

DNA microarray profiling identified a new role of growth hormone in vascular remodeling of rat ductus arteriosus

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Abstract The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the aorta, has a character distinct from the adjacent arteries. We compared the transcriptional profiles of the DA and the aorta of Wistar rat fetuses on embryonic day 19 (preterm) and day 21 (near-term) using DNA microarray analyses. We found that 39 genes were expressed 2.5-fold greater in the DA than in the aorta. Growth hormone (GH) receptor (GHR) exhibited the most significant difference in expression. Then, we found that GH significantly promoted migration of DA smooth muscle cells (SMCs), thus enhancing the intimal cushion formation of the DA explants. GH also regulated the expression of cytoskeletal genes in DA SMCs, which may retain a synthetic phenotype in the smooth muscle-specific cytoskeletal genes. Thus, the

present study revealed that GH-GHR signal played a role in the vascular remodeling of the DA.

Keywords Growth hormone · Gene expression · Vascular remodeling · Premature infant · Congenital heart disease

Introduction

The ductus arteriosus (DA), a fetal arterial connection between the pulmonary artery and the descending aorta, is essential to fetal life. The morphology and function of the DA dramatically change during development [1]. In particular, during late gestation, the deposition of extracellular matrix in the subendothelium is increased, and the smooth muscle cells (SMCs) of the media migrate into this region, resulting in intimal thickening [2]. This vascular remodeling of the DA is essential for its postnatal closure and is not observed in adjacent arteries. Thus, the DA has distinct characteristic features that differ from those of the adjacent arteries (the aorta and pulmonary arteries). This characteristic of the DA is largely dependent on the expression of the distinct subsets of genes involved in the developmental vascular remodeling that occurs during gestation. To understand the precise transcriptional network in the DA, genome-wide analysis is a powerful approach that can be utilized. In this context, several studies, including ours, have been carried out to identify the effects of oxygen [3] or maternal administration of vitamin A [4] on the transcriptional profiles of the DA. Although the study by Costa et al. [3] is the only one that demonstrated the characteristic differences in the transcriptional profiles between rat DA and the aorta of premature fetuses and neonates, they analyzed their transcriptional profiles on embryonic day 19 (e19) only; they did not examine the changes during later gestation.

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Because the morphological and physiological characteristics of the DA differ significantly between premature and mature fetuses [1], it is of great interest to investigate the transcriptional profiles of the DA and the adjacent aorta in the remodeling process that occurs during late gestation.

Materials and methods

Tissue collection for DNA microarray and quantitative reverse transcription polymerase chain reaction analyses

Pooled tissues from the DA or the aorta were obtained from Wistar rat embryos on e19 ($n \geq 120$) and e21 ($n \geq 120$). Reverse transcription polymerase chain reaction (RT-PCR) analysis was performed as described previously [2]. The information on PCR primers for RT-PCR analyses is provided in Supplemental data 1.

Total RNA preparation and DNA microarray analysis

Total RNA preparation and DNA microarray analysis were performed as described previously [4]. Briefly, total RNA was converted to biotin-labeled cRNA that was hybridized to rat genome U34A GeneChip DNA microarray (Affymetrix, Santa Clara, CA). The hybridization experiments were performed in duplicate and the intensities were averaged. If the difference in the signal intensities of a given sequence tag was equal to the cutoff ($=2.5$ -fold) or more, and if the “Comparison Analysis” of the Microarray Suite Software indicated “increased” or “decreased” with the ≥ 2.5 -fold difference at any developmental stage, that sequence tag was considered to exhibit a significant difference between the DA and the aorta.

Primary culture of rat DA SMCs

Vascular SMCs in primary culture were obtained from the DAs of Wistar rat embryos at e21. The tissues were minced and transferred to a 1.5-ml centrifuge tube that contained 800 μ l of collagenase-dispase enzyme mixture [1.5 mg/ml collagenase-dispase (Roche), 0.5 mg/ml elastase type II-A (Sigma Immunochemicals, St. Louis, MO), 1 mg/ml trypsin inhibitor type I-S (Sigma), and 2 mg/ml bovine serum albumin fraction V (Sigma) in Hanks' balanced salt solution (Sigma)]. The digestion was carried out at 37°C for 15–20 min. Then cell suspensions were centrifuged, and the medium was changed to the collagenase II enzyme mixture [1 mg/ml collagenase II (Worthington), 0.3 mg/ml trypsin inhibitor type I-S, and 2 mg/ml bovine serum albumin fraction V in Hanks' balanced salt solution]. After 12 min of incubation at 37°C, cell suspensions were

transferred to growth medium in 35-mm poly-L-lysine (Sigma)-coated dishes in a moist tissue culture incubator at 37°C in 5% CO₂, 95% ambient mixed air. The growth medium contained Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Sigma). The confluent cells were used at passages 4–6.

SMC migration assay

The migration assay was performed using 24-well Transwell culture inserts with polycarbonate membranes (8- μ m pores; Corning Inc.) coated with fibronectin. The DA SMCs were harvested with trypsin-ethylenediamine tetraacetic acid (EDTA), resuspended in serum-free DMEM, and distributed at a density of 1×10^5 cells/100 μ l in the inserts. The cells were allowed to settle in serum-free DMEM for 1 h before the addition of GH (20 and 200 ng/ml) in the lower chamber. Under basal conditions, the lower chambers were filled with 600 μ l serum-free DMEM. SMCs were then allowed to migrate to the underside of the insert's membrane at 37°C/5% CO₂. At the end of the experiment, the cells were fixed in 10% buffered formalin. SMCs were stained with Cyto Quick (Muto Pure Chemicals), and cells on the upper surface of the membrane were mechanically removed with a cotton swab. Cells that migrated onto the lower surface of the membrane were manually counted from three different fields (0.5 mm²/field) under a microscope.

Cell proliferation assays

[³H]thymidine incorporation was used to measure cell proliferation in DA SMCs. The SMCs were reseeded into a 24-well culture plate at an initial density of 1×10^5 cells per well for 24 h before serum starvation with DMEM containing 0.1% FBS. Cells were then incubated with or without GH (20 and 200 ng/ml) for 24 h in the starvation medium before addition of 1 μ Ci of [*methyl*-³H]thymidine (specific activity 5 Ci/mM; Amersham International, Bucks, UK) for 4 h at 37°C. After fixation with 1.0 ml of 10% trichloroacetic acid, the cells were solubilized with 0.5 ml of 0.5 M NaOH and then neutralized with 0.25 ml of 1 N HCl. A liquid scintillation counter was used to measure [³H]thymidine incorporation. Data obtained from triplicate wells were averaged.

Quantitation of hyaluronan

The amount of hyaluronan in the cell culture supernatant was measured by a latex agglutination method based on the specific interaction of hyaluronan with the latex-labeled hyaluronan-binding protein from bovine cartilage (Fujirebio Inc.). Hyaluronan was quantified in duplicate according to

the manufacturer's instructions using 2.5- μ l aliquots of the conditioned cell culture medium using the HITACHI 7070 analysis system (Hitachi) at an 800-nm wavelength.

Organ culture

Fetal arteries including the DA and the aortic arch arteries were incubated with GH (200 ng/ml) for 72 h in serum-free DMEM as described previously [2]. Explants were then fixed in 10% buffered formalin and embedded in paraffin. The sectioned segments in the middle portion of the DA were analyzed histochemically.

Immunohistochemistry

Tissue staining and immunohistochemistry were performed as described previously [5, 6]. Mouse monoclonal anti-GHR antibody (MAB263) was purchased from Abcam (Tokyo, Japan).

