Identification of differentially regulated genes in human patent ductus arteriosus

Pratik Parikh¹, Haiqing Bai¹, Michael F Swartz², George M Alfieris^{1,2} and David A Dean^{1,3}

¹Division of Neonatology, Department of Pediatrics, University of Rochester Medical Center, Rochester, NY 14642, USA; ²Department of Surgery, University of Rochester Medical Center, Rochester, NY 14642, USA; ³Department of Biomedical Engineering, University of Rochester Medical Center, Rochester, NY 14642, USA

Corresponding author: David A Dean. Email: david_dean@urmc.rochester.edu

Abstract

In order to identify differentially expressed genes that are specific to the ductus arteriosus, 18 candidate genes were evaluated in matched ductus arteriosus and aortic samples from infants with coarctation of the aorta. The cell specificity of the gene's promoters was assessed by performing transient transfection studies in primary cells derived from several patients. Segments of ductus arteriosus and aorta were isolated from infants requiring repair for coarctation of the aorta and used for mRNA quantitation and culturing of cells. Differences in expression were determined by quantitative PCR using the $\Delta\Delta C_t$ method. Promoter regions of six of these genes were cloned into luciferase reporter plasmids for transient transfection studies in matched human ductus arteriosus and aorta cells. Transcription factor AP-2b and phospholipase A2 were significantly up-regulated in ductus arteriosus compared to aorta in whole tissues and cultured cells, respectively. In transient transfection experiments, Angiotensin II type 1 receptor and Prostaglandin E receptor 4 promoters consistently gave higher expression in matched ductus arteriosus versus aorta cells from multiple patients. Taken together, these results demonstrate that several genes are differentially expressed in ductus arteriosus and that their promoters may be used to drive ductus arteriosus-enriched transgene expression.

Keywords: Neonatology, cardiovascular, gene, heart, gene therapy, pediatric

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Introduction

The ductus arteriosus (DA) represents a persistence of the terminal portion of the left sixth branchial arch.¹ The major function of the ductus arteriosus during fetal life is to redirect the fetal circulation from the placenta directly to the aorta, bypassing the lungs. During the transition from intra to extra uterine life, the DA undergoes functional and structural changes leading to ductal closure in the neonatal period. In full-term infants, functional closure of the ductus results from vasoconstriction in response to exposure to oxygen, regulatory hormones, and cell signaling mediators.¹ Anatomical closure follows by smooth muscle migration and endothelial proliferation to form ligamentum arteriosum within the first few weeks of life.¹

In the preterm infant, the ductus arteriosus frequently fails to constrict.² This may be due to many factors including decreased tone due to presence of immature smooth muscle myosin isoforms and impairment of calcium entry through L-type calcium channels, increased sensitivity to prostaglandin E₂ (PGE₂) and nitric oxide, or increased levels of PGE₂.^{3–5} The clinical consequences of a patent ductus

arteriosus (PDA) are related to the degree of left-to-right shunt through the PDA with its associated change in blood flow to the lungs, kidneys, and intestines.² Preterm infants of <30 weeks gestation with severe respiratory distress have a 65% incidence of persistent ductus patency beyond the fourth day of life.⁶

The standard of care for infants with PDA is administration of a non-selective prostaglandin inhibitor such as Indomethacin. While successful in the majority of instances, a drawback of such prostaglandin inhibitors is generalized vasoconstriction that can affect cerebral, mesenteric, and renal perfusion, thereby aggravating the insult caused by the PDA. When patients do not respond to prostaglandin inhibitors, the remaining option is surgical PDA ligation which has been associated with its own set of morbidities such as thoracotomy, pneumothorax, chylothorax, scoliosis, and infection. One alternative to pharmacological or surgical intervention could be the application of gene therapy to promote constriction and closure of the PDA. Our laboratory has a long-standing interest in developing general and cell-specific gene delivery strategies for the vasculature, and the potential for application of these approaches to develop

a treatment for PDA is intriguing. However, any approach to preferentially constrict and close the DA would necessitate a high degree of DA specificity, either in gene delivery or transgene expression, so that the surrounding vasculature, namely the aorta, would not be subject to the same fate. As such, it is necessary to identify a set of genes whose expression is enriched in the DA compared to the aorta to enable any type of gene therapy to be developed.

