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Patterns of Gene Expression in the Ductus Arteriosus Are Related to Environmental and Genetic Risk Factors for Persistent Ductus Patency

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ABSTRACT: Three independent risk factors (immature gestation, absence of antenatal glucocorticoid exposure, and presence of the *rs2817399(A) allele* of the gene *TFAP2B*) are associated with patent ductus arteriosus (PDAs) that fail to close during prostaglandin inhibition. We hypothesized that these three factors may affect a common set of genes that increase the risk of persistent PDA after birth. We studied baboon ductus from term, preterm, and glucocorticoid-treated preterm fetuses and found that both immature gestation and absence of antenatal glucocorticoid exposure decreased RNA expression of calcium- and potassium-channel genes involved in oxygen-induced constriction, and phosphodiesterase genes (that modulate cAMP/cGMP signaling). Ductus obtained from second trimester human pregnancies were genotyped for *TFAP2B* polymorphisms. When present, the *rs2817399(A) allele* also was associated with decreased expression of calcium- and potassium-channel genes. In contrast, alleles of two other *TFAP2B* polymorphisms, rs2817419(G) and rs2635727(T), which are not related to the incidence of PDA after birth, had no effect on RNA expression. In conclusion, three calcium- and potassium-channel genes (*CACNA1G*/alpha1G, *CACNB2*/CaL-beta2, and *KCNA2*/Kv1.2) were similarly affected by each of the PDA risk factors. We speculate that these channels may play a significant role in closing the preterm ductus during prostaglandin inhibition and may be potential targets for future pharmacologic manipulations. **(*Pediatr Res* 68: 292–297, 2010)**

In contrast with the full term newborn, preterm infants frequently fail to close their ductus arteriosus after birth. Persistent ductus patency alters mesenteric blood flow, impairs pulmonary mechanics, increases the risk of pulmonary hemorrhage, and prolongs the need for mechanical ventilation (1,2).

In preterm infants, persistent ductus patency appears to be the result of alterations in the balance between developmentally regulated vasoconstricting and vasodilating pathways. Alterations in prostaglandin signaling seem to account for most persistent patent ductus arteriosus (PDA); 70% of preterm infants will close their PDA when prostaglandin production is inhibited by indomethacin or ibuprofen. However, approximately 30% of PDAs are the result of factors that are independent of prostaglandin signaling. In these infants, the PDAs fail to close when prostaglandin production is inhibited (3,4). Discovering the pathways that prevent ductus closure (when prostaglandin signaling is not involved) may lead to the development of new therapeutic approaches.

We recently identified three perinatal/neonatal risk factors that are independent of each other and predict the presence of PDAs that fail to close when prostaglandin signaling is inhibited (5): 1) immature gestation at birth, 2) absence of antenatal glucocorticoid exposure, and 3) racial/genetic variation (5). We hypothesized that these environmental (immature gestation and absence of antenatal glucocorticoid exposure) and genetic risk factors may be associated with altered mRNA expression of genes that are important for ductus constriction. We also hypothesized that these three independent risk factors may affect mRNA expression of a common set of genes, making their combined presence more likely to increase the risk of PDA after birth.

We designed the following studies to identify genes from both human and nonhuman primates that are affected by gestation, antenatal glucocorticoid exposure, and genetic variation. We focused our attention on the expression of genes that have been found to play an important role in ductus contractility in other species (6–18). We used nonhuman primates to study the effects of advancing gestation and antenatal glucocorticoids on ductus mRNA expression.