Statistics

Data are presented as mean \pm standard error (SEM) of independent experiments. Statistical analysis was performed between two groups by unpaired two-tailed Student's *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

Genes differentially expressed between the DA and the aorta

All the microarray data in the present study were deposited at the Gene Expression Omnibus (GEO) repository (<http://www.ncbi.nlm.nih.gov/projects/geo/>; accession no. GSE3422). A total of 117 genes (142 probe sets) showed a significant difference (≥ 2.5 -fold) between the DA and the aorta at e19 or e21. Among 117 genes, 39 (43 probe sets) exhibited a DA-dominant expression pattern (Table 1), and 78 (99 probe sets) exhibited an aorta-dominant expression pattern (Table 2).

Of 39 genes in the DA-dominant expression pattern (Table 1), 34 had a known function, and 3 were homologous to known genes. Although several genes, such as prostaglandin E receptor 4 (subtype EP4) (*Ptger4*) and endothelin-1, are known to play an important role in the regulation of vascular tone of the DA [1], the role of most of the other genes in the DA has not been identified. We found that growth hormone (GH) receptor exhibited the

highest difference in the expression between the DA and the aorta among 39 DA-dominant genes.

Of the 39 genes, 9 encode proteins related to cytoskeleton and the extracellular matrix, including sarcomeric genes such as *Myh11* (myosin heavy chain 11), *Myl6* (myosin light chain, polypeptide 6, alkali, smooth muscle and non-muscle), *Actg2* (actin, gamma 2), *Tpm1* (tropomyosin 1, alpha), *Tnn* (tenascin N, predicted), and *Lamb2* (laminin beta2). Three membrane ion channels, ATPase Na⁺/K⁺ transporting b1 polypeptide and potassium inwardly rectifying channel (*Atp1b1*), subfamily J, member 8 (*Kcnj8*), which is known as ATP-sensitive potassium channel K_{ATP}-1, and Ca²⁺ channel, voltage-dependent, $\alpha 2/\delta$ subunit 1 (*Cacna2d1*), were also strongly expressed in the DA.

We also identified 79 genes in the aorta-dominant expression pattern (Table 2). Of the 79 genes, 14 genes encode proteins related to cytoskeleton and the extracellular matrix. Cardiac sarcomeric genes such as *Myh6* (myosin heavy chain, polypeptide 6), *Myh7* (myosin heavy chain, polypeptide 7), *Myl7* (myosin, light polypeptide 7, regulatory), *Myl2* (myosin regulatory light chain 2, ventricular/cardiac muscle isoform), *Actc1* (alpha, actin alpha cardiac 1), *Tnnt2* (troponin T2, cardiac), *Tnni3* (troponin I, cardiac), and *Fn1* (fibronectin 1) were more highly expressed in the aorta than in the DA. Accordingly, there was a marked difference in the composition of the genes related to the cytoskeleton and the extracellular matrix between the DA and the aorta. Sixteen genes were expressed 2.5-fold more in the aorta at both e19 and e21 than in the DA, whereas 24 genes were expressed 2.5-fold more in the aorta than in the DA only at e21. To confirm the results of the DNA microarray, we performed RT-PCR (Supplemental data 2).

Growth hormone receptor mRNA and protein were dominantly expressed in the developing DA

As mentioned above, GH receptor (GHR) exhibited the highest difference of expression between the DA and the aorta (Fig. 1a), suggesting that GH-GHR signal plays a distinct role in the vascular remodeling of the DA from the aorta. The expression of GH mRNA was very low, and there was no difference between the DA and the aorta (Fig. 1b). Interestingly, the expression levels of insulin-like growth factor (IGF)-I and IGF-II mRNAs were higher in the aorta than in the DA, whereas the expression levels of IGF-I receptor (IGF-IR) and IGF-IIR mRNAs did not differ (Fig. 1c–f). In addition, the expression levels of IGF binding protein (IGFBP) 2 and IGFBP5 mRNAs were also higher in the aorta than in the DA at e21 and at e19, respectively (Table 2).

The expression of GHR mRNA was also confirmed by quantitative RT-PCR analyses. We found that the expression levels of GHR mRNA were higher in the rat DA than

Table 1 DA-dominant genes

	Probe set ID	RefSeq Transcript ID	Gene title	Gene symbol	Fold difference between DA and aorta	
					e19	e21
1	rc_AI104225_at	NM_017094	Growth hormone receptor	Ghr	5.0	8.7
2	Z83757mRNA_at	XM_222794	Tenascin N (predicted)	Tnn_predicted	2.3	7.4
3	L16764_s_at/// Z75029_s_at	NM_031971/// NM_212504	Heat shock 70kD protein 1A///heat shock 70kD protein 1B	Hspa1a///Hspa1b	8.6	7.1
4	rc_AA891527_at	NM_022531	Desmin	Des	1.4	6.3
5	rc_AA893846_at	NM_053591	Dipeptidase 1 (renal)	Dpep1	3.4	5.6
6	X73524_at	NM_013129	Interleukin 15	Il15	2.0	5.6
7	D28561_s_at	NM_031677	Four and a half LIM domains 2	Fhl2	3.4	4.9
8	rc_AA894200_at	XM_342032	Proprotein convertase subtilisin/kexin type 5	Pcsk5	1.4	4.8
9	AF002281_at	NM_012870	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	Tnfrsf11b	1.2	4.7
10	M22323_at	NM_013086/// NM_017334	cAMP responsive element modulator	Crem	1.8	3.5
11	U94330_at	NM_001007678	Mss4 protein	Mss4	1.5	3.1
12	rc_AA799773_at	NM_134410	Mg87 protein	Mg87	1.3	2.9
13	X82152_at	NM_001002287	MAS-related G protein-coupled receptor, member B4	Mrgprb4	2.5	2.9
14	M64711_at	NM_080698	Fibromodulin	Fmod	1.6	2.8
15	D28860_s_at	NM_012893	Actin, gamma 2	Actg2	3.7	2.8
16	U69272_g_at	NM_017099	Potassium inwardly rectifying channel, subfamily J, member 8	Kcnj8	2.8	2.8
17	rc_AA859578_at	NM_021587	Latent transforming growth factor beta binding protein 1	Ltbp1	2.0	2.7
18	rc_AA859954_at	NM_012751	Solute carrier family 2 (facilitated glucose transporter), member 4	Slc2a4	2.3	2.7
19	rc_AI014135_g_at	NM_013113	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	Atp1b1	2.1	2.7
20	rc_AI176662_s_at	NM_019131	Tropomyosin 1, alpha	Tpm1	2.3	2.7
21	AB020504_g_at	NM_032076	Prostaglandin E receptor 4 (subtype EP4)	Ptger4	2.4	2.6
22	rc_AI232078_at	NM_012827	Bone morphogenetic protein 4	Bmp4	2.8	2.6
23	U02553cds_s_at	NM_033485	PRKC, apoptosis, WT1, regulator	Pawr	1.4	2.6
24	X63253cds_s_at	NM_080902	Hypoxia induced gene 1	Hig1	1.7	2.6
25	M60921_g_at	XR_086177	PMF32 protein (predicted)	Pmf31	2.8	2.5
26	S66024_at	XM_343144	Myosin, light polypeptide 6, alkali, smooth muscle and non-muscle (predicted)	Myl6_predicted	2.7	2.5
27	M86621_at	NM_012887	Thymopoietin	Tmpo	1.2	2.5
28	X54686cds_at/// rc_AA891041_at	XM_573030	Myosin heavy chain 11	Myh11	3.7	2.3
29	U17254_g_at	NM_012974	Laminin, beta 2	Lamb2	2.5	2.3
30	Z22607_at	NM_019620	Zinc finger protein 386 (Kruppel-like)	Znf386	7.5	2.2
31	rc_AA891422_at	NM_053650	PDZ and LIM domain 3	Pdlim3	2.6	1.8
32	rc_AI144767_s_at/// rc_AA875132_at	NM_024162	Fatty acid binding protein 3	Fabp3	3.5	1.7
33	M63656_s_at	NM_012919	Calcium channel, voltage-dependent, alpha2/delta subunit 1	Cacna2d1	2.7	1.6
34	rc_AI014163_at	NM_012548	Endothelin 1	Edn1	2.8	1.4
35	rc_AI639161_at	XM_346029	Similar to KIAA1411 protein (predicted)	RGD1304927_predicted	2.7	1.2
36	rc_AA866345_at	NM_012531	Catechol- <i>O</i> -methyltransferase	Comt	4.6	1.2
37	rc_AI230614_s_at/// rc_AI112173_at	NM_001002829	RAS-like family 11 member A	Rasl11a	3.0	1.1
38	rc_AA799511_g_at	–	–	–	1.8	2.6
39	rc_AA893871_at	–	–	–	1.4	2.5