To date, many studies have investigated pathophysiological aspects and pharmacological modulation of DA.⁷ Interestingly, the smooth muscle cells of ductus are derived entirely from cardiac neural crest cells, whereas smooth muscle cells of other vascular beds are mostly mesenchymal in origin.^{8,9} While the aorta also is primarily derived from mesenchymal cells, some cells in the ascending aorta region also include a neural crest origin.^{10,11} This may account for some of the DA's unique properties, including proliferation, intimal cushion formation, and migration, all of which may contribute to constriction and be the result of expression of distinct subsets of genes involved in the developmental vascular remodeling that occurs during gestation.^{8,12} Studies also have looked at the genetic expression of DA during development and compared the genetic expression to that in surrounding vessels such as aorta in animals.^{13–16} Early studies used pooled whole vessel preparations of DA and aorta in microarray analyses. A more recent study used laser capture microdissection to isolate smooth muscle from frozen sections and analyzed the profile in late fetal (day 18) and near term (day 21) rats.¹⁶ Similar studies in the human neonatal population are valuable in understanding the mechanisms of the failure of DA constriction and could be used as the basis for the development of new strategies to treat PDA.

In this study, we examined differentially expressed genes identified previously in animal models of patent ductus arteriosus directly in human samples with the hypotheses that there is differential gene expression between human ductus and aorta similar to that found in animals and that expression of these genes is developmentally regulated.

Results

Differential expression of genes in human ductus vs. aorta

To identify a set of genes that was differentially regulated in human DA compared to surrounding vascular smooth muscle (e.g. aorta), the expression of 18 genes shown to be DA-specific in the rat was assessed.¹³ For this analysis, tissue from 11 matched human DA and aorta samples, isolated from patients undergoing surgery for coarctation of the aorta, was used. Aortic tissue was from the aortic arch (transverse), distal to the ductus arteriosus insertion. Patient age ranged from 6 days of life to 1 year of life (Table 1), including three premature infants. Of these patients, four were on prostaglandins when the tissues were collected. Finally, the ductus was still patent in five of the patients, among whom three were on prostaglandin infusion therapy.

Table 1 Human tissue samples

Samples	Tissue Type	Gender	Age	Premie	PGE	PDA
1	Cells	F	6 days	N	Y	Y
2	Cells	M	21 days	Y	N	N
3	Cells	M	2 months	N	N	N
4	Cells	M	6 months	N	N	N
5	Cells	F	8 months	N	N	Y
6	RNA	M	6 days	N	N	Y
7	RNA	M	7 days	N	Y	N
8	RNA	F	25 days	Y	Y	Y
9	RNA	F	31 days	Y	N	N
10	RNA	M	32 days	N	Y	Y
11	RNA	M	1 year	N	N	N

Note: The aorta and the DA from each patient were dissected within 1 h of removal from the patient and used for either cell culture (and mRNA isolation at cell passage 3) or direct mRNA isolation from the fresh tissue. The post-gestational age of the patients at the time of surgery, whether the infants were delivered prematurely, on prostaglandin infusion, and whether the DA was patent at the time of surgery are also indicated.

Following surgery, the tissues were dissected to separate DA from aorta and then either immediately frozen as whole tissues for subsequent RNA isolation ($n = 6$) or dissected to isolate and establish smooth muscle cell cultures from the fresh tissue ($n = 5$). Gene expression was determined using quantitative PCR and values normalized to GAPDH as an internal housekeeping gene. All comparisons were between matched pairs of tissues (from the same patients). The results were calculated as fold difference of DA compared to aorta (Tables 2 and 3). There was consistency in upregulation of gene expression in the earlier part of infancy and as the age advanced, gene expression became variable (Tables 2 and 3). This may be attributed to the change in the gene expression occurring during ductal transition.

To determine whether expression levels were different in samples in which the RNA was isolated directly from the tissue compared to those in which RNA was isolated from cells cultured for 3–4 passages, sample groups were divided accordingly. Only transcription factor AP2b was significantly expressed in the ductus compared to the aorta in the whole tissue sample group, whereas only phospholipase A2 gene was significantly expressed in ductus compared to aorta in the cultured cells.

