Introduction

A wide variety of vascular biology studies in the past several decades have verified that the vascular endothelium plays a critical role in the homeostasis of the cardiovascular system. Endothelial cells (ECs) lining blood vessels form monolayers to cover the inner lumens of all types of the vessels in the normal state. The vascular endothelium regulates vasoactive responses via secretion of nitric oxide and maintains the vascular tone by secretion of reparative factors such as tissue plasminogen activator. The endothelium also mediates leukocyte trafficking and monocyte activation to control platelet adhesion and coagulation. In addition, it interacts with vascular smooth muscle to mediate proliferation and differentiation of smooth muscle cells (SMCs). ECs respond diversely to a variety of external or internal stimuli to alter membrane permeability, transcellular transport systems, membrane adhesive molecules, various growth factor secretions, and so on [1,2]. These responses occur in order to fulfill the needs of tissues and maintain the homeostasis of the circulatory system. Endothelial phenotypic heterogeneity also plays an important role in the remodeling of the cardiovascular system where specific ECs are localized [3,4].

The ductus arteriosus (DA), a fetal shunt artery between the pulmonary artery and the aorta, closes promptly after birth, although its connecting arteries remain open. The DA exhibits sensitivity to changes in circulating oxygen concentration and prostaglandin E2 [5–7]. Since the changes in circulating oxygen concentration and prostaglandin E2 directly transduce the intravascular lumen where ECs surround its surface, ECs of the DA must play an important role in regulating these distinct characteristics. Accordingly, several studies have demonstrated the endothelium-dependent or independent vasoreaction of the DA [8–11]. Nonetheless, the majority of previous studies investigating molecular events in the DA utilized the whole DA tissue or cultured SMCs. Therefore, the role of DA ECs remains largely unknown. We hypothesized that the ECs of the DA exhibit a unique gene profile involved in DA-specific functional and morphologic characteristics.
specific vasoconstriction and vascular remodeling. Recently, Weber et al. reported that they successfully isolated ECs from fetal rat DA using the immunomagnetic cell separation method and harvested the isolated ECs to further confirm their purity by flow cytometry analysis [12]. In the present study, we investigated gene expression differences in ECs between the DA and the aorta by using a combination of fluorescence-activated cell sorter (FACS) and DNA microarray experiments followed by further enrichment analysis using MetaCore GeneGo software.

Materials and Methods

Antibodies

FITC-conjugated anti-CD31 antibody was obtained from Abcam (Cambridge, MA, USA). APC/Cy7-conjugated anti-CD45, FITC-conjugated anti-control IgG, and APC/Cy7-conjugated anti-control IgG antibodies were obtained from Biolegend (San Diego, CA, USA).

Animals

Timed-pregnant Wistar rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Rat fetuses at the 21st day of gestation as full-term were divided into two groups: fetuses before breathing (F group) and neonates obtained 30 minutes after breathing (N group). Animals in both groups were delivered by cesarean section. All animals were cared for in compliance with the American Physiological Society. The experiments were approved by the Ethical Committee on Animal Experiments of Waseda University.

Fluorescence Activated Cell Sorter (FACS)

Pooled tissues from the DA or the aorta were obtained from three litters of timed-pregnant Wistar rats, which accounted for approximately thirty fetuses. Tissues were treated with collagenase-dispase enzyme mixture as described previously [13]. Approximately 1.0 x 10^6 cells were obtained from combined whole DA tissues from the three litters. These cells were reacted with FITC-conjugated anti-CD31 and APC/Cy7-conjugated anti-CD45 antibodies as cell surface markers for EC and hematopoietic derivation cells, respectively. In order to confirm the nonspecific binding of antibodies to cells, we also prepared the cells reacted with CD45 antibodies as cell surface markers for EC and hematopoietic derivation cells, respectively. In order to confirm the nonspecific binding of antibodies to cells, we also prepared the cells reacted with CD45, FITC-conjugated anti-control IgG, and APC/Cy7-conjugated anti-control IgG antibodies were obtained from Biolegend (San Diego, CA, USA).

Quantitative real-time PCR (qRT-PCR)

The cell suspensions sorted by FACS were centrifuged at 300 x g for 15 minutes, and the precipitation was quickly frozen in liquid nitrogen. Total RNA was extracted from the collected cells using an RNeasy micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). For quantitative RT-PCR analysis, sequences for PCR primers are listed in Table 1. qRT-PCR was performed using a Step One Real-time PCR System (Applied Biosystems) with Fast SYBR Green Master Mix (Applied Biosystems). The abundance of each gene was determined relative to an internal control using 18S rRNA. For each qRT-PCR experiment, which included an RT-negative control, we confirmed there was no non-specific amplification in any reaction.

DNA microarray procedure

We repeated FACS sorting ten times for each developmental group (30 liters used in total) in order to accumulate enough total RNA (~100 ng). Then, cDNA was generated using the WT Expression Kit (Ambion, Austin, TX, USA) in accordance with the manufacturer's protocol. Briefly, a total of 100 ng of total RNA was reverse-transcribed to cDNA, which was subsequently used as a template for an in vitro transcription reaction. Sense-strand cDNA that contains dUTP was synthesized by amplified cRNA. We used the Affymetrix GeneChip® WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA) to recognize the dUTP and to fragment the cDNA with uracil-DNA glycosylase.