To study the role of genetic variation, we examined the influence of single-nucleotide polymorphisms (SNPs) on the mRNA expression of genes that regulate human ductus contractility. Several genes have sequence polymorphisms that have been associated with the presence of isolated (nonsyndromic) PDAs in preterm infants: *AGTR1*/angiotensin II type

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Abbreviations: CT, cycle threshold; **ΔCT,** the difference in cycle threshold between the expression of the housekeeping gene MDH and the gene of interest; **MDH,** malate dehydrogenase; **PDA,** patent ductus arteriosus; **SNP,** single-nucleotide polymorphism; **TFAP2B,** transcription factor AP-2 beta

1 receptor (19), *IFNG*/IFNγ (20), estrogen receptor-alpha *Pvu*II pP (21), *TFAP2B*/transcription factor AP-2 beta, *PTGIS*/prostacyclin synthase, and *TRAF1* (22). We examined SNPs in the gene *TFAP2B* because polymorphisms in this gene seem to act in concert with premature birth to delay ductus closure (see "Discussion") (9,13,22).

We were particularly interested in genes expressed in the ductus arteriosus that were similarly affected by all three risk factors (immature gestation, absence of glucocorticoid exposure, and *TFAP2B DNA* polymorphisms) because they might represent common targets for future pharmacologic manipulations.

METHODS

Tissue. Studies involving baboons (*Papio papio*) were performed at the Southwest National Primate Research Center, Southwest Foundation for Biomedical Research in San Antonio, TX. Study procedures were approved by the Institutional Animal Care and Use Committee. Details of animal care have been published elsewhere (23–25). Briefly, fetuses from time-dated pregnant dams were delivered by elective caesarean section [at either 125 ± 2 ($n = 20$) or 175 ± 2 ($n = 10$) d gestation; full term = 185 d] and euthanized at the time of delivery. Ten of the dams were treated with 6 mg of intramuscular betamethasone 48 and 24 h before elective delivery at 125 ± 2 d gestation.

Human tissue was obtained under the oversight of the Institutional Review Board at University of New Mexico (which determined that the study did not constitute human subject research because no identifiable patient data were collected). Mid-gestation (11–22 wk, $n = 57$) human fetal ductus arteriosus and ascending aorta were obtained at elective terminations of pregnancy in healthy women. Prostaglandins were not used during the terminations. Cervical ripening was performed using laminaria (compressed seaweed). Ductus and aorta samples were isolated and snap frozen in liquid nitrogen within 30 min of termination. Gestational age was determined by fetal foot length (26).

Preparation of total RNA, reverse transcription, and quantitative polymerase chain reaction. Total RNA was isolated from each individual ductus as described elsewhere (27). We used the TaqMan Universal PCR master mix of PE Applied Biosystems (Foster City, CA) to quantify gene expression. Taqman probes were designed using the Primer Express program and labeled with fluorophores FAM (6-caboxy-fluorescein) and TAMRA (6 carboxy-tetramethyl-rhodamine) as reporter and quencher dyes, respectively. An ABI PRISM 7500 Sequence detection system was used to determine the cycle threshold (CT). Reactions were carried out in triplicates. Data were analyzed using the Sequence Detector version 1.6.3 program. The degree of expression of the gene of interest was determined using the relative gene expression method. Malate dehydrogenase (MDH) was used as an internal control to normalize the data (28). ΔCT represents the difference in CT between the expression of the housekeeping gene (MDH) and the gene of interest. Each unit of ΔCT represents a twofold change in a gene's mRNA. The more negative the ΔCT, the fewer the number of starting copies of a gene (mRNA).

DNA genotyping. DNA was extracted from the ascending aorta of each of human fetal samples using the QIAamp DNA mini kit (QIAGEN Inc, Valencia, CA). DNA was quantified spectrophotometrically. Allelic variation was determined by using the TaqMan genotyping system (Applied Biosystems, Foster City, CA), as described previously (29). Allele scoring was performed using the Sequence Detection Systems 2.2 software (Applied Biosystems). We studied several sequence polymorphisms of the *TFAP2B* gene because recent findings suggest that they may be associated with delayed ductus closure in preterm infants (22). The *TFAP2B* SNPs were selected using data from the International HapMap project (available at: www.hapmap.org). To ensure that an adequate number of individuals within the population would be carriers of the minor allele, we chose a minor allele frequency of 0.1 as a lower cutoff for a SNP. We examined one *TFAP2B* SNP (rs2817399: A-allele) that has been positively associated with delayed ductus closure (even in the presence of indomethacin), with a p value of <0.01. We also examined two *TFAP2B* SNPs that are unrelated to the timing of ductus closure (rs2817419: G-allele, $p > 0.90$ and rs2635727: T-allele, $p > 0.90$ for association with the development of a PDA) (22) (unpublished results, Dagle *et al.*).