Differential expression of DA-specific promoters

To test whether the promoters for these differentially expressed genes could drive similar ductus-enriched expression themselves, we carried out a series of transient transfections. Approximately 2.5 kb of each of six promoters were amplified from human genomic DNA and cloned into luciferase reporter plasmids. Plasmids were then transfected into cultured smooth muscle cells isolated from ductus or aorta of the same patients as used for qPCR analysis. Matched ductus and aorta smooth muscle were cultured from three different patients (Figure 1). The transfections were done on cells cultured from a 6-day infant with a patent ductus (Figure 1(a)), a 2-month-old

Table 2 Ratio of expression of candidate genes in ductus arteriosus compared to aortic smooth muscle in whole tissues isolated from each patient as a function of age

Gene	Post-gestational age						P
	6 days	7 days	25 days	31 days	32 days	1 year	
FHL2	7.654	3.529	3.151	0.496	0.890	1.055	0.156
AGTR1	13.300	3.296	7.249	0.385	0.983	0.639	0.218
CPA3	11.840	2.770	5.736	0.461	0.695	0.887	0.218
CREM	1.140	4.902	1.698	0.352	1.357	2.960	0.109
DES		4.806	4.587	0.315	0.748	18.800	0.218
DLX1	76.030	9.337	9.029	0.160	0.885	0.170	0.218
GAL		9.540	3.731	0.202	1.006	0.062	0.500
GATA2	3.060	5.847	4.513	0.308	1.279	0.883	0.312
IL15	0.669	1.483	1.763	0.384	0.617	2.920	0.500
PTGER4	18.990	4.332	4.498	0.461	0.732	1.200	0.156
PCSK5	4.120	7.092	0.402	0.357	1.968	0.133	0.500
PLA2		0.497	8.347	0.587	0.161	0.254	0.500
RGS5		3.535	6.392	0.512	0.868	0.569	0.218
RHOB		8.395	1.231	0.573	1.077	2.750	0.156
SELP	3.820	3.951	5.959	0.489	0.880	0.827	0.218
TNN	0.800	3.025	3.075	0.109	0.705	1.840	0.422
TFAP2B	26.190	1.371	5.645	1.024	0.969	4.260	0.047
TNFRSF11B	1.283	3.909	6.650	0.342	0.978	0.742	0.344

Note: Quantitative PCR was used to determine levels of gene expression in dissected aortic or ductus arteriosus smooth muscle tissue. Most candidate gene expression is elevated in each patient until approximately 1 month of age, after which time expression becomes more variable. The fold difference between gene expression (determined by the ΔCt method) in ductus compared to aorta was determined. Statistical significance was determined by Wilcoxon signed ranked test.

Table 3 Ratio of expression of candidate genes in ductus arteriosus cells compared to aortic smooth muscle cells cultured from each patient as a function of age

Gene	Post-gestational age					P
	6 days	21 days	60 days	6 months	8 months	
FHL2	1.401	0.200	0.282	1.190	0.284	0.156
AGTR1	2.787	2.741	5.655	0.200	2.670	0.219
CPA3	8.107	9.440	1.621	10.150	3.620	0.062
CREM	2.233	4.210	0.481	0.550	0.443	0.406
DES	2.759	0.220	1.023	1.970	169.000	0.219
DLX1	2.195	2.770	13.767	0.983	2.950	0.062
GAL	0.796	3.280	1.298	0.220	0.233	0.312
GATA2	2.614	8.610	2.515	1.130	4.040	0.062
IL15	1.403		0.215	11.990	1.770	0.188
PTGER4	1.370	5.630	1.928	0.930	0.836	0.500
PCSK5	4.147	0.450	0.651	0.350	2.330	0.500
PLA2	1.238	0.870	1.113	2.420	1.580	0.031
RGS5	2.429	0.830	3.564	9.520	0.569	0.500
RHOB	0.874	1.360	0.711	1.150	2.750	0.406
SELP	3.283	2.260	0.155	1.990	0.827	0.312
TNN	2.095	0.540	0.985	3.540	1.840	0.500
TFAP2B	2.638	16.600	3.096	1.290	4.260	0.094
TNFRSF11B	0.403	1.340	0.168	3.450	0.742	0.218

Note: Quantitative PCR was used to determine levels of gene expression in cultured cells at matched passage number. Most candidate gene expression is elevated in each patient until approximately 1 month of age, after which time expression becomes more variable. The fold difference between gene expression (determined by the ΔCt method) in ductus compared to aorta was determined. Statistical significance was determined by Wilcoxon signed ranked test.