Table 1. List of primer sequences used for quantitative RT-PCR.

| Gene         | NCBI Accession No. | Primer sequence (forward) | Reverse sequence |
|--------------|--------------------|----------------------------|-----------------|
| Tie2         | NM_001105737.1     | GAGACAGTGCTCACAACAAAAAT    | CATCCCCAAAGTGAAGGCTCA |
| β-actin      | NM_012893.1        | ATGGTTGACAGGACAGGAGGAGAG  | GGTCTTAATGATGCTGAGTGGGA |
| Ednra        | NM_012550.2        | CACGCGCAGCTCCGAGCGAGCTTGG | GAGAGCCAGCCAGCAGGCTGAGC |
| Scl38a1      | NM_138832.1        | GTCCTGCAATCTACAGGAGCA      | GTACCCAAAGTAGGGCTCA |
| Lrat         | NM_0679408.1       | CAGCCGAGGAGGAACTTCAAGGGAA | CATACCACAGACTGACGAGGGG |
| 18S rRNA     | NR_003720.3        | AGCCCTGAGAGGAGGCTACC       | TCCCCAGTAGCAAACCTGAGAG |
| GAPDH        | NM_017008.4        | AGTTGGTGGTTAGAAGGATTTTG    | TGTTAGACCTGATGGAAGGGTC |

*The mRNA descriptions are listed below:

Tie2: TEK tyrosine kinase, endothelial; β-actin: actin, gamma 2, smooth muscle, enteric; Ednra: endothelin receptor type A; Scl38a1: solute carrier family 38, member 1; Lrat: lecithin-retinol acyltransferase; 18S: 18S ribosomal RNA; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

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Figure 1. **CD31+ cells successfully divided from whole tissue by FACS.** A) Population of cells reacted with FITC-conjugated anti-CD31 antibody (CD31) and APC/Cy7-conjugated anti-CD45 antibody (CD45). CD31⁺/CD45⁻: consisting mainly of SMCs, CD31⁺/CD45⁺: consisting entirely of ECs. B) Population of cells reacted with fluorescence conjugated anti-control IgG antibodies in order to confirm the nonspecific binding of antibodies.

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Figure 2. **Obvious differences in gene expression between sorted ECs and SMCs.** A) The expression levels of Tie2 mRNA were significantly higher in ECs than in SMCs. (*p<0.05, n = 5) B) The expression levels of β2-actin mRNA were significantly lower in ECs than in SMCs. (**p<0.001, n = 5) C) The expression levels of Ednra mRNA were significantly lower in ECs than in SMCs. (***p<0.001, n = 3).

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(UDG) and apurinic/apyrimidinic endonuclease 1 (APE1). These fragmented cDNAs were then labeled through a terminal deoxytransferase reaction and hybridized to the Affymetrix GeneChip® Rat Gene 1.0 ST Array (Affymetrix). The hybridization experiments were performed in triplicate (approximately 180 litters were needed in total), and the intensities were averaged.

### Microarray data analysis

Of the 26,469 genes on the microarray, 14,944 were excluded based on aberrant low signals as determined by the poly-A spike of "lys" (probe set ID: 10700066) expression, the smallest composition out of the poly-A RNA control cocktail, which was added in each total RNA sample. All remaining gene probes were analyzed for their differential expression between the DA and the aorta at each developmental stage. Initially, we calculated the p value by Student’s t-test across each group, and the data were cut off at

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**Figure 3. Color scale table imitating heat maps of DA dominant genes and Ao dominant genes.** The listed genes in A) and B) are the same as in Table 2 and Table 3, respectively. The color scale is based on their expression intensities. The green or red color indicates the lowest or the highest expression levels, respectively. The midpoint shown as a dark color represents 235 since it is the average of whole gene expression. doi:10.1371/journal.pone.0073685.g003
Table 2. DA endothelium-dominant genes.

