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Effects of advancing gestation and non-Caucasian race on ductus arteriosus gene expression

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Abstract

Objective—To identify genes affected by advancing gestation and racial/ethnic origin in human ductus arteriosus (DA).

Study design—We collected three sets of DA tissue (n=93, n=89, n=91; total = 273 fetuses) from second trimester pregnancies. We examined four genes, with DNA polymorphisms that distribute along racial lines, to identify "*Caucasian*" and "*Non-Caucasian*" DA. We used RT-PCR to measure RNA expression of 48 candidate genes involved in functional closure of the DA, and used multivariable regression analyses to examine the relationships between advancing gestation, "*Non-Caucasian*" race, and gene expression.

Results—Mature gestation and Non-Caucasian race are significant predictors for identifying infants who will close their patent DA when treated with indomethacin. Advancing gestation consistently altered gene expression in pathways involved with oxygen-induced constriction (e.g., calcium-channels, potassium-channels, and endothelin signaling), contractile protein maturation, tissue remodeling, and prostaglandin and nitric oxide signaling in all three tissue sets. None of the pathways involved with oxygen-induced constriction appeared to be altered in "*Non-Caucasian"* DA. Two genes, *SLCO2A1* and *NOS3*, (involved with prostaglandin reuptake/metabolism and nitric oxide production, respectively) were consistently decreased in "*Non-Caucasian*" DA.

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The authors declare no conflicts of interest.

Conclusions—Prostaglandins and nitric oxide are the most important vasodilators opposing DA closure. Indomethacin inhibits prostaglandin production, but not nitric oxide production. Because decreased *SLCO2A1* and *NOS3* expression can lead to increased prostaglandin and decreased nitric oxide concentrations, we speculate that prostaglandin-mediated vasodilation may play a more dominant role in maintaining the "*Non-Caucasian*" PDA, making it more likely to close when inhibited by indomethacin.

Keywords

nitric oxide synthase; Race; Caucasian; Prostaglandin transporter; gene expression; ductus arteriosus

In contrast with full term infants, those born before 28 weeks gestation frequently fail to close their ductus arteriosus (DA) after birth. DA patency is determined by a balance between developmentally regulated vasoconstriction and vasodilation pathways. Prostaglandins and nitric oxide appear to be the most important vasodilators influencing DA tone in utero. After birth, the newborn ductus continues to be sensitive to the vasodilating effects of NO, but gradually loses its ability to respond to prostaglandins (1, 2). Although the vasodilating effects of prostaglandins begin to wane after birth, prostaglandin signaling still seems to play a dominant role in the majority of persistent preterm patent DA (PDA), because inhibitors of prostaglandin production (indomethacin and ibuprofen) will close the PDA in most preterm infants. Unfortunately, approximately 30% of PDAs fail to close when prostaglandins are inhibited, which suggests that alterations in the balance of vasodilators, or factors other than vasodilator signaling, may be preventing PDA closure in these infants (3, 4). Discovering which factors or pathways prevent PDA closure when prostaglandin production is inhibited may lead to the development of new therapeutic approaches.

Several groups have previously shown that immature gestation and racial/ethnic origin are the two most consistent risk factors for identifying infants who fail to close their PDA with inhibitors of prostaglandin production (5-8). Premature infants born to Non-Caucasian or Hispanic mothers are more likely to close their PDA following prostaglandin inhibition than are infants born to Caucasian mothers (5-8). The racial differences in the DA's response to prostaglandin inhibition cannot be explained by differences in polymorphisms of cytochrome P450 CYP2C (6); nor can they be explained by differences in other factors that control the rate of indomethacin/ibuprofen metabolism. Serum levels of indomethacin/ibuprofen appear to be similar in the two racial groups (8). Prostaglandin serum levels also appear to be similar in the two racial groups following prostaglandin inhibition (8).

We hypothesized that the identification of genes that differ in their expression between Caucasian and Non-Caucasian fetal ductus may identify pathways involved with DA closure that are critical for its closure and independent of prostaglandin production. Therefore, we designed a study to identify genes from human DA that are affected by advancing gestation and racial/ethnic origin. We focused our attention on a group of candidate genes that have both a unique pattern of expression in the DA, as well as a functional role in DA closure (Table I; available at www.jpeds.com).

Methods

Human tissue was obtained under the oversight of the Institutional Review Board at University of California San Francisco and was given exempt status. Mid-gestation ($13^{0/7}$– $23\ ^{6/7}$ weeks) human fetal DA and ascending aorta were collected from elective pregnancy terminations in healthy women (legal in California up to 24 weeks). Consent for the use of fetal tissue for research purposes is obtained from the mothers by the clinic staff, who have been trained in human subjects protections. The consent for the use of fetal tissue for research purposes is separate from the consent for the clinical procedure. Researchers have no patient contact and only receive de-identified tissues.