Figure 1 Ability of promoters to drive DA-specific transgene expression in transiently transfected primary human ductus and aorta-derived smooth muscle cells. Luciferase-expressing plasmids driven by the indicated promoters were transfected into matched primary human DA and aorta smooth muscle cells at passage 4 using Lipofectamine 2000 and assayed for gene expression 48 h later. CMV and SV40 promoter-driven plasmids were used as positive controls for transfection efficiency. All expression was normalized to total cellular protein and expressed as relative to expression of the CMV constructs in ductus cells. All transfections were carried out in triplicate using cells isolated from (a) 6-day-old, (b) 2-month-old, and (c) 8-month-old patients. * $P \leq 0.05$ for expression in DA compared to aorta

patient with muscular ductus and closed lumen (Figure 1(b)), and an 8-month old with a patent ductus (Figure 1(c)). When the cultured cells were stained for a fibroblast marker (FSP1), no positive staining was observed, but all of the cultured cells stained positive for smooth muscle alpha actin (not shown). For comparison and determination of smooth muscle specificity, plasmids were also

transfected into the A549 pulmonary adenocarcinoma epithelial cell line. Transfection of two control plasmids driving gene expression from the ubiquitously active CMV immediate early promoter/enhancer or the SV40 early promoter gave robust gene expression in all cells tested. When reporter constructs for *CPA3*, *CREM*, *GATA2*, and *TNFRSF11b* promoters were transfected into matched aorta or ductus smooth muscle cells from two different patients, no gene expression was detected (Figure 1(a) and (b)). However, when transfected into matched cells from a third patient, all of these promoters except *CPA3* drove gene expression in cells from the ductus, but not those from aorta (Figure 1(c)). By contrast, when plasmids driving luciferase expression from the *AGTR1* or *PTGER4* promoters were transfected into cells from all three patients, both plasmids expressed gene product (Figure 1). Moreover, both promoters showed between 1.1- and 11-fold greater expression in smooth muscle cells from the ductus compared to those from the aorta.

Discussion

To our knowledge, this is the first study to look at the differential expression of candidate genes in human ductus arteriosus compared to surrounding vascular tissue, during the first year of life. Building on a study in rats, we looked at the expression of 18 genes in human samples that had been shown to be upregulated in the DA of this species.¹⁵ In the current study, we saw consistently higher differential expression of several candidate genes in ductus arteriosus *vs.* aorta in the neonatal period, but as the age of the patients increased, expression of all of the genes became more variable. When expression patterns from whole tissue were analyzed, we found that transcription factor AP 2b (*TFAP2B*) was significantly higher in DA compared to aorta from the same patients. By contrast, when expression was measured in smooth muscle cells that were isolated and cultured from tissues, phospholipase A2 (*PLA2*) was significantly up in DA versus aorta. When promoters for several of these genes were cloned and transiently transfected into the cultured DA or aorta smooth muscle cells from three different patients, several promoters drove consistent DA-enriched gene expression. These promoters included the *AGTR1* and the *PTGER4*, as well as the *TNFRSF11b*, *CREM*, and *GATA2* promoters. Taken together, these studies identify several genes that are differentially expressed in the DA versus aorta of human subjects.

Previous animal studies assessed gene expression in the ductus arteriosus during development and compared this expression to surrounding vessels including the aorta.^{13,15-19} For example, Costa et al. examined the effects of oxygen and birth on ductus gene expression at E19 and at 3 h of life in mice using microarrays and found a number of genes upregulated in DA, including angiotensin II receptor type 1a.¹⁵ In another study, Jin et al. compared gene expression profiles of the ductus and aorta E19 and E21 in rats.¹³ Their results revealed that a number of genes were upregulated in ductus, but a significant limitation of all of these studies was that pooled whole vessel preparations of ductus and aorta (containing smooth muscle, endothelial,

and adventitial cells) were used for the microarray analysis. A recent study that overcomes this has used laser capture microdissection to isolate DA or aorta smooth muscle from frozen sections and analyzed the profile from late fetal (day 18) and near term (day 21) rats.¹⁶ Of the top 16 most DA-specific genes in this study, only *DLX1* was also identified in the top 18 hits from the Jin study.¹³ By contrast to animal studies, to date, only one microarray study analyzing the human ductus has been described,²⁰ although a number of studies have evaluated the differential expression of individual genes in human DA versus the surrounding vasculature (reviewed in Hajj et al.⁶). In the microarray study by Mueller et al., a comparative analysis of ductus and pulmonary artery was performed using vessels obtained from patients ranging from 1 to 807 days, and included vessels that were stented for different indications.²⁰ However, not all of the ductus vessels that were removed and analyzed were open, limiting direct comparisons to the present data.

