

**ORIGINAL****Characterization of follistatin-related gene as a negative regulatory factor for activin family members during mouse heart development**

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**Abstract :** Follistatin-related gene (FLRG) encodes a secretory glycoprotein that has characteristic cysteine-rich follistatin domains. FLRG protein binds to and neutralizes several transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily members, including myostatin (MSTN), which is a potent negative regulator of skeletal muscle mass. We have previously reported that FLRG was abundantly expressed in fetal and adult mouse heart. In this study, we analyzed the expression of FLRG mRNA during mouse heart development. FLRG mRNA was continuously expressed in the embryonic heart, whereas it was very low in skeletal muscles. By contrast, MSTN mRNA was highly expressed in embryonic skeletal muscles, whereas the expression of MSTN mRNA was rather low in the heart. *In situ* hybridization and immunohistochemical analysis revealed that FLRG expressed in smooth muscle of the aorta and pulmonary artery, valve leaflets of mitral and tricuspid valves, and cardiac muscles in the ventricle of mouse embryonic heart. However, MSTN was expressed in very limited areas, such as valve leaflets of pulmonary and aortic valves, the top of the ventricular and atrial septa. Interestingly, the expression of MSTN was complementary to that of FLRG, especially in the valvular apparatus. Biochemical analyses with surface plasmon resonance biosensor and reporter assays demonstrated that FLRG hardly dissociates from MSTN and activin once it bound to them, and efficiently inhibits these activities. Our results suggest that FLRG could function as a negative regulator of activin family members including MSTN during heart development. *J. Med. Invest.* 54 : 276-288, August, 2007

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**INTRODUCTION**

The TGF- $\beta$  superfamily consists of multifunctional proteins including TGF- $\beta$ s, activins, and bone morphogenetic proteins (BMPs), which regulate the growth, differentiation, metabolism, and apoptosis of

various cell types (1-4). The cellular functions and signaling of these proteins are regulated in diverse manners, intracellularly and extracellularly. During investigations of regulatory mechanisms for activin functions, we have identified a mouse follistatin-like protein, which was most likely a mouse homologue of the human follistatin-related gene (FLRG) (5, 6). Although FLRG was originally cloned at the breakpoint of the chromosomal rearrangement in a B cell chronic lymphocytic leukemia carried at t(11; 19)(q13; p13) translocation (7), the physiological function has not been identified.

Follistatin (FS) is a single chain protein and has three cysteine-rich FS domains, which are presumed to be growth factor binding motifs (8, 9). In fact, FS binds to activins with high affinity, and prevents their binding to receptors, thereby neutralizing all aspects of biological activities of activins (4, 10, 11). FS also functions as a BMP binding protein, although the affinity for BMPs is much lower than that for activins (12). We have previously reported that FLRG protein has domain structures similar to FS and shows high affinity for activins and BMPs like FS (5, 13).

Recently, both FS (14, 15) and FLRG (14, 16) protein have been shown to bind to myostatin (MSTN), which is another member of the TGF- $\beta$  superfamily. MSTN, also known as growth and differentiation factor 8 (GDF-8), is almost exclusively expressed in developing and adult skeletal muscle, and functions as a negative regulator of skeletal muscle mass by controlling myoblast proliferation (17, 18); however, several lines of evidences suggested that MSTN plays a broader role other than the inhibition of skeletal muscle cell growth. Sharma, *et al.* showed that MSTN mRNA was detected in fetal and adult heart tissues, and this gene was upregulated in cardiomyocytes bordering an infarct area (19). Moreover, Cook, *et al.* found that MSTN regulated cardiomyocyte growth through modulation of serine-threonine kinase AKT signaling by using MSTN-null mice (20, 21).

In this study, we explored the role of FLRG in the heart during mouse development. We studied the expression of FLRG in heart by RNA *in situ* hybridization and immunohistochemistry. We also analyzed the expression of FLRG and MSTN genes by using *in vitro* P19CL6 cardiomyocyte differentiation system. Biochemical analyses with reporter assay and binding assay suggested that FLRG could function as a negative regulator of activin family members during heart development.

## MATERIALS AND METHODS

### *Animals*

Pregnant wild-type ICR mice were purchased from Japan CLEA (Tokyo, Japan). Embryos were retrieved by Caesarian section from pregnant female mice anesthetized with diethylether, followed by cervical dislocation. All animal experiments in this study were performed in accordance with the guideline principles of The Institutional Animal Care and Oversight Committee and were approved by The Committee for the Care and Use of Animals in The University of Tokushima Faculty of Medicine.

Hearts and skeletal muscles of hind limbs were dissected from each embryo (E10.5, E12.5 and E14.5), newborn (P1, P3, P8, and P15) and adult (pregnant female). The hearts were rinsed with sterile RNase free phosphate-buffered saline (PBS) containing 10 U/ml heparin, and then used for RNA isolation.

### *Cell culture and cardiomyocyte differentiation*

P19CL6 cells were purchased from RIKEN Gene Bank (Tsukuba, Japan). Cells were cultured essentially as described previously (22). Briefly, the cells were grown in a 100-mm tissue culture grade dish under adherent conditions in growth medium ( $\alpha$ -MEM) supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml). For the induction of cardiomyocyte differentiation, P19CL6 cells were plated at a density of  $3.7 \times 10^5$  in a 60-mm tissue culture grade dish in growth medium containing 1% dimethyl sulphoxide (DMSO). The medium was changed every two days. The first day of DMSO treatment was designated day 0, and days of differentiation were counted consecutively.

### *RNA isolation and RT-PCR analysis*

For the semi-quantitative analysis of FLRG and MSTN mRNA expression, total RNA was extracted from mouse hearts, skeletal muscles, or cultured P19CL6 cells with TRIzol reagent (Gibco BRL), and each sample was treated with RNase-free DNase I. First-strand cDNA was synthesized in a 20  $\mu$ l reverse transcriptase (RT) reaction with 3  $\mu$ g of total RNA using SuperScript III (Invitrogen) according to the manufacturer's protocol. One  $\mu$ l of the RT reaction was amplified by PCR with 0.25 U of ExTaq DNA polymerase (Takara) in 25  $\mu$ l of a reaction mixture containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP and 0.5  $\mu$ M of oligonucleotide primers. The primers used for RT-PCR analysis are shown in Table 1.  $\beta$ -actin amplification was

**Table 1** Oligonucleotide primers used for RT-PCR in this study

Gene	GenBank Accession No.	Primer sequence
FLRG	AB024429	F: 5'-ACTCTGTGGCAACAACAACG-3' (621-640) R: 5'-TTCTCTTCCTCTCTGCTGG-3' (742-761)
MSTN	NM_010834	F: 5'-GCACTGGTATTTGGCAGAGTA-3' (702-722) R: 5'-CACACTCTCCTGAGCAGTAAT-3' (1026-1046)
Nkx 2.5	X75415	F: 5'-TCTCCGATCCATCCACTTTATTG-3' (1368-1391) R: 5'-TTGCGTTACGCACTCACTTTAATG-3' (1566-1589)
GATA4	NM_008092	F: 5'-AGCAGGACTCTTGAACAGC-3' (1872-1891) R: 5'-CAGGCAGGTGGAGAATAAGG-3' (2214-2233)
$\beta$ -actin	NM_007393	F: 5'-CCTGGAGAAGAGCTATGAGCTGC-3' (785-807) R: 5'-TGCTGATCCACATCTGCTGGAAG-3' (1133-1155)

The sequence of forward (F) and reverse (R) strand primers are shown together with GenBank accession numbers relating to the DNA sequences from which they were obtained.

used as an internal control. Full-length cDNA clone for FLRG in pBluescript vector (Stratagene) and a HindIII-BamHI fragment of MSTN in pBluescript vector were used as positive controls (data not shown). PCR programs were as follows: 94°C for 5 min, 30 cycles of 94°C for 1 min, 60°C for 1 min (for FLRG cDNA amplification, the annealing temperature was set at 57°C) and 72°C for 1 min. Amplified products (20  $\mu$ l) were resolved by 2% agarose gels, stained with ethidium bromide, and photographed under UV light.

