

Letter to the Editor

Reduced placental FOXP3 associated with subsequent infant allergic disease

To the Editor:

As the immunologically active interface between the mother and the fetus, the placenta has enormous potential to influence fetal immune development. Complex immunologic mechanisms have evolved to allow the fetal and maternal immune systems to coexist during pregnancy. In addition to well-recognized subtle adaptations of humoral and cellular immunity, there is an emerging role of CD4⁺CD25⁺ regulatory T cells (Treg)¹ in suppressing alloimmune responses between the mother and the fetus. During pregnancy, human chorionic gonadotrophin attracts Treg cells to the maternal-fetal interface² and collectively these fork-head box P3 (FOXP3)⁺ cells are important in maintaining the tolerogenic cytokine milieu critical for successful pregnancy.

As the fetus is sensitive to events in the placenta, we explored whether variations in placental *FOXP3* gene expression are associated with subsequent infant susceptibility to allergy disease. While maternal FOXP3⁺ Treg cells have been identified in the maternal placenta tissues (decidua), this is the first study to identify FOXP3⁺ cells in the fetal placenta tissues and to relate *FOXP3* expression to subsequent allergic outcomes.

We measured the FOXP3 mRNA expression in placental samples of nonsmoking mothers (n = 63) collected from a previously described birth cohort³ and compared levels in infants with allergic disease at 1 year (n = 32) with levels in infants without allergic disease (n = 31). Placental RNA was extracted from homogenized frozen placenta (collected from the fetal portion below the amniotic membrane) as described previously.⁴ Quantitative real-time RT-PCR was used to determine placental FOXP3 mRNA levels normalized against β -actin housekeeping gene.⁴ Locus-specific methylation analysis (*FOXP3* promoter and enhancer) was carried out by using Sequenom MassARRAY Epi-TYPING as previously described.⁵ We confirmed FOXP3 protein in placental tissue by immunohistochemistry (as described in this article's Online Repository at www.jacionline.org).

Infant allergic disease was defined by doctor-diagnosed eczema and/or IgE-mediated food allergy with sensitization to that food (skin prick test ≥ 3 mm) as previously described.³ In the group with allergic disease, 72% of infants had eczema (all with allergen sensitization) and 37% had food allergy (most commonly to egg). The infants without allergic disease had no evidence of sensitization or allergy symptoms. Maternal allergic disease (asthma or allergic rhinitis) was more common in the infants with allergic disease (100%) than in the infants without allergic disease (42%; $P < .001$).

FOXP3 was expressed in all placentas tested, with reduced expression in infants who developed allergic disease as compared with infants who did not develop allergic disease ($P = .013$) (Fig 1, A). Placenta *TGF*- β expression, a key regulatory cytokine inducing *FOXP3* gene expression, showed parallel trends (as FOXP3 mRNA) for lower levels in infants with allergic disease, but this was not statistically significant (see Fig E1 in this article's Online Repository at www.jacionline.org). FOXP3 mRNA was lower in placentas of female infants ($P = .029$), both with and without allergic disease (Fig 1, B; see Fig E2 in this article's Online Repository at www.jacionline.org), supporting previously

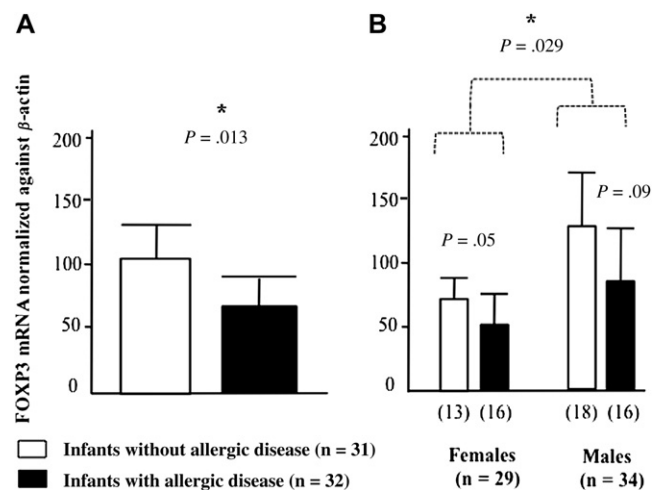


FIG 1. Placental *FOXP3* expression in infants with and without allergic disease. **A**, FOXP3 mRNA expression (normalized to housekeeping gene, β -actin) as means and 95% CIs for infants with allergy (solid bars) and those without allergy (open bars). **B**, Levels are also shown separately for male and female infants according to allergic status (* $P < .05$).