| Probe set ID | Description                                      | Gene Symbol | Fold change (DA/Ao) |
|--------------|--------------------------------------------------|-------------|---------------------|
| 10906592     | solute carrier family 38, member 1               | Slc38a1     | 7.31 6.81           |
| 10932759     | calpain 6                                        | Capn6       | 8.18 6.04           |
| 10823949     | lecithin-retinol acyltransferase                 | Lrat        | 6.98 5.15           |
| 10875375     | carbonic anhydrase 8                             | Car8        | 5.88 4.94           |
| 10925936     | erythrocyte protein band 4.1-like 3              | Epb4.1L3    | 5.05 4.27           |
| 10764702     | similar to glycosyltransferase 25 domain containing 2 | Glk25d2   | 3.90 4.18           |
| 10903177     | G protein-coupled receptor 182                   | Gpr182      | 4.12 4.12           |
| 10712171     | interferon induced transmembrane protein 1       | Ifitm1      | 4.89 3.99           |
| 10867593     | growth differentiation factor 6                  | Gdf6        | 4.82 3.89           |
| 10752295     | T-box 1                                          | Tbx1        | 4.69 3.80           |
| 10932726     | transient receptor potential cation channel, subfamily C, member 5 | Trpc5 | 4.73 3.78 |
| 10869158     | similar to zinc finger protein 462               | Zfp462      | 4.52 3.53           |
| 10840076     | prion protein                                    | Prnp        | 3.70 3.52           |
| 10826561     | similar to N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3 | Ndst3 | 3.69 3.36 |
| 10749983     | coxsackie virus and adenovirus receptor          | Cxadr       | 3.65 3.36           |
| 10937327     | similar to zinc finger, CCHC domain containing 5  | Zcchc16     | 3.63 3.32           |
| 10783648     | solute carrier family 7, member B                | Slc7a8      | 3.95 3.25           |
| 10776676     | shisa homolog 3 (Xenopus laevis)                 | Shisa3      | 3.76 3.16           |
| 10815785     | pentraxin related gene                           | Ptx3        | 4.28 3.14           |
| 10829418     | poly(C) binding protein 3                        | Pcbp3       | 3.01 3.10           |
| 10714323     | aldehyde dehydrogenase 1 family, member A1       | Aldh1a1     | 2.19 3.08           |
| 10924824     | SP100 nuclear antigen                            | Spi100      | 2.83 3.00           |
| 10863549     | actin, gamma 2, smooth muscle, enteric            | Actg2       | 2.76 2.96           |
| 10852378     | GATA binding protein 5                           | Gata5       | 3.60 2.91           |
| 10931308     | prolyl 4-hydroxylase, alpha polypeptide 1        | P4ha1       | 2.86 2.89           |
| 10804750     | similar to Actin-binding LIM protein 3           | Ablim3      | 3.15 2.88           |
| 10902420     | leucine rich repeat containing G protein coupled receptor 5 | Lgr5 | 3.49 2.84 |
| 10862554     | 31 kDa protein                                   | Hoxa4       | 2.42 2.77           |
| 10767388     | Cd55 molecule                                    | Cd55        | 2.75 2.76           |
| 10868627     | similar to GLI pathogenesis-related 2            | Glipr2      | 2.87 2.75           |
| 10934173     | ephrin B1                                        | Efnb1       | 2.35 2.66           |
| 10876507     | similar to F-box only protein 10                  | Fbobox10     | 2.56 2.65           |
| 10935038     | brain expressed gene 4                           | Bex4        | 2.2 2.62            |
| 10837351     | solute carrier family 43, member 1               | Slc43a1     | 2.52 2.60           |
| 10707862     | similar to ADAM metallopeptidase with thrombospondin type 1 motif, 17 preproprotein | Adams17 | 2.61 2.60 |
| 10880095     | serine incorporator 2                            | Serinc2     | 2.87 2.56           |
| 10838117     | peptidase domain containing associated with muscle regeneration 1 | Paml1  | 2.95 2.55 |
| 10886162     | transmembrane protein 63c                        | Tmem63c     | 3.49 2.53           |
| 10745095     | aldolase C, fructose-bisphosphatase               | Aldoc       | 2.22 2.51           |
| 10833152     | cysteine and glycine-rich protein 2              | Cspr2       | 2.31 2.48           |
| 10739927     | C1q and tumor necrosis factor related protein 1  | C1qtn1f1    | 2.15 2.48           |
| 10858499     | microfibrillar associated protein 5               | Mfap5       | 2.42 2.46           |
| 10792421     | plasmoglobin activator, tissue                   | Plat        | 2.41 2.46           |
| 10853819     | met proto-oncogene                               | Met         | 2.17 2.44           |
| 10791504     | heart and neural crest derivatives expressed 2   | Hand2       | 2.52 2.42           |
| 10713857     | fatty acid desaturase 1                          | Fads1       | 2.46 2.38           |
| 10899023     | calcium channel, voltage-dependent, beta 3 subunit | Cacnb3 | 2.67 2.38 |
| 10863777     | anthrax toxin receptor 1                         | Antx1       | 2.70 2.37           |
| Probe set ID | Description | Symbol | F | N |
|-------------|-------------|--------|---|---|
| 10813172    | fibroblast growth factor 10 | Fgf10 | 2.58 | 2.37 |
| 10939764    | glypican 3 | Gpc3 | 2.04 | 2.36 |
| 10766082    | kinesin family member 26B | Kif26b | 2.65 | 2.35 |
| 10896751    | metastasis suppressor 1 | Mts1 | 2.27 | 2.35 |
| 10921772    | vascular endothelial growth factor A, transcript variant 1 | Vegfa | 2.45 | 2.34 |
| 10732358    | ADAM metallopeptidase with thrombospondin type 1 motif, 2 | Adamts2 | 2.40 | 2.30 |
| 10910473    | hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 | Hcn4 | 2.81 | 2.29 |
| 10751190    | zinc finger, DHHC-type containing 23 | Zdhhc23 | 2.96 | 2.24 |
| 10772332    | CD38 molecule | Cd38 | 2.34 | 2.21 |
| 10919175    | T-box18 | Tbx18 | 2.23 | 2.21 |
| 10803323    | cadherin 2 | Cdh2 | 2.28 | 2.20 |
| 10939725    | similar to Heparan-sulfate 6-O-sulfotransferase 2 | Hs6st2 | 2.44 | 2.19 |
| 10922964    | similar to esophageal cancer related gene 4 protein | RGD1305645 | 2.60 | 2.17 |
| 10770577    | transforming growth factor, beta 2 | Tgfβ2 | 2.50 | 2.17 |
| 10875588    | solute carrier family 26, member 7 | Slc26a7 | 2.18 | 2.17 |
| 10734242    | microfibrillar-associated protein 4 | Mfap4 | 2.51 | 2.15 |
| 10889263    | tribbles homolog 2 (Drosophila) | Trib2 | 2.55 | 2.11 |
| 10767077    | GLI family zinc finger 2 | Gli2 | 2.29 | 2.11 |
| 10921428    | similar to inhibitor of MyoD family-a | RGD1560271 | 2.34 | 2.10 |
| 10819269    | solute carrier family 39 (zinc transporter), member 8 | Slc39a8 | 2.23 | 2.08 |
| 10784579    | scavenger receptor class A, member 3 | ScarA3 | 2.04 | 2.05 |
| 10849327    | fibrillin 1 | Fbn1 | 2.15 | 2.04 |
| 10729667    | dickkopf homolog 1 (Xenopus laevis) | Dkk1 | 2.32 | 2.02 |
| 10767597    | similar to transmembrane and coiled-coil domains 2 | Tmcc2 | 1.54 | 2.48 |
| 10888610    | similar to limb-bud and heart | LOC683626 | 1.71 | 2.24 |
| 10818989    | paired-like homeodomain 2, transcript variant 2 | Pitx2 | 1.96 | 2.14 |
| 10917034    | transgelin | Tagln | 1.95 | 2.13 |
| 10708399    | similar to ring finger and KH domain containing 3 | Mex3b | 1.68 | 2.12 |
| 10846013    | transmembrane protein 90B | Tmemb90b | 1.85 | 2.11 |
| 10807601    | syntrophin, beta 2 | Snrb2 | 1.93 | 2.02 |
| 10862547    | homeo box A2 | Hoxa2 | 1.76 | 2.01 |
| 10898022    | cold shock domain containing C2, RNA binding | Csd2 | 1.97 | 2.00 |
| 10802375    | phorbol-12-myristate-13-acetate-induced protein 1 | Pmaip1 | 1.99 | 2.00 |
| 10849700    | mal, T-cell differentiation protein | Mal | 2.71 | 1.51 |
| 10754454    | semaphorin 5B | Sema5b | 2.44 | 1.93 |
| 10862541    | homeo box A1 | Hoxa1 | 2.44 | 1.95 |
| 10717233    | connective tissue growth factor | Ctgf | 2.19 | 1.29 |
| 10903979    | similar to breast cancer membrane protein 101 isoform 1 | Fam84b | 2.15 | 1.51 |
| 1077242     | bone marrow stromal cell antigen 1 | Bst1 | 2.12 | 1.68 |
| 10899405    | Keratin, type II cytoskeletal 7 | Krt7 | 2.09 | 1.66 |
| 10848165    | cholinergic receptor, muscarinic 5 | Chrm5 | 2.09 | 1.89 |
| 10871043    | similar to C05G5S5 | LOC689914 | 2.07 | 1.89 |
| 10821486    | ISL LIM homeobox 1 | Isl1 | 2.05 | 1.93 |
| 10878845    | microtubule associated serine/threonine kinase 2 | Mast2 | 2.02 | 1.93 |