#### RNA *in situ* hybridization

Mouse embryos were fixed in 4% paraformaldehyde and dehydrated before embedding in paraffin. *In situ* hybridization was performed on 5- $\mu$ m-thick paraffin sections as described (23). The full-length cDNA clone for FLRG in pBluescript vector, a HindIII-BamHI fragment of MSTN in pBluescript vector, full-length cDNA clone for mouse activin A in pGEM-T-Easy vector (Promega), a NcoI-SpeI fragment of mFS in pGEM-T-Easy vector, and a BamHI-HindIII fragment of GDF-11 in pBluescript vector were used to prepare the probe. Digoxigenin-labeled RNA probes were prepared according to the standard procedure. In parallel with antisense probes, the corresponding sense probes were used as negative controls.

#### Immunohistochemistry

Mouse embryos and hearts dissected from adult mice were fixed in 4% paraformaldehyde and dehydrated before embedding in paraffin wax. Serial sections were then cut at 8  $\mu$ m by cryostat, and mounted on 3-aminopropyltriethoxysilane-coated glass slides. After deparaffinization and rehydration, sections were treated with 3% H<sub>2</sub>O<sub>2</sub> in 100% methanol for 30 min at -20°C, and then boiled in 10 mM sodium citrate

buffer (pH 6.0) for 5 min. The sections were washed by TBST (0.1% Tween-20 in Tris-buffered saline) and then blocked with 1.5% BSA in PBS for 1 hr at room temperature. The samples were then incubated with 1 : 100 diluted anti-mouse FLRG monoclonal antibody or 1 : 100 diluted anti-MSTN monoclonal antibody (clone JA16; Wyeth Research) in blocking buffer overnight at 4°C. Staining without primary antibodies was used as a negative control. The sections were washed with three changes of TBST, incubated with horseradish peroxidase-conjugated secondary antibody for 45 min at room temperature, and then visualized by ImmunoPure Metal Enhanced DAB substrate Kit (PIERCE, Rockford, IL). The sections were also counterstained with hematoxylin.

#### Purification of human FLRG protein

Bovine activin A was conjugated to affigel-10 column (BioRad) in 0.5 M NaHCO<sub>3</sub> (pH 8.0) buffer at room temperature for 1 hr and then at 4°C overnight. The activin A-conjugated column was equilibrated with a binding buffer (20 mM Tris-HCl (pH 7.5), 0.15 M NaCl and 0.03% CHAPS) before use.

CHO cells stably expressing human FLRG were grown in  $\alpha$ -MEM supplemented with 10% FCS until confluency, and then conditioned with serum-free EC-CELL301 medium. The conditioned medium was first fractionated by ammonium sulfate precipitation (30-80%), and then passed through an activin A-conjugated column at a flow rate of 0.2 ml/min. Columns were washed with binding buffer, and then eluted with elution buffer (20 mM Tris-HCl (pH 7.5), 1.0 M NaCl, 0.03% CHAPS containing 1 M Guanidine-HCl). Eluted proteins were concentrated by ultrafiltration, and the buffer was changed to PBS containing 0.03% CHAPS. Samples were resolved by 12.5% SDS-PAGE and blotted onto a PVDF membrane.

FLRG protein was detected with anti-human FLRG antibody followed by enhanced chemiluminescence (ECL) reaction.

#### Surface plasmon resonance (SPR) biosensor analysis

All measurements were performed using a BIACORE X system (Biacore AB). For immobilized samples, proteins were dissolved in 20 mM sodium acetate (pH 4.5) at a concentration of 10  $\mu$ g/ml and immobilized on sensor chip CM5 at a flow rate of 5 ml/min at 25°C. The immobilized ligands were regenerated after each cycle using a 40 ml injection of 10 mM HEPES (pH 7.4) / 2 M guanidine-HCl. The kinetic parameters, association rate constant ( $k_{on}$ ) and dissociation rate constant ( $k_{off}$ ) were determined using BIA evaluation software version 3.0 (Biacore AB).

FLRG protein was purified as described above. Bovine activin A and human FS-288 were purified as described previously (24). Human MSTN, GDF-11 and BMP proteins were either from R&D systems or Wyeth Research.

#### Luciferase assays

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma), supplemented with 10% FCS. Cells plated at a density of  $4 \times 10^4$  in a 24-well dishes were transfected with 500 ng of CAGA-luc reporter DNA, and 200 ng of the cytomegalovirus promoter- $\beta$ gal (CMV- $\beta$ gal) DNA by the calcium phosphate precipitation method (25). Twenty-four hours after transfection, the cells were stimulated with activin A (20 ng/ml), TGF- $\beta$ 1 (15 ng/ml), MSTN (20 ng/ml) or GDF-11 (20 ng/ml) in media containing 1% FCS for 24 hr. In order to study whether FS or FLRG has inhibitory effects on activin A, TGF- $\beta$ 1, MSTN or GDF-11, FS or FLRG proteins were simultaneously added to these stimulants. Luciferase activity was normalized to  $\beta$ -galactosidase activity as described previously (26).

## RESULTS

### Expression of FLRG and MSTN mRNAs in heart and limbs during mouse development

First, we analyzed the expression of FLRG during cardiac muscle development in mouse. For this purpose, we purified hearts mRNAs from mouse embryos, newborn littermates and adult mice, and performed RT-PCR analysis with the set of primers listed in Table 1. In adult mice, FLRG mRNA was

abundantly expressed in the heart (Fig. 1A), as we reported previously (5). During development, FLRG mRNA was continuously expressed in the heart from the early embryonic stage (E10.5) to adult (Fig. 1A, upper panel). When we analyzed the expression of MSTN, one of the binding partners for FLRG, MSTN mRNA was also expressed in the heart during the early embryonic stage (E10.5-14.5). The expression, however, dropped after birth, and then later gradually increased but did not reach to the expression level at E10.5 (Fig. 1A, middle panel).

When the expressions of these genes were analyzed in skeletal muscle obtained from the hind limbs of the same animals, we found that skeletal muscle constantly expressed MSTN mRNA during development (Fig. 1B, middle panel). In contrast to the heart, however, a very limited amount of FLRG mRNA was expressed in skeletal muscle during embryogenesis. The expression of FLRG mRNA was transiently increased in embryos at E14.5, and then decreased after birth (Fig. 1B, upper panel). These results raised the possibility that FLRG may regulate the development and maintenance of heart tissue, similarly as MSTN regulates the growth and differentiation of skeletal muscles.