observed sex-specific differences in placental gene regulation.⁴ The strong association between maternal and infant allergy in this population makes it difficult to dissect the relative influence of the maternal phenotype, which has been previously associated with reduced neonatal Treg cell function.⁶

Although *FOXP3* is expressed by maternal Treg cells recruited to the maternal-fetal interface, these are found largely in decidual (maternal) tissue.⁷ For the first time, we now demonstrate, by using immunohistochemistry, the presence of FOXP3 protein in fetal placental tissue (see Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, we found significant demethylation of the *FOXP3* promoter (particularly cytosine phosphoguanine [CpG]8; 40% methylated) and the *FOXP3* enhancer (particularly CpG1; 40% methylated) in the placenta (Fig 2) and purified cytotrophoblast (cytokeratin-7 positive) cells. Placental methylation levels were significantly lower than those in cord blood CD4⁺ T cells, which were generally more than 90% methylated ($P < .05$). Previous studies have demonstrated that neonatal *FOXP3* gene expression is epigenetically modified by environmental exposures in pregnancy.⁸ Specifically, the allergy-protective effect of microbial exposure in pregnancy has been associated with increased neonatal Treg cell activity through demethylation of the *FOXP3* gene.⁸ Given the demonstrated regulation of *FOXP3* expression in the Treg cell lineage through decreasing methylation of these regions, we conclude that the placental expression of this gene is similarly regulated by DNA methylation.

In combination, these findings suggest that *FOXP3* is expressed by resident cells of the placenta derived from fetal tissues, not from contaminating cells of the fetal circulation or the maternal compartment. Ongoing studies will also examine individual epigenetic variations of specific cell types in relation to environmental exposures in pregnancy.

Our findings also provide further support for a role of FOXP3⁺ regulatory populations in early immune programming.^{6,8} We

Perinatal variation in FOXP3 gene methylation

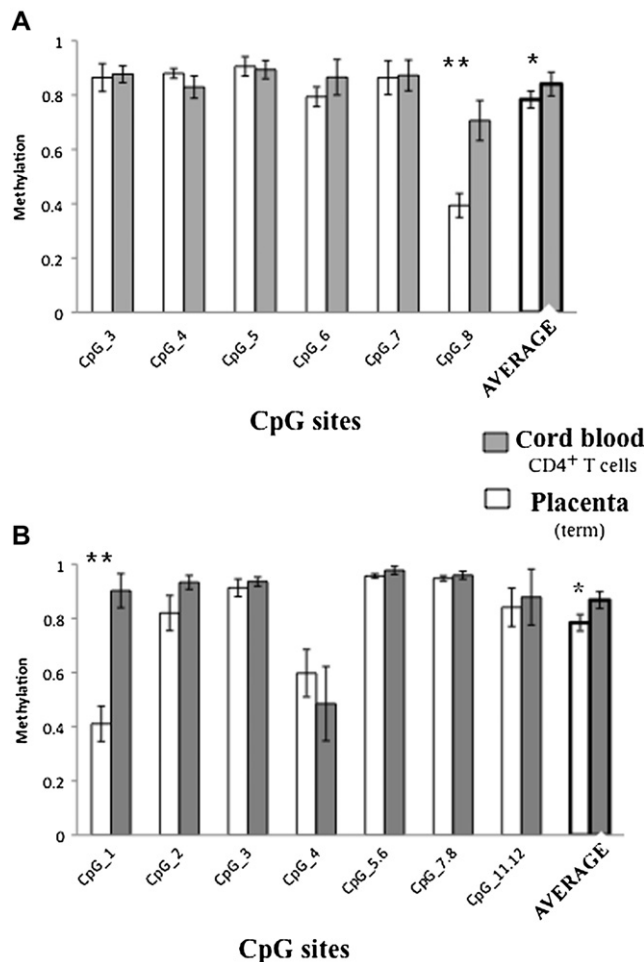


FIG 2. Comparison of *FOXP3* gene methylation patterns in cord blood and placenta. Methylation of CpG sites in the *FOXP3* promoter (A) and *FOXP3* enhancer (B) for placental samples ($n = 11$) and matched cord blood samples ($n = 11$) as means and 95% CIs (* $P < .05$; ** $P < .01$).

speculate that the differences in placental *FOXP3* gene expression associated with allergy observed in this study reflect combined effects of maternal phenotype, fetal genotype, and environmental exposures *in utero*. Further studies are needed to examine the functional significance of *FOXP3* expression in the placenta.

