Transcriptome Analysis Reveals Differential Gene Expression between the Closing Ductus Arteriosus and the Patent Ductus Arteriosus in Humans

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Abstract: The ductus arteriosus (DA) immediately starts closing after birth. This dynamic process involves DA-specific properties, including highly differentiated smooth muscle, sparse elastic fibers, and intimal thickening (IT). Although several studies have demonstrated DA-specific gene expressions using animal tissues and human fetuses, the transcriptional profiles of the closing DA and the patent DA remain largely unknown. We performed transcriptome analysis using four human DA samples. The three closing DA samples exhibited typical DA morphology, but the patent DA exhibited aorta-like elastic lamellae and poorly formed IT. A cluster analysis revealed that samples were clearly divided into two major clusters, the closing DA and patent DA clusters, and showed distinct gene expression profiles in IT and the tunica media of the closing DA samples. Cardiac neural crest-related genes such as JAG1 were highly expressed in the tunica media and IT of the closing DA samples compared to the patent DA sample. Abundant protein expressions of jagged 1 and the differentiated smooth muscle marker calponin were observed in the closing DA samples but not in the patent DA sample. Second heart field-related genes such as ISL1 were enriched in the patent DA sample. These data indicate that the patent DA may have different cell lineages compared to the closing DA.

Keywords: ductus arteriosus; neointima; tunica media; congenital heart disease; transcriptome; neural crest

1. Introduction
The ductus arteriosus (DA) is a fetal vascular shunt that connects the pulmonary artery and the aorta, and it is essential for maintaining fetal circulation. The DA begins to close immediately after birth. This closing process is characterized by several DA-specific features including differentiated contractile smooth muscle cells (SMCs), fragmentation of internal elastic laminae, sparse elastic fiber formation in the tunica media, and intimal thickening (IT) formation [1–6]. Histological analyses of human DA samples and several animal models indicated that these DA-specific features gradually develop throughout the fetal and neonatal periods [7]. Patent DA is a condition where the DA does not close properly after birth. Patent DA occurs more frequently in premature neonates [8]. PDA samples exhibit fewer DA-specific structural features [9]. Comprehensive analysis of gene expression comparing the closing DA and the patent DA is necessary in order to better understand DA-specific remodeling and to explore methods to regulate patency of the DA.
Several research groups have previously reported the gene expression profiles of mouse, rat, and ovine DAs [10–17]. However, transcriptome analyses of human DA tissues are limited [18,19]. Yarboro et al. performed RNA sequencing using human DA tissues of 21 weeks gestation and identified genes that were distinctly expressed in the DA tissue compared to the aorta [18]. Additionally, they compared their human RNA sequencing data with previously reported rodent microarray data and demonstrated transcriptional commonalities between human and rodent DAs [18]. A report by Mueller et al. [19] is presently the only published study that demonstrated the differences of transcriptional profiles using postnatal human DAs. They compared gene expression between stent-implanted open DAs on postnatal days 222 and 239, closed ligamentous DAs on day 147, and an open unstented DA on day 1. To the best of our knowledge, transcriptomic comparisons of the closing DA and the patent DA in human infants have not been previously reported.

Using four postnatal human DA tissues, we performed an unbiased transcriptome analysis using the IT and the tunica media of each DA tissue. We found differential gene expression between the tunica media of the closing DA and that of the patent DA. Additionally, we investigated genes enriched in the IT or the tunica media of closing DA tissues to identify genes that potentially contribute to DA-specific remodeling.

2. Materials and Methods

2.1. Study Subjects and Ethics Statements

The protocol for using human DA tissues was approved by the Research Ethics Committees at Tokyo Medical University and Kanagawa Children’s Medical Center (reference numbers: T2020-0238 and 1502-05, respectively). The protocol conformed to the principles outlined in the Declaration of Helsinki. After receiving written informed parental consent, human DA tissues were obtained from four patients with congenital heart diseases, during cardiac surgeries in Kanagawa Children’s Medical Center. The patient information for each of the four cases included in this study is summarized in Table 1.

| Case Number | Diagnosis                                                                 | Gestational Age (Weeks) | Birth Weight (g) | Age at Operation (Days) | Duration of PGE1 Administration (Days) | Closing Tendency of the DA |
|-------------|---------------------------------------------------------------------------|-------------------------|------------------|-------------------------|----------------------------------------|---------------------------|
| 1           | Polysplenia, intermediate AVSD, CoA, TAPVC (cardiac type), PDA, IVC interruption (azygos connection) | 38                      | 2714             | 17                      | 0                                      | No                        |
| 2           | DORV (subpulmonary VSD), hypoplastic distal arch, CoA, PFO                | 41                      | 2948             | 5                       | 5                                      | Yes                       |
| 3           | HLHS, TR, cor triatriatum, PLSVC                                          | 40                      | 3352             | 24                      | 24                                     | Yes                       |
| 4           | HLHS (MA, AA), PLSVC                                                     | 37                      | 2654             | 98                      | 98                                     | Yes                       |

Abbreviations: PGE1, prostaglandin E1; DA, ductus arteriosus; AVSD, atrioventricular septal defect; CoA, coarctation of the aorta; TAPVC, total anomalous pulmonary venous connection; PDA, patent ductus arteriosus; IVC, inferior vena cava; DORV, double outlet right ventricle; VSD, ventricular septal defect; PFO, patent foramen ovale; HLHS, hypoplastic left heart syndrome; TR, tricuspid regurgitation; PLSVC, persistent left superior vena cava; MA, mitral atresia; AA, aortic atresia.

2.2. Total RNA Preparation and Microarray Analysis

Four human DA tissues were subjected to tissue staining and transcriptome analysis. Each DA tissue was divided into two pieces. One piece was fixed with 10% buffered formalin (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) for tissue staining. The other piece of tissue was prepared for microarray analysis as follows. After the
adventitia was removed, each DA tissue was divided into two parts: the inner part and the outer part, which mainly contained IT and the tunica media, respectively, as indicated by the yellow dotted lines in Figure 1A. The tissues were immediately frozen in liquid nitrogen and stored at $-80\,^\circ\text{C}$ until all patient samples were collected. Total RNA preparation and microarray analysis were performed as described previously [10,11]. Briefly, the frozen tissues were disrupted by a multi-bead shoker instrument (Yasui Kikai, Osaka, Japan). After buffer RLT with $\beta$-mercaptoethanol was added to the tissues, they were sonicated to ensure the samples were uniformly homogeneous. Total RNA was isolated using a RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). Microarray experiments were carried out using a SurePrint G3 Human GE 8 × 60 K v2 Microarray (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol.

Figure 1. Histological analysis of the human ductus arteriosus (DA) tissues. (A) Lower magnification images of the Elastica van Gieson stain of the human DA tissues. The yellow dotted lines indicate the border between the intimal thickening (IT) and the tunica media and the border between the tunica media and the adventitia. Scale bars: 200 $\mu$m. (B) Magnified images of red boxes in (A). Scale bars: 100 $\mu$m.

2.3. Generation of a Dendrogram, Venn Diagrams, and a Heatmap

The dendrogram was generated with Ward’s method using the hclust and dendrogram functions in R. The packages gplots and genefilter in R were used to create a heatmap in which data was normalized into a z-score. The mapping grids were subsequently colored according to their z-score. Venn diagrams of the number of differentially expressed genes in each sample group were generated using the gplots package in R.

2.4. Gene Set Enrichment Analyses (GSEAs)

Gene set enrichment analyses (GSEAs) were conducted to investigate the functions of genes that significantly correlated with each sample group. GSEAs ranked the gene list by the correlation between genes and phenotype, and an enrichment score was calculated to as-
sess the gene distribution. Each analysis was carried out with 1000 permutations. Gene sets were considered significantly enriched if the false discovery rate (FDR) \( q \)-value < 0.25 [20].

2.5. Tissue Staining and Immunohistochemistry

Paraffin-embedded blocks containing the human DA tissues were cut into 4 µm thick sections and placed on glass slides. Elastica van Gieson staining was performed for morphological analysis to evaluate IT and the tunica media, as described previously [21,22]. Immunohistochemistry was performed using primary antibodies for jagged 1 (sc-390177, Santa Cruz Biotechnology, Dallas, TX, USA) and calponin (M3556, DakoCytomation, Glostrup, Denmark). Biotinylated rabbit antibody (Vectastain Elite ABC IgG kit, Vector Labs, Burlingame, CA, USA) was used as a secondary antibody, and the presence of targeted proteins was determined by 3,3′-diaminobenzidine tetrahydrochloride (DAB) (DakoCytomation, Glostrup, Denmark). Negative staining of immunohistochemistry was confirmed by the omission of primary antibodies.

3. Results

3.1. DA-Related Clinical Course of Each Participant

Four patients with congenital heart diseases were analyzed in this study. Patient profiles are presented in Table 1. Case 1 was considered a patent DA case because the DA did not exhibit closing tendency throughout the clinical course. The DA tissue was isolated during an operation for an atrioventricular septal defect closure and repairs of the aortic arch and pulmonary venous returns. The other three cases (Cases 2–4) exhibited complex congenital heart diseases that required DA patency to maintain systemic circulation. Cases 2–4 were administered prostaglandin E1 (PGE\(_1\)) because they exhibited closing tendency of the DA.

In Case 2, lipo-PGE\(_1\) (1 ng/kg/min) was administered 8 h after birth when an echocardiography indicated narrowing of the DA. Case 2 continued lipo-PGE\(_1\) treatment until the operation. The patient had an aortic repair and pulmonary artery banding (PAB) conducted on postnatal day 5.

Case 3 showed closing tendency of the DA soon after birth and was administrated lipo-PGE\(_1\) (2 ng/kg/min). The dose of lipo-PGE\(_1\) was increased (4 ng/kg/min) 8 h after birth due to further closing tendency. The patient had PAB conducted on postnatal day 3. The closing tendency of the DA remained and required PGE\(_1\)-cyclodextrin (30 ng/kg/min) on postnatal day 4. The patient received PGE\(_1\)-cyclodextrin until the Norwood operation was conducted on postnatal day 24.

