inflammatory response syndrome versus saline controls

			ANOVA Model		IL-6 Model	
Probe ID	Gene Name	Symbol	Log2 Fold Change	p value	Model Coefficient*	p value
13782964	Myosin Heavy Chain 6	MYH6	-2.72	0.05**	-0.47	2.0×10^{-5}
13728400	Secreted frizzled-related protein 4	SFRP4	-2.13	0.002	-0.28	1.6×10^{-5}
13746553	Phospholipase A2, Group VII	PLA2G7	-1.58	0.02	-0.23	8.8×10^{-4}
13807788	Glutamate receptor, Ionotropic, AMPA 3	GRIA3	-1.53	7×10^{-4}	-0.19	3.3×10^{-5}
13593196	Natriuretric peptide A	NPPA	-1.51	0.001	-0.14	0.01
13789400	Collectin sub-family member 10	COLEC10	-1.49	0.002	-0.14	0.02
13728244	Myosin regulatory light chain 2, atrial isoform-like	MYL7	-1.49	0.005	-0.19	5.5×10^{-4}
13580672	Angiopoietin-like 7	ANGPTL7	-1.35	0.002	-0.1	0.005
13765171	Iroquois homeobox 4	IRX4	1.16	8×10^{-4}	0.11	0.008
13729406	STEAP family member 4	STEAP4	1.20	9×10^{-4}	0.14	6.1×10^{-4}
13730121	Cystic fibrosis transmembrane conductance regulator	CFTR	1.44	8×10^{-4}	0.18	5.5×10^{-5}
13593639	Phospholipase A2, Group IIA	PLA2G2	1.50	0.04	0.19	0.01
13809224	Angiotensin I converting enzyme 2	ACE2	1.75	0.009	0.12	0.1**
13690264	Family with sequence similarity 9, member c	FAM69C	1.84	0.004	0.17	0.02
13599257	S100 calcium binding protein A8	S100A8	2.64	0.004	0.30	0.001

*The model coefficient estimates the log change in gene expression for a doubling of fetal plasma IL-6 concentration.

**NS, nonsignificant

Table 3. Select differentially expressed gene sets and Ingenuity Pathway Analysis pathways downregulated in the fetal heart of animals with the fetal inflammatory response syndrome

Gene Set Analysis			
Gene Set	**Number of Genes**		**p value**
Cellular Morphogenesis During Differentiation	37		0.008
Axonogenesis	31		0.008
Neurite Development	40		0.008
Neuron Differentiation	57		0.008
Neuron Development	46		0.008
Nervous System Development	292		0.01
Regulation of DNA Replication	16		0.03
Hallmark TGF Beta Signaling	41		0.04
Negative Regulation of Cellular Component Organization and Biogenesis	23		0.04
Ingenuity Pathway Functional Analysis of a Network*			
Diseases and Biological Functions	**Number of Molecules**		**p value**
Cellular Movement, Migration of Cells	150		7×10^{-21}
Development of Vasculature	94		2×10^{-15}
Morphology of Blood Vessel	40		1×10^{-13}
Proliferation of Smooth Muscle Cells	36		$3x\ 10^{-12}$
Growth of Muscle Tissue	42		$1x\ 10^{-11}$
Morphology of Cardiovascular System	69		4×10^{-11}
Vasculogenesis	66		1×10^{-10}
Abnormal Morphology of Cardiovascular System	50		5×10^{-10}
Migration of Endothelial Cells	33		4×10^{-8}
Ingenuity Pathway Transcription Factor Analysis			
Top Transcription Factors	**Predicted Activation State**	**Activation Z-Score**	**p-value of overlap**
TNF	Activated	4.2	2×10^{-14}
IL1B	Activated	3.7	1×10^{-11}
TGFB1	Inhibited	-2.0	2×10^{-10}

Gene Set and Ingenuity Pathway Analyses were based on the IL-6 model and select results are reported in the table.

*The Ingenuity Pathway Functional Analysis of a Network identified biological functions and/or diseases most significant to the molecules in the network using a right-tailed Fisher's exact test.

**Transcription factor analysis is based on prior knowledge of expected effects between transcription factors and their target genes stored in the Ingenuity Pathway Analysis library. The overlap p-value measures whether there is a statistically significant overlap between the dataset genes and the genes regulated by a transcription factor using Fisher's Exact Test.

Figure Legends.

Figure 1. Bacterial quantities, cytokines and gene expression in fetal heart tissues. (A) Quantitation of bacterial and cytokine protein levels in the fetal heart. Quantities of GBS and fetal cardiac cytokines in cases with the fetal inflammatory response syndrome (N=4, GBS) and controls (N=4) are shown (*, $p<0.05$). FIRS: fetal inflammatory response syndrome, CFU: colony forming units. (B, C) Hematoxylin and eosin staining of fetal cardiac tissues is shown to demonstrate cytoplasmic pallor, but otherwise an absence of neutrophilic infiltrates or significant inflammation in E. coli 1 (B) versus Saline 3 (C). (D) Comparison of the microarray and qRT-PCR analysis. The x-axis represents individual genes and the y-axis fold-change in expression by either microarray (gray bars) or qRT-PCR (black bars). All genes shown were significant in the unadjusted microarray analysis. Eight of ten genes selected for validation by qRT-PCR (black bars) were significantly up- or downregulated (*, $p<0.05$; two-sided t-test). (E) A heatmap depicting gene expression for the "cellular morphogenesis during differentiation" gene set is shown ($p=0.008$; Table 3).

Figure 2. (A) Heatmap of gene expression for the Ingenuity Pathway Analysis gene set, "morphology of cardiovascular system", is shown across all samples. The rows represent genes and the columns represent either fetal inflammatory response syndrome cases (red) or controls (blue). (B) A visual construct of the "morphology of cardiovascular system" gene set depicts the regulatory network of genes identified in the nonhuman primate fetal cardiac tissues. "Morphology of cardiovascular system" was a significantly downregulated gene set identified through Ingenuity Pathway Analysis in the IL-6 model ($p=4 \times 10^{-11}$, right-tailed Fisher's exact test). Each node represents a gene with different colored lines showing types of connectivity and functional relationships. Green and red nodes represent significant gene down- and up-regulation, respectfully. Protein-protein interactions from the Ingenuity Knowledge base were used to connect genes. Network data was then exported into cytoscape (version 3.2.1) to make custom network figures.

Figure 3. Conceptual model of events leading to fetal cardiac injury *in utero*. First, bacteria from the vagina traffic into the choriodecidual space, modeled in our experiments by inoculation of bacteria into this space using an implanted catheter in a nonhuman primate. Inflammatory cytokines are then produced by the decidua and chorioamniotic membranes, which diffuse into the amniotic fluid and fetal lung leading to a placental and fetal inflammatory response. When fetal inflammation is severe, which is typical in cases of early preterm birth complicated by infection, disruptions in the expression of gene networks for morphogenesis and vasculogenesis may occur in the fetal heart.