Prostaglandins were not used during the terminations. Cervical ripening was performed with laminaria (compressed seaweed). Fetal tissue was immediately submerged in calcium- and magnesium-free phosphate buffered saline at 4°C following delivery. The DA and aorta were dissected in the chilled buffer solution and the isolated DA and aorta were snap frozen in liquid nitrogen (between 1.5 and 2 hours after delivery). Gestational age was determined by fetal foot length (9).

Three separate sets of DA and aorta tissues were collected and independently analyzed between July 2011 and May 2014: tissue set #1 (July 2011-May 2012, n=93 fetuses, gestation: 19.8±3.0 weeks (mean±sd)), tissue set #2 (June 2012-May 2013, n=89 fetuses, gestation: 19.6±2.9 weeks), and tissue set #3 (June 2013-May 2014, n=91 fetuses, gestation: 20.0±3.0 weeks). De-identified tissue samples were individually labeled and stored for later analysis. Samples were then processed at the end of each sample group collection period. The data from each tissue sample were analyzed individually – there was no "pooling" or combining of tissues during the analyses.

During the period of the study, women who donated tissue self-identified their racial/ethnic origins to the clinic staff as Caucasian=21%, Non-Caucasian=76% (Hispanic=31%, African=28%, Asian=14%, Native American/Hawaiian/Pacific Islander=2%, Other=1%), or Unknown=3%. Data on self-reported race/ethnic origin was not linked to de-identified tissues samples. No other clinical information was available for analysis.

Preparation of total RNA, reverse transcription and quantitative polymerase chain reaction

We examined the RNA expression of 48 candidate genes in each of the 273 human DAs. The candidate genes were chosen because: 1) their expression in the DA has previously been shown to differ from their expression in the aorta, and 2) their mutations or polymorphisms (or their pharmacologic inhibition) has been shown to affect DA closure (Table I).

Total RNA was isolated from each individual DA and cDNA was generated as described elsewhere (10, 11). We used the TaqMan Universal PCR master mix of PE Applied Biosystems (Foster City, CA) to quantify gene expression in a 96-well format. 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 triplicate. 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 (11, 12). ΔCT represents the difference in cycle threshold (CT) between the expression of the housekeeping gene (MDH) and the gene of interest. Each unit of ΔCT represents a 2-fold change in a gene's mRNA. The more negative the ΔCT, the fewer the number of starting copies of a gene's mRNA.

We examined the RNA expression of the 48 candidate genes in each of the 3 tissue sample sets. The analyses for each sample set were performed once all of the tissues in a set had been collected. The expression analyses for each sample set were separated in time from the analyses of the other sample sets by at least a year.

DNA genotyping to determine racial origins of fetal tissues

DNA was extracted from the ascending aorta of each of the 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 previously described (13). Allele scoring was performed using the Sequence Detection Systems 2.2 software (Applied Biosystems).

To identify the race of the fetal tissues, we used an established approach for determining Non-Caucasian race. We examined four genes whose sequence polymorphisms were distributed along racial lines between Caucasian and Non-Caucasian (African, Chinese, and Japanese) populations (14-16). These include: *SLC24A5* SNP rs1426654 (100% Caucasian = **A**-allele; 98% Non-Caucasian = **G**-allele) (14, 15); *SLC45A2* SNP rs16891982 (100% Caucasian = **G**-allele; 100% Non-Caucasian = **C**-allele) (14, 15); *DARC* SNP rs2814778 (100% Caucasian = **A**-allele; 85-100% Non-Caucasian = **G**-allele) (16); and, *HERC2* SNP rs12913832 (79% Caucasian = **G**-allele; 100% Non-Caucasian = **A**-allele) (15). Each fetal sample possessed between 0 and 8 of the Non-Caucasian alleles. The distribution of Non-Caucasian alleles among the 273 tissue samples in the three tissue sample sets was: 0-alleles = 9%, 1-allele = 8%, 2-alleles = 7%, 3-alleles = 10%, 4-alleles = 20%, 5-alleles = 13%, 6-alleles = 18%, 7-alleles = 8%, and 8-alleles = 7%. Therefore, 76% of the samples had three or more Non-Caucasian alleles. Because 76% of the women who donated tissue self-identified their racial/ethnic origin as "Non-Caucasian" (see above), we defined tissues with three or more Non-Caucasian alleles as "Non-Caucasian origin," and those with two or less Non-Caucasian alleles as "Caucasian origin."

Statistical analyses

Univariate linear regression models were used to test for possible associations between gestational age and mRNA expression in 48 candidate genes involved with DA closure. These models determined the increase or decrease in gene expression for a one-week increase in gestational age. Additional analyses were conducted using multivariable linear regression models to determine the effect of race on mRNA expression of each individual gene, while adjusting for gestational age. Coefficients derived from these models were thus interpreted as the difference (positive or negative) between the mRNA expression of "non-

Caucasians" and that of "Caucasians" while holding gestational age constant. Models were run individually for each candidate gene. Statistical significance was defined using an α-level of 0.05.