### Expression of FLRG and MSTN mRNAs in differentiating P19CL6 cells

To verify the characteristic expression of FLRG and MSTN mRNAs during heart development, we took advantage of the P19CL6 *in vitro* cardiomyocyte differentiation system. The P19CL6 cell line is a sub-

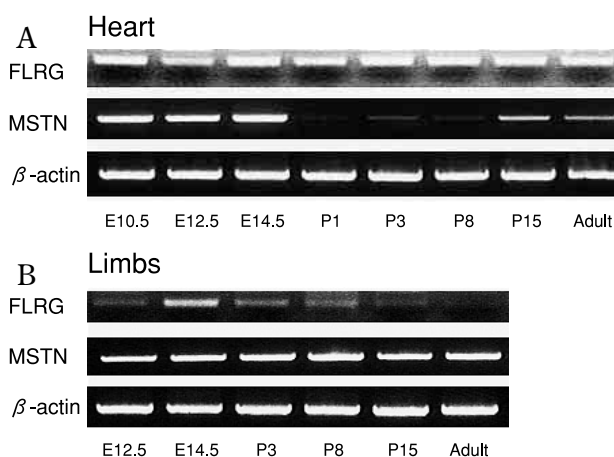


Fig. 1 Expression profiles of FLRG and MSTN mRNAs in heart and limb during mouse development.

Mouse heart ventricles and skeletal muscle of hind limbs were collected from embryos (E10.5, E12.5, and E14.5), newborns (P1, P3, P8 and P15) and adults (pregnant female). Total RNAs were isolated from each sample and RT-PCR was performed to detect FLRG and MSTN mRNAs expression.  $\beta$ -actin was used as the internal control.

line of P19 embryonic carcinoma stem cells. This cell line shows efficient differentiation into beating cardiac myocytes after induction with DMSO (22). When we cultured P19CL6 in the presence of 1% DMSO, the first contraction was observed at some part of the restricted area of the sheet on day 12. On day 14, almost all parts of the sheet beat synchronously. Under these conditions, the expression of FLRG, MSTN, and early cardiac-specific regulatory genes, *nkx2.5* and *gata4*, were analyzed by RT-PCR in four stages (day 4, 8, 12, 16). As previously reported (27), cells expressed *gata4* as early as 4 days after DMSO treatment, and then *nkx2.5* was detected at day 8 (Fig. 2), suggesting that P19CL6 cells specifically differentiated to cardiomyocytes. FLRG mRNA was detected at day 4, and continued to be expressed after synchronous beating occurred. On the other hand, the expression of MSTN mRNA was observed after day 12, concurrently with spontaneous beating. In control P19CL6 cells, we failed to detect either FLRG mRNA expression by RT-PCR, or FLRG protein by Western blotting, although we detected the expression of FLRG protein in cardiogenic P19CL6 cells on day 6 by immunofluorescence (data not shown). These results further strengthen the hypothesis that MSTN and the binding protein, FLRG, are involved in the cardiomyogenesis of this

cell line.

#### RNA *in situ* hybridization of FLRG and MSTN mRNAs in the mouse embryonic heart

In order to analyze the localization of FLRG and MSTN mRNAs in the mouse embryonic heart, *in situ* hybridization was performed with E14.5 mouse embryos, which express both genes (Fig. 1A).

FLRG mRNA was clearly detected in the smooth muscle of the aorta (Fig. 3G, H, I) and pulmonary

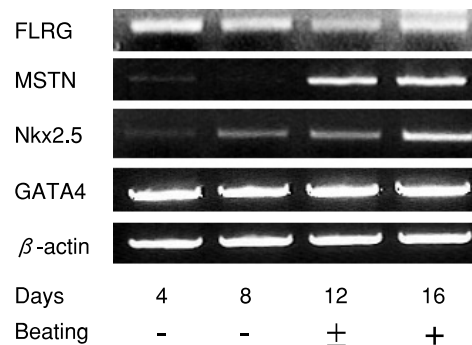


Fig. 2 Expression of FLRG and MSTN mRNAs during *in vitro* cardiomyocyte differentiation in P19CL6 cells. Cardiomyocyte differentiation was induced in P19CL6 cells by adding 1% DMSO to the growth medium. Twelve days after DMSO treatment, the first contraction was observed in some part of the restricted area of cells. Temporal changes of the expression of FLRG, MSTN, Nkx2.5, and GATA4, mRNAs were analyzed by RT-PCR.  $\beta$ -actin was used as the internal control.

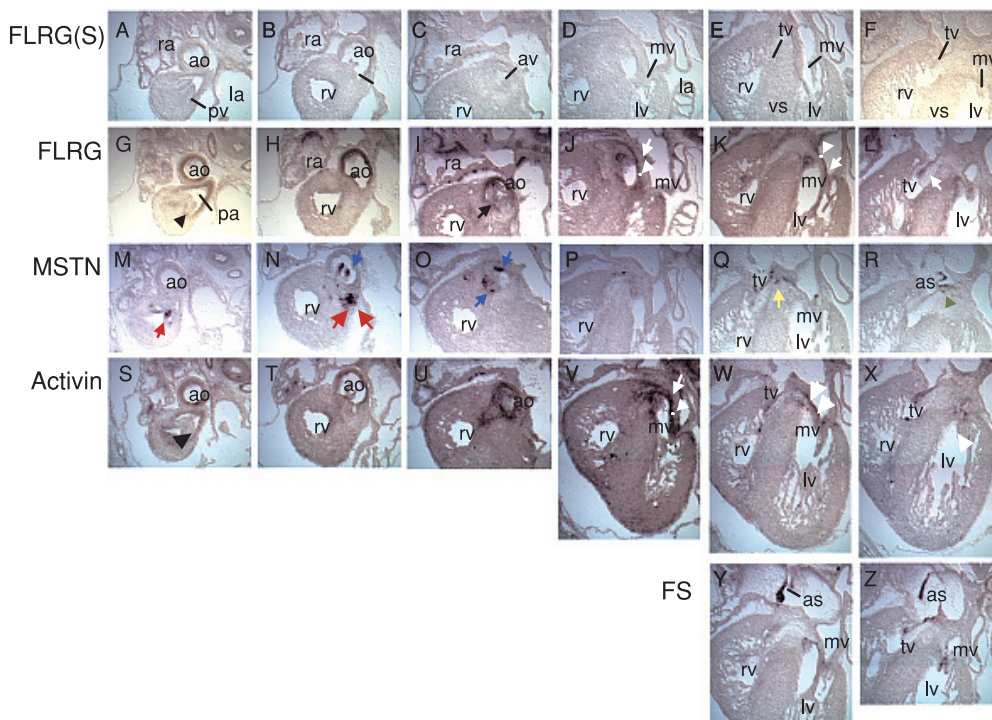


Fig. 3 Localization of FLRG, MSTN, activin, and FS mRNAs in mouse embryonic heart. Serial sections of E14.5 mouse heart were analyzed by RNA *in situ* hybridization. Localization of FLRG (G-L), MSTN (M-R), activin (S-X), and FS (Y, Z) mRNAs is shown. Sense signals of FLRG (A-F) were shown as the controls. ao indicates aorta ; as, atrial septum ; av, aortic valve ; la, left atrium ; lv, left ventricle ; mv, mitral valve ; pa, pulmonary artery ; pv, pulmonary valve ; tv, tricuspid valve ; ra, right atrium ; rv, right ventricle ; vs, ventricular septum.

artery (Fig. 3G). The mRNA was also detected in valve leaflets of the mitral valve (Fig. 3J, K, white arrow) and tricuspid valve (Fig. 3L, white arrow). Besides these tissues, FLRG mRNA was widely expressed in cardiac muscles of the ventricle. Since FLRG protein binds to MSTN and neutralizes the functions, we expected that these two mRNAs either colocalized or were adjacent to each other. The expression of MSTN mRNA was, however, quite distinct and was detected in the valve leaflets of the pulmonary valve (Fig. 3M, N, red arrow) and aortic valve (Fig. 3N, O, blue arrow). FLRG mRNA was not detected in these tissues (Fig. 3G, I, black arrows). MSTN mRNA was also detected at the top of the ventricular septum (Fig. 3Q, yellow arrow) and in the atrial septum (Fig. 3R, brown arrow).