Eighty two genes in the F group and 81 genes in the N group were expressed more than 2-fold in ECs of the DA than in ECs of the aorta ($p<0.05$). Among these DA dominant genes, 71 genes were expressed more than 2-fold in ECs of the DA in both groups (above the thick line). F: fetuses before breathing; N: neonates obtained 30 minutes after breathing.

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### Table 3. Aorta endothelium-dominant genes.

| Probe set ID | mRNA Description | Gene Symbol | Fold change (DA/Ao) F | N |
|--------------|------------------|-------------|-----------------------|---|
| 10722992     | alanyl (membrane) aminopeptidase | Anpep | 0.16 | 0.16 |
| 10716939     | similar to G protein-coupled receptor 126 | Gpr126 | 0.15 | 0.19 |
| 10816144     | secreted frizzled-related protein 2 | Sfrp2 | 0.25 | 0.25 |
| 10810778     | dipeptidase 2 | Dpep2 | 0.25 | 0.28 |
| 10730266     | NK2 transcription factor related, locus 3 (Drosophila) | Nkx2-3 | 0.33 | 0.28 |
| 10731622     | similar to MGC45438 protein | RGD1565166 | 0.26 | 0.28 |
| 10810631     | tubulin polymerization-promoting protein family member 3 | Tppp3 | 0.26 | 0.29 |
| 10787517     | growth differentiation factor 15 | Gdf15 | 0.32 | 0.31 |
| 10896020     | syndecan 2 | Sdc2 | 0.31 | 0.33 |
| 10769370     | flavin containing monoxygenase 2 | Fmo2 | 0.35 | 0.34 |
| 10826249     | vascular cell adhesion molecule 1 | Vcam1 | 0.35 | 0.35 |
| 10768269     | complement factor H | Cfh | 0.34 | 0.35 |
| 10903816     | syntrophin, beta 1 | Sntb1 | 0.27 | 0.36 |
| 10801683     | proline rich 16 | Prr16 | 0.42 | 0.36 |
| 10769476     | ATPase, Na+/K+ transporting, beta 1 polypeptide | Atp1b1 | 0.41 | 0.37 |
| 10801761     | similar to PR-domain zinc finger protein 6 | Pdm6 | 0.34 | 0.37 |
| 10744939     | serine (or cysteine) peptidase inhibitor, clade F, member 1 | Serpinf1 | 0.37 | 0.38 |
| 10859799     | interleukin 6 | Il6 | 0.27 | 0.39 |
| 10892352     | similar to Jagged-2 precursor | Jag2 | 0.44 | 0.40 |
| 10800696     | LIM and senescent cell antigen like domains 2 | Lim3 | 0.40 | 0.41 |
| 10883686     | neurexin 1 | Nrnx1 | 0.37 | 0.41 |
| 10916228     | neurogranin | Ngrn | 0.40 | 0.42 |
| 10932211     | monoamine oxidase B, nuclear gene encoding mitochondrial protein | Mabo | 0.35 | 0.42 |
| 10808274     | cadherin 13 | Cdh13 | 0.38 | 0.42 |
| 10738676     | formin-like 1 | Fmnl1 | 0.42 | 0.42 |
| 10863068     | cytochrome P450, family 26, subfamily b, polypeptide 1 | Cyp26b1 | 0.46 | 0.42 |
| 10785846     | ATP-binding cassette, sub-family C (CFTR/MRP), member 4 | Abcc4 | 0.41 | 0.44 |
| 10892330     | similar to AHNAK nucleoprotein isoform 1 | Ahnak2 | 0.38 | 0.45 |
| 10837310     | similar to KIAA1946 | Faml171b | 0.37 | 0.45 |
| 10896405     | polycystic kidney and hepatic disease 1-like 1 | Pkhd1l1 | 0.40 | 0.45 |
| 10791552     | glycoprotein m6a | Gmp6a | 0.46 | 0.45 |
| 10764862     | angiprotein-1 like 1 | Angpt1 | 0.37 | 0.46 |
| 10782454     | thyroid hormone receptor beta | Trh | 0.48 | 0.46 |
| 10822007     | PDZ domain containing 2 | Pdzd2 | 0.46 | 0.47 |
| 10726371     | similar to ADAM 12 precursor | Adam12 | 0.50 | 0.48 |
| 10811956     | signal-induced proliferation-associated 1 like 2 | Sipa1l2 | 0.41 | 0.48 |
| 10739364     | somatostatin receptor 2 | Sstr2 | 0.47 | 0.48 |
| 10790939     | similar to KIAA1683 | LOC306346 | 0.49 | 0.48 |
| 10797499     | receptor tyrosine kinase-like orphan receptor 2 | Ror2 | 0.48 | 0.49 |
| 10704840     | similar to protein 7 transactivated by hepatitis B virus X antigen | LOC686809 | 0.47 | 0.49 |
| 10805996     | plasma membrane proteolipid (plasmolipin) | Plp | 0.45 | 0.49 |
| 10776608     | similar to Probable phospholipid-transporting ATPase VD | Atpl1d | 0.45 | 0.5 |
| 10859886     | dipeptidylpeptidase 6 | Dpp6 | 0.48 | 0.50 |
| 10724315     | hemoglobin, beta | Hb | 1.21 | 0.44 |
| 10896028     | plasma glutamate carboxypeptidase | Pgc | 0.53 | 0.47 |
| 10771655     | chemokine (C-X-C motif) ligand 10 | Cxcl10 | 0.61 | 0.48 |
| 10737730     | homeo box B3 | Hoxb3 | 0.64 | 0.48 |
| 10845767     | Cobl-like 1 | Cobl1 | 0.54 | 0.49 |
For a more robust differential analysis between the DA and the aorta, we selected the genes that had more than a 2.0-fold change (\(|FC|\geq 2.0\)). Genes that went through these analyses were considered significant. Genes were further analyzed for enriched biological themes and pathways using the MetaCore program (GeneGo, a division of Thomson Reuters, St. Joseph, MI, USA). The program ranked the significant ontology and pathways dominant in DA ECs in each developmental stage by importing whole expression results (excluding aberrantly low expressed genes) from the microarray. MetaCore is an established program that includes a manually annotated database of gene interactions and metabolic reactions obtained from scientific literature. The enrichment analysis of the biological process was based on the hypergeometric distribution algorithm and relevant pathway maps were then prioritized according to their statistical significance [14]. The complete data set of the DNA microarray is available in the GEO database (accession number: GSE40500).