Results

Effects of advancing gestation on ductus arteriosus gene expression in human fetuses

We previously found that advancing gestation alters a diverse number of gene pathways in the human DA (11). Our current study was designed to test the reproducibility of these results and to expand the list of genes that might be affected by advancing gestation in DA tissues obtained from a different geographic area. In order to determine how reproducible our observations were, we examined the relationship between advancing gestation and mRNA expression in three separate sets of DA tissues (total = 273 fetuses) collected at three separate periods of time. When a gene's expression was significantly (p<0.05) associated with advancing gestation in each of the three separate tissue sets, we classified the gene as having a *HIGHLY Consistent* association with advancing gestation; when the association was significant in two of the three tissue sets, we classified the gene as having a *MODERATELY Consistent* association. When the association was significant in only one of the three tissue sets (or when no significant association was found in any of the tissue sets), we classified the gene as having *LESS or NO Consistent* association with advancing gestation, respectively. Similar to our prior observations, we found that advancing gestation altered the mRNA expression of DA genes that are involved in oxygen-induced constriction (eg, calcium-channels, potassium-channels, and endothelin signaling), contractile protein maturation, prostaglandin and nitric oxide mediated relaxation, and tissue inflammation and remodeling (Tables II and III; Table III available at www.jpeds.com).

Effects of racial origins on ductus arteriosus gene expression in human fetuses

Premature infants born to Non-Caucasian or Hispanic mothers are more likely to close their patent DA after indomethacin treatment than Caucasian infants (5-8); therefore, we were interested in identifying a set of genes whose expression within the DA was affected by the racial origin of the tissue. Because no identifiable patient data were collected with the tissues, we genotyped the DNA of each of the human fetuses as a means of assigning racial origin. We based our classification system on the observation that 76% of the fetuses had three or more Non-Caucasian alleles (see Methods), and 76% of the clinic population, from whom the tissue came, self-identified their racial origin as being of "*Non-Caucasian origin*" (see Methods). Therefore, we classified tissues from fetuses with three or more Non-Caucasian alleles as being of "*Non-Caucasian origin*" (those with two or fewer Non-Caucasian alleles were classified as being of *"Caucasian origin"*).

Because advancing gestational age affected most of the candidate genes we were studying (Table II), we used a multivariable model adjusted for gestational age to examine the independent effects of "*Non-Caucasian origin*" on mRNA expression. After controlling for advancing gestation, we found two genes, *NOS3* and *SLCO2A1*, (which are involved with nitric oxide signaling and prostaglandin uptake, respectively) which were altered by "*Non-Caucasian origin*" in a *HIGHLY Consistent* manner (Tables IV and V). We also found five

genes (involved with muscle contractility (*CNN1*, *SM2/SM1* ratio) and tissue remodeling (*EPAS1, PDGFB, SMARCA4*)) that were altered in a *MODERATELY Consistent* manner by "*Non-Caucasian origin*" (Table IV).

Discussion

In the current study, we used human fetal DA tissue to identify a group of genes whose mRNA expression is affected by advancing gestation and racial/ethnic origin in a highly or moderately reproducible manner. We found that advancing gestation consistently altered gene pathways involved with oxygen-induced constriction (eg, calcium-channels, potassium-channels, and endothelin signaling), contractile protein maturation, tissue remodeling and inflammation, and prostaglandin and nitric oxide signaling (Table II). Our findings are consistent with earlier reports that examined changes in gene pathways during advancing gestation in the human DA (11, 17).

Our primary objective was to identify a set of genes whose expression (within the fetal DA) is altered by the racial origin of the tissue. In prior studies, although race was not a consistent risk factor for identifying infants who were likely to develop a PDA, it was a significant risk factor for identifying infants who failed to close their PDA after prostaglandin inhibition: premature infants born to Non-Caucasian mothers were more likely to close their PDA (following prostaglandin inhibition) than were infants born to Caucasian mothers (5, 6, 8). We found two genes that were altered by "*Non-Caucasian origin*" in a *HIGHLY Consistent* manner: *NOS3* (which regulates nitric oxide production) and *SLCO2A1* (which regulates prostaglandin reuptake) (Tables IV and V). DA from fetuses of "*Non-Caucasian origin*" had lower *NOS3* and *SLCO2A1* expression than DA from "*Caucasian origin*" fetuses. . The significance of these relationships persisted, even when we adjusted for the effects of advancing gestational age in multivariable statistical models (Tables IV and V).

The decreased expression of *SLCO2A1* and *NOS3* in the "*Non-Caucasian*" DA is intriguing because it suggests that the balance of influence between the vasodilators prostaglandins and nitric oxide may be altered in the "*Non-Caucasian*" DA. *SLCO2A1* is a lactate/prostaglandin exchanger, which regulates fetal pericellular prostaglandin E2 (PGE2) levels via reuptake and catabolism (18). We speculate that decreased *SLCO2A1* expression leads to increased PGE2 concentrations surrounding the "*Non-Caucasian*" DA smooth muscle cells (due to the decreased PGE2 reuptake and metabolism). This would make the "*Non-Caucasian*" DA more susceptible to prostaglandin-mediated relaxation and more likely to be affected by alterations in prostaglandin production (18).