Case 4 showed closing tendency of the DA at 9 h after birth and received a lipo-PGE\(_1\) infusion (1 ng/kg/min). The patient underwent PAB on postnatal day 3. The DA was gradually narrowed and required an increased dose of lipo-PGE\(_1\) (5 ng/kg/min) on postnatal day 70. The patient underwent the Norwood operation on postnatal day 98. On the basis of their clinical courses, Cases 2–4 were considered as closing DAs.

3.2. Histological Differences between the Patent DA and the Closing DA Tissues

The Elastica van Gieson stain demonstrated that Case 1 had well-organized layered elastic fibers in the tunica media and a poorly formed IT (Figure 1A, upper panel). In Case 1, there was no overt fragmentation of the internal elastic laminae (Figure 1B, upper panel). Case 2 and Case 3 showed prominent IT formation that protruded into the lumen (Figure 1A, middle panels). Circumferentially oriented layered elastic fibers in the tunica media were sparsely formed and the internal elastic laminae were highly fragmented (Figure 1B, middle panels). Similarly, Case 4, who received PGE\(_1\) administration for more than 3 months, had a prominent IT (Figure 1A, lower panel). However, the entire tunica media consisted of sparse elastic fibers radially oriented toward the internal lumen, and circumferentially oriented elastic fibers were not recognized (Figure 1B, lower panel). These findings indicated that the closing DA had well-recognized, DA-specific morphological features, including prominent IT formation, fragmented internal elastic laminae, and
less elastic fibers in the tunica media, which seemed to reflect a normal closing process. On the other hand, the patent DA tissue (Case 1) was devoid of these structures and exhibited aortification of the vascular wall, which was consistent with previously reported morphological characteristics of the patent DA [9].

3.3. Microarray Analysis of the IT and the Tunica Media of Human DA Tissues

To elucidate a differential gene expression profile between the patent DA (Case 1) and the closing DA tissues (Cases 2–4), we performed an unbiased transcriptomic analysis using these human DA tissues. Each DA sample was divided into the IT and the tunica media in Cases 1–3. In Case 4, circumferentially oriented SMCs and layered elastic laminae could not be identified; therefore, the IT-like wall was divided into the inner part and the outer part (Figure 1A, lower). These samples were subjected to microarray analysis.

The dendrogram demonstrated that the human DA tissues were clearly divided into two major clusters, A and B (Figure 2). Cluster A consisted of both the IT and the tunica media from Case 1. Cluster B consisted of the samples from Cases 2–4, and this cluster was further divided into two subgroups, B1 and B2. Cluster B1 consisted of the IT tissues from Cases 2 and 3 and both the inner and outer IT-like parts from Case 4. Cluster B2 consisted of the tunica media samples from Cases 2 and 3. These data suggested that the patent DA tissue (Case 1) had a distinct gene expression pattern compared to the other closing DA samples (Cases 2–4). In Cases 2 and 3, the gene expression patterns of the tunica media samples were relatively similar. Additionally, the IT samples showed similar gene expression profiles, which were distant from the tunica media samples of Cases 2 and 3. In agreement with histological analysis showing that two parts of the DA tissue from Case 4 (inner and outer parts) exhibited an IT-like structure, these two samples of Case 4 showed similar gene patterns, which were close to that of the IT of Cases 2 and 3.

![Figure 2](https://example.com/figure2.png)

**Figure 2.** A dendrogram of the gene expressions from human ductus arteriosus tissues. IT: intimal thickening.

3.4. Transcriptomic Differences between the Tunica Media of Closing DA Tissues and the Patent DA Tissue

Both the histological assessment and the cluster analysis of DA tissues demonstrated that the gene expression profile of the tunica media of the patent DA tissue (Case 1) was markedly different from that of the closing DA tissues (Cases 2 and 3). We, thus, compared gene expressions between the tunica media of the patent DA and closing DA tissues.

The GSEAs between the tunica media of the closing DA and the patent DA tissues, using all gene sets related to biological processes in the Gene Ontology (GO) (size > 300), revealed that the closing DA tissues were significantly correlated to 87 biological processes...
(FDR < 0.25, Table 2). Notably, vascular development-related gene sets (GO_REGULATION_OF_VASCULATURE_DEVELOPMENT and GO_BLOOD_VESSEL_MORPHOGENESIS) were highly enriched in the tunica media of closing DA tissues (Figure 3). Kinase activation-related gene sets (GO_REGULATION_OF_MAP_KINASE_ACTIVITY, GO_ACTIVATION_OF_PROTEIN_KINASE_ACTIVITY, and GO_POSITIVE_REGULATION_OF_PROTEIN_SERINE_THREONINE_KINASE_ACTIVITY) and three catabolic process-related gene sets, including GO_REGULATION_OF_PROTEIN_CATABOLIC_PROCESS, were positively correlated with the closing DA tunica media tissue. This suggested that intracellular signaling was more actively regulated in the closing DA tissues compared to the patent DA tissue.

Protein secretion-related gene sets (GO_POSITIVE_REGULATION_OF_SECRETION and GO_GOLGI_VESICLE_TRANSPORT) and adhesion-related gene sets (GO_POSITIVE_REGULATION_OF_CELL_ADHESION, GO_REGULATION_OF_CELL_CELL_ADHESION, and GO_CELL_SUBSTRATE_ADHESION) were also enriched in the closing DA tissues, which support previous reports which found that multiple extracellular matrices and cell–matrix interactions play roles in DA-specific physiological remodeling [5,21,22]. The gene set GO_RESPONSE_TO_OXYGEN_LEVELS was positively correlated to the closing DA tissues (Figure 3). In this gene set, EGR1, which was previously shown to increase immediately after birth in rat DA tissues [10], was upregulated in the tunica media of the closing DA tissues. Enrichment of the gene set GO_ACTIN_FILAMENT_ORGANIZATION in the closing DA tissues (Figure 3) contained the Rho GTPase RHOD, which regulates directed cell migration [23]. This may support the migratory feature of SMCs in the closing DA tissue.

Table 2. Gene Ontology biological process terms (size > 300) that were significantly upregulated (FDR < 0.25) in the tunica media of the closing human DA tissues (Cases 2 and 3) compared to that of the patent DA tissue (Case 1).

| Gene Set Name                                      | Size | NES  | FDR q-Value | Rank at Max |
|---------------------------------------------------|------|------|-------------|-------------|
| GO_REGULATION_OF_VASCULATURE_DEVELOPMENT          | 310  | 1.61 | 0.000       | 6660        |
| GO_BLOOD_VESSEL_MORPHOGENESIS                      | 564  | 1.61 | 0.000       | 6712        |
| GO_GOLGI_VESICLE_TRANSPORT                        | 350  | 1.61 | 0.000       | 8448        |
| GO_REGULATION_OF_HEMPOIESIS                       | 441  | 1.60 | 0.000       | 6338        |
| GO_NCRNA_PROCESS                                  | 340  | 1.59 | 0.000       | 5889        |
| GO_VIRAL_LIFE_CYCLE                               | 315  | 1.58 | 0.000       | 7537        |
| GO_REGULATION_OF_MAP_KINASE_ACTIVITY              | 332  | 1.55 | 0.000       | 6500        |
| GO_LEUKOCYTE_CELLCELL_ADHESION                    | 339  | 1.54 | 0.000       | 5082        |
| GO_NEGATIVE_REGULATION_OF_IMMUNE_SYSTEM_PROCESS   | 405  | 1.54 | 0.000       | 4982        |
| GO_NEGATIVE_REGULATION_OF_PHOSPHORYLATION         | 424  | 1.53 | 0.000       | 5671        |
| GO_POSITIVE_REGULATION_OF_CELL_ADHESION           | 410  | 1.53 | 0.000       | 4942        |
| GO_NEGATIVE_REGULATION_OF_CELL_CYCLE_PROCESS      | 312  | 1.53 | 0.000       | 5276        |
| GO_NUCLEAR_TRANSPORT                              | 337  | 1.52 | 0.000       | 7197        |
| GO_T_CELL_ACTIVATION                              | 458  | 1.51 | 0.000       | 5293        |
| GO_IN_UTERO_EMBRYONIC_DEVELOPMENT                 | 372  | 1.47 | 0.000       | 6660        |
| GO_REGULATION_OF_INFLAMMATORY_RESPONSE            | 348  | 1.47 | 0.000       | 5237        |
| GO_PEPTIDYL_LYSINE_MODIFICATION                   | 353  | 1.46 | 0.000       | 8228        |
| GO_REGULATION_OF_PROTEIN_CATABOLIC_PROCESS        | 372  | 1.45 | 0.000       | 6333        |
| GO_IMMUNE_RESPONSE_REGULATING_SIGNALING_PATHWAY   | 385  | 1.40 | 0.000       | 7472        |
| GO_CELLULAR_RESPONSE_TO_EXTERNAL_STIMULUS         | 305  | 1.40 | 0.000       | 7120        |
| GO_REGULATION_OF_CELL_CELL_ADHESION               | 406  | 1.39 | 0.000       | 5082        |
| GO_RNA_SPlicING                                   | 423  | 1.39 | 0.000       | 7328        |
| GO_ACTIVATION_OF_PROTEIN_Kinase_ACTIVITY          | 321  | 1.39 | 0.000       | 5553        |
### Table 2. Cont.