Statistical treatment

Data are presented as mean ± standard error (SEM) or independent experiments. Statistical analyses were performed between two groups by unpaired two-tailed \(t\) test or unpaired \(t\) test with Welch correction, and among multiple groups by one-way analysis of variance (ANOVA) followed by Neuman-Keuls multiple comparison test. A \(p\) value of <0.05 was considered significant.

**Results**

**Endothelial cells were purely isolated from rat DA tissues**

At least 10,000 of the cells (approximately 1% of the initially isolated cells) were sorted in anti-CD31 positive and anti-CD45 negative areas from the pooled DA tissues of three litters of timed-pregnant Wistar rats (Figure 1A). No cell in the CD31\(^+\)/CD45\(^-\) cells reacted with an anti-IgG antibody (Figure 1B), indicating that no false positive cells were contained in the CD31\(^+\)/CD45\(^-\) cells believed to be ECs. We also assumed that CD31\(^-\)/CD45\(^-\) cells mainly consisted of SMCs. The detailed gating strategies of FACS sorting are shown in Figure S1. To confirm the purity of FACS isolation, we examined the expression levels of EC-specific and SMC-specific genes by qRT-PCR. The expression levels of Tie2 mRNA, an EC-specific gene, were significantly higher in CD31\(^+\)/CD45\(^-\) cells than in CD31\(^-\)/CD45\(^+\) cells (\(p<0.05, n=5\)) (Figure 2A). The expression levels of

### Table 3. Cont.

| Probe set ID | mRNA Description | Gene Symbol | Fold change (DA/Ao) |
|-------------|------------------|-------------|--------------------|
| 1088616     | cDNA clone IMAGE:8372043. RGD1566401 | 0.5        | 0.49               |
| 10908328    | intercellular adhesion molecule 5, telencephalin Icam5 | 0.56 | 0.49               |
| 10876069    | aquaporin 3 Aqp3 | 0.51 | 0.50               |
| 1076646     | semaphorin 3G Sema3g | 0.56 | 0.50               |
| 10736520    | active BCR-related gene Abr | 0.46 | 0.54               |
| 10889560    | B-cell receptor-associated protein 29 Bcap29 | 0.47 | 0.63               |
| 10787757    | chondroitin sulfate N-acetylgalactosaminytransferase 1 Cgalact1 | 0.37 | 0.52               |
| 10834031    | dual specificity phosphatase 14 Dusp14 | 0.46 | 0.54               |
| 10926651    | ectonucleotide pyrophosphatase/phosphodiesterase 5 Enpp5 | 0.46 | 0.52               |
| 10855387    | GTPase, IMP family member 4 Gimap4 | 0.45 | 0.58               |
| 10853229    | guanine nucleotide binding protein (G protein), alpha inhibiting 1 Gna1 | 0.45 | 0.55               |
| 10761128    | heat shock protein 1 Hspb1 | 0.45 | 0.62               |
| 10833346    | cDNA clone MGC:188337 IMAGE:7453022 LOC100365935 | 0.42 | 0.66               |
| 10785523    | similar to Protocadherin 9 precursor isoform 3 Pcdh9 | 0.43 | 0.58               |
| 10768373    | phospholipase A2, group IVA (cytosolic, calcium-dependent) Pla2g4a | 0.50 | 0.63               |
| 10811347    | phospholipase C, gamma 2 Plcg2 | 0.49 | 0.51               |
| 10919554    | plasmin 1 (I isoform) Pls1 | 0.41 | 0.57               |
| 10910204    | proline-serine-threonine phosphatase-interacting protein 1 Ptstpip1 | 0.49 | 0.51               |
| 10764551    | prostaglandin-endoperoxide synthase 2 Plgs2 | 0.48 | 0.52               |
| 10934662    | riboflavin kinase Rfk | 0.48 | 0.56               |
| 10753629    | 126 kDa protein RGD1562717 | 0.49 | 0.55               |
| 10783537    | solute carrier family 7, member 7 Slc7a7 | 0.49 | 0.56               |
| 10902696    | similar to CG3996-PA Tbc1d30 | 0.50 | 0.63               |
| 10725253    | similar to Tmc7 protein Tmc7 | 0.49 | 0.51               |
| 10877532    | tumor necrosis factor (ligand) superfamily, member 15 Tnfsf15 | 0.48 | 0.54               |
| 10875363    | thymocyte selection-associated high mobility group box Tox | 0.48 | 0.51               |

Sixty-five genes in the F group and 52 genes in the N group were expressed more than 2-fold in ECs of the aorta than in ECs of the DA (\(p<0.05\)). Among these aorta dominant genes, 43 genes were expressed more than 2-fold in ECs of the aorta in both groups (above the thick line). F: fetuses before breathing; N: neonates obtained 30 minutes after breathing.

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2-actin and endothelin-1 receptor Ednra mRNAs, SMC-specific genes, were significantly lower in CD31^+/CD45^2 cells than in CD31^2/CD45^2 cells (p < 0.001, n = 3), respectively (Figures 2B and 2C). Therefore, we concluded that a FACS could isolate pure ECs in the CD31^+/CD45^2 area without contamination.