According to the previous report, activin and FS mRNAs were also expressed in the E10.5-12.5 mouse embryonic heart (28). Thus, we further analyzed the expression of these genes with the same samples. In our study with E14.5 mouse embryos, activin mRNA was strongly expressed in the smooth muscle of the aorta (Fig. 3S, T, U) and pulmonary artery (Fig. 3S), and the valve leaflets of the mitral valve (Fig. 3V, W, white arrow) and tricuspid valve (Fig. 3W, X, white arrow). On the other hand, FS mRNA was detected at the atrial septum, as described previously (28), and was also detected at mitral and tricuspid valves (Fig. 3Y and Z). These results indicated that expression profiles of FLRG and activin mRNAs resembled each other, and that the expression of MSTN mRNA was complementary to that of FLRG.

#### *Immunohistochemical analysis of FLRG protein in fetal and adult mouse hearts*

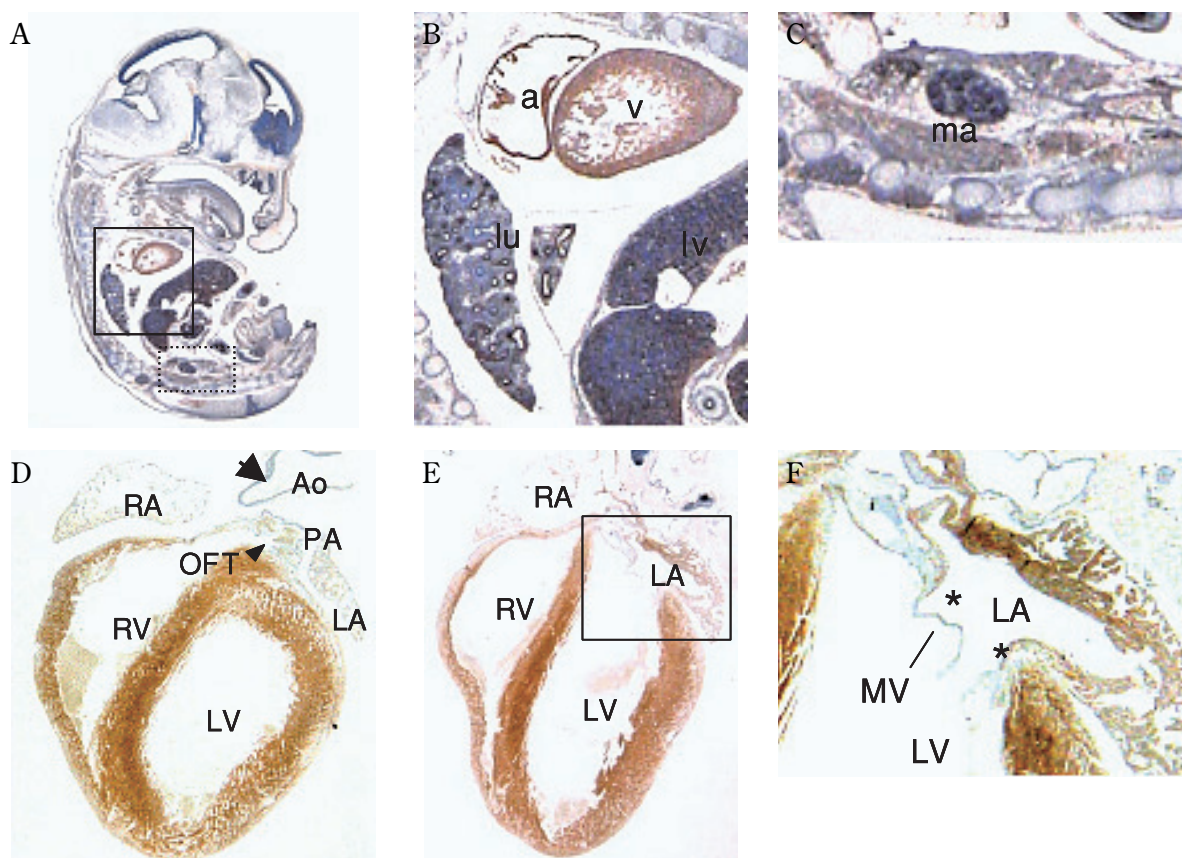
We further analyzed the tissue distribution of FLRG protein in the fetal and adult mouse heart by immunohistochemistry with a monoclonal anti-mouse FLRG antibody (5, 29). In E13.5 mouse embryos, which also express both FLRG and MSTN mRNA, FLRG protein was detected clearly in the heart muscle of the atrium with anti-FLRG antibody, and rather diffusely in the ventricle (Fig. 4A and B). Other tissues, such as the lung (Fig. 4B) and metanephros (Fig. 4C), were also stained but rather weakly. In adult mouse hearts, similar immunoreactivity was observed as in embryonic hearts, although the expression of FLRG in the ventricle was as strong as in the atrium (Fig. 4E and F). As in our previous report (29), FLRG was widely detected in adult heart muscles but not in the nuclei of cardiomyocytes

(data not shown). On the other hand, no signal was detected in the vascular smooth muscle of the aorta (Fig. 4D, arrow) and pulmonary artery (Fig. 4D), or in the pulmonary valve leaflets (Fig. 4D, black arrowhead) of adult mice. In the mitral valve, however, FLRG staining was detected in the upper layer of the valve leaflets (Fig. 4F, asterisks).

In order to confirm the expression of FLRG and MSTN protein in valvular apparatus in the mouse embryonic heart, we further analyzed the localization of these proteins by immunohistochemistry (Fig. 5). To do this, we used the JA16 monoclonal antibody against MSTN, which was previously reported (14, 16, 30). In the E12.5 embryonic heart, both FLRG and MSTN proteins were clearly detected in cardiomyocytes of the atrium and ventricles (Fig. 5A and D), and their respective immunoreactivity resembled each other. In the region of the outflow tract cushion, however, the subcellular localization of FLRG protein was different from that of MSTN. FLRG protein was mainly detected in the nucleus (Fig. 5B and C, yellow-dotted area) as previously reported with human granulosa cells and several other cell lines (13, 31). In contrast, MSTN protein was found in the cytosol of the outflow tract cushion cells (Fig. 5E and F, yellow-dotted area). The role of FLRG localized in the nuclei of the cells in this tissue remains to be determined. Interestingly, conspicuous FLRG signals were also detected at the top of the outflow cushion, where the pulmonary valve (Fig. 5B, pink arrowheads) and aortic valve (Fig. 5C, green arrowheads) would be formed, and MSTN message was also detected at the surface of these valve leaflets (Fig. 5E, pink arrows and Fig. 5F, green arrows). These findings suggest that FLRG and MSTN could regulate not only cardiomyogenesis but also valvulogenesis during cardiac development.