NOS3 regulates the production of nitric oxide, which also is important for preventing DA closure in preterm infants. We have previously shown that inhibitors of nitric oxide production can increase the rate of PDA closure in preterm newborns that fail to close their PDA with indomethacin (19, 20). The decreased expression of *NOS3* in the "*Non-Caucasian*" DA is interesting because Non-Caucasians are known to have decreased nitric oxide bioavailability, which makes them more likely to develop systemic hypertension

(21-23). Non-Caucasians are also more likely to develop pulmonary hypertension during the newborn period (24, 25).

PGE2 and nitric oxide are reciprocally coupled in the fetal DA (26, 27). During fetal life, *NOS3* expression is upregulated when prostaglandins are inhibited and downregulated when prostaglandins are increased (27). If the decreased *SLCO2A1* expression leads to increased fetal pericellular PGE2 concentrations (as we hypothesized above), it may contribute to the decreased *NOS3* expression in the "*Non-Caucasian*" DA.

We speculate that the decrease in *NOS3* and *SLCO2A1* expression in the "*Non-Caucasian*" DA may alter the relative importance of nitric oxide and prostaglandins in maintaining DA patency. Because decreased *SLCO2A1* and *NOS3* expression can lead to increased prostaglandin concentrations and decreased nitric oxide concentrations, we speculate that prostaglandin-mediated vasodilation may play a more dominant role in the "*Non-Caucasian*" PDA, making it more likely to close when inhibited by indomethacin. Conversely, we speculate that a PDA that persists in a "*Caucasian*" infant is more likely to be under the influence of nitric oxide (than under the influence of prostaglandins) and therefore less likely to respond to indomethacin (5-8).

In addition to *NOS3* and *SLCO2A1*, we found several genes that were associated with "*Non-Caucasian origin*" in a *MODERATELY Consistent* manner (Table IV). These genes are involved with the contractile apparatus and with tissue remodeling. They include *CNN1*/ Calponin and smooth muscle myosin heavy chain isomers (*SM1* and *SM2*) (both of these genes are associated with advanced differentiation of the contractile apparatus (28)). We also observed changes in the expression of *SMARCA4*/BRG1 (Brahma-related gene 1), which plays a role in smooth muscle development and differentiation (29), and in *EPAS1*/ HIF2alpha and *PDGFB* /PDGF-B chain, which have important roles in neointimal formation, anatomic remodeling, and permanent closure of the DA (30). We speculate that increased PGE2 concentrations in utero (due to decreased *SLCO2A1* expression) may also contribute to changes in these genes, because recent studies indicate that PGE2 may regulate genes capable of modulating the contractile apparatus (31, 32).

None of the pathways that are usually involved with oxygen-induced constriction of the DA (e.g., calcium signaling, endothelin signaling, or potassium-channel regulation) appeared to be altered by "*Non-Caucasian origin*".

Our study has several limitations. The tissues were from pregnancy terminations, which may have altered the gene expression in the DA before we were able to isolate the tissues. We explored a limited number of candidate genes and may have missed others that might have been detected by genome-wide association studies or pathway-based analyses. There was a relatively low proportion of Caucasian samples in our study populations and substantial variability in the CT results between the tissue sets. This may have limited the ability of our analyses to identify small effects on other genes.

Our findings provide biologic plausibility to the observation that newborns of "*Non-Caucasian origin*" are more likely to close their PDA following indomethacin or ibuprofen than are infants born to Caucasian mothers. We do not have an explanation for the changes

in gene expression that occur within the "*Non-Caucasian*" DA. Nor do we know if the changes in gene expression have a direct effect on DA closure, or if they are merely an indirect effect of other events that are responsible for its closure. We speculate that decreased expression of both *SLCO2A1* and *NOS3* may alter the balance of vasodilators within the "*Non-Caucasian*" DA and make it more likely to close after indomethacin treatment.

Acknowledgments

Abbreviations

BRG1	Brahma-related gene 1
CT	cycle threshold
CT	the difference in cycle threshold between the expression of the housekeeping gene MDH and the gene of interest
DA	ductus arteriosus
EPAS1	endothelial PAS domain protein 1
HIF2alpha	hypoxia inducible factor 2 alpha
MDH	Malate dehydrogenase
PDA	patent ductus arteriosus
PDGFB	platelet derived growth factor B-chain
PGE2	prostaglandin E2
PGT	prostaglandin transporter
SM2/SM1	smooth muscle myosin heavy chain isomers SM2/SM1 ratio
SNP	single nucleotide polymorphism