| Gene Set Name                                                                 | Size | NES | FDR \(q\)-Value | Rank at Max |
|------------------------------------------------------------------------------|------|-----|------------------|-------------|
| GO_REGULATION_OF_CELLULAR_RESPONSE_TO_STRESS                                 | 690  | 1.39| 0.000            | 6338        |
| GO_RAS_PROTEIN_SIGNAL_TRANSDUCTION                                            | 330  | 1.39| 0.000            | 7040        |
| GO_POSITIVE_REGULATION_OF_PROTEIN_SERINE_THREONINE_KINASE_ACTIVITY            | 329  | 1.38| 0.000            | 6543        |
| GO_AMEBOIDAL_TYPE_CELL_MIGRATION                                             | 386  | 1.38| 0.000            | 7624        |
| GO_REGULATION_OF_DNA_BINDING_TRANSCRIPTION_FACTOR_ACTIVITY                    | 415  | 1.37| 0.000            | 6196        |
| GO_REGULATION_OF_LYMHPOCYTE_ACTIVATION                                       | 420  | 1.35| 0.000            | 5268        |
| GO_RNA_SPLICING_VIA_TRANSESTERIFICATION_REACTIONS                            | 341  | 1.35| 0.000            | 7328        |
| GO_REGULATION_OF_T_CELL_ACTIVATION                                           | 316  | 1.35| 0.000            | 5256        |
| GO_LYMHPOCYTE_DIFFERENTIATION                                                | 354  | 1.34| 0.000            | 5436        |
| GO_ORGANELLE_FISSION                                                         | 414  | 1.34| 0.000            | 4640        |
| GO_POSITIVE_REGULATION_OF_CATABOLIC_PROCESS                                  | 430  | 1.34| 0.000            | 4648        |
| GO_EPITHELIAL_CELL_PROLIFERATION                                             | 379  | 1.33| 0.011            | 6543        |
| GO_REGULATION_OF_PROTEIN_SERINE_THREONINE_KINASE_ACTIVITY                     | 495  | 1.33| 0.010            | 6256        |
| GO_MRNA_PROCESSING                                                            | 477  | 1.33| 0.010            | 7113        |
| GO_EMBRYO_DEVELOPMENT_ENDING_IN_BIRTH_OR_EGG_HATCHING                        | 644  | 1.32| 0.010            | 6259        |
| GO_NEGATIVE_REGULATION_OF_INTRACELLULAR_SIGNAL_TRANSDUCTION                  | 475  | 1.32| 0.009            | 7981        |
| GO_PROTEIN_POLYUBIQUITINATION                                                 | 327  | 1.31| 0.014            | 6212        |
| GO_RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS                                       | 405  | 1.31| 0.013            | 6510        |
| GO_MAINTENANCE_OF_LOCATION                                                    | 305  | 1.31| 0.013            | 5837        |
| GO_LEUKOCYTE_DIFFERENTIATION                                                 | 514  | 1.31| 0.013            | 7110        |
| GO_POSITIVE_REGULATION_OF_CELL_CYCLE                                         | 357  | 1.31| 0.013            | 5009        |
| GO_POSTTRANSCRIPTIONAL_REGULATION_OF_GENE_EXPRESSION                          | 554  | 1.29| 0.012            | 6212        |
| GO_RESPONSE_TO_OXYGEN_LEVELS                                                  | 370  | 1.29| 0.012            | 6459        |
| GO_POSITIVE_REGULATION_OF_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION              | 371  | 1.28| 0.012            | 6982        |
| GO_REGULATION_OF_METAL_ION_TRANSPORT                                         | 363  | 1.28| 0.012            | 4661        |
| GO_PROTEASOMAL_PROTEIN_CATABOLIC_PROCESS                                      | 455  | 1.28| 0.011            | 5596        |
| GO_NEGATIVE_REGULATION_OF_PHOSPHORUS_METABOLIC_PROCESS                        | 530  | 1.26| 0.011            | 5671        |
| GO_POSITIVE_REGULATION_OF_GTPASE_ACTIVITY                                     | 375  | 1.26| 0.011            | 7037        |
| GO_REGULATION_OF_GTPASE_ACTIVITY                                              | 447  | 1.26| 0.011            | 7095        |
| GO_POSITIVE_REGULATION_OF_RESPONSE_TO_EXTERNAL_STIMULUS                      | 496  | 1.25| 0.010            | 6529        |
| GO_COVALENT_CHROMATIN_MODIFICATION                                           | 436  | 1.25| 0.010            | 6473        |
| GO_NEURON_DEATH                                                              | 338  | 1.25| 0.010            | 7040        |
| GO_POSITIVE_REGULATION_OF_PROTEOLYSIS                                        | 340  | 1.24| 0.010            | 6359        |
| GO_POSITIVE_REGULATION_OF_CELLULAR_PROTEIN_LOCALIZATION                      | 307  | 1.24| 0.010            | 6982        |
| GO_POSITIVE_REGULATION_OF_CYTOKINE_PRODUCTION                                | 432  | 1.24| 0.010            | 5082        |
| GO_PROCESS_UTILIZING_AUTOPHAGIC_MECHANISM                                     | 495  | 1.23| 0.009            | 5327        |
| GO_VESICLE_ORGANIZATION                                                      | 315  | 1.23| 0.009            | 7253        |
| GO_POSITIVE_REGULATION_OF_CELL_ACTIVATION                                    | 324  | 1.23| 0.009            | 7094        |
| GO_POST_TRANSLATIONAL_PROTEIN_MODIFICATION                                   | 352  | 1.23| 0.009            | 7885        |
| GO_LEUKOCYTE_MIGRATION                                                       | 428  | 1.22| 0.009            | 7094        |
| GO_EMBRYO_DEVELOPMENT_ENDING_IN_BIRTH_OR_EGG_HATCHING                        | 397  | 1.22| 0.009            | 6914        |
| GO_CANONICAL_WNT_SIGNALING_PATHWAY                                           | 315  | 1.20| 0.009            | 5210        |
| GO_REGULATION_OF_CELLULAR_AMIDE_METABOLIC_PROCESS                            | 385  | 1.19| 0.008            | 6376        |
| GO_REGULATION_OF_AUTOPHagy                                                    | 327  | 1.18| 0.008            | 4699        |
| GO_REGULATION_OF_CHROMOSOME_ORGANIZATION                                     | 321  | 1.17| 0.014            | 6695        |
| GO_REGULATION_OF_SMALL_GTPASE_MEDIATED_SIGNAL_TRANSDUCTION                   | 312  | 1.16| 0.024            | 7040        |
| GO_REPRODUCTIVE_SYSTEM_DEVELOPMENT                                           | 428  | 1.16| 0.026            | 3453        |
| GO_REGULATION_OF_SUPRAMOLECULAR_FIBER_ORGANIZATION                           | 339  | 1.15| 0.028            | 6891        |
| GO_POSITIVE_REGULATION_OF_DEFENSE_RESPONSE                                   | 360  | 1.14| 0.048            | 5237        |
| GO_POSITIVE_REGULATION_OF_NERVOUS_SYSTEM_DEVELOPMENT                         | 513  | 1.14| 0.047            | 4697        |
| GO_REGULATION_OF_APOPTOTIC_SIGNALING_PATHWAY                                 | 383  | 1.13| 0.047            | 5796        |
| GO_MYELOID_CELL_DIFFERENTIATION                                              | 375  | 1.12| 0.061            | 6268        |
| GO_RESPONSE_TO_VIRUS                                                          | 315  | 1.12| 0.060            | 5563        |
| GO_ACTIN_FILAMENT_ORGANIZATION                                               | 400  | 1.11| 0.077            | 6891        |
| GO_RESPONSE_TO_MOLECULE_OF_BACTERIAL_ORIGIN                                   | 326  | 1.10| 0.097            | 6178        |
Table 2. Cont.

| Gene Set Name                                                                 | Size | NES  | FDR q-Value | Rank at Max |
|-------------------------------------------------------------------------------|------|------|-------------|-------------|
| GO_REGULATION_OF_PROTEIN_CONTAINING_COMPLEX_ASSEMBLY                           | 408  | 1.10 | 0.098       | 6574        |
| GO_OSSIFICATION                                                              | 378  | 1.10 | 0.106       | 6790        |
| GO_POSITIVE_REGULATION_OF_SECRETION                                         | 358  | 1.09 | 0.109       | 6953        |
| GO_EPITHELIAL_TUBE_MORPHOGENESIS                                             | 326  | 1.09 | 0.126       | 6477        |
| GO_ACTIVATION_OF_IMMUNE_RESPONSE                                             | 433  | 1.09 | 0.138       | 7472        |
| GO_CELL_SUBSTRATE_ADHESION                                                   | 342  | 1.08 | 0.172       | 8285        |
| GO_REGULATION_OF_BINDING                                                     | 349  | 1.08 | 0.178       | 6143        |
| GO_REGULATION_OF_DEVELOPMENT_GROWTH                                          | 324  | 1.08 | 0.178       | 4701        |
| GO_CELLULAR_RESPONSE_TO CHEMICAL_STRESS                                     | 329  | 1.07 | 0.229       | 4800        |

Abbreviations: NES, normalized enrichment score; FDR, false discovery rate.

Figure 3. Gene set enrichment analyses (GSEAs) of the tunica media of the closing human ductus arteriosus (DA) and the patent DA tissues. (A) GSEAs revealed positive correlations between the tunica media of the closing human DA tissues and vascular development-related genes, oxygen level response-related genes, and actin filament organization-related genes. On the x-axis, the genes in each gene set are ranked from the left side (positively correlated) to the right side (negatively correlated). The vertical black lines that look like barcodes indicate each gene in the gene set. The y-axis displays the calculated enrichment score of each gene (green color). NES, normalized enrichment score; FDR, false discovery rate. (B) The top 25 genes that comprise the leading edge of the enrichment score in (A) are shown in each heatmap.
Although we demonstrated several genes that were highly expressed in the tunica media of the closing DAs compared to that of the patent DA (Figure 3, and Tables 2 and 3), postnatal PGE\(_1\) administration possibly affected these gene expressions of the tunica media. To address this issue, we compared gene expressions of the tunica media between shorter-term PGE\(_1\)-treated DAs (less than one month of administration, Cases 2 and 3) and a longer-term PGE\(_1\)-treated DA (more than three months of administration, Case 4). The GSEAs revealed that the outer part of longer-term PGE\(_1\)-treated DA was significantly correlated to eight biological processes related to cell-cycle regulation (FDR < 0.25, Table S1 and Figure S1A,B, Supplementary Materials) compared to the tunica media of shorter-term PGE\(_1\)-treated DAs. Among these gene sets, the gene sets GO_ORGANELLE_FISSION and GO_NEGATIVE_REGULATION_OF_CELL_CYCLE_PROCESS belonged to gene sets that were highly expressed in the tunica media of the closing DAs (Table 2). These two gene sets may be associated with PGE\(_1\) administration, but not with specific features of the closing DA. However, the remaining 85 gene sets in Table 2 seemed to be independent of duration of PGE\(_1\) administration.