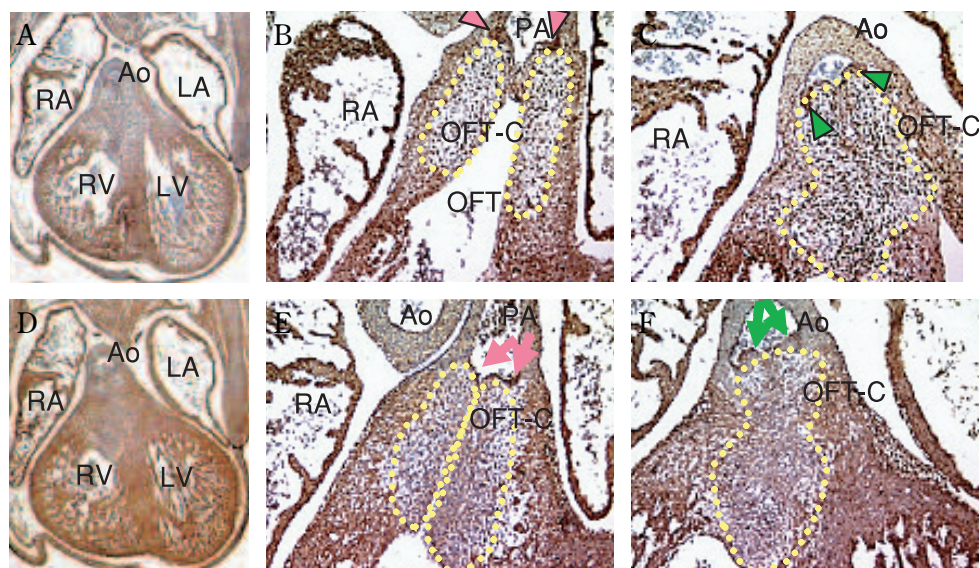
In order to test the involvement of other activin and FS members for valve formation, we carefully compared these expressions by *in situ* hybridization with the E14.5 embryonic heart (Fig. 6). The expression patterns of activin and FLRG were very similar (Fig. 6A and E), but not identical (Fig. 6G). Interestingly, FLRG mRNA was localized rather complementary to MSTN mRNA around the aortic valve (Fig. 6D, E, G). Similarly, the expression of GDF-11 mRNA was complementary to that of activin (Fig. 6A and B). This mRNA expression profiling suggests that activin family members including MSTN and two regulatory factors, FLRG and FS, are cooperatively involved in valvulogenesis during heart development.





**Fig. 4** Immunohistochemical analysis of FLRG protein in fetal and adult mouse heart.

A-C : Sagittal sections of E13.5 mouse embryo (A) and enlargement of boxed area in A (B), dotted area in A (C). D, E : Coronal sections of mouse adult heart. F : Enlargement of mitral valve of adult mouse (boxed area in E). RA indicates right atrium ; LA, left atrium ; RV, right ventricle ; LV, left ventricle ; Ao, aorta ; PA, pulmonary artery ; OFT, outflow tract ; MV, mitral valve ; a, atrium ; v, ventricle ; lu, lung ; lv, liver ; ma, metanephros.



**Fig. 5** Localization of FLRG and MSTN proteins in valvular apparatus of E12.5 embryonic heart.

Immunohistochemical analysis of FLRG (A-C) and MSTN (D-F) proteins in embryonic heart. A, D : Coronal sections of E12.5 mouse embryonic heart. B, E : Enlargement of outflow tract of right ventricle. C, F : Enlargement of aortic valve. RA indicates right atrium ; LA, left atrium ; RV, right ventricle ; LV, left ventricle ; Ao, aorta ; PA, pulmonary artery ; OFT, outflow tract ; OFT-C, outflow tract cushion.

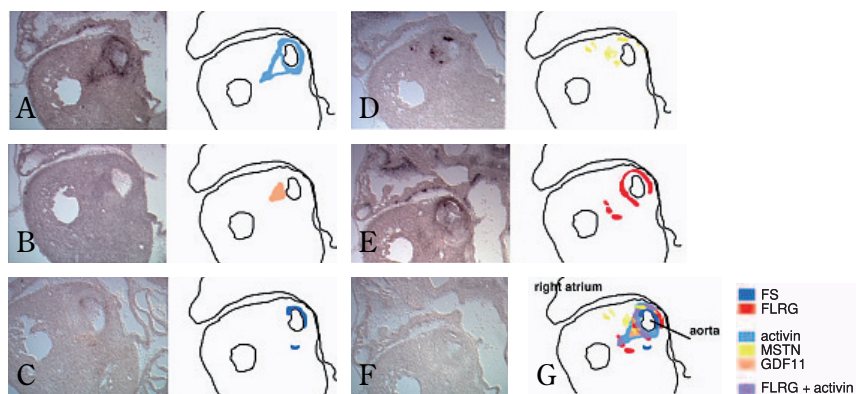


Fig. 6 mRNA expression of activin and FS members around aorta and aortic valve leaflets. RNA *in situ* hybridization analysis of serial sections around aorta of E14.5 mouse heart. Localization of activin (A), GDF-11 (B), FS (C), MSTN (D), and FLRG (E) mRNAs are shown. Sense signal of FLRG (F) is shown as the control. Localization of each mRNA is respectively indicated in the illustration next to each photograph. These expression patterns were merged as shown in G.

*Inhibitory effect of FLRG protein on MSTN and other activin family member signaling*

To elucidate the roles of FLRG, we compared the affinity of FLRG for MSTN and other TGF-β superfamily member proteins. First, we prepared recombinant FLRG protein by transient transfection of Chinese hamster ovary (CHO) cells with a human FLRG (hFLRG) expression plasmid followed by partial purification by passing the conditioned medium through an activin A-conjugated column. Purified hFLRG protein was resolved by SDS-PAGE and immunoblotted with the monoclonal anti-human FLRG antibody. As shown in Fig. 7, recombinant hFLRG protein with an apparent molecular mass of 37 kDa was generated. Next, we performed surface plasmon resonance (SPR) biosensor analysis using a BIACORE system. We also used human FS-288, which is the splicing isoform of FS that has stronger inhibitory activity against activin than the other isoform, FS-315 (32, 33). FLRG or FS-288 was immobilized on a BIACORE sensor chip, and then MSTN protein and other TGF-β superfamily members were injected to flow over the sensor chip as analytes. As shown in Table 2, the affinity ( $K_d$ ) of MSTN for FLRG was comparable with that for FS-288. Activin showed around 10 times higher affinity than MSTN for FLRG or FS-288. In

contrast, BMP2 showed 10 times lower affinity for these proteins. Interestingly, the dissociation constant ( $k_{off}$ ) of MSTN indicated that MSTN slowly dissociates from FLRG or FS-288 than other TGF-β superfamily members. These results suggested that MSTN did not efficiently bind to FLRG or FS as activin did due to its low association constant ( $k_{on}$ ),

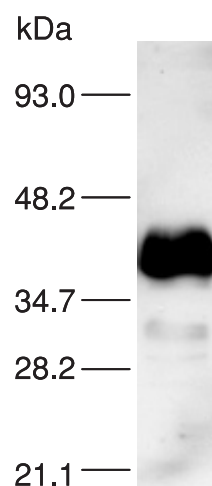


Fig. 7 Recombinant human FLRG protein purified through an immobilized activin column. Human FLRG protein, secreted by CHO cells, was partially purified by passing through the activin column. The sample was resolved by 12.5% SDS-PAGE and blotted with anti-human FLRG antibody. The signal was detected by enhanced chemiluminescence. Molecular mass markers are indicated on the left.