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Table 1

Studies supporting the choice of candidate genes

Genes/Aliases	References			Genes/Aliases	References	
	Mutations & Inhibitors[A]	Ductus versus Aorta transcription[B]			Mutations & Inhibitors[A]	Ductus versus Aorta transcription[B]
Ca++ signaling				Prostaglandin Signaling		
CACNA1G/ Cav3.1	(33)	(34)		CYP8A1/PTGIS	(35, 36)	(37)
ATP2A3/SERCA	(38, 39)	(40)		PTGER4/EP4	(41, 42)	(34, 37, 40, 43, 44)
CACNA1C/ CaV1.2	(11, 32, 45, 46)			PTGS1/COX1	(47)	(37, 43)
CACNB2/ Cavbeta2	(11, 32, 45, 46)			SLCO2A1/PG transporter	(18)	(48)
RHOB	(38, 49)	(43, 48)		PDE1B	(32, 50)	(34, 44)
ROCK1	(38, 49, 51)	(43)		PDE3B	(32, 50)	(34, 40, 43)
				PTGS2/ COX2	(47)	(37)
K+ channels				PDE4D	(32, 50)	(34, 44)
ABCC9/ SUR2B	(52, 53)	(34, 43, 44)				
KCNA2/ Kv1.2	(45)	(34, 43)		Nitric Oxide Signaling		
KCNB1/ Kv2.1	(45, 54)	(34, 40, 44)				
KCNS3/ Kv9.3	(45)	(34)		NOS3/eNOS	(20)	(40)
KCNJ8/ Kir6.1	(32, 52, 53)	(34, 44, 55)		PDE5A	(32, 50)	(34, 40, 43)
KCNA5/ Kv1.5	(32, 45, 54)	(34, 43)				
Contractile proteins				Inflammation and Remodeling		
ACTA2	(56)	(34, 43, 44, 48)		AGTR2	(48, 57, 58)	(43, 44, 55)
CNN1/ Calponin	(28)	(44)		EPAS1/ HIF2 alpha	(59)	(37, 40, 44, 48, 55)
MYH11/ SM1	(60, 61)	(34, 43, 44)		SMARCA4/BRG1	(29)	(34, 43)
MYH11/ SM2	(60, 61)			TGFB1/ TGF beta1	(62-64)	(34, 37, 40, 44)
MYLK	(65)	(40, 44, 48)		AGTR1	(48, 57, 58)	(43, 44)
MYOCD/ Myocardin	(66)	(37, 40)		BMP4	(67)	(34, 37, 43)
TPM1/Tropomyosin	(28)	(40, 44, 48, 55)		IGF1	(68)	(43, 44, 48)
				ILK	(69)	(43)
Endothelin signaling				JAG1	(70)	(43, 44)
EDNRA/ EtA- receptor	(71, 72)	(34, 40, 44, 48)		MAPK1/ ERK2	(73)	(43)
EDNRB/ EtB- receptor	(72, 74)	(34, 40, 44)		PDGFB/ PDGF-B chain	(75)	

Genes/Aliases	References			Genes/Aliases	References	
	Mutations & Inhibitors[A]	Ductus versus Aorta transcription[B]			Mutations & Inhibitors[A]	Ductus versus Aorta transcription[B]
ECE1	(72, 74)	(34)		TFAP2B/ TFAP2 beta	(66, 67, 76)	(34, 37, 43, 44)
				FN1	(35, 77)	(48)
				PTPN11	(78)	(44)
				TRAF1	(35, 36)	

[A]Studies demonstrating that a mutation/polymorphism in the gene is associated with a patent ductus arteriosus or that pharmacologic inhibition of the gene's product alters ductus arteriosus contractility

[B]Studies demonstrating that the gene's mRNA expression in the ductus arteriosus differs from its expression in the aorta

Table 2

Regression models examining the effects of gestational age on the mRNA expression of genes involved with ductus closure in three separate tissue sets of second trimester human ductus.

Genes	Aliases	Regression Coefficients [§][*]		
		Sample set #1 (n=93)	Sample set #2 (n=89)	Sample set #3 (n=91)
HIGHLY Consistent association between RNA expression and Gestational Age:				
ABCC9	SUR2B	0.074	0.050	0.075
ACTA2	ACTA2	0.073	0.071	0.064
AGTR2	AT2R2	0.142	0.094	0.078
CACNA1G	Cav3.1	−0.105	−0.144	−0.165
CNN1	Calponin	0.142	0.097	0.124
CYP8A1	PTGIS	0.049	0.028	0.046
EDNRA	EtA-receptor	−0.032	−0.084	−0.083
EDNRB	EtB-receptor	−0.036	−0.111	−0.093
EPAS1	HIF2 alpha	0.064	0.044	0.068
MYH11	SM1	0.146	0.099	0.076
MYH11	SM2	0.137	0.148	0.162
MYLK	MYLK	0.082	0.050	0.056
NOS3	eNOS	0.089	0.057	0.070
PTGER4	EP4	0.086	0.049	0.085
PTGS1	COX1	0.101	0.120	0.044
SLCO2A1	PG transporter	0.055	0.050	0.094
SMARCA4	BRG1	−0.028	−0.038	−0.031
TGFB1	TGF beta1	0.080	0.044	0.038
MODERATELY Consistent association between RNA expression and Gestational Age:				
AGTR1	AT2R1	-	−0.233	−0.178
ATP2A3	SERCA	-	−0.053	−0.063
BMP4	BMP4	-	−0.073	−0.086
CACNA1C	CaV1.2	-	−0.064	−0.104
CACNB2	Cavbeta2	-	−0.080	−0.087
ECE1	ECE1	0.034	-	0.035
IGF1	IGF1	-	−0.335	−0.310
ILK	ILK	0.090	-	0.086
JAG1	JAG1	-	−0.073	−0.052
KCNA2	Kv1.2	-	−0.355	−0.264
KCNB1	Kv2.1	-	−0.147	−0.117
KCNS3	Kv9.3	-	−0.187	−0.327