Table 2 Aorta-dominant genes

Probe set ID	RefSeq transcript ID	Gene title	Gene symbol	Fold difference between aorta and DA	
				e19	e21
1 X15939_r_at//rc_AI104924_f_at//rc_AI103920_f_at//rc_AA891522_f_at	NM_017239	Myosin heavy chain, polypeptide 6, cardiac muscle, alpha	Myh6	2.2	23.8
2 X80130cds_i_at//rc_AI104567_g_at//rc_AA866452_s_at	XM_215801	Actin alpha cardiac 1	Acte1	3.0	18.4
3 M93638_at	NM_183333	Keratin complex 2, basic, gene 5	Krt2-5	6.9	16.8
4 rc_AA891242_g_at//rc_AA891242_at	NM_001106017	Myosin, light polypeptide 7, regulatory	Myl7	3.2	15.3
5 X15939_f_at	NM_017240	Myosin, heavy polypeptide 7, cardiac muscle, beta	Myh7	1.9	13.2
6 D78159mRNA_s_at	NM_001008806	Type II keratin Kb4	Kb4	1.5	9.1
7 X15512_at	NM_012824	Apolipoprotein C-I	Apoc1	0.5	7.4
8 U67914_at	XM_342219	Carboxypeptidase A3	Cpa3	1.7	7.2
9 rc_AI169372_g_at	NM_031839	Cytochrome P450, family 2, subfamily c, polypeptide 23	Cyp2c23	0.5	5.5
10 K01933_at	NM_012582	Haptoglobin	Hp	0.5	5.1
11 M80829_at	NM_012676	Troponin T2, cardiac	Tnnt2	1.3	4.8
12 X00975_g_at//X07314cds_at	NM_001035252	Myosin, light polypeptide 2	Myl2	5.1	4.8
13 M24852_at	NM_013002	Purkinje cell protein 4	Pcp4	4.7	4.8
14 M92074_g_at	NM_017144	Troponin I, cardiac	Tnni3	2.1	4.6
15 rc_AA945054_s_at	NM_022245	Cytochrome b-5	Cyb5	0.8	4.1
16 S76779_s_at	NM_138828	Apolipoprotein E	Apoe	1.3	3.9
17 AF014503_at	NM_053611	Nuclear protein 1	Nupr1	3.2	3.9
18 X02412_at	NM_019131	Tropomyosin 1, alpha	Tpm1	1.0	3.7
19 D00752_at	NM_182474	Serine protease inhibitor	Spin2a	0.5	3.6
20 D89730_at	NM_001012039	Epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (predicted)	Efemp1_predicted	4.0	3.6
21 M14656_at	NM_012881	Secreted phosphoprotein 1	Spp1	4.7	3.4
22 X81448cds_at//rc_AI072634_at	NM_053976	Keratin complex 1, acidic, gene 18	Krt1-18	1.5	3.1
23 rc_AA946368_at	XM_575338	Similar to fatty acid translocase/CD36	LOC499984	1.9	3.1
24 M91595exon_s_at//J04486_at//A09811cds_s_at	NM_013122	Insulin-like growth factor binding protein 2	Igfbp2	2.1	3.1
25 U30938_at	NM_013066	Microtubule-associated protein 2	Mtap2	2.1	3.0
26 Y12502cds_at	NM_021698	Coagulation factor XIII, A1 subunit	F13a	2.8	2.9
27 M84719_at	NM_012792	Flavin containing monooxygenase 1	Fmo1	4.4	2.8
28 M91652complete_seq_at	NM_017073	Glutamine synthetase 1	Glul	1.8	2.8
29 J03752_at	NM_134349	Microsomal glutathione S-transferase 1	Mgst1	1.1	2.7
30 AF072411_g_at//rc_AA925752_at	NM_031561// XM_575338// XM_575339	CD36 antigen//similar to fatty acid translocase/CD36//similar to fatty acid translocase/CD36	Cd36//LOC499984// LOC499985	1.7	2.7
31 L19998_g_at//L19998_at	NM_031834	Sulfotransferase family 1A, phenol-preferring, member 1	Sult1a1	2.9	2.7
32 L25387_g_at	NM_206847	Phosphofructokinase, platelet	Pfkip	1.7	2.6
33 rc_AI230247_s_at	NM_019192	Selenoprotein P, plasma, 1	Sepp1	2.2	2.6
34 rc_AI237731_s_at//L03294_g_at//L03294_at	NM_012598	Lipoprotein lipase	Lpl	0.9	2.6
35 M83680_at	NM_053589	RAB14, member RAS oncogene family	Rab14	2.6	2.6
36 rc_AI639532_at	XM_215935	Troponin C2, fast (predicted)	Tnnc2_predicted	4.7	2.5
37 X71127_g_at	NM_019262	Complement component 1, q subcomponent, beta polypeptide	C1qb	3.2	2.5
38 AB000113_at	NM_017217	Solute carrier family 7 (cationic amino acid transporter, y + system), member 3	Slc7a3	1.6	2.5
39 rc_AA894092_at	XM_342245	Periostin, osteoblast specific factor (predicted)	Postn_predicted	2.5	2.5
40 M32062_g_at	NM_053843// XM_573502// XM_573503	Fc receptor, IgG, low affinity III//Fc gamma receptor II beta//similar to low affinity immunoglobulin gamma Fc region receptor III precursor (IgG Fc receptor III) (Fc-gamma RIII) (FcRIII)	Fcgr3// LOC498276// LOC498277	3.2	2.4
41 M24353_g_at	XM_343636	Mannosidase 2, alpha 1	Man2a1	4.7	2.3