Table 3. Sixteen genes that overlapped and were enriched (>8-fold) in the tunica media of the closing DA tissues (Cases 2 and 3) compared to that of the patent DA tissue (Case 1).

| Gene Name | Description | Case 2 vs. Case 1 | Case 3 vs. Case 1 |
|-----------|-------------|------------------|------------------|
| CD83      | CD83 molecule | 29.9             | 29.9             |
| AP1S3     | adaptor-related protein complex 1 subunit sigma 3 | 26.5             | 18.0             |
| GSTT1     | glutathione S-transferase theta 1 | 21.6             | 10.7             |
| BCL2L13   | BCL2-like 13 | 12.9             | 13.1             |
| NEDD9     | neural precursor cell expressed, developmentally downregulated 9 | 12.1             | 13.6             |
| HLA-DMA   | major histocompatibility complex, class II, DM alpha | 9.1              | 16.3             |
| GHRL      | ghrelin and obestatin prepropeptide | 13.1             | 12.2             |
| FLCN      | folliculin | 12.2             | 9.1              |
| TCF7      | transcription factor 7, T-cell-specific | 11.0             | 9.0              |
| ELOVL5    | ELOVL fatty-acid elongase 5 | 9.0              | 9.9              |
| APLN      | apelin | 9.2              | 9.6              |
| MAFF      | MAF bZIP transcription factor F | 9.5              | 8.8              |
| AURKAP51  | aurora kinase A pseudogene 1 | 9.7              | 8.4              |
| MIS12     | MIS12 kinetochore complex component | 9.5              | 8.3              |
| CEMIP2    | cell migration inducing hyaluronidase 2 | 8.4              | 9.1              |
| GMCL1     | germ cell-less 1, spermatogenesis associated | 8.5              | 8.5              |

3.5. Vascular Development-Related Genes in Human DA Tissues

The vascular development-related gene sets noted above (Table 2 and Figure 3) contain cardiovascular cell lineage-related genes. A heatmap composed of cardiovascular cell lineage-related genes demonstrated distinct gene expression patterns between the closing DA tissues (Cases 2 and 3) and the patent DA tissue (Case 1) (Figure 4A). The genes SEMA5A, SFRP1, NRG1, CTNNB1, PHACTR4, and JAG1 were highly expressed in the ITs of the closing DA tissues. Among these genes, the expression of PHACTR4 and JAG1, which are cardiac neural crest-related genes, was greater in the tunica media of the closing DA tissues than the patent DA tissue. The expression levels of CFL1, TWIST1, EDNRB, SMO, and MAPK1 were greater in the tunica media of the patent DA tissue compared to the closing DA tissues. Similarly, expressions of SEMA4F, NR3F1, LTBP3, EDN3, and FGF8 were enriched in the tunica media of the patent DA tissue. SEMA3G, ALX1, SOX8, ALDH1A2, and SEMA7A were relatively highly expressed in the entire tissue of the patent DA. WNT8A, KLHL12, FBXL17, and ISL1, which is a second heart field-related gene, were relatively enriched in the patent DA tissue, and the expression levels of these genes were higher in the IT than in the tunica media.
Figure 4. Differential gene expression between the tunica media of the closing human ductus arteriosus (DA) tissues and that of the patent DA tissue. (A) A heatmap of vascular cell lineage-related genes is depicted. (B) A Venn diagram shows the number of probe sets that were highly expressed (>8-fold) in the tunica media of closing DA tissues (Cases 2 and 3) compared to the patent DA tissue (Case 1). (C) A Venn diagram shows the number of probe sets highly expressed (>8-fold) in the tunica media of the patent DA tissue (Case 1) compared to that of the closing DA tissues (Cases 2 and 3). IT, intimal thickening.

3.6. The Closing or Patent DA Tissue-Specific Gene Expression

Figure 4B presents a Venn diagram that shows probe sets that were upregulated (>8-fold) in the tunica media of the closing DA tissues (Cases 2 and 3) compared to the patent DA tissue (Case 1). Twenty-one overlapped probe sets consisted of 16 genes (Table 3). APLN, CEMIP2, and GHRL are related to vascular development [24–26]. There were several genes related to adhesion and protein secretion such as APLN, CD83, FLCN, and NEDD9 [27–30]. GHRL and NEDD9 were reported to regulate actin filament organization [31,32]. APLN was reported to promote proliferation and migration of vascular SMCs, as well as promote SMC contraction [27]. NEDD9 is involved in embryonic neural crest cell development and promotes cell migration, cell adhesion, and actin fiber formation [31]. To examine the effect of PGE1 administration on the human DAs, we compared gene expressions of the tunica media between shorter-term PGE1-treated DAs (Cases 2 and 3) and a longer-term PGE1-treated DA (Case 4) using a Venn diagram (Figure S1C, Supplementary Materials). We identified 20 probe sets that overlapped and were enriched in the outer part of a longer-term PGE1-treated DA compared to the IT of shorter-term PGE1-treated DAs (Table S2, Supplementary Materials). These genes did not belong to the genes in Table 3, suggesting that the genes presented in Table 3 did not seem to have been strongly influenced by PGE1 administration.

In the Venn diagram in Figure 4C, 116 probe sets are presented, which were upregulated (>8-fold) in the tunica media of the patent DA tissue (Case 1) compared to the
closing DA tissues (Cases 2 and 3). These probe sets contained 52 genes (Table 4). Latent transforming growth factor beta-binding protein 3 (LTBP3) was upregulated in the tunica media of the patent DA tissue. LTBP3 is related to extracellular matrix constituents [33] and second heart field-derived vascular SMCs [34]. Expression of PRSS55, identified as an aorta-dominant gene in rodent microarray data [11], was elevated in the patent DA tissue.

Table 4. Fifty-two genes that overlapped and were enriched (>8-fold) in the tunica media of the patent DA tissue (Case 1) compared to that of the closing DA tissues (Cases 2 and 3).

| Gene Name | Description                                      | Fold Change Case 1 vs. Case 2 | Fold Change Case 1 vs. Case 3 |
|-----------|--------------------------------------------------|-------------------------------|-------------------------------|
| MYH16     | myosin heavy chain 16 pseudogene                 | 70.1                          | 68.9                          |
| PRDM12    | PR/SET domain 12                                 | 69.8                          | 68.5                          |
| CXXC4     | CXXC finger protein 4                             | 62.8                          | 61.5                          |
| VENTX1    | VENT homeobox pseudogene 1                       | 60.4                          | 57.3                          |
| MKRN3     | makorin ring finger protein 3                     | 70.9                          | 43.7                          |
| CLEC3A    | C-type lectin domain family 3 member A           | 26.1                          | 251.3                         |
| TEX43     | testis expressed 43                               | 51.3                          | 26.7                          |
| SCN11A    | sodium channel, voltage-gated, type XI, alpha subunit 11 | 38.0                          | 26.5                          |
| CYP3A43   | cytochrome P450 family 3 subfamily A member 43   | 24.5                          | 22.3                          |
| MROH2A    | maestro heat-like repeat family member 2A         | 21.1                          | 21.4                          |
| MUC12     | mucin 12, cell-surface-associated                 | 30.1                          | 15.3                          |
| MSA4A6A   | membrane-spanning 4-domains subfamily A member 6A | 46.8                          | 11.7                          |
| RBPFOX3   | RNA-binding fox-1 homolog 3                       | 15.5                          | 20.4                          |
| SLC2A1    | solute carrier family 2 member 1                  | 30.4                          | 10.7                          |
| CFAP299   | cilia- and flagella-associated protein 299        | 15.4                          | 15.8                          |
| PSSS5     | pregnancy-specific beta-1-glycoprotein 5         | 16.3                          | 14.6                          |
| LTBP3     | latent transforming growth factor beta-binding protein 3 | 13.7                          | 16.9                          |
| ZF5P7     | zinc finger protein 57                            | 21.8                          | 11.0                          |
| MFSD4     | major facilitator superfamily domain-containing 4A | 13.8                          | 13.8                          |
| HOXA11    | homeobox A11                                     | 10.2                          | 19.1                          |
| ALLC      | allantocase                                      | 24.8                          | 8.8                           |
| SLC6A14   | solute carrier family 6 member 14                 | 13.1                          | 12.1                          |
| SLC4A4    | solute carrier family 4 member 4                 | 12.7                          | 11.7                          |
| MAS1      | MAS1 proto-oncogene, G-protein-coupled receptor   | 12.2                          | 12.2                          |
| CARD18    | caspase recruitment domain family member 18       | 12.9                          | 11.5                          |
| LCE1C     | late cornified envelope protein 1C                | 12.2                          | 10.6                          |
| PAMR1     | peptidase-domain-containing associated with muscle regeneration 1 | 8.1                           | 18.7                          |
| PRSS55    | serine protease 55                               | 11.4                          | 10.5                          |
| RINDC3B   | RIN domain-containing 3B                         | 10.0                          | 11.8                          |
| LINC00114 | long intergenic non-protein-coding RNA 114        | 8.9                           | 13.8                          |
| TFA4      | transcription factor AP-4                        | 11.2                          | 10.1                          |
| PLIN2     | perilipin 2                                      | 9.8                           | 11.1                          |
| CCR2      | C-C motif chemokine receptor 2                   | 11.7                          | 9.3                           |
| CDKN2B-AS | CDR2B antisense RNA 1                             | 10.0                          | 9.8                           |
| MYO7A     | myosin VIIA                                      | 10.4                          | 9.3                           |
| PDILT     | protein disulphide isomerase like, testis expressed | 9.6                           | 9.6                           |
| SPA17     | sperm autoantigenic protein 17                    | 10.8                          | 8.5                           |
| SLITRK2   | SLIT and NTRK-like family member 2               | 9.5                           | 9.5                           |
| SLC9B1    | solute carrier family 9 member B1                | 9.3                           | 9.7                           |
| PANX2     | panxin 2                                         | 11.4                          | 8.0                           |
| PLPPR1    | phospholipid phosphatase-related 1               | 10.1                          | 8.8                           |
| ASTE1     | asteroide homolog 1                              | 9.2                           | 9.4                           |
| MUC16     | mucin 16, cell-surface-associated                 | 9.5                           | 8.7                           |
| OR51B2    | olfactory receptor family 51 subfamily B member 2 | 9.2                           | 8.6                           |
| ARHGAP36  | Rho GTPase-activating protein 36                  | 8.8                           | 8.7                           |
| KRTAP4-8  | keratin-associated protein 4-8                   | 8.8                           | 8.5                           |
| METTL21CIP1 | methyltransferase-like 21E, pseudogene                | 9.1                          | 8.3                           |
| BPIFB6    | BPI fold-containing family B member 6            | 8.9                           | 8.4                           |
| HABP2     | hyaluronan-binding protein 2                     | 9.0                           | 8.2                           |
| DUSP13    | dual-specificity phosphatase 13                  | 8.9                           | 8.2                           |
| Cxorf51A  | chromosome X open reading frame 51A              | 8.2                           | 8.0                           |
| MTMI      | myotubularin 1                                   | 8.2                           | 8.1                           |
3.7. Jagged 1 Was Highly Expressed in the Closing DA Tissues