Genes	Aliases	Regression Coefficients § *		
		Sample set #1 (n=93)	Sample set #2 (n=89)	Sample set #3 (n=91)
MAPK1	ERK2	0.044	-	0.030
MYH11-ratio	SM2/SM1 ratio	-	0.048	0.086
MYOCD	Myocardin	−0.056	-	−0.062
PDE1B	PDE1B	-	−0.070	−0.091
PDE3B	PDE3B	0.044	0.059	-
PDGFB	PDGF-B chain	-	−0.093	−0.054
PTGS2	COX2	0.070	0.049	-
RHOB	RhoB	0.046	0.058	-
TFAP2B	TFAP2 beta	0.042	-	0.093
TPM1	Tropomyosin	-	0.056	0.047
LESS Consistent association between RNA expression and Gestational Age:				
FN1	FN1	-	0.049	-
KCNJ8	Kir6.1	-	-	0.030
PDE4D	PDE4D	-	-	0.067
NO Consistent association between RNA expression and Gestational Age:				
KCNA5	Kv1.5	-	-	-
PDE5A	PDE5A	-	-	-
PTPN11	PTPN11	-	-	-
ROCK1	ROCK1	-	-	-
TRAF1	TRAF1	-	-	-

§Regression coefficients are derived from univariate linear regression models. Positive regression coefficients represent the increase in a gene's CT for every increased week of gestation; negative regression coefficients represent the decrease in a gene's DCT for every increased week of gestation.

*Regression coefficients are listed in the table if the association between RNA expression and Gestation is significant ($p < 0.05$)

Table 3

Effects of advancing gestation on the Real Time polymerase chain reaction (PCR) measurements of genes involved with ductus arteriosus closure in three separate tissue sets of second trimester human ductus arteriosus.

Genes/Aliases [§]	CT [¶] Sample set #1 (n=93)			CT Sample set #2 (n=89)			CT Sample set #3 (n=91)		
	13-16 wks (n=16)	17-20 wks (n=39)	21-24 wks (n=38)	13-16 wks (n=19)	17-20 wks (n=37)	21-24 wks (n=33)	13-16 wks (n=15)	17-20 wks (n=35)	21-24 wks (n=41)
ABCC9/SUR2B	-2.70 ±1.08	-2.07 ±0.99	-2.23 ±0.80	-0.78 ±0.56	-0.66 ±0.34	-0.59 ±0.38	-0.37 ±0.97	0.07 ±0.49	0.18 ±0.33
ACTA2	3.78 ±0.54	4.31 ±0.42	4.41 ±0.57	4.02 ±0.59	4.34 ±0.40	4.51 ±0.45	4.02 ±0.32	4.20 ±0.46	4.50 ±0.37
AGTR2/AT2R2	-3.16 ±0.78	-2.22 ±0.98	-2.09 ±1.44	-2.07 ±0.91	-1.40 ±0.83	-1.29 ±0.87	-1.96 ±1.38	-0.83 ±0.68	-0.99 ±0.77
CACNA1G/Cav3.1	-2.39 ±0.45	-2.72 ±0.54	-3.30 ±0.53	-3.06 ±0.49	-3.24 ±0.49	-4.11 ±0.69	-1.88 ±0.49	-2.67 ±0.61	-3.2 ±0.55
CNN1/Calponin	0.93 ±1.07	1.88 ±0.57	2.11 ±0.60	1.27 ±0.58	1.63 ±0.42	1.94 ±0.43	0.96 ±0.74	1.48 ±0.51	1.93 ±0.42
CYP8A1/PTGIS	-0.49 ±0.60	0.06 ±0.48	-0.07 ±0.45	0.56 ±0.51	0.71 ±0.34	0.80 ±0.26	0.18 ±0.48	0.40 ±0.35	0.54 ±0.27
EDNRA/EtA-receptor	-1.84 ±0.55	-1.71 ±0.43	-2.13 ±0.49	-0.69 ±0.49	-0.66 ±0.36	-1.25 ±0.47	-1.46 ±0.37	-1.71 ±0.42	-2.08 ±0.37
EDNRB/EtB-receptor	-4.89 ±0.63	-4.93 ±0.48	-5.20 ±0.71	-4.83 ±1.05	-4.97 ±0.77	-5.53 ±0.80	-3.64 ±0.64	-3.78 ±0.49	-4.18 ±0.56
EPAS1/HIF2alpha	-2.29 ±0.38	-1.87 ±0.35	-1.76 ±0.26	-0.97 ±0.41	-0.85 ±0.32	-0.66 ±0.30	-1.19 ±0.39	-0.90 ±0.31	-0.68 ±0.30
MYH11/SM1	0.23 ±1.80	0.94 ±1.30	0.95 ±1.29	4.42 ±0.70	4.77 ±0.68	5.12 ±0.54	1.76 ±0.81	1.88 ±0.85	2.22 ±0.91
MYH11/SM2	-0.76 ±0.95	-0.01 ±0.49	0.23 ±0.70	0.02 ±0.82	0.63 ±0.68	1.11 ±0.38	0.68 ±1.34	1.63 ±0.65	2.01 ±0.61
MYLK	-2.77 ±0.67	-2.16 ±0.48	-2.14 ±0.56	-1.49 ±0.51	-1.15 ±0.47	-1.10 ±0.41	-0.84 ±0.39	-0.67 ±0.37	-0.43 ±0.33
NOS3/eNOS	-5.36 ±0.69	-4.87 ±0.96	-4.78 ±0.86	-4.01 ±0.75	-3.70 ±0.68	-3.55 ±0.56	-2.99 ±0.65	-2.52 ±0.54	-2.37 ±0.60
PTGER4/EP4	-1.44 ±0.90	-0.76 ±0.84	-0.80 ±0.84	2.29 ±0.46	2.44 ±0.57	2.61 ±0.43	1.80 ±0.83	2.28 ±0.43	2.47 ±0.43