Identification of DA-specific genes in ECs

Among over 26,469 gene-level probe sets, we found that 82 genes in the F group and 81 genes in the N group were expressed more than 2-fold in ECs of the DA than in ECs of the aorta (p < 0.05) (Table 2, Figure 3A). Among these DA dominant genes, 71 genes were expressed more than 2-fold in ECs of the DA in both groups. On the other hand, 65 genes in the F group and 52 genes in the N group were expressed more than 2-fold in ECs of the aorta than in ECs of the DA (p < 0.05) (Table 3, Figure 3B). Among these aorta dominant genes, 43 genes were expressed more than 2-fold in ECs of the aorta in both groups. Importantly, the majority of the genes in Table 2 and 3 have never been reported previously as DA-related genes. We found only a limited number of the well-known endothelium-related genes such as transforming growth factor-beta 2 (Tgfb2) and vascular endothelial growth factor A (Vegfa). Validation of the results from the DNA microarray, qRT-PCR was performed with Slc38a1 and Lrat, the genes with the most significant difference between the DA and the aorta at both developmental stages (Figure S2).

Surprisingly, the present results showed remarkably low variations of transcription profiles before and after birth. Although there were 178 genes of which expression levels significantly differed between both developmental stages (p < 0.05), arrestin domain containing 3 (Arrdc3) and TBC1 domain family, member 30 (Tbc1d30) were the only two genes that had more than a 2.0-fold change (|FC| ≥ 2.0) between both developmental stages. Among 178 genes, Table 4 shows 25 genes of which the p values were less than 0.01. Among these 25 genes, Ctgf and Tbc1d30 are listed in Table 2 and Table 3, respectively. F: fetuses before breathing; N: neonates obtained 30 minutes after breathing.

Enrichment analysis of DA dominant genes using GeneGo MetaCore software

In the MetaCore systems, there are about 110 cellular and molecular processes whose content is defined and annotated by
GeneGo. The top 10 ranked regulatory biological processes were listed in each stage of the DA ECs based upon their p values (Table 5). Most of the categories indicate morphogenesis and development. Four processes (anatomical structure morphogenesis, cardiovascular system development, circulatory system development, and locomotion) are ranked in both the F and N groups. Interestingly, excluding processes related to morphogenesis and development, regulation of phosphatidylinositol dephosphorylation is an enriched process that is listed only in the F group. On the other hand, response to external stimulus, response to vitamin A stimulus, and axon guidance were listed only in the top 10 ranked biological processes in the N group. In these GeneGo biological processes, 322 and 172 genes were listed in the F and N groups, respectively. The genes included in each category are shown in Figure 4.

Figure 4. Color scale table imitating heat maps of the DA dominant genes categorized by GeneGo processes. DA dominant genes are identified using GO analysis (MetaCore). The whole expression data set was processed by importing it into the MetaCore system. The MetaCore system lined up the top 10 (based on p-value) sets of categorized genes according to their GO biological processes (Table 5). The color scale table imitating heat maps was created manually based on the genes in GO biological processes. A) The genes in all the top processes except the development or morphogenesis processes that emerged in both F and N. B) The genes categorized in the processes related to the development and morphogenesis in both F and N. C) The genes categorized only in cardiovascular or circulatory specific development processes. The color scale is the same as that used in Figure 3. All heat maps were created manually based on the genes in the GeneGo biological processes. a) The genes in all the processes in Table 4, except the development or morphogenesis processes which emerged in both F and N. b) The genes categorized in the processes related to development and morphogenesis in both F and N. c) The genes categorized only in cardiovascular or circulatory specific development processes.
appared in more than five processes of the top 10 ranking as active genes. These genes are likely to be involved in the network by potential interactions with many of the identified genes to form DA-specific endothelium.

Furthermore, there are over 1200 pathway maps in MetaCore, comprehensively covering signaling and metabolism, selected diseases and some drug targets mechanisms. All maps are accurately drawn by GeneGo annotators and manually curated and edited. The canonical pathway maps and GeneGo process networks, validated by statistical values, were evaluated by MetaCore and are listed in Table 7 together with the top 10 ranking for each pathway significantly worked in the DA ECs. As we found that the gene expression profiles exhibited remarkably low variations at both time points, nine of the top 10 ranked pathway maps were listed in both F and N groups. These categories are related to regulation of epithelial-to-mesenchymal transition (EMT), cell adhesion, and retinol metabolism.

**Discussion**

To date, the characteristic features of the DA endothelium have remained largely unknown. Several studies have demonstrated the endothelium-dependent or independent vasomotor reaction of the DA [8–11]. Rabinovitch et al. made great efforts to identify the role of the DA endothelium in vascular remodeling of the DA. They found that the increase in the expression of transforming growth factor-beta (Tgfb) in the DA endothelium promoted the synthesis of glycosaminoglycan such as hyaluronan that is a critical regulator of neointimal formation of the DA [15,16]. The present comprehensive gene expression analysis identified a DA endothelium-dominant rat gene profile during a perinatal period for the first time. It should be noted that we collected DA ECs from more than 30 litters to obtain a sufficient amount of mRNA for one sample. Additionally, the hybridization experiments were performed in triplicate and the intensities were averaged. To avoid an unexpected artificial bias, we did not use cultured ECs or any amplification method to increase mRNA from the endothelium. Therefore, the present study represents the transcription profile of the freshly isolated endothelium from the rat DA. Importantly, most of the genes that were expressed greater or lower in the DA endothelium than in the aortic endothelium have not yet been investigated in the DA. In addition to the up-regulated or down-regulated genes that met the 2-fold threshold in Table 2 and 3, one may be interested in genes that showed a statistical significance but a lower than 2.0 difference. We therefore also listed the genes with a statistical significance (p < 0.001) in Table S1 and Table S2. Since the endothelium plays a critical role not only in vascular tone but also in vascular remodeling, the newly identified genes should be of great interest for further investigation of the molecular mechanisms of DA-specific differentiation and function.