Statistics. Values are expressed as mean ± standard deviation. The t test was used to compare means. When appropriate, multivariate linear or logistic regression analyses were performed to determine the independent effects of gestational age, *TFAP2B* mRNA expression, and *TFAP2B* allele frequency on the mRNA expression of genes involved with ductus contractility. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of immature gestation and antenatal glucocorticoid exposure on ductus arteriosus gene expression in fetal baboons. Immature gestation altered the mRNA expression of several baboon ductus genes that are involved in oxygen-induced constriction [calcium channels (*CACNA1G*/Ca-alpha1G, *CACNB2*/CaLbeta2, and *CACNB3*/CaLbeta3), calcium pumps (*ATP2A3*/SERCA3), and Potassium-channels (*KCNA2*/Kv1.2, *KCNA5*/Kv1.5, *KCNB1*/Kv2.1, *KCNS3*/Kv9.3, *KCNAB2*/Kvbeta1.2, *KCNAB1*/Kvbeta1.3, *KCNMB1*/BKCa-beta1, and *ABCC9*/SUR2)] (Table 1). The mRNA expression of several of the genes involved with endothelin signaling (ECE1 and *EDNRA*/ETA (endothelin A) receptor), prostaglandin signaling (*PTGS1*/COX1, *PTGIS*/PGI2-synthase, phospodiesterases 1B, 3A, 3B, and 4D), contractile proteins (*CNN1*/Calponin, *CALD1*/Caldesmon, and *TPM1*/Tropomyosin), and inflammation and remodeling (*EPAS1*/HIF2alpha, *PDGFB*/PDGF-B chain, and *VEGFA*) were also altered by immature gestation (Table 1).

On the other hand, immature gestation had no effect on mRNA expression of the following genes: *CACNA1C*/CaLalpha1c, *SLC8A1*/NCX-1, *RHOA, RHOB, KCNMA1*/BKCa, *KCNJ8*/Kir6.1, *EDN1*/ET1, *EDNRB*/ETB (endothelin B)-receptor, *PTGS2*/COX2, *PTGER2*/EP2, *PTGER3*/EP3, *PTGER4*/EP4, *PDE1C, CD40LG*/CD154, *NOS3*/eNOS, *HAS2, IFNG*/IFNgamma, *IL6, IL8, NOS2*/iNOS, *CSF1*/MCSF, *MMP2, MMP3, MMP9, TGFB3*/TGFbeta3, *TNF*/TNFalpha, *PLAU*/UPA, and *VCAM* (data not shown).

We were interested in identifying a set of genes whose expression was similarly affected by the risk factors immature gestation and absence of antenatal glucocorticoid exposure because both risk factors are associated with an increased incidence of ductus patency after birth. Absence of antenatal glucocorticoid exposure affected mRNA expression of a similar, but more limited, set of the same genes that were altered by immature gestation (Table 1). In particular, both immature gestation and absence of antenatal glucocorticoid exposure altered mRNA expression of calcium-channel and potassium-channel genes that are involved in oxygen-induced constriction and phosphodiesterase genes that modulate second messenger (cAMP/cGMP) signaling.