Table 2 continued

Probe set ID	RefSeq transcript ID	Gene title	Gene symbol	Fold difference between aorta and DA	
				e19	e21
42 U77931_at	NM_147136	rRNA promoter binding protein	RGD:727924	3.0	2.3
43 M12098_s_at	NM_012604	Myosin, heavy polypeptide 3, skeletal muscle, embryonic	Myh3	2.7	2.1
44 AJ005396_at	XM_342325	Procollagen, type XI, alpha 1	Col11a1	3.1	2.1
45 rc_AA893230_at	XM_236325	Ceroid-lipofuscinosis, neuronal 6 (predicted)	Cln6_predicted	2.8	2.1
46 rc_AA875172_at	NM_053360	SH3-domain kinase binding protein 1	Sh3kbp1	2.6	2.1
47 AB012235_at//AB012234_g_at//AB012234_at	XM_213849	Nuclear factor I/X	Nfix	4.9	1.9
48 X00722_at	XM_578812	Similar to testin	LOC503278	2.8	1.9
49 M32062_at	NM_053843//XM_573502	Fc receptor, IgG, low affinity III//Fc gamma receptor II beta	Fcgr3//LOC498276	2.8	1.8
50 rc_AI013472_at//rc_AA924925_at	NM_138905	ER transmembrane protein Dri 42	Ppap2b	2.6	1.8
51 U62897_at	NM_012836	Carboxypeptidase D	Cpd	3.0	1.8
52 Z12298cds_s_at//X59859_r_at//X59859_i_at	NM_024129	Decorin	Dcn	2.7	1.7
53 M15797_at	XM_213954	Nidogen (entactin)	Nid	13.4	1.7
54 AF041066_at	NM_020082	Ribonuclease, RNase A family 4	Rnase4	2.9	1.7
55 U50842_at	XM_343427	Neural precursor cell expressed, developmentally downregulated gene 4A	Nedd4a	3.9	1.6
56 rc_AA866443_at	NM_001008560	Protease, serine, 35 (predicted)	Prss35	4.2	1.6
57 S66184_s_at//rc_AI234060_s_at//rc_AI102814_at//rc_AA875582_at	NM_017061	Lysyl oxidase	Lox	3.2	1.6
58 rc_AI639314_at	XM_238213	Delangin (predicted)	NIPBL_predicted	2.5	1.6
59 rc_AA800908_at	XM_344450	Potassium channel tetramerisation domain containing 12 (predicted)	Kctd12_predicted	3.6	1.6
60 rc_AI029920_s_at	NM_012817	Insulin-like growth factor binding protein 5	Igfbp5	4.0	1.5
61 L10326_at	NM_019132	GNAS complex locus	Gnas	2.7	1.5
62 E00988mRNA_s_at	NM_031511	Insulin-like growth factor 2	Igf2	3.0	1.4
63 U01908cds_s_at//D43778exon#3_s_at//D16840_s_at	NM_012494	Angiotensin II receptor, type 2	Agtr2	4.4	1.4
64 rc_AI176461_s_at	NM_017211	Golgi apparatus protein 1	Glg1	3.3	1.4
65 U23146cds_s_at	NM_057103	A kinase (PRKA) anchor protein (gravin) 12	Akap12	2.9	1.4
66 rc_AA900750_s_at	NM_012760	Pleiomorphic adenoma gene-like 1	Plagl1	2.5	1.4
67 Z17223_at	NM_017149	Mesenchyme homeo box 2	Meox2	2.6	1.3
68 rc_AI171966_at	NM_198740	Major histocompatibility complex, class II, DM beta	RGD:735096	2.5	1.2
69 U35775_g_at//U35775_at	NM_031552	Adducin 3 (gamma)	Add3	5.3	1.2
70 U43534_at	NM_012894	Adenosine deaminase, RNA-specific, B1	Adarb1	2.7	0.8
71 AF004811_at	NM_030863	Moesin	Msn	2.9	0.7
72 X05831cds_at//U82612cds_at//M28259cds_at	NM_019143	Fibronectin 1	Fn1	3.9	0.7
73 U17604_at	NM_053865	Reticulon 1	Rtn1	2.6	0.7
74 X51531cds_g_at//X51531cds_at	–	–	–	4.4	30.2
75 rc_AA799865_at	–	Transcribed locus	–	1.3	2.7
76 X05472cds#2_at	–	–	–	7.2	2.4
77 rc_AA799406_at	XM_578859	Hypothetical protein LOC503325	LOC503325	2.6	1.3
78 rc_AA859921_at	–	–	–	3.3	0.9

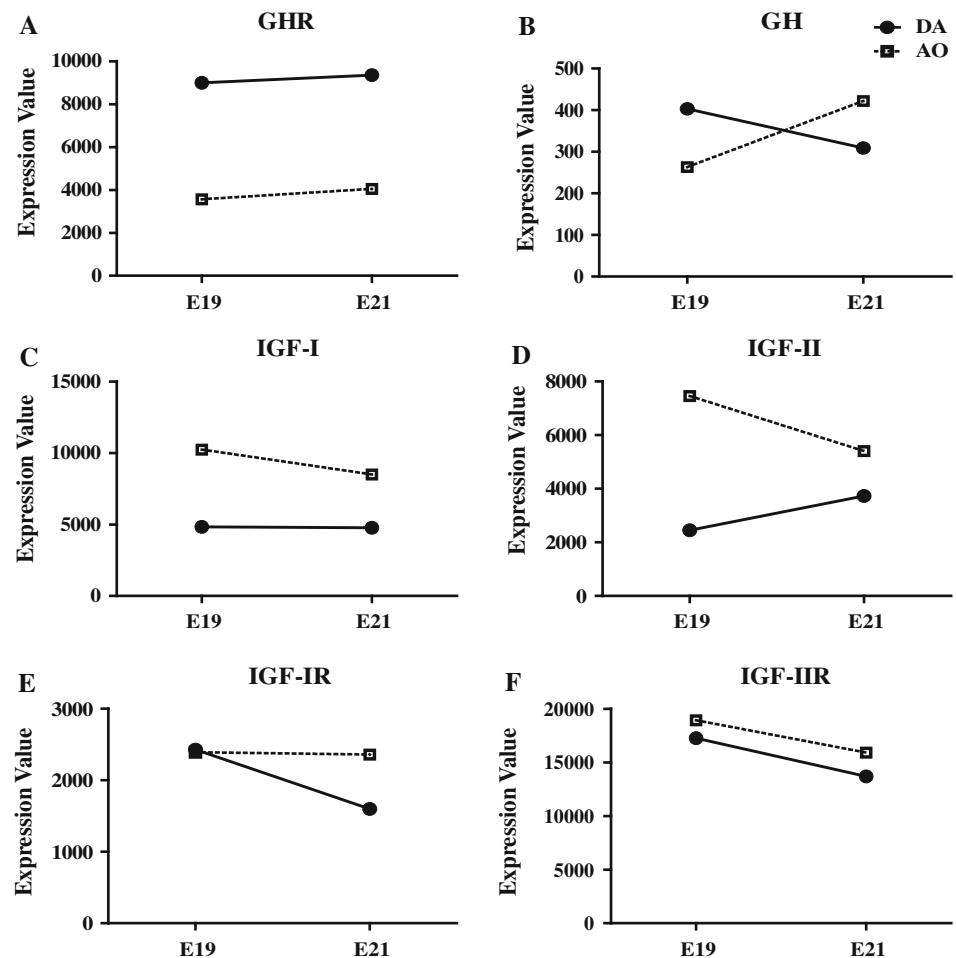
in the aorta at e19 and at e21 ($p < 0.05$ and $p < 0.001$, $n = 6–8$, respectively) (Fig. 2a).

We then examined the localization of GHR in the DA and the aorta at e19 and e21. GHR immunoreactivity was detected abundantly in the SMC layer and less in the endothelial cells of the DA (Fig. 2b).