Although two studies, including our previous one, have identified DA-dominant genes using DNA microarray analysis [17,18], the present study revealed that the transcription profile of DA ECs are quite different from those of DA whole tissues of which the majority is composed of SMCs. These data suggest that the transcription profiles of the DA endothelium are tightly regulated in a cell-specific manner. Furthermore, local interaction between ECs and SMCs may contribute to establishing each unique transcription profile in the DA. It would be beneficial to further investigate how ECs and SMCs interact locally with each other.
To our surprise, the present study also demonstrated that the transcription profile of DA ECs did not change much before and after birth, although the DA does dramatically alter its morphology during the perinatal period. After birth, the change in oxygen and PGE2 content in circulating blood induces functional closure of the DA. Costa et al. also demonstrated that the transcription profile of DA tissues significantly differs before and after birth [18].

In their experiment, DA samples were collected 3 hours after spontaneous delivery. We used neonatal DA ECs 30 minutes after delivery by cesarean section, because we aimed to detect an initial change in the transcription profile of DA ECs after birth. This period, however, may not be long enough to investigate the alternation in its transcription profile, although functional closure of the rat DA had mostly occurred in our previous studies [19,20].

It is very important to investigate the roles of the newly identified genes in the morphology and function of the DA. Unfortunately, an in vitro experiment using rat DA ECs is technically very difficult because of the limited amount of tissue.

Table 6. Thirty overlapping genes that appeared in more than five processes of the top ten ranking as active genes.

| ID       | Gene Symbol | mRNA- Description                        | Number of overlapped processes |
|----------|-------------|------------------------------------------|-------------------------------|
|          |             |                                          | F    | N    |
| Receptor ligand                      |             |                                          |      |      |
| 10770577 | Tgfb2       | transforming growth factor, beta 2 (Tgfb2)| 9    | 9    |
| 10921772 | Vegfa       | vascular endothelial growth factor A (Vegfa), transcript variant 1 | 9    | 8    |
| 10813172 | Fgf10       | fibroblast growth factor 10 (Fgf10)      | 9    | 8    |
| 10934173 | Efnb1       | ephrin B1 (Efnb1)                        | 9    | 6    |
| 10717233 | Ctgf        | connective tissue growth factor (Ctgf)   | 9    | 0    |
| 10849327 | Fbn1        | fibrillin 1 (Fbn1)                       | 8    | 3    |
| Receptor                                      |             |                                          |      |      |
| 10749883 | Cxadr       | coxsackie virus and adenovirus receptor (Cxadr) | 7    | 5    |
| 10853819 | Met         | met proto-oncogene (Met)                 | 6    | 6    |
| 10848165 | Chrm5       | cholinergic receptor, muscarinic 5 (Chrm5)| 5    | 0    |
| Voltage-gated ion channel                   |             |                                          |      |      |
| 10899023 | Cacnb3      | calcium channel, voltage-dependent, beta 3 subunit (Cacnb3) | 6    | 6    |
| 10932726 | Trpc5       | transient receptor potential cation channel, subfamily C, member 5 (Trpc5) | 6    | 4    |
| Binding protein                             |             |                                          |      |      |
| 10863549 | Actg2       | actin, gamma 2, smooth muscle, enteric (Actg2) | 9    | 9    |
| 10803323 | Cdh2        | cadherin 2 (Cdh2)                        | 8    | 6    |
| 10939764 | Gpc3        | glypican 3 (Gpc3)                        | 8    | 0    |
| 10804750 | Ablim3      | actin-binding LIM protein 3 gene:ENSRNOG00000019365 | 6    | 4    |
| 10840076 | Prnp        | prion protein (Prnp)                     | 6    | 4    |
| 10766027 | Kif26b      | kinesin family member 26B (Kif26b)       | 6    | 2    |
| 10921428 | RGD1560271  | similar to inhibitor of MyoD family-a    | 6    | 1    |
| 10858499 | Mfap5       | microfibrillar associated protein 5 (Mfap5)| 5    | 1    |
| Transcriptional factor                     |             |                                          |      |      |
| 10767077 | Gli2        | GLI family zinc finger 2 (Gli2)          | 9    | 7    |
| 10752295 | Tbx1        | T-box 1 (Tbx1)                           | 9    | 7    |
| 10821486 | Isl1        | ISL LIM homeobox 1 (Isl1)                | 9    | 0    |
| 10791504 | Hand2       | heart and neural crest derivatives expressed 2 (Hand2) | 8    | 6    |
| 10919175 | Tbx18       | T-box18 (Tbx18)                          | 7    | 6    |
| 10862541 | Hoxa1       | homeo box A1 (Hoxa1)                     | 7    | 0    |
| 10862554 | Hoxa4       | homeo box A4 (Hoxa4)                     | 6    | 0    |
| 10818989 | Pitx2       | paired-like homeodomain 2 (Pitx2), transcript variant 2 | 0    | 8    |
| enzyme                                      |             |                                          |      |      |
| 10792421 | Plat        | plasminogen activator, tissue (Plat)     | 8    | 4    |
| 10714323 | Aldh1a1     | aldehyde dehydrogenase 1 family, member A1 (Aldh1a1) | 6    | 4    |
| 10745095 | Aldoc       | aldolase C, fructose-bisphosphatase (Aldoc) | 6    | 1    |

Thirty genes that frequently appeared in more than five processes of the top ten ranking in Table 5 are regarded as active genes. These genes are listed in accordance with their function. F: fetuses before breathing; N: neonates obtained 30 minutes after breathing.

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or cells obtainable from small animals. Currently, bioinformatic technology has developed to the point that it is now possible to attribute functions to genes and their encoded proteins, and to identify the regulatory networks controlling metabolic, protein synthesis and signal transduction pathways. To facilitate the analysis of experiments using post-genomic technologies, newly developed knowledge-based gene set enrichment analysis provides a powerful analytical method to link the vast amount of raw data to biological pathways [14,21]. Pathway analysis by MetaCore is based on the concept that the function of a gene depends directly on the context in which it acts, and MetaCore correlates genes identified by DNA microarray with the cellular pathways that are hypothetically activated dominantly in the DA endothelium. It has also been shown that Isl1, Fgf10-positive cells and the receptor ligand Fgf10 are enriched in the DA [28].