Effects of genetic variations, or polymorphisms, on ductus arteriosus gene expression in human fetuses. We examined how genetic variations in the *TFAP2B* gene might alter mRNA expression of genes that were affected by both immature gestation and absence of antenatal glucocorticoid exposure. Because of the small size of the second trimester human ductus, we were limited in the number of candidate genes we could evaluate. Between 11 and 22 wk of gestation, there was a significant increase in *TFAP2B* mRNA expression in the human ductus (for every 1 wk increase in gestation, there was a 0.16 increase in *TFAP2B* ΔCT, $p = 0.000$). Because *TFAP2B* mRNA expression was significantly linked to gestational age, we used a multivariate model (which included gestational age) to examine the independent effects of *TFAP2B* mRNA expression on the mRNA expression of the candidate genes that affect ductus contractility. We found a

Table 1. *Effects of gestational age and antenatal betamethasone on real-time polymerase chain reaction (PCR) measurements of genes involved with ductus arteriosus closure in fetal baboons*

Gene	125-d gestation, ΔCT		125 d + betamethasone, ΔCT		175-d gestation, ΔCT		Risk factors	
	Mean	SD	Mean	SD	Mean	SD	Immaturity, $p < 0.05$	No betamethasone, $p < 0.05$
Ca++ signaling								
CACNA1G/Ca-alpha1G	−2.08	0.88	−1.35	0.72	−1.22	0.66	▼	▼
CACNB2/CaLbeta2	−0.68	0.88	0.55	0.97	0.90	0.67	▼	▼
CACNB3/CaLbeta3	−1.84	0.17	−1.90	0.20	−2.00	0.12	▲	–
ATP2A3/SERCA3	−3.28	1.09	−1.74	0.82	−1.40	0.72	▼	▼
K+ channels								
KCNA2/Kv1.2	−3.34	1.37	−1.85	1.28	−1.16	0.92	▼	▼
KCNA5/Kv1.5	1.15	0.22	1.04	0.46	0.75	0.22	▲	—
KCNB1/Kv2.1	−3.80	0.81	−3.30	0.74	−3.00	0.51	▼	—
KCNS3/Kv9.3	−6.51	1.60	−5.11	1.18	−4.72	1.05	▼	▼
KCNAB2/Kvbeta1.2	−5.10	1.25	−3.70	1.29	−3.34	0.82	▼	▼
KCNAB1/Kvbeta1.3	−3.02	1.21	−1.57	1.17	−1.29	0.81	▼	▼
KCNMB1/BKCa-beta1	−0.46	0.37	0.31	0.29	0.05	0.37	▼	▼
ABCC9/SUR2	−0.86	0.29	−1.18	0.38	−1.19	0.19	▲	—
Contractile proteins								
CNN1/calponin	−3.78	0.37	−4.04	0.65	−2.54	0.25	▼	—
CALD1/caldesmon	0.90	0.31	1.02	0.43	2.77	0.55	▼	—
TPM1/tropomyosin	3.09	0.42	3.03	0.46	4.08	0.23	▼	—
Endothelin signaling								
ECE1	−7.45	0.65	−7.16	0.83	−6.79	0.81	▼	—
EDNRA/ETA-receptor	−1.03	0.36	−0.99	0.36	−0.56	0.64	▼	—
Prostaglandin signaling								
PTGS1/COX1	−2.75	0.70	−2.59	0.40	−2.05	0.62	▼	—
PDE1B	−3.80	2.52	−0.85	2.13	−0.50	1.26	▼	▼
PDE3A	−2.18	0.21	−2.08	0.33	−1.81	0.22	▼	—
PDE3B	−2.83	0.67	−2.10	0.82	−1.77	0.57	▼	▼
PDE4D	−4.19	0.45	−4.29	0.39	−3.61	0.35	▼	—
PTGIS/PGI2-synthase	2.38	0.31	2.46	0.45	2.83	0.38	▼	—
Inflammation/remodeling								
EPAS1/HIF2alpha	0.67	0.42	0.89	0.55	2.75	0.33	▼	—
PDGFB/PDGF-B chain	−2.78	0.90	−1.49	1.05	−1.13	0.66	▼	▼
TFAP2B	0.65	0.31	0.53	0.39	0.22	0.22	▲	—
VEGFA	0.37	0.19	0.07	0.46	1.66	0.48	▼	—