Most of the candidate genes chosen for our study based on the rat study by Jin et al.¹³ have been speculated to play an important role in ductal transition and neuro-hormonal mechanism.^{1,7} Several others have a role in cellular growth and vascular remodeling. Several of these genes, including *TFAP2B*, *PLA2*, *PTGER4*, *DLX1*, and *AGTR1*, were found in our study to be either DA-enriched at the mRNA level or drove DA-enriched transgene expression when their promoters were cloned and used in transient transfection studies in cultured DA or aorta SMCs from patients. Transcription factor AP2b is part of the TFAP2 family involved in the transcriptional regulation of many cellular genes required during embryonic development. *TFAP2B* is expressed in the DA precursor and the wall of ductus of middle stage mouse embryos,²¹ suggesting a role in the regulation of genes essential for ductal remodeling and closure at birth.^{22,23} This is consistent with a number of other studies implying that it may play an important role in ductal transition.^{21–24} In addition, in several human gene polymorphism studies, specific *TFAP2B* and *PTGER4* SNPs were associated with PDA in term and preterm infants,^{5,25,26} although another study detected no such association of *TFAP2B* or *AGTR1* SNPs with PDA.²⁷ Another gene, *DLX1*, a transcription factor expressed in the embryonic caudal pharyngeal arch complex, where the ductus develops, has been hypothesized to direct BMP4 expression to cause vascular remodeling in DA during late gestation.¹⁶ Prostaglandin E receptor 4 (*PTGER4*) also has shown higher expression in the DA compared to aorta and pulmonary artery in mice and rats, and studies with conditional knockout mice and cell lines support both a dilatory and developmental function of *PTGER4* on smooth muscle.^{12,13,20} Finally, phospholipase A2 (*PLA2*) which plays an important enzymatic role in prostaglandin formation may be upregulated in concert with the increased prostaglandin activity seen in ductal smooth muscle cells during ductal transition.²⁸

An ultimate goal of our group is to develop gene-based approaches to treat symptomatic PDA that fail medical therapy. The success of any type of gene therapy relies first and foremost on safety of the approach. One approach

to genetically treat PDA would be to deliver genes to the PDA to promote and facilitate vessel constriction and closure. For this approach to succeed, expression of any transferred gene would have to be restricted to the DA smooth muscle as opposed to any surrounding vascular bed. The studies carried out here describe our first step toward this goal and suggest that several candidate promoters could be used to drive such expression. The *ATGR1* and *PTGER4* promoters drove higher reporter gene expression in DA compared to aorta smooth muscle cells in cells from three different patients. While the differences in expression were variable, they were consistently higher in the DA. In contrast, although expression from the *TNFRSF11b*, *GATA2*, and *CREM* promoters were more enriched for ductus expression, they did not express in cells from all patients and their levels of expression were less than 10% that of either the *ATGR1* or *PTGER4* promoters. Unfortunately, multiple attempts to clone the *DLX1* and *TFAP2B* promoters were unsuccessful; based on their DA expression in qPCR studies, we would expect that these promoters may be both strong and restricted to ductus.

A major limitation of our study was the small sample size, due to limited availability of human samples. However, our findings confirm that the human ductus arteriosus has a similar gene expression pattern and changes during ductal transition as seen in multiple animal models and studies. Another limitation is that the human ductus and aorta samples were obtained from patients with coarctation of aorta, which by itself will likely alter gene expression in these tissues. Despite this, our study showed consistently higher expression of almost all candidate genes in ductus compared to aorta, suggesting that the candidate expression was not altered due to the diagnosis of coarctation of aorta.

Methods

Collection of tissue, cell isolation, and cell culture

Human tissue was obtained under the oversight of the Research Subjects Review Board of the University of Rochester, which approved the collection as part of ongoing study entitled “Basis for Re-Coarctation in Patients with Coarctation of the Aorta”. Matched tissue of ductus arteriosus and aorta were obtained from patients undergoing repair of coarctation of the aorta. Aortic tissue was from the aortic arch (transverse), distal to the ductus arteriosus insertion. The patient age ranged from 6 days to 1 year of life (Table 1). The tissue samples were collected as a part of ongoing research project after obtaining signed consent. The tissues collected were either frozen immediately for subsequent RNA isolation or used for cell isolation by placing in culture media. Fresh tissues were dissected within 1 h of collection for smooth muscle isolation and cell culture.