Genes/Aliases [§]	CT [¶] Sample set #1 (n=93)			CT Sample set #2 (n=89)			CT Sample set #3 (n=91)		
	13-16 wks (n=16)	17-20 wks (n=39)	21-24 wks (n=38)	13-16 wks (n=19)	17-20 wks (n=37)	21-24 wks (n=33)	13-16 wks (n=15)	17-20 wks (n=35)	21-24 wks (n=41)
PTGS1/COX1	−5.80 ±0.61	−5.12 ±0.71	−5.03 ±0.75	−6.76 ±0.76	−6.13 ±0.66	−5.84 ±0.62	−3.90 ±0.44	−3.57 ±0.40	−3.52 ±0.36
SLCO2A1/PG transporter	−4.80 ±0.60	−4.40 ±0.55	−4.45 ±0.84	−4.00 ±0.55	−3.85 ±0.65	−3.66 ±0.55	−4.35 ±1.26	−3.64 ±0.58	−3.55 ±0.64
SMARCA4/BRG1	−1.89 ±0.44	−1.86 ±0.35	−2.13 ±0.34	−2.00 ±0.47	−2.10 ±0.32	−2.27 ±0.31	−1.81 ±0.33	−1.92 ±0.23	−2.08 ±0.22
TGFB1	−8.67 ±1.12	−8.25 ±0.88	−8.22 ±0.71	−5.42 ±0.78	−4.91 ±0.54	−5.02 ±0.49	−3.51 ±0.50	−3.45 ±0.42	−3.33 ±0.30

[§] The genes displayed in the Table have a *Highly Consistent* association between RNA expression and Gestation (i.e., the RNA expression is significantly (p<0.05) associated with advancing gestation in each of the 3 separate tissue sets).

[¶] CT 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 CT represents a 2-fold change in a gene's mRNA. The more negative the CT, the fewer the number of starting copies of a gene (mRNA).

Table 4

Multivariable regression models examining the independent effects of "Non-Caucasian origin" on the mRNA expression of genes involved with ductus closure in three separate tissue sets of second trimester human ductus.

Genes	Aliases	Regression Coefficients [‖]*		
		Sample set #1 (n=93) (Non-Caucasian=82%)	Sample set #2 (n=89) (Non-Caucasian=76%)	Sample set #3 (n=91) (Non-Caucasian=67%)
HIGHLY Consistent association between RNA expression and Non-Caucasian Race:				
NOS3	eNOS	−0.322	−0.337	−0.303
SLCO2A1	PG transporter	−0.376	−0.369	−0.522
MODERATELY Consistent association between RNA expression and Non-Caucasian Race:				
CNN1	Calponin	-	0.166	0.197
EPAS1	HIF2 alpha	-	−0.236	−0.183
MYH11-ratio	SM2/SM1 ratio	0.548	0.363	-
PDGFB	PDGF-B chain	−0.032	−0.402	-
SMARCA4	BRG1	-	−0.244	−0.111
LESS Consistent association between RNA expression and Non-Caucasian Race:				
ABCC9	SUR2B	-	−0.202	-
AGTR1	AT2R1	-	−0.976	-
ATP2A3	SERCA	-	−0.277	-
CACNB2	Cavbeta2	-	−0.187	-
EDNRB	EtB-receptor	-	−0.856	-
FN1	FN1	-	0.243	-
IGF1	IGF1	-	−0.843	-
KCNA2	Kv1.2	-	−0.981	-
KCNA5	Kv1.5	-	-	−0.268
MAPK1	ERK2	-	-	−0.202
MYH11	SM1	−0.678	-	-
MYH11	SM2	-	0.251	-
MYLK	MYLK	-	-	−0.245
PTGS1	COX1	-	-	−0.221
PTGS2	COX2	0.491	-	-
PTGER4	EP4	-	0.198	-
RHOB	RhoB	0.310	-	-
TFAP2B	TFAP2 beta	-	0.221	-
TGFB1	TGF beta1	-	-	−0.220