Fetal ductus from 125-d gestation baboons (not exposed to betamethasone in utero: 125 d gestation, $n = 10$ separate animals) were compared with ductus from 125-d gestation fetuses exposed to 48 h of betamethasone before necropsy (125 d + betamethasone, $n = 10$ separate animals) and with ductus from 175-d gestation fetuses (not exposed to betamethasone in utero: 175 d gestation, $n = 10$ separate animals). ΔCT represents the difference in cycle threshold (CT) between the expression of the housekeeping gene malate dehydrogenase (MDH) and the gene of interest. Each unit of ΔCT represents a twofold change in a gene's mRNA. The more negative the ΔCT, the fewer the number of starting copies of a gene (mRNA). Immaturity: fetal ductus from 125-d gestation baboons were compared with ductus from 175-d gestation baboons. Betamethasone: fetal ductus from 125-d gestation baboons, not exposed to betamethasone in utero, were compared with ductus from 125-d gestation baboons exposed to 48 h of betamethasone before necropsy. ▲: $p < 0.05$, ΔCT of immature (125-d gestation) ductus is significantly greater than ΔCT of either the mature (175-d gestation) ductus or the immature ductus exposed to antenatal betamethasone; ▼: $p < 0.05$, ΔCT of immature ductus is significantly less; and —: $p > 0.05$, ΔCT of immature ductus is not significantly different.

Ca-alpha1G, calcium T-channel; CaL, calcium L-channel; SERCA, sarcoplasmic reticulum Ca^{++}-ATPase; K channels (Kv, BKCa, Kir), potassium channels; ECE, endothelin converting enzyme; ETAreceptor, endothelin receptor A; COX, cyclooxygenase; PDE, phosphodiesterase; PTGIS, prostacyclin (PGI2)-synthase; HIF, hypoxia inducible factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.

positive correlation between *TFAP2B* mRNA expression and the mRNA expression of most of the candidate genes we examined (even after controlling for gestation) (Table 2).

We genotyped the DNA of individual human ductus samples to determine the allele frequency of several *TFAP2B* polymorphisms. The A allele of the *TFAP2B* polymorphism *rs2817399* (which is associated with an increased incidence of PDA in preterm infants (22) (unpublished results, Dagle *et al.*) was present in 25 of 57 (44%) of fetal ductus. Because expression of many of the candidate genes that alter ductus closure is regulated by gestational age (Table 3), we used multivariate models (which included gestational age) to examine the independent effects of the *rs2817399(A)-allele* on their mRNA expression. We found that the *rs2817399(A)-allele* was significantly associated with decreased mRNA expression of genes involved with oxygen-induced constriction of the ductus arteriosus: the *CACNB2*/calcium L-channel beta2 subunit, the *CACNA1G*/alpha1G (calcium T-channel), and the *KCNA2*/Kv1.2 potassium-channel (Table 2).

Alleles of two other *TFAP2B* polymorphisms, rs2817419(G) and rs2635727(T) (neither are associated with an increased incidence of PDA), were present in 20 of 57 (35%) and 19 of 57 (33%) of second trimester fetal ductus samples, respectively. In contrast to the rs2817399(A) allele,

Table 2. *Multivariate regression models examining the independent effects of gestational age, TFAP2B mRNA expression, and TFAP2B allele polymorphisms on the mRNA expression of genes involved with ductus closure in second trimester human ductus*