Among over 1200 pathways, the MetaCore systems defined the top 10 ranked pathways that were dominantly worked in each stage of the DA ECs based upon their p values. F: fetuses before breathing; N: neonates obtained 30 minutes after breathing. (p-value (DA vs Ao) Developmental stage

| GeneGo Pathway Maps | p-value (DA vs Ao) | Developmental stage |
|---------------------|--------------------|---------------------|
| Development_Regulation of epithelial-to-mesenchymal transition (EMT) | 7.52E-05 | F |
| Cell adhesion_Cadherin-mediated cell adhesion | 1.04E-04 | F |
| Cell adhesion_Plasmink signaling | 2.56E-04 | F |
| Development_TGF-beta-dependent induction of EMT via SMADs | 2.56E-04 | F |
| Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK | 5.79E-04 | F |
| Retinol metabolism/Rodent version | 1.97E-03 | F |
| Retinol metabolism | 2.31E-03 | F |
| Development_SIP2 and SIP3 receptors in cell proliferation and differentiation | 3.87E-03 | F |
| Cell adhesion_Chemokines and adhesion | 5.41E-03 | F |
| Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling | 7.23E-03 | F |
| Development_Regulation of epithelial-to-mesenchymal transition (EMT) | 7.52E-05 | N |
| Cell adhesion_Cadherin-mediated cell adhesion | 1.04E-04 | N |
| Development_SIP2 and SIP3 receptors in cell proliferation and differentiation | 1.04E-04 | N |
| Cell adhesion_Plasmink signaling | 2.56E-04 | N |
| Development_TGF-beta-dependent induction of EMT via SMADs | 2.56E-04 | N |
| Development_TGF-beta-dependent induction of EMT via RhoA, PI3K and ILK | 5.79E-04 | N |
| Retinol metabolism/Rodent version | 1.97E-03 | N |
| Retinol metabolism | 2.31E-03 | N |
| Muscle contraction_nNOS Signaling in Skeletal Muscle | 4.49E-03 | N |
| Cell adhesion_Chemokines and adhesion | 5.41E-03 | N |

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The development of the DA, the transcriptional regulation of DA myocardin play an important role in ductal smooth muscle. Hypothetically activated dominantly in the DA endothelium. The great arteries and outflow tract of the heart [28].

Development_TGF-beta-dependent induction of EMT via SMADs [29]. The cells that comprise the pharyngeal arch arteries are of pharyngeal mesoderm origin. The mesodermal core of the arches is continuous with the mesoderm derived from the second heart field (SHF) [25]. To date, the majority of the cells that constitute the DA media are known to derive from cardiac neural crest cells (NCCs) at the somite 1 to somite 3 level [26,27]. The importance of this neural crest origin in understanding specific DA differentiation lies in the segmental nature of the pharyngeal arches themselves and of the origin of the NCCs that invade them. Accordingly, transcription factors related to NCCs such as Hoxa1, Hoxa3, and heart and neural crest derivatives expressed 2 (Hand2) [28–30] are listed in Table 6. Although a previous study suggested that Hoxb5 may be involved in DA differentiation [31], the expression level of Hoxb5 mRNA was not increased in the DA ECs in the present study. A recent study in humans revealed that mutations in Hoxa1 can cause severe cardiovascular malformations in patients with Bosley-Salih-Alorainy Syndrome [32]. Furthermore, Hoxa1 null mice show defects such as interrupted aortic arch, aberrant subclavian artery and tetralogy of Fallot, demonstrating that Hoxa1 is required for patterning of the great arteries and outflow tract of the heart [28].

In addition, MetaCore enrichment analysis revealed that the SHF-related transcription factors T-box (Tbx) 1, Tbx18, and Is1, and the receptor ligand Fgf10 are enriched in the DA endothelium. It has also been shown that Is1, Fgf10-positive...
ductal closure in premature infants [47]. Indeed, there are several studies demonstrating that vitamin A induces various embryonic developments via many different pathways such as Tgfβ2, Cdh2, or Pitx2 [48]. Recently, Amengual at al. identified that Lrat is critical for cellular uptake of vitamin A from serum retinol-binding protein [49].

In conclusion, the present comprehensive transcription analysis identified the novel DA endothelium-dominant genes during a perinatal period that are highly related with biological processes involved in morphogenesis and development. Moreover, we found that regulation of epithelial-to-mesenchymal transition, cell adhesion, and retinol metabolism are the active pathways that form DA-specific endothelium. Newly identified DA endothelium-dominant genes may play an important role in DA-specific functional and morphologic characteristics.

Supporting Information

Figure S1 The representative figures of FACS gating strategy. Cell debris and doublets were removed by light scattering; forward-scattered light (FSC) and side-scattered light (SSC). FSC and SSC are the parameter of cell-surface area/size and cell-internal complexity, respectively. A. The primary gating was done by removing the factors that affected FSC- and SSC-area. B. The secondary gating with FSC-height and width. C. The third gating with SSC-height and width. D. After those three gating steps by light scattering, dead cells were detected and removed by propidium iodide (PI) staining. E. Population of cells reacted with FITC-conjugated anti-CD31 antibody and APC-Cy7-conjugated anti-CD45 antibody. F. Population of cells reacted with fluorescence conjugated anti-control IgG antibodies to confirm nonspecific binding of antibodies. (TIFF)

Figure S2 Validation using quantitative RT-PCR. (TIFF)

Table S1 The genes that have $p<0.001$ but range between 0.5< Fold change<2.0 in F. (DOCX)

Table S2 The genes that have $p<0.001$ but range between 0.5< Fold change<2.0 in N. (DOCX)

Author Contributions

Conceived and designed the experiments: NML, TK, S. Minamisawa. Performed the experiments: NML, TY, PL, S. Maekawa, IT. Analyzed the data: NML, TY, S. Maekawa, IT, TK. Contributed reagents/materials/analysis tools: S. Maekawa, IT, TK. Wrote the paper: NML, PL, S. Minamisawa.

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