Table 2 Kinetic rate constants of FLRG/FS-288 and Activin A, BMP-2, MSTN, GDF-11 binding

Analyte	Ligand	$k_{on}$	$k_{off}$	$K_d$
MSTN	FLRG	$5.73 \times 10^4$	$9.40 \times 10^{-4}$	$1.64 \times 10^{-8}$
Activin A	FLRG	$1.68 \times 10^6$	$1.24 \times 10^{-3}$	$7.36 \times 10^{-10}$
BMP-2	FLRG	$3.26 \times 10^5$	$3.95 \times 10^{-2}$	$1.21 \times 10^{-7}$
GDF-11	FLRG	$9.14 \times 10^4$	$4.10 \times 10^{-3}$	$4.49 \times 10^{-8}$
MSTN	FS-288	$5.29 \times 10^4$	$6.51 \times 10^{-4}$	$1.23 \times 10^{-8}$
Activin A	FS-288	$8.31 \times 10^5$	$1.43 \times 10^{-3}$	$1.72 \times 10^{-9}$
BMP-2	FS-288	$4.48 \times 10^5$	$6.90 \times 10^{-2}$	$1.36 \times 10^{-7}$
GDF-11	FS-288	$2.61 \times 10^5$	$1.29 \times 10^{-3}$	$4.95 \times 10^{-9}$

General curve fitting was performed for 12.5-200 nM of analyte. A 1 : 1 binding with mass transfer was used as a model.

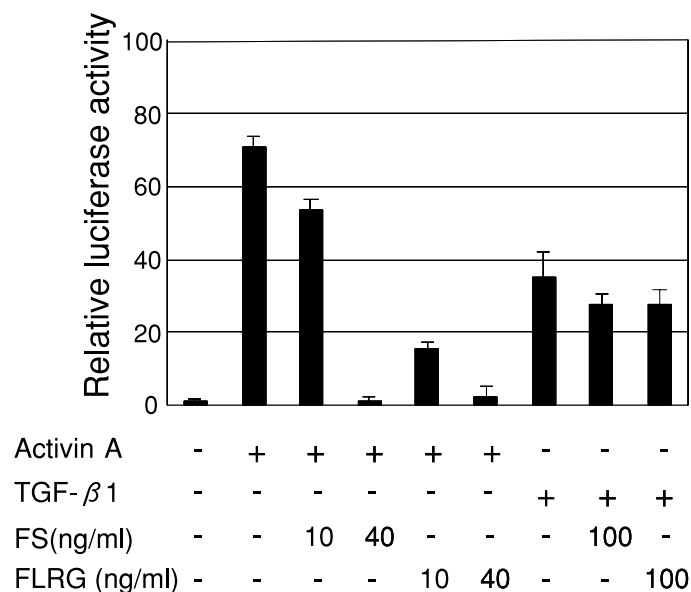


whereas once MSTN bound to FLRG or FS, these complexes could be stable.

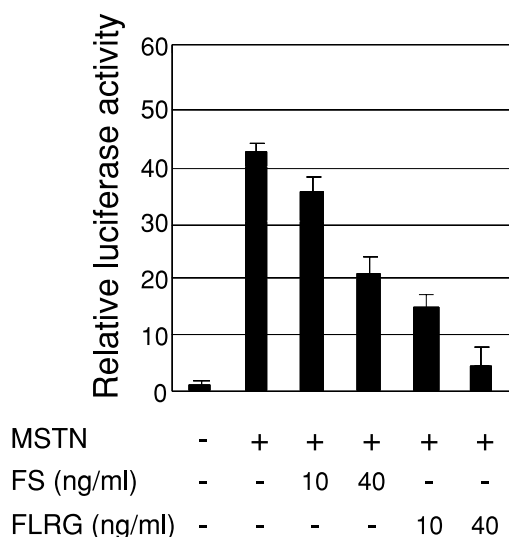
Next, we examined the inhibitory effect of hFLRG and hFS on activin A- or TGF- $\beta$ 1-induced signaling with human embryonic kidney (HEK293) cells transfected with a Smad-specific reporter plasmid, 12 $\times$ CAGA-luc. Both hFLRG and hFS decreased

the activin A-induced 12 $\times$ CAGA-luc transcriptional activities in a dose-dependent manner, whereas these proteins failed to inhibit the TGF- $\beta$ 1-induced signal (Fig. 8A). We also observed that hFLRG has a stronger inhibitory effect than hFS on activin signaling. Similar inhibitory effects of FLRG either on MSTN- or GDF-11-induced signaling were observed

A



B



C

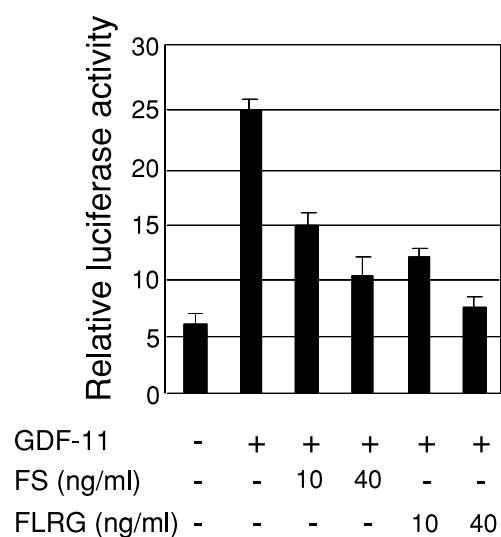


Fig. 8 Inhibition of ligand-dependent transcription by FLRG or FS.

A, Inhibition of activin A or TGF- $\beta$ 1 signaling by hFLRG or hFS protein in HEK293 cells. HEK293 cells were transfected with CAGA-luc and CMV- $\beta$ gal, and cultured with activin A (20 ng/ml) or TGF- $\beta$ 1 (15 ng/ml) with or without different concentrations of hFLRG or hFS proteins for 24 h. The luciferase activity of each cell lysate was measured and normalized to  $\beta$ -galactosidase activity. The mean  $\pm$  SE of triplicate determination is shown.

B, C, Inhibition of MSTN or GDF-11 signaling by hFLRG or hFS protein in HEK293 cells. HEK293 cells were transfected with CAGA-luc and CMV- $\beta$ gal, and cultured with MSTN (20 ng/ml) or GDF-11 (20 ng/ml) as described in A.

in a dose-dependent manner (Fig. 8B and C). These results suggested that FLRG expressed in the heart could negatively regulate the activin family members including MSTN during heart development.