	Regression coefficient (95% confidence interval)				
			TFAP2B DNA polymorphism (present vs absent)‡		
Gene	Gestation*	TFAP2B mRNA†	rs2817399(A)	*rs2817419(G)*	*rs2635727(T)*
Calcium signaling					
CACNA1G/ Ca-alpha1G	−0.07 (−0.14 to 0.01)	**0.33**§ (0.07 to 0.60)	**−0.59**§ (−1.12 to −0.05)	0.41 (−0.02 to 0.84)	0.33 (−0.10 to 0.78)
CACNA1C/ CaLalpha1c	**−0.08**§ (−0.15 to −0.01)	**0.41**§ (0.16 to 0.66)	−0.36 (−0.90 to 0.19)		
CACNB2/CaLbeta2	**−0.10**§ (−0.18 to −0.02)	**0.42**§ (0.14 to 0.71)	**−0.63**§ (−1.22 to −0.03)	0.44 (−0.04 to 0.92)	0.34 (−0.16 to 0.83)
RHOB	**0.23**§ (0.16 to 0.30)	**0.37**§ (0.12 to 0.62)	−0.12 (−0.67 to 0.43)		
K+ channels					
KCNA2/Kv1.2	0.13 (−0.23 to 0.02)	0.14 (−0.41 to 0.68)	**−1.09**§ (−2.15 to −0.03)	0.76 (−0.01 to 0.62)	−0.62 (−0.25 to 1.50)
KCNA5/Kv1.5	−0.02 (−0.07 to 0.04)	**0.68**§ (0.49 to 0.87)	−0.29 (−0.82 to 0.25)		
Contractile proteins					
CNN1/calponin	**0.09**§ (0.04 to 0.13)	**0.88**§ (0.72 to 1.03)	0.32 (−.25 to 0.89)		
Endothelin signaling					
ECE1	**0.06**§ (0.01 to 0.12)	**0.40**§ (0.21 to 0.58)	0.11 (−0.32 to 0.55)		
Prostaglandin signaling					
PDE1B	0.05 (−0.12 to 0.03)	**0.44**§ (0.17 to 0.70)	0.46 (−1.02 to 0.11)		
PDE3B	0.00 (−0.07 to 0.07)	**0.72**§ (0.46 to 0.98)	0.11 (−0.01 to 0.62)		
PDE4D	**0.09**§ (0.01 to 0.17)	**0.29**§ (0.01 to 0.57)	0.16 (−0.75 to 0.42)		
PTGIS/PGI2-synthase	**0.04**§ (>0.01 to 0.07)	**0.67**§ (0.55 to 0.78)	0.01 (−0.42 to 0.44)		
Inflammation/ remodeling					
EPAS1/HIF2alpha	**0.08**§ (0.02 to 0.13)	**0.31**§ (0.11 to 0.50)	0.20 (−0.29 to 0.69)		
TFAP2B	**0.16**§ (0.09 to 0.22)	—	0.19 (−0.36 to 0.73)		

* Regression coefficient represents the increase in a gene's ΔCT for every increased week of gestation (multivariate analysis with TFAP2B ΔCT in the statistical model).

† Regression coefficient represents the increase in a gene's ΔCT for every increase in a unit of TFAP2B ΔCT (multivariate analysis with gestational age in the statistical model).

‡ Regression coefficient represents the increase in a gene's ΔCT when a TFAP2B allele (*rs2817399(A), rs2817419(G)*, or *rs2635727(T)*) was present in the tissue (compared with when it was absent) (multivariate analysis with gestational age in the statistical model).

§ Values presented in bold: $p < 0.05$.

alleles of the two other *TFAP2B* polymorphisms, rs2817419(G) and rs2635727(T), were not associated with changes in candidate gene expression (Table 2).