Previous reports using genetically modified mice clearly demonstrated that SMCs of the DA are derived from cardiac neural crest cells, and these cells contribute to SMC differentiation in the DA [35,36]. Since the transcriptome analysis revealed that the neural crest cell-related gene JAG1 was abundantly expressed in the closing DA tissues compared to the patent DA tissue (Figure 4A), we performed immunohistochemistry to examine protein expression of jagged 1. In agreement with the transcriptome data, jagged 1 was highly expressed in the closing DA tissues (Cases 2–4) (Figure 5A). Calponin is well recognized as a differentiated SMC marker [1], and it was decreased in the DA tissues of Jag1-deficient mice [37]. A strong immunoreaction for calponin was observed in the closing DA tissues but was not as strong in the patent DA tissue (Figure 5B).

Figure 5. Immunohistochemistry for jagged 1 (A) and calponin (B) in the human ductus arteriosus tissues from Cases 1–4. A brown color indicates positive immunostaining. Scale bars: 100 µm.

3.8. Transcriptomic Characteristics of the IT and the Tunica Media in the Closing DA Tissues

Lastly, we investigated the difference in gene expression between the IT and the tunica media in the closing DA tissues. IT formation is partly attributed to migration and proliferation of the tunica media-derived SMCs [2–5,7]. Gene expression analysis indicated that there were different transcriptomic characteristics between the IT and the tunica media in the closing DA tissues (Clusters B1 and B2 in Figure 2). We, thus, compared the expression of genes between the IT and the tunica media of the closing DA tissues (Cases 2 and 3).

The GSEAs between the IT and the tunica media, using all gene sets which related to biological processes in the Gene Ontology (GO) (size > 300), were performed. The analyses revealed that the IT was significantly correlated to 89 biological processes (FDR < 0.25, Table 5), and that the tunica media correlated to 81 biological processes (FDR < 0.25, Table 6). The IT of the closing DAs was significantly correlated to more than 10 migration- and proliferation-related gene sets (GO_MICROTUBULE_CYTOSKELETON_ORGANIZATION and GO_CELL_DIVISION, etc.) (Figure 6A,B). Wnt signaling-related gene sets (GO_REGULATION_OF_WNT_SIGNALING_PATHWAY, GO_CANONICAL_WNT_SIGNALING_PATHWAY, and GO_CELL_CELL_SIGNALING_BY_WNT) were also enriched in the IT of the closing DA tissues. The tunica media of the closing DAs was significantly correlated to vascular development-related gene sets (GO_REGULATION_OF_VASCULAR_DEVELOPMENT, GO_BLOOD_VESSEL_MORPHOGENESIS, GO_VASCULAR_DEVELOPMENT, and GO_CIRCULATORY_SYSTEM_DEVELOPMENT) (Figure 6C,D). Five adhesion-related gene sets, including GO_BIOLOGICAL_ADHESION, were enriched in the tunica media compared to the IT in the closing DA tissues.
Table 5. Gene Ontology biological process terms (size > 300) that were significantly upregulated (FDR < 0.25) in the intimal thickening compared to the tunica media of the closing DA tissues (Cases 2 and 3).

| Gene Set Name                                                                 | Size | NES | FDR q-Value | Rank at Max |
|-------------------------------------------------------------------------------|------|-----|-------------|-------------|
| GO_MICROTUBULE_CYTOSKELETON_ORGANIZATION                                       | 526  | 1.67| 0.000       | 6797        |
| GO_MICROTUBULE_BASED_PROCESS                                                  | 757  | 1.59| 0.000       | 6797        |
| GO_CELL_DIVISION                                                              | 549  | 1.58| 0.000       | 6799        |
| GO_PROTEIN_POLYUBIQUITINATION                                                 | 327  | 1.56| 0.000       | 7778        |
| GO_MITOTIC_CELL_CYCLE                                                         | 954  | 1.54| 0.000       | 6055        |
| GO_MODIFICATION_DEPENDENT_MACROMOLECULE_CATABOLIC_PROCESS                     | 604  | 1.51| 0.000       | 6933        |
| GO_NEGATIVE_REGULATION_OF_CELL_CYCLE                                         | 566  | 1.51| 0.000       | 6548        |
| GO_NEGATIVE_REGULATION_OF_CELL_CYCLE_PROCESS                                 | 312  | 1.50| 0.001       | 6055        |
| GO_MRNA_PROCESSING                                                            | 477  | 1.50| 0.001       | 6995        |
| GO_RNA_SPLITTING_VIA_TRANSESTERIFICATION_REACTIONS                            | 341  | 1.48| 0.001       | 6758        |
| GO_ESTABLISHMENT_OF_ORGANELLE_LOCALIZATION                                   | 397  | 1.47| 0.001       | 6823        |
| GO_REGULATION_OF_MRNA_METABOLIC_PROCESS                                       | 326  | 1.47| 0.001       | 9158        |
| GO_ORGANELLE_LOCALIZATION                                                     | 602  | 1.46| 0.001       | 6823        |
| GO_RNA_SPLITTING                                                             | 423  | 1.45| 0.002       | 6007        |
| GO_CELL_CYCLE                                                                | 1681 | 1.44| 0.002       | 6059        |
| GO_ORGANELLE_FISSION                                                          | 414  | 1.44| 0.002       | 5836        |
| GO_CELL_CYCLE_PROCESS                                                         | 1251 | 1.43| 0.002       | 6470        |
| GO_REGULATION_OF_MITOTIC_CELL_CYCLE                                          | 600  | 1.43| 0.002       | 6548        |
| GO_MUSCLE_TISSUE_DEVELOPMENT                                                 | 368  | 1.42| 0.003       | 4069        |
| GO_CELLULAR_PROTEIN_CATABOLIC_PROCESS                                        | 733  | 1.41| 0.005       | 6933        |
| GO_REGULATION_OF_CELL_CYCLE_PROCESS                                         | 706  | 1.41| 0.005       | 6450        |
| GO_CELL_CYCLE_PHASE_TRANSITION                                               | 578  | 1.40| 0.005       | 6055        |
| GO_REGULATION_OF_CELL_CYCLE                                                 | 1110 | 1.40| 0.004       | 6548        |
| GO_MICROTUBULE_BASED_MOVEMENT                                                  | 321  | 1.37| 0.007       | 5942        |
| GO_PROTEIN_MODIFICATION_BY_SMALL_PROTEIN_CONJUGATION_OR_REMOVAL              | 1033 | 1.36| 0.008       | 6585        |
| GO_PROTEIN_CATABOLIC_PROCESS                                                 | 876  | 1.36| 0.008       | 7607        |
| GO_PROTEASOMAL_PROTEIN_CATABOLIC_PROCESS                                     | 455  | 1.34| 0.011       | 7536        |
| GO_POST_TRANSLATIONAL_PROTEIN_MODIFICATION                                   | 352  | 1.33| 0.013       | 4520        |
| GO_VESICLE_ORGANIZATION                                                      | 315  | 1.33| 0.013       | 6530        |
| GO_PROTEIN_MODIFICATION_BY_SMALL_PROTEIN_CONJUGURATION                        | 864  | 1.32| 0.014       | 7634        |
| GO_PROTEIN_CONTAINING_COMPLEX_DISASSEMBLY                                    | 310  | 1.32| 0.014       | 6434        |
| GO_CHROMOSOME_ORGANIZATION                                                   | 1059 | 1.32| 0.015       | 6004        |
| GO_RNA_PROCESSING                                                            | 1149 | 1.31| 0.016       | 7021        |
| GO_REGULATION_OF_CHROMOSOME_ORGANIZATION                                     | 321  | 1.29| 0.024       | 7299        |
| GO_REGULATION_OF_CELL_CYCLE_PHASE_TRANSITION                                 | 424  | 1.29| 0.024       | 6055        |
| GO_MRNA_METABOLIC_PROCESS                                                    | 789  | 1.28| 0.026       | 7021        |
| GO_REGULATION_OF_INTRACELLULAR_TRANSPORT                                    | 325  | 1.28| 0.026       | 6449        |
| GO_MUSCLE_SYSTEM_PROCESS                                                     | 423  | 1.28| 0.028       | 3948        |
| GO_CELLULAR_MACROMOLECULE_CATABOLIC_PROCESS                                  | 1100 | 1.26| 0.034       | 7104        |
| GO_REGULATION_OF_AUTOPHAGY                                                   | 327  | 1.25| 0.041       | 6901        |
| GO_CELLULAR_PROTEIN_CONTAINING_COMPLEX.Assembly                               | 909  | 1.25| 0.041       | 6799        |
| GO_REGULATION_OF_WNT_SIGNALING_PATHWAY                                       | 347  | 1.25| 0.042       | 3438        |
| GO_RIBONUCLEOPROTEIN_COMPLEX_BIogenesis                                       | 405  | 1.24| 0.042       | 8496        |
| GO_PROCESS_UTILIZING_AUTOPHAGIC_MECHANISM                                    | 495  | 1.23| 0.049       | 6585        |
| GO_ANATOMICAL_STRUCTURE_HOMEOSTASIS                                           | 426  | 1.23| 0.048       | 6332        |
| GO_ORGANOPHOSPHATE_BIOSYNTHETIC_PROCESS                                      | 526  | 1.23| 0.049       | 3613        |
| GO_REGULATION_OF_CELLULAR_CATABOLIC_PROCESS                                  | 812  | 1.23| 0.051       | 6913        |
| GO_PROTEIN_CONTAINING_COMPLEX_SUBUNIT_ORGANIZATION                           | 1716 | 1.23| 0.052       | 5624        |
| GO_REGULATION_OF_SYSTEM_PROCESS                                              | 571  | 1.22| 0.057       | 3069        |
| GO_GLYCEROPHOSPHOLIPID_METABOLIC_PROCESS                                     | 325  | 1.22| 0.055       | 2842        |
| GO_ORGANELLE.Assembly                                                        | 780  | 1.22| 0.055       | 5055        |
| GO_MUSCLE_CONTRACTION                                                        | 339  | 1.20| 0.070       | 3948        |
| GO_REGULATION_OF_CELLULAR_LOCALIZATION                                      | 939  | 1.20| 0.073       | 6537        |
| GO_CYTOSKELETOR_4TOMIZATION                                                   | 1278 | 1.20| 0.072       | 5064        |
| GO_REGULATION_OF_CATABOLIC_PROCESS                                           | 960  | 1.20| 0.075       | 7638        |
| GO_MACROMOLECULE_CATABOLIC_PROCESS                                           | 1319 | 1.19| 0.077       | 7614        |
### Table 5. Cont.