In summary, this is the first study to identify *FOXP3* expression in placental tissue and to demonstrate reduced *FOXP3* expression in placentas of infants who subsequently develop allergic disease. This supports our hypothesis that variations in the placental

activity of this key regulatory gene influence fetal immune development and disease susceptibility.

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METHODS

Study population

This study used samples collected as part of previously described^{E1} longitudinal cohort studies, as approved by the Princess Margaret Hospital Ethics Committee. Briefly, healthy pregnant women aged ≥ 18 and ≤ 44 years were recruited from the private clinics of obstetricians in the Perth metropolitan area, Western Australia. Only healthy term pregnancies were included, and all women were nonsmokers. The women were recruited from 20 weeks' gestation and reviewed regularly through their pregnancy and the postpartum period. Their infants were seen for allergy assessments and skin prick testing at 1 year of age (see below).

Definitions of allergy

Maternal allergy was defined as a doctor-diagnosed clinical history of asthma, eczema, or allergic rhinitis plus a positive skin prick test (SPT) result to one or more common allergens. Mothers without allergic disease had no history of allergic disease and negative SPT result to all tested allergens.

Infants were clinically evaluated at 12 months of age with a detailed history and examination by the same pediatric allergist (Prof Susan Prescott). The main allergic outcomes were eczema, IgE-mediated food allergy, and sensitization. A diagnosis of eczema was based on typical skin lesions responsive to topical steroids. *IgE-mediated food allergy* was defined as a history of immediate (within 60 minutes) symptoms following contact with and/or ingestion of food (such as egg, dairy products, and nuts) and a positive SPT result to the implicated food. Symptoms of acute food allergy included skin reactions (hives, rashes, or swelling), respiratory symptoms (cough, wheeze, stridor), gastrointestinal symptoms (abdominal pain, vomiting, loose stools), and/or cardiovascular symptoms (collapse). Sensitization was determined by the presence of a positive SPT result to allergen extracts (egg, milk, peanut, house dust mite, cat; Hollister-Stier Laboratories) and a histamine control. A wheal diameter of ≥ 3 mm was considered positive.

Detection of FOXP3 protein in placental tissue by immunohistochemistry

Formalin-fixed paraffin-embedded sections (4 μ m) were dewaxed and taken to distilled water and then treated with microwave heat-mediated antigen retrieval with in-house EDTA buffer (pH 8.0). Sections were incubated with FOXP3 mouse monoclonal antibody (1 mg/mL used at 1:50) (clone 236A/E7) (Abcam, San Francisco, Calif) for 60 minutes at room temperature. Detection was performed by using Dako EnVision+ Dual Link System-HRP (K4061, 30 minutes) (Dako, Houston, Tex), and the reaction product was visualized with Dako Liquid DAB+ Substrate-Chromogen System (K3468, 5 minutes). Positive control tissue was run in parallel with the test sections, consisting of a 4- μ m section of appendix with lymphoid patches. For negative controls, the primary FOXP3 antibody was replaced with concentration- and isotype-matched mouse IgG1 control antibody (code X 0931) (Dako). Positive cells appeared brown under bright field illumination.

Placental RNA extraction

Total placental RNA was extracted from 0.1 g of crushed frozen placenta by using the TRIzol method (Invitrogen) and homogenization in a Precellys 24 Homogenizer, as described previously.^{E2} Following extraction, the RNA was purified by using Qiagen RNeasy Mini Kit and DNase-treated by using Qiagen RNase-free DNase kit (Qiagen, Clifton Hill, Australia) according to the manufacturer's directions. The quality of the purified placental RNA samples was verified by running samples on an Agilent 2100 Bioanalyzer, and visualization of the 18S and 28S rRNA bands verified that the extracted RNA was intact.

Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was used to determine placental mRNA expression (FOXP3 and TGF- β) compared with levels of the housekeeping gene β -actin. Two micrograms of purified total RNA was reverse transcribed

by using the Invitrogen SuperScript III Kit for RT-PCR with random hexamers as primers, according to the manufacturer's instructions. All samples were diluted 1:5. Quantitect primer assays were used to detect both targets. Following optimization of primer concentration and volumes, real-time PCR was performed using an ABI Prism 7900HT Sequence Detector (Applied Biosystems, Foster City, Calif). Each mRNA sequence was amplified in duplicate in 20- μ L reactions containing SYBR green PCR master mix (Applied Biosystems), cDNA corresponding to 40 ng of reverse-transcribed RNA, and forward and reverse primers at a concentration between 200 and 800 nM, depending on the optimal primer concentration. Both no-template and no-reverse transcriptase controls were included for each amplification reaction, and the homogeneity of the amplified products was confirmed routinely by melting curve analysis and agarose gel electrophoresis. Dissociation curve analysis was used to confirm the specificity of amplification, and relative copy numbers were determined from PCR product standard curves. Copy numbers were normalized against β -actin copy numbers for each cDNA sample.