GH promoted DA SMC migration, but not proliferation

SMC migration and proliferation play an essential role in intimal cushion formation of the DA, especially during late gestation. Therefore, we investigated the effects of GH on migration and proliferation using DA SMCs in primary

Fig. 1 The expression of growth hormone receptor and its related genes in the developing DA by DNA microarray analysis. **a** GHR (growth hormone receptor), **b** GH (growth hormone), **c** IGF (insulin-like growth factor)-I, **d** IGF-II, **e** IGF-IR (type I receptor), and **f** IGF-IIR (type II receptor) mRNA. *E19* Embryonic day 19, *E21* embryonic day 21, *DA* ductus arteriosus, *AO* aorta



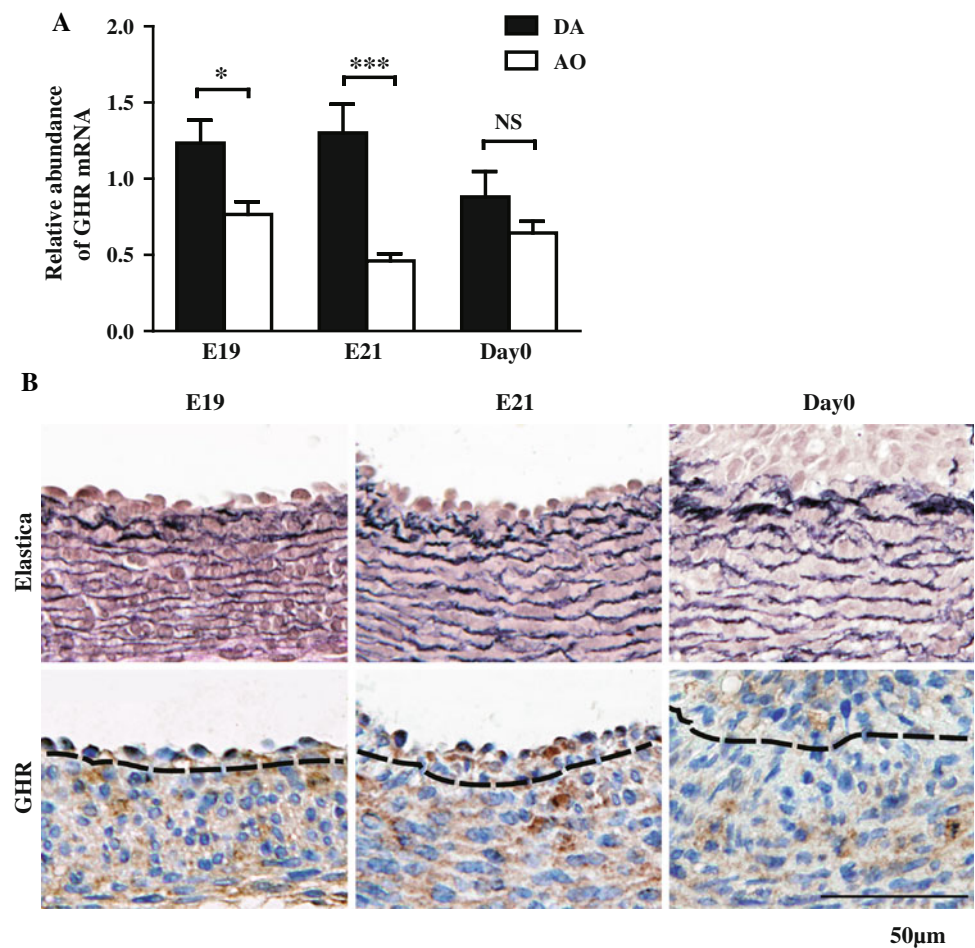
culture from rat DA at e21. We found that recombinant rat GH promoted migration of DA SMCs in a dose-dependent manner (Fig. 3a). However, the same amount of GH did not promote migration of aortic SMCs (Supplemental data 3). When DA SMCs were treated with platelet-derived growth factor BB (PDGF-BB) (10 ng/ml), a potent stimulator for SMC migration, SMC migration was significantly increased by 141% in DA SMCs. In contrast, [³H]thymidine incorporation was unchanged in DA SMCs in the presence of recombinant rat GH (up to 200 ng/ml) (Fig. 3b). When DA SMCs were treated with 10% FBS, a potent stimulator for SMC proliferation, [³H]thymidine incorporation was significantly increased by 112% in DA SMCs. Hyaluronan is an important component of the intimal cushion, and hyaluronan-rich matrices are essential for cell migration and proliferation in the DA [2]. Because our recent study revealed that PGE₁ is a potent stimulator for hyaluronan production in DA SMCs [2], we investigated whether or not GH altered hyaluronan production in DA SMCs. We found that GH had no effect on hyaluronan production in DA SMCs (data not shown).

Effect of GH on tissue-specific cytoskeletal genes in the DA and the aorta

Our DNA array analyses revealed that the expression of cytoskeletal genes was markedly different between the DA and the aorta. Because GH is known to regulate cytoskeletal organization [7], we examined whether or not GH affected such a tissue-specific expression of cytoskeletal genes in DA SMCs. We found that GH decreased the expression of DA-dominant cytoskeletal genes such as desmin, Fhl2, Actg2, and Myh11 in DA SMCs (Fig. 4a–d). Among the aorta-dominant sarcomere genes, we also found that GH decreased the expression of Myl2 and Tnnt2 mRNAs, increased the expression of Tnni3 mRNA, and exhibited no change in Myh7, Actc1, and Tnnc2 mRNAs (Fig. 5).

Because GH is known to inhibit the expression of skeletal muscle-specific proteins in a dose-dependent manner in satellite cells [8], we examined the effect of GH on the expression of smooth muscle-specific genes. We found that GH significantly decreased the expression of SM1, SM2, SM22, and h-caldesmon mRNAs, whereas GH did not

Fig. 2 Expression of GHR mRNA and protein in rat DA.
a Quantitative RT-PCR analyses of GHR. The expression level of GHR mRNA was maximal at E19 and E21 in the DA ($n = 6–8$).
b Immunohistological analysis of GHR protein in the rat DA at E19, E21, and Day0. GHR was detected in a *brown color*. Dark blue stain was counterstained with Mayer's hematoxylin. Scale bars 50 μ m. * $p < 0.05$, *** $p < 0.001$. Data are expressed as means \pm SEM. E19 Embryonic day 19, E21 embryonic day 21, Day0 at birth, DA ductus arteriosus, AO aorta, GHR growth hormone receptor, NS not significant



change the expression of SMemb mRNA (Fig. 6a–e). We also found that GH decreased the expression of myocardin mRNA (Fig. 6f), a transcriptional factor, which is sufficient for a smooth muscle-like contractile phenotype. To investigate whether the effect of GH on the expression of cytoskeletal genes is found in aortic SMCs, we also did the same experiment using SMCs from the rat aorta at e21. We also found a similar effect of GH on the expression of cytoskeletal genes in cultured rat aortic SMCs (Supplemental data 4, 5, and 6).

GH promoted intimal thickening of immature rat DA explants

To examine to what extent GH contributes to the intimal thickening of the DA, we administrated GH into the premature vessel explants containing the DA, the aorta, and the main pulmonary artery from fetuses at e19 (Fig. 7). We found that GH significantly promoted intimal thickening of the DA, but not the aorta when compared with the control (Fig. 7). It should be noted that the effect of GH on the intimal thickening was greater in the DA than in the aorta.