| Gene Set Name                                                                 | Size | NES | FDR \(q\)-Value | Rank at Max |
|------------------------------------------------------------------------------|------|-----|-----------------|-------------|
| GO_ORGANONITROGEN_COMPOUND_CATABOLIC_PROCESS                                 | 1233 | 1.19| 0.080           | 6337        |
| GO_CANONICAL_WNT_SIGNALING_PATHWAY                                           | 315  | 1.18| 0.084           | 3438        |
| GO_DIVALENT_INORGANIC_CATION_TRANSPORT                                      | 443  | 1.18| 0.087           | 2865        |
| GO_MUSCLE_STRUCTURE_DEVELOPMENT                                              | 606  | 1.18| 0.094           | 4139        |
| GO_POSITIVE_REGULATION_OF_ESTABLISHMENT_OF_PROTEIN_LOCALIZATION             | 371  | 1.18| 0.092           | 5436        |
| GO_DNA_METABOLIC_PROCESS                                                    | 822  | 1.18| 0.091           | 7381        |
| GO_CELL_CELL_SIGNALING_BY_WNT                                                | 488  | 1.17| 0.102           | 3490        |
| GO_INTRACELLULAR_TRANSPORT                                                  | 1599 | 1.16| 0.108           | 6629        |
| GO_MUSCLE_ORGAN_DEVELOPMENT                                                 | 360  | 1.16| 0.108           | 4772        |
| GO_REGULATION_OF_PEPTIDE_TRANSPORT                                          | 641  | 1.16| 0.109           | 5020        |
| GO_SECOND_MESSENGER_MEDIATED_SIGNALING                                       | 412  | 1.15| 0.125           | 3308        |
| GO_PEPTIDE_SECRETION                                                        | 495  | 1.15| 0.125           | 3880        |
| GO_REGULATION_OF_CYTOSKELETON_ORGANIZATION                                  | 513  | 1.15| 0.128           | 3944        |
| GO_SIGNAL_RELEASE                                                            | 577  | 1.14| 0.130           | 3898        |
| GO_DNA_REPAIR                                                                | 480  | 1.14| 0.132           | 6578        |
| GO_MUSCLE_CELL_DIFFERENTIATION                                              | 338  | 1.14| 0.131           | 4061        |
| GO_NEGATIVE_REGULATION_OF_PROTEIN_MODIFICATION_PROCESS                      | 565  | 1.14| 0.132           | 5343        |
| GO_NEGATIVE_REGULATION_OF_PHYOSPHORYLATION                                   | 424  | 1.14| 0.138           | 5364        |
| GO_MITOCHONDRION_ORGANIZATION                                                | 459  | 1.14| 0.138           | 7936        |
| GO_NEGATIVE_REGULATION_OF_PHOSPHORUS_METABOLIC_PROCESS                      | 530  | 1.13| 0.144           | 5394        |
| GO_HORMONE_TRANSPORT                                                        | 309  | 1.12| 0.165           | 3880        |
| GO_REGULATION_OF_PROTEIN_CATABOLIC_PROCESS                                   | 372  | 1.12| 0.176           | 7675        |
| GO_REGULATION_OF_PROTEIN_LOCALIZATION                                       | 905  | 1.11| 0.184           | 6577        |
| GO_RIBOSE_PHYOSPHATE_METABOLIC_PROCESS                                       | 373  | 1.11| 0.183           | 4526        |
| GO_NUCLEOBASE_CONTAINING_SMALL_MOLECULE_METABOLIC_PROCESS                   | 545  | 1.11| 0.196           | 3618        |
| GO_PHOSPHOLIPID_METABOLIC_PROCESS                                            | 420  | 1.10| 0.215           | 5395        |
| GO_REGULATION_OF_ORGANELLE_ORGANIZATION                                      | 1209 | 1.10| 0.220           | 5212        |
| GO_ORGANOPHOSPHATE_METABOLIC_PROCESS                                         | 950  | 1.09| 0.238           | 3618        |
| GO_REGULATION_OF_CELL_RESPONSE_TO_STRESS                                     | 690  | 1.09| 0.235           | 6943        |
| GO_CELLULAR_RESPONSE_TO_DNA_DAMAGE_STIMULUS                                  | 761  | 1.09| 0.246           | 6578        |
| GO_GLYCEROLIPID_METABOLIC_PROCESS                                            | 409  | 1.09| 0.246           | 5591        |
| Abbreviations: NES, normalized enrichment score; FDR, false discovery rate   |      |     |                 |             |

### Table 6. Gene Ontology biological process terms (size > 300) that were significantly upregulated (FDR < 0.25) in the tunica media compared to the intimal thickening of the closing DA tissues (Cases 2 and 3).

| Gene Set Name                                                                 | Size | NES | FDR \(q\)-Value | Rank at Max |
|------------------------------------------------------------------------------|------|-----|-----------------|-------------|
| GO_EXTRACELLULAR_STRUCTURE_ORGANIZATION                                      | 376  | 1.67| 0.002           | 5349        |
| GO_SKELETAL_SYSTEM_DEVELOPMENT                                              | 494  | 1.55| 0.012           | 5310        |
| GO_REGULATION_OF_VASCATURE DEVELOPMENT                                      | 310  | 1.49| 0.020           | 7236        |
| GO_EMBRYONIC_ORGAN_DEVELOPMENT                                              | 443  | 1.49| 0.023           | 6270        |
| GO_PATTERN_SPECIFICATION_PROCESS                                            | 442  | 1.48| 0.021           | 6393        |
| GO_INFLAMMATORY_RESPONSE                                                    | 706  | 1.45| 0.029           | 7279        |
| GO_TAXIS                                                                     | 612  | 1.45| 0.027           | 7278        |
| GO_NEGATIVE_REGULATION_OF_CELL_DEVELOPMENT                                 | 311  | 1.44| 0.030           | 6296        |
| GO_BLOOD_VESSEL_MORPHOGENESIS                                               | 564  | 1.43| 0.030           | 7164        |
| GO_NEGATIVE_REGULATION_OF_CELL_DIFFERENTIATION                             | 668  | 1.42| 0.032           | 6377        |
| GO_POSITIVE_REGULATION_OF_NERVOUS_SYSTEM_DEVELOPMENT                       | 513  | 1.42| 0.036           | 6270        |
| GO_REGREGIONALIZATION                                                       | 347  | 1.40| 0.043           | 6393        |
| GO_VASCULATURE_DEVELOPMENT                                                 | 676  | 1.39| 0.050           | 6711        |
| GO_EMBRYONIC_MORPHOGENESIS                                                  | 578  | 1.39| 0.050           | 5668        |
| GO_POSITIVE_REGULATION_OF_CELL_DEVELOPMENT                                 | 528  | 1.38| 0.052           | 5938        |
| GO_REGULATION_OF_NERVOUS_SYSTEM_DEVELOPMENT                                | 888  | 1.37| 0.058           | 6409        |
| GO_BIOLOGICAL_ADHESION                                                     | 1379 | 1.37| 0.062           | 7362        |
Table 6. Cont.