DISCUSSION

We hypothesized that different environmental and genetic risk factors may negatively impact a similar cohort of candidate genes involved in ductus closure. Recent findings support the concept that environmental risk factors (such as immature gestation and absence of antenatal glucocorticoid exposure) directly affect ductus closure through mechanisms that are independent of prostaglandin signaling. Advancing gestation affects pathways that regulate intracellular calcium concentrations (15,17) and alters the sensitivity of ductus smooth muscle to vasodilators, such as nitric oxide (4,30). Glucocorticoids, similar to advancing gestation, also affect intracellular calcium concentrations and alter the balance of vasodilators in the ductus (31–36). As a result, antenatal glucocorticoid exposure increases fetal ductus contractility even when prostaglandin production has already been inhibited (37). Our current experiments demonstrate that, in nonhuman primates, immature gestation and absence of antenatal glucocorticoid exposure alter the mRNA expression of calcium-channel and potassium-channel genes involved in oxygen-induced constriction and phosphodiesterase genes that modulate cAMP/cGMP signaling (Table 1).

To study the effects of genetic variation on candidate gene expression, we examined the *TFAP2B* gene because DNA polymorphisms in *TFAP2B* seem to act in concert with premature birth to delay ductus closure (22). *TFAP2B* is a member of the AP2 transcription factor family, a family of retinoic acid-responsive genes that play an important role in development, apoptosis, cell-cycle control, and complex morphogenic processes (38,39). *TFAP2B* is expressed in cells derived from the neural crest and is uniquely expressed in ductus smooth muscle (compared with the surrounding pulmonary artery and aorta) (9,40). *TFAP2B* regulates several genes that are important for ductus smooth muscle development in mice (*e.g.* calponin, endothelin, and HIF2alpha). Targeted deletions of *TFAP2B* prevent the ductus from constricting in full term, newborn mice (9). Similarly, the dominant-negative mutations and intronic splicing mutations in *TFAP2B* (found in Char syndrome) prevent the ductus from constricting in full term human newborns (13,41).

TFAP2B mRNA is strongly expressed by the beginning of the third trimester, in the mouse ductus, and then declines

Table 3. *Effects of immature gestational age, absence of betamethasone exposure, and presence of the TFAP2B* rs2817399(A) *allele on mRNA expression in the baboon and human ductus arteriosus*

Risk factor, gene	Immaturity,* ΔCT	No betamethasone,† ΔCT	TFAP2B polymorphism rs2817399(A),‡ regression coefficient
Calcium signaling			
CACNA1G/Ca-alpha1G	▼	▼	▼
CACNA1C/CaLalpha1c	—	—	—
CACNB2/CaLbeta2	▼	▼	▼
RHOB	—	—	—
K+ channels			
KCNA2/Kv1.2	▼	▼	▼
KCNA5/Kv1.5	▲	—	—
Contractile proteins			
CNN1/Calponin	▼	—	—
Endothelin signaling			
ECE1	▼	—	—
Prostaglandin signaling			
PDE1B	▼	▼	—
PDE3B	▼	▼	—
PDE4D	▼	—	—
PTGIS/PGI2-synthase	▼	—	—
Inflammation/remodeling			
EPAS1/HIF2alpha	▼	—	—
TFAP2B	▲	—	—

* ▲, ▼, and–: ΔCT of immature ductus is significantly ($p < 0.05$) larger, smaller, or no different from the ΔCT of mature ductus (data from baboons, Table 1).

† ▲, ▼, and–: ΔCT of immature ductus (not exposed to betamethasone) is significantly ($p < 0.05$) larger, smaller, or no different from the ΔCT of ductus exposed to betamethasone (data from baboons, Table 1).

‡ ▲, ▼, and–: regression coefficients that represent a significant ($p < 0.05$) increase, decrease, or no significant change in a gene's ΔCT when the TFAP2B allele *rs2817399(A)* is present in the tissue (compared with when it is absent) (data from humans, Table 2).

toward term (9). We observed a similar pattern of *TFAP2B* expression in the baboon ductus during the third trimester (Table 1).