Genes	Aliases	Regression Coefficients [//][*]		
		Sample set #1 (n=93) (Non-Caucasian=82%)	Sample set #2 (n=89) (Non-Caucasian=76%)	Sample set #3 (n=91) (Non-Caucasian=67%)
TPM1	Tropomyosin	-	-	−0.222
NO Consistent association between RNA expression and Non-Caucasian Race:				
ACTA2	ACTA2	-	-	-
AGTR2	AT2R2	-	-	-
BMP4	BMP4	-	-	-
CACNA1C	CaV1.2	-	-	-
CACNA1G	Cav3.1	-	-	-
CYP8A1	PTGIS	-	-	-
ECE1	ECE1	-	-	-
EDNRA	EtA-receptor	-	-	-
ILK	ILK	-	-	-
JAG1	JAG1	-	-	-
KCNB1	Kv2.1	-	-	-
KCNJ8	Kir6.1	-	-	-
KCNS3	Kv9.3	-	-	-
MYOCD	Myocardin	-	-	-
PDE1B	PDE1B	-	-	-
PDE3B	PDE3B	-	-	-
PDE4D	PDE4D	-	-	-
PDE5A	PDE5A	-	-	-
PTPN11	PTPN11	-	-	-
ROCK1	ROCK1	-	-	-
TRAF1	TRAF1	-	-	-

[//]Regression coefficients were derived from multivariable linear regression models adjusted for gestational age. A positive regression coefficient represents the increase in a gene's CT when ductus from "Non-Caucasian origin" fetuses were compared with those from Caucasian fetuses; a negative regression coefficient represents the decrease in a gene's CT when ductus from "Non-Caucasian origin" fetuses were compared with those from Caucasian fetuses.

[*] Regression coefficients are listed in the table if the association between RNA expression and "Non-Caucasian origin" is significant (p<0.05).

Table 5

Effects of Non-Caucasian race on the Real Time polymerase chain reaction (PCR) measurements of NOS3 and SLCO2A1 genes in three separate tissue sets of second trimester human ductus arteriosus. (n) The number of "Caucasian" and "Non-Caucasian" tissues examined at each gestation.

Genes/Aliases §	Sample set #1 (n=93)			Sample set #2 (n=89)			Sample set #3 (n=91)		
	13-16 wks	17-20 wks	21-24 wks	13-16 wks	17-20 wks	21-24 wks	13-16 wks	17-20 wks	21-24 wks
Caucasian (n) **Non-Caucasian (n)**	(5) (11)	(6) (33)	(6) (32)	(5) (14)	(6) (31)	(11) (22)	(5) (10)	(9) (26)	(16) (25)
NOS3/eNOS									
Caucasian - CT ¶	-5.13 ±0.68	-4.37 ±0.92	-4.50 ±0.63	-3.17 ±0.56	-3.49 ±0.73	-3.16 ±0.47	-2.75 ±0.30	-2.08 ±0.34	-1.92 ±0.45
Non-Caucasian - CT ¶	-5.46 ±0.75	-5.18 ±0.94	-4.96 ±1.07	-4.20 ±0.73	-3.81 ±0.62	-3.88 ±0.58	-3.13 ±0.72	-2.74 ±0.38	-2.50 ±0.61
- CT §	-0.33	-0.81	-0.46	-1.03	-0.32	-0.72	-0.38	-0.66	-0.58
SLCO2A1/PG transporter									
Caucasian - CT ¶	-4.45 ±0.45	-3.94 ±0.58	-3.75 ±0.77	-3.45 ±0.03	-3.36 ±0.78	-3.25 ±0.48	-3.35 ±0.43	-2.80 ±0.44	-2.91 ±0.69
Non-Caucasian - CT ¶	-4.96 ±0.68	-4.58 ±0.50	-4.70 ±0.83	-4.24 ±0.64	-3.99 ±0.60	-3.91 ±0.50	-4.70 ±0.90	-3.90 ±0.57	-3.90 ±0.58
-DCT §	-0.51	-0.64	-0.95	-0.79	-0.63	-0.66	-1.35	-1.10	-0.99

¶ CT 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 CT represents a 2-fold change in a gene's mRNA. The more negative the CT, the fewer the number of starting copies of a gene (mRNA).

§ - CT represents the difference in CT between Caucasian and Non-Caucasian infants. In each of the 3 sample sets, and at each of the gestational epochs, "Non-Caucasian" infants had fewer copies of NOS3 and SLCO2A1 mRNA than "Caucasian" infants.