Discussion

Our microarray analyses uncovered gene expression profiles of the DA distinct from those of the aorta during fetal development. These gene expression profiles are considered to be the primary determinant of the different functional and morphological characteristics of the DA from the adjacent arteries. In fact, several unexpected genes that are known to be involved in tissue differentiation were identified as having a DA-dominant expression pattern. It is of note that among them GHR exhibited the highest difference in expression between the rat developing DA and the adjacent aorta. Although the expression levels of GH mRNA were slightly higher in the aorta than in the DA at e21, the difference did not reach statistical significance. We think that total GH-GHR signals are higher in the DA than in the aorta due to the predominant expression of GHR in the DA during gestation. Accordingly, we hypothesized that GH stimulation via GHR may be involved in DA remodeling and that it may play a role in the specification of the DA from other arteries. During gestation, the serum GH concentration of fetuses increased gradually as

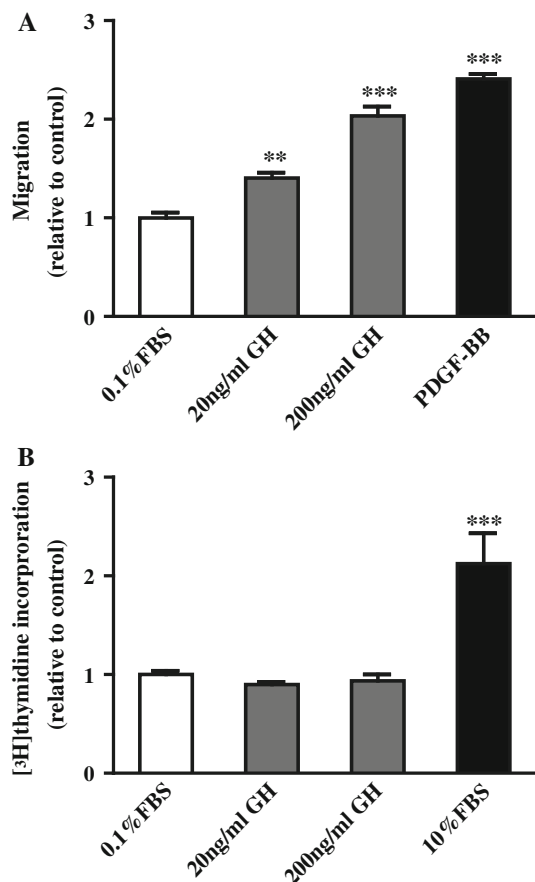


Fig. 3 GH promoted DA SMC migration, but not proliferation. **a** Effects of growth hormone (GH) stimulation on DA SMC migration. DA SMC migration was significantly increased in the presence of GH at a concentration of 20 or 200 ng/ml ($n = 6$). PDGF-BB at a concentration of 10 ng/ml was used as a positive control. **b** The effect of GH on proliferation of rat DA SMCs. GH did not promote cell proliferation in DA SMCs ($n = 6-8$); 10% FBS was used as a positive control. ** $p < 0.01$, *** $p < 0.001$ versus 0.1% FBS. Data are expressed as means \pm SEM. FBS Fetal bovine serum

pregnancy advanced and declined before parturition in many species [9–11]. In rodents, the fetal pituitary gland starts to secrete GH from e15, whereas many tissues, including vascular endothelial and SMCs, locally produce GH beginning in relatively early embryogenesis [12]. Even though the level of serum GH in a fetus is almost comparable to or slightly less than that in an adolescent [9–11], GH has been regarded as of little functional significance in fetal growth [13]. A growing body of evidence, however, has revealed the importance of GH in tissue differentiation during fetal development. First, many fetal tissues, including vascular endothelium and smooth muscle, express GHR [14, 15]. Therefore, GH likely activates an intracellular signal pathway through GHR in fetuses. Second, a considerable number of studies have demonstrated that fetal tissues indeed respond to GH in vitro [12, 16], which was observed in the present study. Third,

GHR knockout mice exhibit functional and morphological changes in both heart and vasculature [17]. These results led us to explore the role of GH in DA development, especially in its vascular remodeling.

Numerous previous studies have demonstrated that GH plays a role in angiogenesis [18] and that GH deficiency or excess increases the risk of cardiovascular morbidity and mortality [19, 20]. However, the role of GH in the vascular remodeling of the developmental arteries has not yet been precisely evaluated. The present study revealed that GH promoted the migration of DA SMCs and then intimal cushion formation in DA explants. In terms of the effect of GH on SMC migration, data from previous studies are very limited [21–23]. Although the precise mechanism still has not been investigated, it can be assumed that the different responses to GH in various cell types are dependent on the GHR expression levels.

Intimal thickness is a hallmark of physiological vascular remodeling of the DA during late gestation [2]. Although the present study demonstrated that GH promotes intimal cushion formation of the ex vivo DA explants, previous clinical studies have shown that the effect of GH on pathological intimal thickness is equivocal. Increases in the carotid intimal media thickness were observed in patients with acromegaly [24]. In contrast, patients with either childhood- or adulthood-onset GH deficiency also exhibited increased intima-media thickness and endothelial dysfunction [19, 25]. Therefore, adequate levels of GH could be important to maintain normal morphology of mature arteries. The present study indicates that GH in the culture media at a concentration of 200 ng/ml is sufficient to promote the physiological intimal cushion formation of the DA through increasing SMC migration.

It is known that the differentiation of DA SMCs precedes that of other arteries [26, 27]. Nevertheless, we were surprised by the considerable number of cardiac-type sarcomere genes expressed in the fetal DA, although it was much less than that in the aorta. The present data suggest that prior to complete differentiation, vascular SMCs may retain a high degree of plasticity, which allows them to modulate their phenotype [28]. Interestingly, through the in vitro experiment, we found that the effect of GH on the expression of the cytoskeletal genes was not always consistent with the tissue-dominant expression patterns that were identified by DNA microarray analysis. Therefore, factor(s) other than GH may determine the tissue-specific expression pattern of cytoskeletal genes in the DA and the aorta.

More importantly, we found that GH downregulated the genes involved in a smooth muscle-like contractile phenotype. Consistent with the result, we also found that GH downregulated myocardin mRNA, which is sufficient for a smooth muscle-like contractile phenotype [28]. In addition

Fig. 4 Effect of GH on the cytoskeletal genes and smooth muscle-specific genes in DA SMCs. **a** Desmin, **b** Fhl2, **c** Actg2, **d** Myh11. ($n = 15$). $*p < 0.05$, $***p < 0.001$. Data are expressed as means \pm SEM. *GH* Growth hormone, *CTRL* control, *DA SMC* ductus arteriosus smooth muscle cell, *NS* not significant