Our human studies used second trimester ductus. During the second trimester, there was a significant increase in *TFAP2B* mRNA expression in the human ductus. As has been observed in mice, *TFAP2B* mRNA expression was positively correlated with calponin, HIF2alpha, and endothelin (endothelin converting enzyme) expression in the human ductus. We also observed a positive correlation between *TFAP2B* mRNA expression and the mRNA expression of several other genes that play important roles in ductus closure at birth (Table 2). The significance of these relationships persisted, even when the effects of advancing gestational age were included in the statistical models (Table 2).

We used multivariate regression models to examine the independent effects of gestational age and genetic variation (in *TFAP2B*) on the mRNA expression of genes that affect ductus closure. We were particularly interested in the A-allele of the *TFAP2B* polymorphism *rs2817399* because it is associated with preterm PDAs that fail to close in the presence of indomethacin (22). The *rs2817399(A)-allele* is located between exons 4 and 5, where mutations reported to cause Char syndrome are located (41). The *rs2817399(A)-allele* is present in a haplotype block that encompasses most of the *TFAP2B* gene, so that the actual genetic variations responsible for PDA in preterm infants could lie anywhere within this haplotype block. Detailed mapping of this region will be necessary to define the actual etiologic polymorphism.

We found that the *rs2817399(A)-allele* was significantly associated with decreased mRNA expression of three genes that could impact ductus closure after birth: *CACNB2* (the calcium L-channel beta2 subunit), *CACNA1G* (the alpha1G (calcium T-channel)), and *KCNA2* (the Kv1.2 potassium-channel) (Table 2) (6–8). To determine whether these changes were specific to the *rs2817399(A)-allele*, we examined two other *TFAP2B* polymorphisms, rs2817419(G) and rs2635727(T), which are unrelated to the incidence of preterm PDA. Both polymorphisms are located beyond exon 7 (the last exon of the *TFAP2B* gene) where no known Char syndrome mutations have been reported (41). Neither polymorphism was associated with changes in any of the candidate genes' mRNA expression (Table 2).

Our findings provide biologic plausibility to the concept that *rs2817399(A)* is a functional polymorphism (or in tight association with a functional polymorphism) that plays an active role in regulating ductus contractility. At this time, we do not have an explanation for the changes in calcium- and potassium-channel gene expression that occur in the presence of the *rs2817399(A)-allele*. The *rs2817399(A)-allele* was not associated with a decrease in *TFAP2B* mRNA expression (as detected by our real-time PCR probe/primers sets) (Table 2). SNPs in or near a gene can affect both the amount and function of the mRNA or protein produced. Future studies will be needed to determine how this polymorphism affects the expression of downstream calcium- and potassium-channel genes.

Our hypothesis was that different environmental and genetic risk factors might negatively impact a similar cohort of genes involved in ductus closure. We found that the same calcium- and potassium-channel genes [*CACNB2*/calcium L-channel beta2 subunit, *CACNA1G*/alpha1G (calcium T-channel), and *KCNA2*/Kv1.2 potassium-channel] that were decreased in humans in the presence of the *TFAP2B* polymorphism *rs2817399(A)* were also decreased in nonhuman primates when the two environmental risk factors (immature gestation and absence of antenatal betamethasone exposure) were present (Table 3). These three calcium- and potassium-channel genes have previously been shown to be involved with oxygen-induced constriction of the ductus arteriosus (6–8). We speculate that calcium- and potassium-channels may play a role in closing the preterm ductus during prostaglandin inhibition because they seem to be altered in PDAs that fail to respond to indomethacin treatment. If this proves to be the case, they may be potential targets for future pharmacologic manipulations. We also speculate that when several PDA risk factors are combined in the same infant, they may have a cumulative effect on the expression of calcium and potassium channels, which may delay ductus closure after birth (even when prostaglandin production has been inhibited). In the future, algorithms incorporating the combinatorial

effects of these risk factors may enable a more targeted use of indomethicin or ibuprofen in infants with a PDA.

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