Methylation studies

Locus-specific methylation analysis was carried out by Sequenom MassARRAY EpiTYPING as previously described^{E3-E5} for quantitation of DNA methylation. In brief, 1 to 2 mg of genomic DNA was bisulfite treated by using the MethylEasy Xceed kit (Human Genetic Signatures, North Ryde, Australia), with converted DNA eluted to a final concentration of 20 ng/ μ L, of which 20 ng was used in each PCR amplification. Amplification primers were directed to sequences of interest following bisulfite conversion. Primers for FOXP3 were designed by using EpiDesigner (<http://epidesigner.com/>) and Methprimer^{E6} software packages. FOXP3 promoter primers were as follows: forward, TATTTTATTTTGTGGT-GAGGGG and reverse, AAATCAACCTAACTTATAAAAACTATCA; and FOXP3 enhancers were as follows: forward, TTTTGATTGGGTTTT-TAGAAGTTG and reverse, ACCAAATAAACATCACCTACCACAT, with the addition of appropriate linkers as previously described.^{E7} The resulting amplicons were then subjected to the EpiTYPER chemistry and analyzed by MALDI-TOF-MS. Methylation ratios (approximating percent cytosine methylation) were obtained by using EpiTYPER v1.0.5 software (Sequenom).

ADDITIONAL INFORMATION

There were no differences in the delivery method in infants with and without allergic disease ($P = .29$), and FOXP3 levels did not vary with the delivery method in this population ($P = .89$). There were also no differences or effects of gravidity (previous pregnancies), or gestational age, birth weight, or maternal age.

RESULTS

Description of FOXP3 immunohistochemistry in the placenta

FOXP3 staining is clearly evident in fetal tissue or cells. Specifically, we found that FOXP3 staining is clearly evident throughout placental (ie, fetal) tissues (Fig E3). There is strong, sporadic staining in the stroma of stem and intermediate villi. In the floating/terminal villi, there is weaker but detectable staining in the syncytiotrophoblast layer, and occasional cells within the stroma are also stained, which may be Hofbauer cells (fetal macrophages). Most interestingly, there are clusters of quite strongly stained cells in regions of dense nuclei in the syncytium suggestive of syncytial sprouts or knots (regions of preapoptotic syncytia that are shed into the maternal circulation) as seen most clearly on samples B and D of Fig E3.

Epigenetic control of regulatory *FOXP3* gene expression in placenta

As one of the most critical immune regulatory genes, we examined the epigenetic control of *FOXP3* in the placenta. In the placenta, *FOXP3* is expressed on regulatory T (Treg) cells recruited from the periphery^{E8} as well as functionally unique populations of Treg cells that have been identified at the maternal-fetal interface.^{E9} We observed significantly lower methylation in the *FOXP3* gene enhancer and promoter in the placenta (Fig 2) compared with neonatal CD4⁺ T cells. For the first time, this shows that relative activation of this gene at the maternal-fetal interface is associated with epigenetic differences (as detailed in the published letter).

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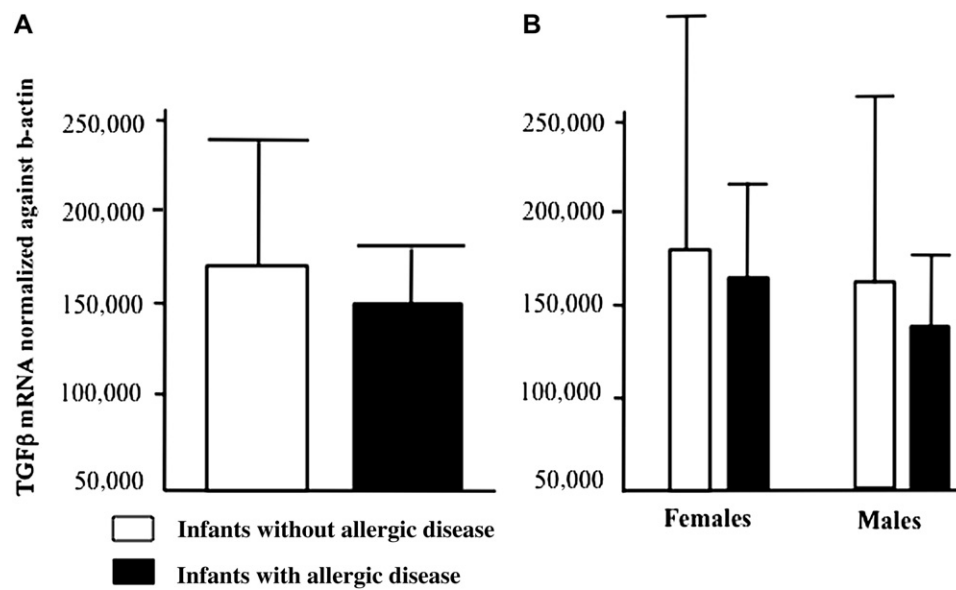