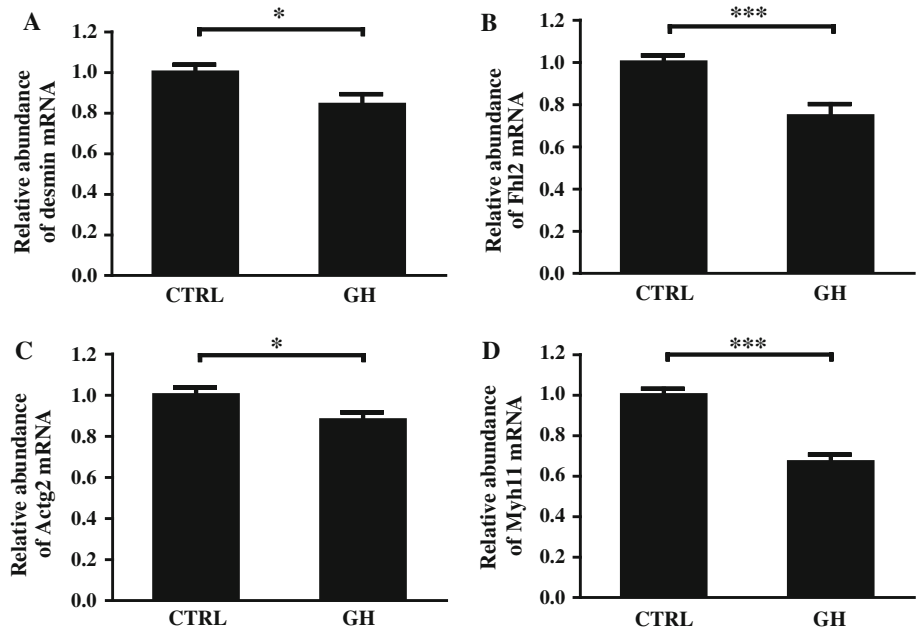


Fig. 5 Effect of GH on the expression of the aorta-dominant sarcomere genes in DA SMCs. **a** Myl2, **b** Tntt2, **c** Tnni3, **d** Myh7, **e** Actc1, and **f** Tnnc2. ($n = 15$). $**p < 0.01$, $***p < 0.001$. Data are expressed as means \pm SEM. *GH* Growth hormone, *CTRL* control, *DA SMC* ductus arteriosus smooth muscle cell, *NS* not significant

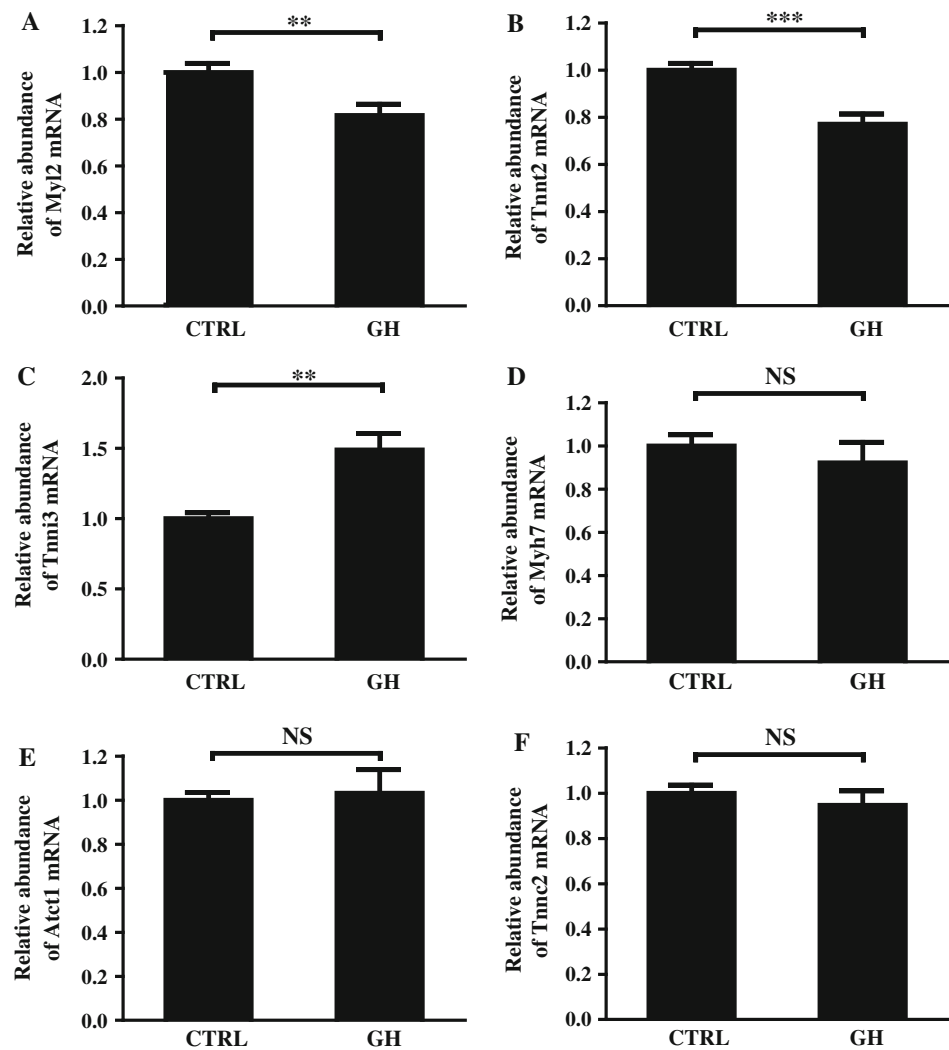
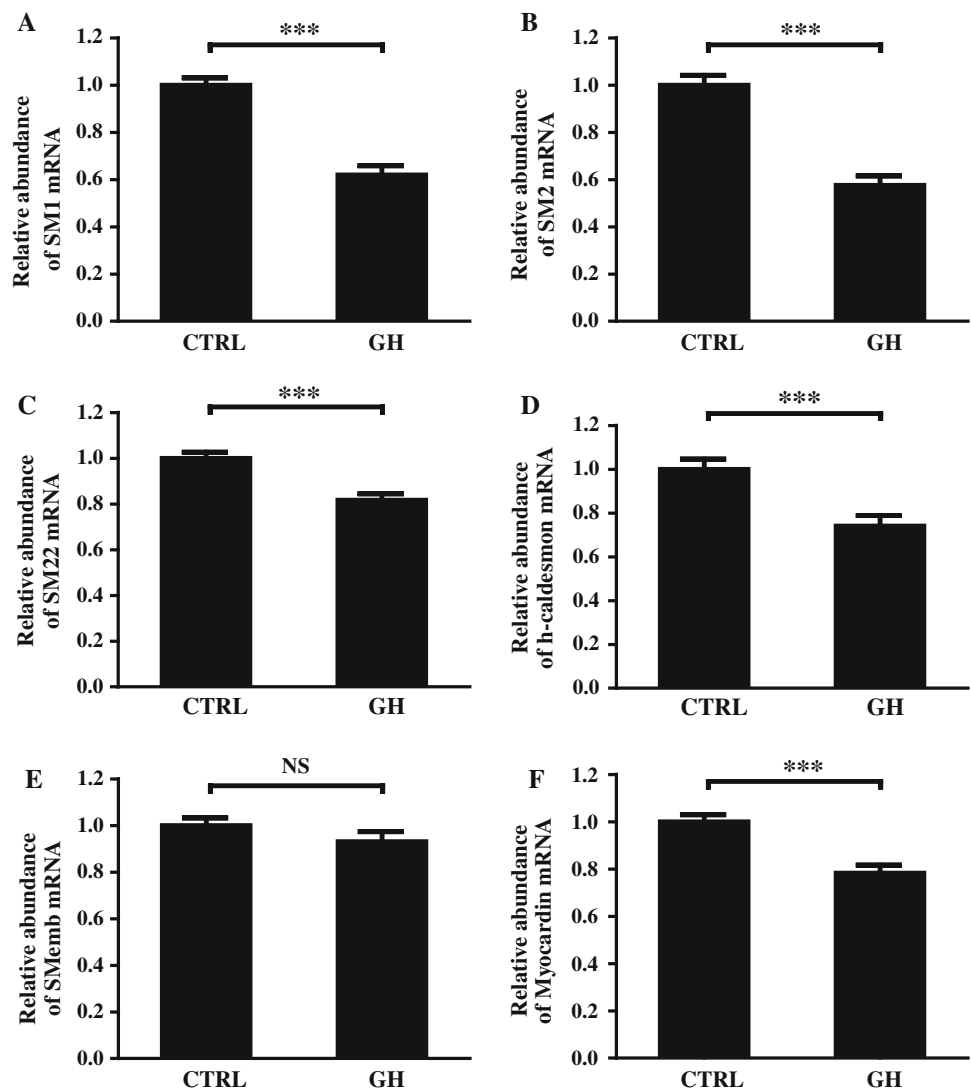