| Gene Set Name                                                                 | Size | NES  | FDR q-Value | Rank at Max |
|-------------------------------------------------------------------------------|------|------|-------------|-------------|
| **GO_EPITHELIAL_TUBE_MORPHOGENESIS**                                         | 326  | 1.36 | 0.069       | 6392        |
| **GO_TUBE_MORPHOGENESIS**                                                     | 808  | 1.35 | 0.075       | 6276        |
| **GO_REGULATION_OF_NEURON_DIFFERENTIATION**                                 | 631  | 1.35 | 0.073       | 4480        |
| **GO_POSITIVE_REGULATION_OF_DEVELOPMENTAL_PROCESS**                          | 1298 | 1.35 | 0.071       | 6726        |
| **GO_REGULATION_OF_INFLAMMATORY_RESPONSE**                                   | 348  | 1.34 | 0.074       | 6671        |
| **GO_POSITIVE_REGULATION_OF_CELL_DIFFERENTIATION**                          | 939  | 1.34 | 0.072       | 6708        |
| **GO_AMEBOIDAL_TYPE_CELL_MIGRATION**                                         | 386  | 1.34 | 0.070       | 3718        |
| **GO_POSITIVE_REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS**              | 1662 | 1.34 | 0.067       | 6427        |
| **GO_REGULATION_OF_CELL_ADHESION**                                           | 682  | 1.34 | 0.071       | 7361        |
| **GO_CELL_MORPHOGENESIS_INVOLVED_IN_NEURON_DIFFERENTIATION**                 | 585  | 1.33 | 0.075       | 6775        |
| **GO_REGULATION_OF_DEVELOPMENTAL_PROCESS**                                  | 905  | 1.33 | 0.075       | 6577        |
| **GO_AXON_DEVELOPMENT**                                                      | 512  | 1.32 | 0.079       | 7161        |
| **GO_TUBE_DEVELOPMENT**                                                      | 998  | 1.32 | 0.080       | 6726        |
| **GO_NEUROGENESIS**                                                          | 1571 | 1.32 | 0.079       | 6708        |
| **GO_REGULATION_OF_ANATOMICAL_STRUCTURE_MORPHOGENESIS**                      | 1032 | 1.32 | 0.079       | 6334        |
| **GO_REGULATION_OF_CELL_DIFFERENTIATION**                                    | 1729 | 1.31 | 0.086       | 6577        |
| **GO_REGULATION_OF_T_CELL_ACTIVATION**                                       | 316  | 1.31 | 0.084       | 5912        |
| **GO_POSITIVE_REGULATION_OF_CELL_ADHESION**                                 | 410  | 1.31 | 0.083       | 6893        |
| **GO_REGULATION_OF_CELL_DEVELOPMENT**                                       | 904  | 1.31 | 0.086       | 6400        |
| **GO_REGULATION_OF_CELL_MORPHOGENESIS**                                      | 474  | 1.30 | 0.102       | 6663        |
| **GO_REGULATION_OF_CELL_CELL_ADHESION**                                      | 406  | 1.29 | 0.105       | 6811        |
| **GO_ANATOMICAL_STRUCTUREFORMATION_INVOLVED_IN_MORPHOGENESIS**                | 1044 | 1.29 | 0.106       | 7374        |
| **GO_EPITHELIAL_CELL_Proliferation**                                         | 379  | 1.28 | 0.119       | 6725        |
| **GO_POSITIVE_REGULATION_OF_NEURON_DIFFERENTIATION**                        | 356  | 1.28 | 0.120       | 6270        |
| **GO_REGULATION_OF_PEPTIDASE_ACTIVITY**                                      | 419  | 1.28 | 0.118       | 6411        |
| **GO_LEUKOCYTE_CELL_ADHESION**                                               | 339  | 1.28 | 0.116       | 6889        |
| **GO_CELL_ADHESION**                                                         | 826  | 1.28 | 0.119       | 7362        |
| **GO_MORPHOGENESIS**                                                         | 996  | 1.27 | 0.118       | 6775        |
| **GO_REGULATION_OF_ANEPITHELIUM**                                            | 539  | 1.27 | 0.125       | 6433        |
| **GO_CIRCULATORY_SYSTEM_DEVELOPMENT**                                       | 1018 | 1.27 | 0.127       | 6433        |
| **GO_REGULATION_OF_HYDROLASE_ACTIVITY**                                     | 719  | 1.26 | 0.132       | 6562        |
| **GO_CELLULAR_PROCESS_INVOLVED_IN_REPRODUCTION_IN_MULTICELLULAR_ORGANISM**   | 330  | 1.26 | 0.131       | 6092        |
| **GO_GLAND_DEVELOPMENT**                                                     | 436  | 1.26 | 0.143       | 6386        |
| **GO_REGULATION_OF_MULTICELLULAR_ORGANISMANAL_PROCESS**                      | 1145 | 1.25 | 0.147       | 6749        |
| **GO_REGULATION_OF_CELLULAR_COMPONENT_SIZE**                                | 360  | 1.25 | 0.152       | 3939        |
| **GO_SENSORY_ORGAN_DEVELOPMENT**                                            | 560  | 1.25 | 0.155       | 5533        |
| **GO_NEURON_DIFFERENTIATION**                                               | 1327 | 1.25 | 0.155       | 6400        |
| **GO_REGULATORY_SYSTEM_DEVELOPMENT**                                        | 428  | 1.25 | 0.154       | 4925        |
| **GO_CELL_PART_MORPHOGENESIS**                                              | 680  | 1.24 | 0.155       | 6775        |
| **GO_REGULATION_OF_MOBILE**                                                 | 1080 | 1.24 | 0.155       | 6705        |
| **GO_REGULATION_OF_T_CELL_ACTIVATION**                                      | 458  | 1.24 | 0.154       | 7224        |
| **GO_REGULATION_OF_CELL_PROJECTION_ORGANIZATION**                          | 366  | 1.24 | 0.169       | 6270        |
| **GO_ANIMAL_ORGAN_MORPHOGENESIS**                                           | 1048 | 1.24 | 0.168       | 6373        |
| **GO_REGULATION_OF_ANION_TRANSPORT**                                        | 491  | 1.22 | 0.168       | 6889        |
| **GO_CELL_MORPHOGENESIS_INVOLVED_IN_DIFFERENTIATION**                       | 728  | 1.22 | 0.168       | 6775        |
| **GO_REGULATION_OF_MULTICELLULAR_ORGANISMANAL_PROCESS**                     | 360  | 1.22 | 0.191       | 8857        |
| **GO_ALCOHOL_METABOLIC_PROCESS**                                            | 362  | 1.22 | 0.192       | 7136        |
| **GO_REGULATION_OF_CELL_PROJECTION_ORGANIZATION**                          | 1235 | 1.22 | 0.195       | 9098        |
| **GO_EMBRYO_DEVELOPMENT**                                                   | 1018 | 1.22 | 0.194       | 6690        |
| **GO_REGULATION_OF_ANION_TRANSPORT**                                        | 628  | 1.21 | 0.204       | 6889        |
| **GO_REGULATION_OF_NEURON_PROJECTION_DEVELOPMENT**                          | 486  | 1.21 | 0.202       | 4480        |
| **GO_REGULATION_OF_CELL_PROJECTION_ORGANIZATION**                          | 652  | 1.21 | 0.203       | 6334        |
| **GO_REGULATION_OF_UROGENITAL_SYSTEM_DEVELOPMENT**                         | 327  | 1.21 | 0.203       | 6013        |
| **GO_REGULATION_OF_ANION_TRANSPORT**                                        | 325  | 1.20 | 0.233       | 6411        |
| **GO_REGULATION_OF_HEMOPOIESIS**                                            | 639  | 1.20 | 0.233       | 6433        |
| **GO_REGULATION_OF_HYDROLYTIC_ACTIVITY**                                   | 441  | 1.20 | 0.236       | 7164        |
| **GO_REGULATION_OF_HYDROLYTIC_ACTIVITY**                                   | 1205 | 1.20 | 0.236       | 6564        |
| **GO_REGULATION_OF_IMMUNE_SYSTEM_PROCESS**                                | 1387 | 1.20 | 0.235       | 7164        |
| **GO_REGULATION_OF_LIPID_CATABOLIC_PROCESS**                               | 354  | 1.19 | 0.243       | 8193        |
| **GO_REGULATION_OF_TISSUE_MORPHOGENESIS**                                  | 1261 | 1.19 | 0.240       | 6313        |
| **GO_REGULATION_OF_HYDROLYTIC_ACTIVITY**                                   | 765  | 1.19 | 0.238       | 7239        |
| **GO_REGULATION_OF_CELL_ADHESION**                                         | 541  | 1.19 | 0.238       | 5944        |
| **GO_REGULATION_OF_CELL_ADHESION**                                         | 695  | 1.19 | 0.245       | 7173        |
| **GO_REGULATION_OF_CELL_ADHESION**                                         | 538  | 1.19 | 0.247       | 7342        |

Abbreviations: NES, normalized enrichment score; FDR, false discovery rate.
Figure 6. Differential gene expression between the intimal thickening (IT) and the tunica media of the closing human ductus arteriosus (DA) tissues. (A) Gene set enrichment analyses (GSEAs) revealed positive correlations between the IT of closing DA tissues and migration- and proliferation–related genes. (B) The top 25 genes that comprise the leading edge of the enrichment score in (A) are shown as a heatmap. (C) GSEAs revealed positive correlations between the tunica media of the closing DA tissues and vascular morphogenesis-related genes. (D) The top 25 genes that comprise the leading edge of the enrichment score in (B) are shown as a heatmap. (E) A Venn diagram shows the number of probe sets that were highly expressed (>8-fold) in the IT compared to the tunica media of closing DA tissues (Cases 2 and 3). (F) A Venn diagram shows the numbers of probe sets that were highly expressed (>8-fold) in the tunica media compared to the IT of closing DA tissues (Cases 2 and 3). NES, normalized enrichment score; FDR, false discovery rate.
Figure 6E presents a Venn diagram that shows probe sets that were upregulated (>8-fold) in the IT of closing DA tissues compared to the tunica media of the same DA tissues (Cases 2 and 3). Eight overlapped probe sets consisted of eight genes (Figure 6E and Table 7). POU4F1, FGF1, and PROCR are related to cell division and cell cycle [38–40]. FGF1 is reportedly involved in proliferation and migration of vascular SMCs [39]. A Venn diagram in Figure 6F shows 12 probe sets that were commonly upregulated (>8-fold) in the tunica media of the closing DA tissues compared to the IT of the same DA tissues (Cases 2 and 3), which consisted of eight genes (Figure 6F and Table 8). There were several genes related to muscle structure development such as BDKRB2, MSC, and DCN [41–43]. DCN is also involved in extracellular constituents and stabilizes collagen and elastic fibers [44,45].

### Table 7. Eight genes that overlapped and were enriched (>8-fold) in the intimal thickening (IT) compared to the tunica media of the closing DA tissues (Cases 2 and 3).

| Gene Name | Description | Fold Change IT vs. the Tunica Media |
|-----------|-------------|------------------------------------|
| POU4F1    | POU class 4 homeobox 1 | 22.1 | 16.4 |
| BMX       | BMX non-receptor tyrosine kinase | 15.9 | 10.6 |
| FGF1      | fibroblast growth factor 1 | 15.9 | 10.0 |
| MPZL2     | myelin protein zero-like 2 | 18.8 | 12.8 |
| FMO3      | flavin-containing dimethylalanine monooxygenase 3 | 13.0 | 9.3 |
| PROCR     | protein C receptor | 13.0 | 9.7 |
| DSP       | desmoplakin | 11.7 | 10.7 |
| NR1I2     | nuclear receptor subfamily 1 group I member 2 | 11.7 | 10.7 |

### Table 8. Eight genes that overlapped and were enriched (>8-fold) in the tunica media compared to the intimal thickening (IT) of the closing DA tissues (Cases 2 and 3).

| Gene Name | Description | Fold Change the Tunica Media vs. IT |
|-----------|-------------|-----------------------------------|
| GAS7      | growth arrest-specific 7 | 52.4 | 9.8 |
| H19       | H19 imprinted maternally expressed transcript | 14.2 | 12.8 |
| BTNL9     | butyrophilin-like 9 | 8.4 | 14.7 |
| SELENOP   | selenoprotein P | 14.8 | 13.4 |
| BDKRB2    | bradykinin receptor B2 | 11.3 | 11.6 |
| CHRDL1    | chordin-like 1 | 9.2 | 23.6 |
| MSC       | musculin | 11.7 | 8.4 |
| DCN       | decorin | 9.0 | 9.4 |

### 4. Discussion

The present study demonstrated that neonatal closing DAs exhibited prominent IT and sparse elastic fiber formation, which are typical human DA characteristics. Postnatal closing DA tissues had abundant expression of cardiac neural crest-related protein jagged 1 and the differentiated smooth muscle marker calponin compared to the patent DA tissue. On the other hand, the patent DA tissue had a distinct morphology (e.g., aorta-like elastic lamellae and a poorly formed IT) and gene profiles, such as second heart field-related genes, compared to the closing DA tissues.