FIG E1. Placental TGF- β mRNA expression in infants with and without allergic disease. **A**, TGF- β mRNA expression (normalized to housekeeping gene, β -actin) as geometric means and 95% CIs for infants with allergic disease (*solid bars*) and those without allergic disease (*open bars*). **B**, Levels are also shown separately for male and female infants according to allergic status.

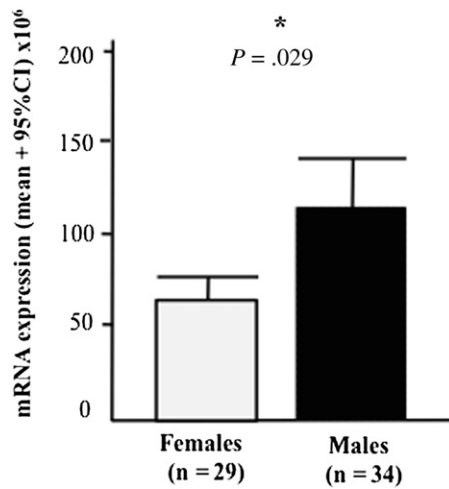


FIG E2. Comparison of placental gene expression in male and female infants: Placental *FOXP3* gene expression (mRNA) is shown (means + 95% CIs) for female (*open bar*) and male (*solid bar*) infants as indicated. Levels are displayed as geometric means and 95% CIs.

FOXP3 staining on placental sections from 4 individuals

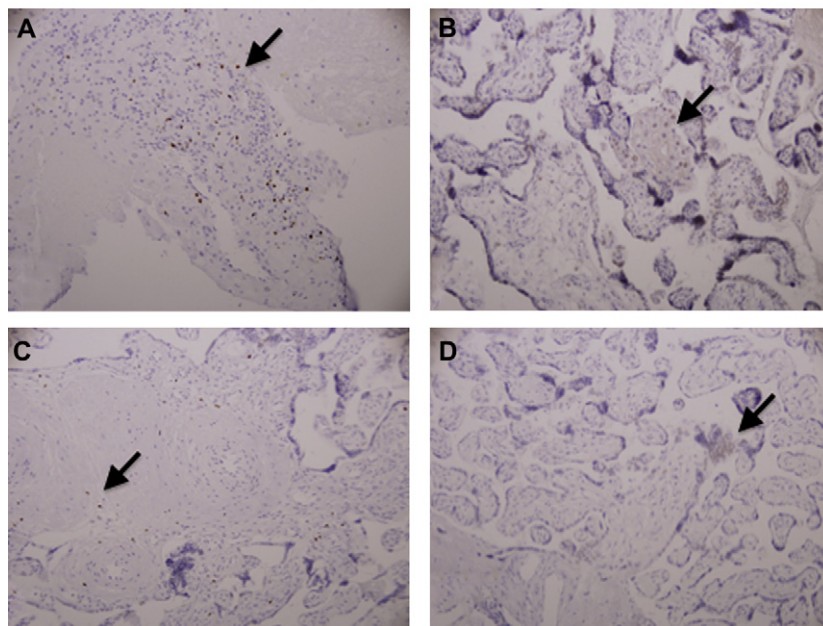


FIG E3. Placental FOXP3 protein detection in fetal tissues. **A-D**, Formalin-fixed paraffin-embedded sections (4 μ m) from 4 individuals are shown under 20 $\times$ magnification after staining for FOXP3 (mouse monoclonal antibody clone 236A/E7 1 mg/mL used at 1:50) as described in the additional [Methods](#) in the Online Repository. Positive cells appear brown under bright field illumination. For further description, refer to [Results](#) provided earlier in the Online Repository.