Fig. 6 Effect of GH on the cytoskeletal genes and smooth muscle-specific genes in DA SMCs. **a** SM1, **b** SM2, **c** SM22, **d** h-caldesmon, **e** SMemb, **f** Myocardin. ($n = 15$). $*p < 0.05$, $***p < 0.001$. Data are expressed as means \pm SEM. *GH* Growth hormone, *CTRL* control, *DA SMC* ductus arteriosus smooth muscle cell, *NS* not significant

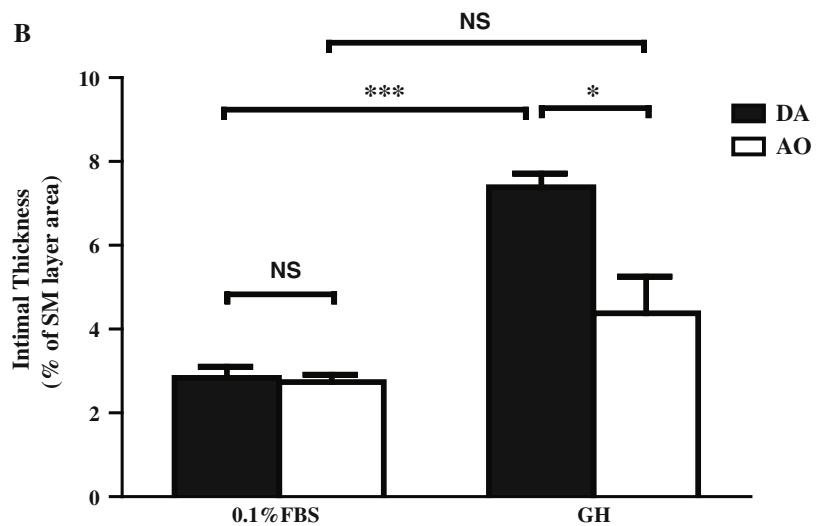
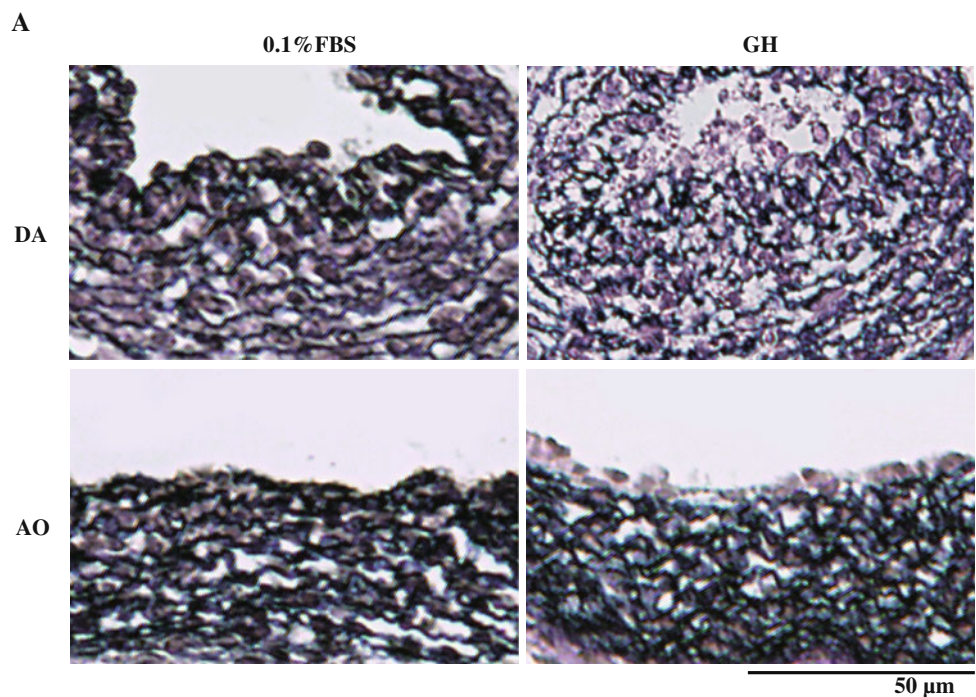


to this observation, Halevy et al. [8] demonstrated that GH inhibited the gene expression of myogenin and the expression of skeletal muscle-specific proteins in a dose-dependent manner in satellite cells. These data suggested that during muscle differentiation, GH inhibited the muscle-specific differentiation at its final stage to retain its synthetic phenotype. We propose that DA SMCs consist of distinct types that depend on their localization. During the progression of intimal cushion formation, DA SMCs in the inner layer are a synthetic phenotype that is highly proliferative and can migrate easily. The GH-GHR signal helps these cells remain a synthetic phenotype. Further studies are required to prove this idea.

Although the present microarray analyses uncovered distinct gene expression profiles of the DA from those of the aorta, several gene profiles are different from a previous report demonstrating the transcriptional profiles between the rat DA and the aorta of premature fetuses

and neonates using the same DNA microarray plates we used [3]. For example, Costa et al. demonstrated that *Myl2* and *Myh7* are predominantly expressed in the rat aorta at e19, but our data showed the opposite result. In addition, the tissue-specific genes that we identified overlap very little with their findings. We do not have a reasonable explanation for this discrepancy. It should be noted that the present study identified several expected genes that are known to be predominantly expressed in the DA, such as prostaglandin E receptor 4, endothelin-1, and *Kcnj8* (potassium inwardly rectifying channel, subfamily J, member 8). In contrast, Costa et al. did not identify this expected DA-dominant gene in the data from their microarray analysis. Furthermore, we also confirmed by quantitative RT-PCR analysis that, using different sets of RNA samples, the expression of several DA-dominant genes was higher in the DA than in the aorta. Therefore, we are confident that we provided

Fig. 7 Effects of GH-mediated intimal thickening of immature rat DA and aorta explants. **a** Elastica staining of the DA and the aorta. GH at a concentration of 200 ng/ml. Scale bars 50 μ m. **b** GH significantly promoted intimal thickening of the DA, but not the aorta. ($n = 4-6$). $*p < 0.05$, $***p < 0.001$. Data are expressed as means \pm SEM. DA Ductus arteriosus, AO aorta, GH growth hormone, NS not significant



reliable data regarding the transcriptional profiles of the developing rat DA.

In conclusion, our study highlighted the distinct transcriptional profiles of the DA. In addition to the expected genes, microarray analysis revealed many genes whose roles were previously unrecognized in the DA. Among them, we found that the GH-GHR signal plays a role in vascular remodeling of the DA by promoting migration of SMCs and the subsequent formation of intimal thickness and regulating the expression of cytoskeletal genes. Although further studies are needed to identify the role of other genes in the DA, our data provide a basis for understanding the molecular mechanisms underlying the

differentiation and remodeling of the DA and for inventing the novel targets that regulate the contraction of the DA in affected children.

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