The DA is originally derived from the sixth left aortic artery and has a unique cell-lineage [46]. SMCs of the DA are derived from cardiac neural crest cells [35,47,48] and the DA endothelial cells (ECs) are from second heart field [48,49], while both SMCs and ECs of the adjacent pulmonary artery are derived from second heart field [48,49]. In the ascending aorta, ECs are derived from second heart field, and SMCs of the inner medial layer and outer layer are derived from neural crest cells and second heart field, respectively [47,48]. The heatmap of transcriptome data indicated that these cell lineage-related genes were differentially expressed between the closing DA tissues and the patent DA tissue. Although it was difficult to clearly classify these genes into each lineage due to some overlap, cardiac
neural crest-related genes such as JAG1 were highly expressed in the closing DA tissues. In contrast, second heart field-related genes, such as ISL1, were enriched in the patent DA tissue.

The DA has been reported to have differentiated SMCs compared to the adjacent great arteries [1,50]. Slomp et al. demonstrated high levels of calponin expression in the tunica media of the fetal human DA [1]. Similarly, Kim et al. reported the presence of highly differentiated SMCs in the fetal rabbit DA, according to SM2 expression [50]. These differentiated SMCs have a high contractile apparatus, which makes them compatible with the postnatal potent DA contraction [1,50]. Additionally, several mutant mice with the patent DA had less differentiated SMCs [35–37]. Ivey et al. reported that mice lacking Tfap2β, which is a neural crest-enriched transcription factor, had decreased expression of calponin on embryonic day 18.5 [36]. Huang et al. utilized mice that harbored a neural crest-restricted deletion of the myocardin gene and demonstrated that decreased SMC contractile proteins were present on embryonic day 16.5 [35]. In addition, SMC-specific Jag1-deficient mice had a limited expression of SMC contractile proteins, even at postnatal day 0 [37]. The transcriptome data of the human DA tissues in the present study exhibited decreased protein levels of Jagged 1 and calponin in the patent DA tissue compared to the closing DA tissues. These altered SMC differentiation markers may contribute to postnatal DA patency in humans.

Prematurity and several genetic syndromes are reported to increase the incidence of the patent DA [51], and the patent DA can be classified into three groups, i.e., (1) patent DA in preterm infants, (2) patent DA as a part of a clinical syndrome, and (3) non-syndromic patent DA. This study included only one case with patent DA who had heterotaxy syndrome (polysplenia), which is a major study limitation. Indeed, in the present study, the expression of Nodal, which plays a primary role in the determination of left–right asymmetry, was positively correlated to the closing DAs compared to the patent DA with heterotaxy (Figure 3B). Therefore, it was not able to conclude that differentially expressed genes between in the closing DAs and the patent DA were associated with DA patency, but not with heterotaxy.

There are several syndromes (mutated genes) associated with the patent DA, such as Cantú (ABCC9), Char (TFAP2B), DiGeorge (TBX1), Holt-Oram (TBX5), and Rubinstein–Taybi (CREBBP) syndromes [52–54]. In addition to these syndromes, heterotaxy syndrome was reported to have a higher incidence of patent DA [55]. Notch signaling pathways have been reported to play a role in the establishment of left–right asymmetry via regulating Nodal expression [56,57]. Mutant for the Notch ligand Dll1 or double mutants for Notch1 and Notch2 exhibited defects in left–right asymmetry [56,57]. Dll1-null mutants die at early embryonic days due to severe hemorrhages [58], and it is not yet elucidated whether this Dll1-mediated Notch signaling is involved in the pathogenesis of patent DA. It has been reported that combined SMC-specific deletion of Notch2 and heterozygous deletion of Notch3 in mice showed the patent DA, but not heterotaxy [59]. In this study, we delineated the low levels of Jag1, which is a Notch ligand, in the patent DA with heterotaxy syndrome. Mice with Jag1-null mutant are early embryonic lethal due to hemorrhage [60], and SMC-specific Jag1-deleted mice are postnatal lethal due to patent DA [37]. These Jag1 mutants were not reported to exhibit heterotaxy. In humans, there is no obvious relationship between Alagille syndrome (JAG1) and patent DA or heterotaxy [61,62]. In mice, phenotypes of patent DA or heterotaxy seem to depend on ligands and isoforms of receptors of Notch signaling. In addition, there are differences in phenotypes caused by genetic mutations between in mice and humans. Analysis of non-syndromic patent DA would provide further insights into molecular mechanisms of closing and patency of the human DA.

Yarboro et al. performed RNA sequencing to determine genes that were differentially expressed in the preterm human DA and aorta at 21 weeks gestation [18], which was a much earlier time point than we used in our study. They found that several previously recognized DA-dominant genes in rodent studies [11,14,15,17] (e.g., ABCC9, PTGER4, and TFAP2B) were also upregulated in the preterm human DA tissues compared to the
aorta [18]. Some DA-dominant genes (e.g., ERG1 and SFRP1) in the preterm human DA [18] were upregulated in the closing DAs compared to the patent DA in the present study. In addition, some aorta-dominant genes, including ALX1 [18], were upregulated in the patent DA compared to the closing DA, which might partly support the aortification phenotype of the patent DA. Jag1 was reported to be the term DA dominant gene rather than the preterm DA dominant gene in rats [10], suggesting that Jag1 contributes to normal DA development. In addition to these previously reported genes, the gene profiles in our study potentially provide novel candidate genes (e.g., APLN and LTBP3) that may contribute to vascular SMC development and function [27,34]. Further study is needed to understand the roles of these genes in DA development.

Mueller et al. performed DNA microarray analysis using postnatal human DAs [19]. Their DA samples were composed of two stent-implanted DAs, one ligamentous DA, and one un-stented open DA [19]. We compared our data to their un-stented open DA dominant genes; however, we could not find obvious overlapped genes among them. One possible reason is that Mueller et al. compared the un-stented open DA on postnatal day 1 to the stented DAs on postnatal days 222 and 239. The stent implantation and time-course of sampling might affect the gene expressions.

This study elucidated the transcriptomic difference between the closing DA tissues and the patent DA tissue in humans. However, one of the limitations in this study pertains to the different durations of PGE1 administration. In utero, the DA is dilated by prostaglandin E2 (PGE2), which is mainly derived from the placenta [63]. After birth, the loss of the placenta and the increased flow of the lung, which is the major site of PGE catabolism, cause a decline in circulating PGE2 [64]. This decline in PGE2 contributes to a postnatal DA contraction [64]. We previously reported PGE2-induced structural DA remodeling via the prostaglandin receptor EP4 (e.g., IT formation [4,5,22] and attenuation of elastic laminae in the tunica media [6]). In humans, Mitani et al. reported that lipo-PGE1 administration increased IT formation in the DA [65]. Gittenberger-de Groot et al. reported that PGE1 treatment induced histopathologic changes (e.g., edema) in the human DA [66]. In this study, Case 4 who received the longest PGE1 administration, for 98 days, had prominent IT formation and less visible layered elastic fibers. This study demonstrated that the duration of PGE1 treatment affected gene expressions such as cell-cycle process-related genes. On the basis of these findings, postnatal PGE1 administration was thought to influence not only structural changes but also gene expression in the postnatal DA.

In 1977, Gittenberger-de Groot et al. performed histological analysis of 42 specimens of postnatal human DAs ranging in age from 12 h after premature delivery to 32 years [9]. An abnormal wall structure of the DA was found in all 14 patients that were over 4 months of age, and the most prominent feature was an aberrant distribution of elastic material, such as unfragmented subendothelial elastic lamina [9]. Three of the 14 patients also showed countable elastic laminae in the tunica media, namely, an aortification [9]. The histological finding of patent DA (Case 1) in the present study was consistent with this aortification type, showing aberrant distribution of elastic materials.

As mentioned above, a major limitation of this study is the use of only one sample of the patent DA, which could not represent the whole entity of patent DA. It is difficult to obtain large numbers of samples with a variety of different congenital heart diseases because isolation of the DA is possible only in the case of a limited number of surgical procedures (e.g., aortic arch repair). However, transcriptome comparisons of different types of patent DA tissues would be more informative to elucidate the pathogenesis of the human patent DA.

5. Conclusions

Transcriptome analysis using the IT and the tunica media of human DA tissue revealed different gene profiles between the patent DA and the closing DA tissues. Cardiac neural crest-related genes such as JAG1 were highly expressed in the tunica media and IT of the closing DA tissues compared to the patent DA. Second heart field-related genes, such as
ISL1, were enriched in the patent DA. The data from this study indicate that patent DA tissue may have different cell lineages from closing DA tissue.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/jcdd8040045/s1: Figure S1. Differential gene expression between the longer-term PGE1-treated human ductus arteriosus (DA) tissue (Case 4) and the shorter-term PGE1-treated DA tissues (Cases 2 and 3); Table S1. Gene Ontology biological process terms (size > 300) that were significantly upregulated (FDR < 0.25) in the outer part of long-term PGE1-treated human ductus arteriosus (DA) tissue (Case 4) compared to the tunica media of short-term PGE1-treated DA tissues (Case 2 and Case 3); Table S2. Twenty genes that overlapped and were enriched (>8-fold) in the outer part of a longer-term PGE1-treated DA tissue (Case 4) compared to the IT of shorter-term PGE1-treated DA tissues (Cases 2 and 3).

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