Smooth muscle cells were isolated by enzymatic digestion of DA and aorta samples as previously described.²⁹ Briefly, dissected aorta and DA were digested with type II collagenase and type I elastase (Worthington Biochemical Corp., NJ, USA) in Dulbecco's Modified Eagle's Medium (DMEM) containing 1% ampicillin/streptomycin/

mycofungin at 37°C for 20 min after which adventitious and endothelial cell layers were removed under a dissecting microscope. The freshly isolated smooth muscle tissue was placed in DMEM containing 20% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA, USA), placed into tissue culture dishes coated with 1% gelatin, and incubated in a humidified incubator at 37°C with a 5% CO₂, 95% ambient air mix. Cells obtained by this method were identified as vascular smooth muscle cells by positive immunostaining for alpha-smooth muscle actin (Sigma-Aldrich, St Louis, MO, USA). Cells were used at passages 3–4 for total RNA isolation and transient transfections.

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

Total RNA was isolated according to the manufacturer's instructions from either minced frozen tissue or cultured DA and aorta cells at passage 3, as indicated, using the QIAGEN RNeasy mini kit (QIAGEN; Valencia, CA, USA). First-strand cDNA was synthesized by reverse transcription of total RNA with 15 units of AMV Reverse Transcriptase (Promega, Madison, WI, USA) with random hexanucleotide primers using the protocol supplied by the manufacturer. cDNAs of each matched DA and aorta sample were used for quantitative real time PCR analysis using the SYBR Green qPCR kit as described by the manufacturer (Bio-Rad, Hercules, CA, USA). Reactions were carried out and quantified with the Bio-Rad CFX Connect Real-Time PCR Detection System. Primers amplified a ~100 base-pair region of each of the 18 candidate genes and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Supplementary Table 1). All samples were run in triplicate. Relative gene expression was calculated by using the 2- $\Delta\Delta C_t$ method, in which C_t indicates the fractional cycle number at which the fluorescent signal reaches the detection threshold. The $\Delta\Delta$ method uses the normalized ΔC_t value of each sample calculated by using GAPDH as an endogenous control gene. Values are presented as an average fold change of 2-(average $\Delta\Delta C_t$ between DA and aorta samples) for genes.

Cloning of promoters and plasmid construction

Approximately 2.5 kb of the promoter regions for seven of the differentially regulated genes were identified and cloned by PCR amplification (Supplementary Table 2). Promoters were identified based on the known transcriptional start sites and roughly 2 kb of upstream sequence and 0.5 kb of downstream sequence were amplified. Following PCR amplification, the products were subcloned into a luciferase reporter construct, pGL3basic (Promega), immediately upstream of the luciferase gene. Plasmids were purified using Qiagen maxipreps. All constructs were verified by restriction digestion and DNA sequencing. For transient transfection studies, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions. Briefly, 4 μ g of Lipofectamine 2000 was complexed with 2 μ g of plasmid prior to addition to cells in 24-well dishes in the presence of serum- and antibiotic-free

growth medium. Four hours later, the medium was replaced with DMEM containing 20% FBS and 1% ampicillin-streptomycin-mycofungin and cells were harvested for transgene expression at 24 h. Plasmids pGL3control (Promega; containing the SV40 promoter and enhancer) and pCMV-Lux-DTS were also used as positive controls, and a promoterless pGL3basic was used as a negative control.³⁰ All transfections were carried out in triplicate and reported as mean $\pm$ standard deviation.

Statistical analysis

The C_t values of matched human DA and aorta samples were used for statistical analysis. Wilcoxon signed ranked test was applied to C_t values of all samples of each gene to find the difference between DA and aorta. We also performed subgroup analysis between neonatal vs. infant population, fresh tissue vs. frozen sections, and prostaglandin exposed vs. prostaglandin non-exposed samples. For transient transfection studies, t -tests were performed using Prism Software (GraphPad, San Diego, CA, USA).

Authors' contribution: The authors PP, HB, MS, GA and DD made a substantial contribution to the concept and design, acquisition of data, and analysis and interpretation of data; PP and DD drafted the article and PP, HB, MS, GA, and DD revised it critically for important intellectual content; and PP, HB, MS, GA, and DD approved the version to be published.

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DECLARATION OF CONFLICTING INTERESTS

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