## DISCUSSION

In this study, we demonstrated that FLRG mRNA was constantly expressed in the heart, whereas it was expressed in skeletal muscles at a very low level, during various stages of mouse development. In contrast, MSTN was stably expressed in skeletal muscles but not in the heart during development (Fig. 1). The high expression of FLRG mRNA in cardiac tissue was also confirmed by *in vitro* cardiomyocyte differentiation using P19CL6 cells (Fig. 2). We have previously shown that human FLRG mRNA was abundantly expressed in the heart, and was detected in skeletal muscle at a low level (5). Further detail expression analyses of these genes during embryogenesis encouraged us to hypothesize that FLRG and MSTN have functional roles during cardiomyogenesis.

Various members of the TGF- $\beta$  superfamily, including activins and BMPs, are involved in cardiac development. In particular, activin has been shown to regulate cardiac myogenesis in the early stage of chick embryogenesis (34, 35). MSTN is well known as a potent negative regulator for skeletal muscles; therefore, MSTN has been considered to function specifically in skeletal muscles. However, other groups have shown that MSTN mRNA is also expressed in the heart (19). Moreover, a recent study revealed that the expression of MSTN increases in hypertrophied cardiomyocytes (20, 36). This raises a possibility that MSTN may function as a negative feedback regulator to counteract these pathological phenomenon. These observations further support our hypothesis that MSTN also plays some roles during heart development.

During chick skeletal muscle development, the activity of MSTN is regulated by a neutralizing factor, FS. FS, which increases muscle mass by inhibiting the MSTN-dependent growth arrest of skeletal muscles (37). Since another neutralizing factor, FLRG, is also expressed and shows a distinct expression during heart development, both FLRG and FS may be cooperatively involved in the regulation of cardiac tissues.

The members of the TGF- $\beta$  superfamily are capable of inducing the transformation of endocardial

cells into mesenchymal cells, which initiates atrioventricular (AV) cushion formation (38-40). Activin  $\beta$ A has been reported to be expressed in mesenchymal cells of the AV cushion, and to induce endothelial-to-mesenchymal transformation (EMT) (41). When we analyzed the expression of activin mRNA in E14.5 mouse embryos by *in situ* hybridization, it was mainly detected in smooth muscle of the aorta and pulmonary artery, some valve leaflets, and cardiac muscles in the ventricle. Intriguingly, the localization of activin mRNA in great arteries or valvular structures was quite similar to that of FLRG mRNA, but did not completely overlap with that of MSTN mRNA (Fig. 6). The localization of MSTN mRNA was rather complementary to that of FLRG mRNA (Fig. 6D and E). Amthor, *et al.* also described similar complementary expressions of MSTN and FS mRNAs during wing and interlimb somite development in chick embryos. They demonstrated that FS prevented the inhibiting effect of MSTN on skeletal muscle development both *in vivo* and *in vitro* (37). Combining these findings, we speculate that during heart development, MSTN could be involved in aortic valve or pulmonary valve formation in cooperation with the negative regulator in the heart, FLRG. We have also shown that GDF-11, a close family member of MSTN, is expressed in similar tissues as MSTN (Fig. 6). No cardiac anomaly of MSTN-knockout mice could be due to compensation of MSTN absence by GDF-11 (17).

By using the surface plasmon resonance biosensor analysis, we have shown that the dissociation constant of MSTN against FLRG or FS-288 was low compared to other TGF- $\beta$  family members. This suggests that MSTN hardly dissociate from FLRG or FS-288, thus MSTN can form a stable complex with these molecules. Intriguingly, Hill, *et al.* previously reported that the MSTN complex immunopurified from serum with anti-MSTN monoclonal antibody contained FLRG but not FS, indicating that MSTN forms a complex with FLRG but not FS *in vivo* (16). FS-288 has much higher affinity against activin than the major form, FS-315 (32, 33). Moreover, FS-288 also binds to heparan sulfate proteoglycans on the cell surface, and therefore functions to sequester activin family members more tightly in the extracellular matrix (32). Based on this information, our data suggest that MSTN is not only neutralized by forming a stable complex with FLRG in serum but also negatively regulated by FS-288 expressed locally during heart development.

Recently, regenerative medicine has attracted

many researchers and is leading to new ways to repair and regenerate cardiac disorders. Therapy by transplantation of bone marrow stem cells or tissue-derived stem cells into damaged cardiac tissue after myocardial infarction has been investigated (42); however, for children with severe congenital heart anomalies or lethal heart diseases like dilated cardiomyopathy, invasive therapies such as surgical operation or heart transplantation are still required. Further understanding of the regulatory factors during cardiac development is needed to enable less-invasive therapy for these patients.

In summary, our results suggest that MSTN, and its negative regulator, FLRG, could be involved in cardiac muscle development and valvulogenesis in cooperation with activin and FS.

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## REFERENCES

- Vale W, Rivier C, Hsueh A, Campen C, Meunier H, Bicsak T, Vaughan J, Corrigan A, Bardin W, Sawchenko P, Petraglia F, Plotsky P, Spiess J, Rivier J : Chemical and biological characterization of the inhibin family of protein hormones. *Recent Prog Horm Res* 44 : 1-34, 1988
- Chang H, Brown CW, Matzuk MM : Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* 23 : 787-823, 2002
- Zimmerman CM, Padgett RW : Transforming growth factor beta signaling mediators and modulators. *Gene* 249 : 17-30, 2000
- Sugino H, Tsuchida K : Activin and follistatin. In : Canalis E ed. *Skeletal Growth Factors*. Lippincott Williams & Wilkins, Philadelphia, 2000, pp.251-263
- Tsuchida K, Arai KY, Kuramoto Y, Yamakawa N, Hasegawa Y, Sugino H : Identification and characterization of a novel follistatin-like protein as a binding protein for the TGF- $\beta$  family. *J Biol Chem* 275 : 40788-40796, 2000
- Tsuchida K, Matsuzaki T, Yamakawa N, Liu Z, Sugino H : Intracellular and extracellular control of activin function by novel regulatory molecules. *Mol Cell Endocrinol* 180 : 25-31, 2001
- Hayette S, Gadoux M, Martel S, Bertrand S, Tigaud I, Magaud JP, Rimokh R : FLRG (follistatin-related gene), a new target of chromosomal rearrangement in malignant blood disorders. *Oncogene* 16 : 2949-2954, 1998
- Shimasaki S, Koga M, Esch F, Cooksey K, Mercado M, Koba A, Ueno N, Ying SY, Ling N, Guillemin R : Primary structure of the human follistatin precursor and its genomic organization. *Proc Natl Acad Sci USA* 85 : 4218-4222, 1988
- Okabayashi K, Shoji H, Onuma Y, Nakamura T, Nose K, Sugino H, Asashima M : cDNA cloning and distribution of the *Xenopus* follistatin-related protein. *Biochem Biophys Res Commun* 254 : 42-48, 1999
- de Winter JP, ten Dijke P, de Vries CJ, van Achterberg TA, Sugino H, de Waele P, Huylebroeck D, Verschueren K, van den Eijnden-van Raaij AJ : Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors. *Mol Cell Endocrinol* 116 : 105-114, 1996
- Nakamura T, Sugino K, Titani K, Sugino H : Follistatin, an activin-binding protein, associates with heparan sulfate chains of proteoglycans on follicular granulosa cells. *J Biol Chem* 266 : 19432-19437, 1991
- Iemura S, Yamamoto TS, Takagi C, Uchiyama H, Natsume T, Shimasaki S, Sugino H, Ueno N : Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early *Xenopus* embryo. *Proc Natl Acad Sci USA* 95 : 9337-9342, 1998
- Tortoriello DV, Sidis Y, Holzman DA, Holmes WE, Schneyer AL : Human follistatin-related protein : a structural homologue of follistatin with nuclear localization. *Endocrinology* 142 : 3426-3434, 2001
- Lee SJ, and McPherron AC : Regulation of myostatin activity and muscle growth. *Proc Natl Acad Sci USA* 98 : 9306-9311, 2001
- Zimmers TA, Davies MV, Koniaris LG, Haynes

- P, Esquela AF, Tomkinson KN, McPherron AC, Wolfman NM, Lee SJ : Induction of cachexia in mice by systemically administered myostatin. *Science* 296 : 1486-1488, 2002
16. Hill JJ, Davies MV, Pearson AA, Wang JH, Hewick RM, Wolfman NM, Qiu Y : The myostatin propeptide and the follistatin-related gene are inhibitory binding proteins of myostatin in normal serum. *J Biol Chem* 277 : 40735-40741, 2002
  17. McPherron AC, Lawler AM, Lee SJ : Regulation of skeletal muscle mass in mice by a new TGF- $\beta$  superfamily member. *Nature* 387 : 83-90, 1997
  18. Sharma M, Langley B, Bass J, Kambadur R : Myostatin in muscle growth and repair. *Exerc Sport Sci Rev* 29 : 155-158, 2001
  19. Sharma M, Kambadur R, Matthews KG, Somers WG, Devlin GP, Conaglen JV, Fowke PJ, Bass JJ : Myostatin, a transforming growth factor- $\beta$  superfamily member, is expressed in heart muscle and is upregulated in cardiomyocytes after infarct. *J Cell Physiol* 180 : 1-9, 1999
  20. Cook SA, Matsui T, Li L, Rosenzweig A : Transcriptional effects of chronic Akt activation in the heart. *J Biol Chem* 277 : 22528-22533, 2002
  21. Morissette MR, Cook SA, Foo S, McKoy G, Ashida N, Novikov M, Scherrer-Crosbie M, Li L, Mastui T, Brooks G, Rosenzweig A : Myostatin regulates cardiomyocyte growth through modulation of Akt signaling. *Circ Res* 99 : 15-24, 2006
  22. Habara-Ohkubo A : Differentiation of beating cardiac muscle cells from a derivative of P19 embryonal carcinoma cells. *Cell Struct Funct* 21 : 101-110, 1996
  23. Schaeren-Wiemers N, Gerfin-Moser A : A single protocol to detect transcripts of various types and expression levels in neural tissues and cultured cells : *in situ* hybridization using digoxigenin-labelled cRNA probes. *Histochemistry* 100 : 431-440, 1993
  24. Hashimoto O, Kawasaki N, Tsuchida K, Shimasaki S, Hayakawa T, Sugino H : Difference between follistatin isoforms in the inhibition of activin signaling : activin neutralizing activity of follistatin isoforms is dependent on their affinity for activin. *Cell Signal* 12 : 565-571, 2000
  25. Tsuchida K, Vaughan JM, Wiater E, Gaddy-Kurten D, Vale WW : Inactivation of activin-dependent transcription by kinase-deficient activin receptors. *Endocrinology* 136 : 5493-5503, 1995
  26. Nakatani M, Yamakawa N, Matsuzaki T, Shimasaki S, Sugino H, Tsuchida K : Genomic organization and promoter analysis of mouse follistatin-related gene (FLRG). *Mol Cell Endocrinol* 189 : 117-123, 2002
  27. Nakamura T, Sano M, Songyang Z, Schneider MD : A Wnt- and beta-catenin-dependent pathway for mammalian cardiac myogenesis. *Proc Natl Acad Sci USA* 100 : 5834-5839, 2003
  28. Feijen A, Goumans MJ, van den Eijnden-van Raaij AJM : Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryo suggests specific developmental functions for different activins. *Development* 120 : 3621-3637, 1994
  29. Tsuchida K, Nakatani M, Matsuzaki T, Yamakawa N, Liu Z, Bao Y, Arai KY, Murakami T, Takehara Y, Kurisaki A, Sugino H : Novel factors in regulation of activin signaling. *Mol Cell Endocrinol* 225 : 1-8, 2004
  30. Bogdanovich S, Krag TO, Barton ER, Morris LD, Whittmore LA, Ahima RS, Khurana TS : Functional improvement of dystrophic muscle by myostatin blockade. *Nature* 420 : 418-421, 2002
  31. Wang HQ, Takebayashi K, Tsuchida K, Nishimura M, Noda Y : Follistatin-related gene (FLRG) expression in human endometrium : sex steroid hormones regulate the expression of FLRG in cultured human endometrial stromal cells. *J Clin Endocrinol Metab* 88 : 4432-4439, 2003
  32. Sugino K, Kurosawa N, Nakamura T, Takio K, Shimasaki S, Ling N, Titani K, Sugino H : Molecular heterogeneity of follistatin, an activin-binding protein. Higher affinity of the carboxyl-terminal truncated forms for heparan sulfate proteoglycans on the ovarian granulosa cell. *J Biol Chem*. 268 : 15579-15587, 1993
  33. Hashimoto O, Nakamura T, Shoji H, Shimasaki S, Hayashi Y, Sugino H : A novel role of follistatin, an activin-binding protein, in the inhibition of activin action in rat pituitary cells. Endocytotic degradation of activin and its acceleration by follistatin associated with cell-surface heparan sulfate. *J Biol Chem* 272 : 13835-13842, 1997
  34. Yatskievych TA, Ladd AN, Antin PB : Induction of cardiac myogenesis in avian pregastrula epiblast : the role of the hypoblast and activin. *Development* 124 : 2561-2570, 1997



35. Ladd AN, Yatskievych TA, Antin PB : Regulation of avian cardiac myogenesis by activin/TGF beta and bone morphogenetic proteins. *Dev Biol* 204 : 407-419, 1998
36. Shyu KG, Ko WH, Yang WS, Wang BW, Kuan P : Insulin-like growth factor-1 mediates stretch-induced upregulation of myostatin expression in neonatal rat cardiomyocytes. *Cardiovasc Res* 68 : 405-414, 2005
37. Anthor H, Nicholas G, McKinnell I, Kemp CF, Sharma M, Kambadur R, Patel K : Follistatin complexes Myostatin and antagonizes Myostatin-mediated inhibition of myogenesis. *Dev Biol* 270 : 19-30, 2004
38. Eisenberg LM, Markwald RR : Molecular regulation of atrioventricular valvuloseptal morphogenesis. *Circ Res* 77 : 1-6, 1995
39. Brown CB, Boyer AS, Runyan RB, Barnett JV : Requirement of type III TGF beta receptor for endocardial cell transformation in the heart. *Science* 283 : 2080-2082, 1999
40. Wang J, Sridurongrit S, Dudas M, Thomas P, Nagy A, Schneider MD, Epstein JA, Kaartinen V : Atrioventricular cushion transformation is mediated by ALK2 in the developing mouse heart. *Dev Biol* 286 : 299-310, 2005
41. Moore CS, Mjaatvedt CH, Gearhart JD : Expression and function of activin beta A during mouse cardiac cushion tissue formation. *Dev Dyn* 212 : 548-562, 1998
42. Fukuda K, Yuasa S : Stem cells as a source of regenerative cardiomyocytes. *Circ Res* 98 : 1002-1013, 2006