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Hepatocyte Nuclear Factor 4 alpha Regulates Expression of the Mouse Female-Specific *Cyp3a41* Gene in the Liver

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Abstract

CYP3A41 is a female-specific cytochrome P450 in mouse liver. A putative HNF4α-binding site was found at -99/-87 in the promoter of *Cyp3a41* by reporter assays performed in the hepatocytes of females. Cotransfection of a HNF4α expression plasmid significantly increased transcription of the reporter gene. Although electrophoretic mobility shift assays with liver nuclear extracts did not show a sex-related difference, chromatin immunoprecipitation (ChIP) assays showed that larger amounts of HNF4α bound to *Cyp3a41* in female than male mice. A relation between the amount of HNF4α on the *Cyp3a41* gene and mRNA expression was observed in hepatic tissue sets which differ in mRNA expression depending on the sex, age or endocrine status of mice. The degree of histone-3-lysine-4 dimethylation and histone-3-lysine-27 trimethylation around the HNF4α-binding site was higher in females and males, respectively. Moreover, the ChIP assay indicated greater acetylation of histone-4-lysine-8 of the *Cyp3a41* chromatin in females than males. HNF4α plays an important role in the transcriptional activation of the *Cyp3a41* gene and a sex difference in chromatin structure may contribute to the female-specific expression of *Cyp3a41* in the liver of mice.

Introduction

Cytochrome P450s (CYPs) are heme-containing enzymes responsible for the oxidative metabolism of various endogenous steroids, bile acids, hormones, and fatty acids, as well as foreign compounds, including environmental chemicals and a large number of active drugs (Guengerich, 1991). The CYP3A subfamily represents the most abundant forms of CYP in the adult human liver, constituting approximately 30% of the total CYP content. This subfamily comprises CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 is the most prevalent and important isoforms in adults, accounting for 95% of the CYP3A mRNA pool in the liver (Koch et al., 2002) and is involved in the metabolism of approximately half of all currently marketed drugs that undergo oxidative biotransformation (Williams et al., 2004).

Sex differences in drug metabolism are well established for some human CYP isoforms. Recent studies have suggested that CYP2B6, CYP2A6 and CYP3A have greater levels of activity in women than men, and CYP2E1 and CYP1A2 have slightly higher activity in men, although significant levels of activity/protein/mRNA expression of these isoforms are detectable in both sexes. (Harris et al., 1995; Anderson, 2005; Cotreau et al., 2005; Scandlyn et al., 2008; Lamba et al., 2003; Nakajima et al., 2006). Differently from humans, the expressions of many isoforms of CYPs in rodents are markedly different between male and female animals (Kato and Yamazoe, 1993); for example, rat *CYP2C12* (MacGeoch et al., 1984; Kamataki et al., 1985) and *CYP3A9* (Kawai et al., 2000) and mouse *Cyp3a41* and *Cyp3a44* (Sakuma et al., 2002) all show female specificity in the adult liver. Sexually dimorphic plasma profiles of growth hormone (GH) have been reported to contribute to the sex-dependent regulation of CYP enzymes (MacGeoch et al., 1984). The intracellular signaling networks that establish

and maintain the sex-dependent patterns of liver gene expression are likely complex, and may involve the integrated actions of an array of liver transcription factors. These transcription factors are termed hepatocyte-enriched nuclear factors, and include HNF1α, HNF3, HNF4α, HNF6 and several CCAT/enhancer binding proteins (C/EBPs). The expression of most hepatocyte-enriched nuclear factors is regulated by GH (Wiwi and Waxman, 2004) and contributes to hepatic *Cyp* expression (Akiyama and Gonzalez, 2003).

HNF4α (NR2A1), a highly conserved hepatocyte-enriched nuclear factor, is required for the hepatic expression of several genes showing sex-specific expression in the liver, notably genes of the *Cyp* superfamily (Wiwi and Waxman, 2004). Male HNF4α-knocked out mice showed decreased expression of several male-specific *Cyp* genes and increased expression of some female-specific *Cyp* genes. By contrast, HNF4α was disclosed to play a dominant, positive role in the regulation of female-specific liver *Cyp*s genes, with the down-regulation of these genes including *Cyp3a41* in female HNF4α-knocked out mice (Wiwi et al., 2004; Holloway et al., 2006).

With respect to the female-specific mouse *Cyp3a41* gene, we reported that the sex-specific pattern of GH secretion is a critical determinant of sexually dimorphic expression (Sakuma et al., 2002) and the expression of *Cyp3a41* is under the cooperative control of GH and glucocorticoid hormone (Sakuma et al., 2004; 2008). Furthermore, in preliminary experiments with a series of reporter constructs containing deletions of the 5’-flanking region of the *Cyp3a41* gene, we had found several enhancer regions, two of which included putative HNF4α-binding sites. Nonetheless, although the nuclear factor HNF4α was suggested to participate in the regulation of female-specific expression of *Cyp3a41*, the precise mechanism by which HNF4α

regulates the sexually dimorphic expression of the gene had not been elucidated. This prompted us to investigate the role of HNF4α in the regulation of female-specific *Cyp3a41* expression.

The present study suggests that sex differences in the chromatin structure including the modification of histones of the *Cyp3a41* gene contribute to the sex specificity of *Cyp3a41* expression by controlling access of the liver-specific transcription factors including HNF4α to the DNA.

Materials and Methods

Materials. Materials for the isolation and culturing of hepatocytes were purchased from Wako Pure Chemical (Osaka, Japan), Gibco-BRL (Grand Island, NY), Invitrogen (Carlsbad, CA), and Sigma-Aldrich (St. Louis, MO). Percoll was obtained from GE Healthcare Biosciences (Buckinghamshire, UK). The TaKaRa RNA PCR Kit (AMV), version 3.0, was obtained from TAKARA Shuzo (Kyoto, Japan). The TaqMan MGB Gene Expression Detection kit was a product of Applied Biosystems (Foster City, CA). TransPass D1 Transfection Reagent was from New England Biolabs (Hercules, CA). Trans IT-EE hydrodynamic delivery solution was obtained from Mirus Bio Corporation (Madison, WI). The TNT®T7 Coupled Reticulocyte Lysate System was from Promega (Madison, WI). The antibodies against HNF4α (sc-8987), acetylated histone H4 (Lys 8) (AcH4K8, sc-8660), and control rabbit IgG (sc-2027) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies against dimethyl-histone H3 (Lys 4) (H3K4me2, 07-030) and trimethyl-Histone H3(Lys27) (H3K27me3, 07-449) were obtained from Millipore (Temecula, CA). [γ-^{32}P] ATP was purchased from MP Biomedicals (Irvine, CA). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Other chemicals were purchased from Wako Pure Chemical, or Sigma-Aldrich, and of the highest grade commercially available.

Animals. ddY mice (6 weeks old) of both sexes, weighing 25 to 30 g, were supplied by Japan SLC (Shizuoka, Japan). Japan SLC also supplied hypophysectomized or sham-operated 6-week-old female ddY mice. At 7 weeks of age, mice received an osmotic infusion (1.5 µg/hr) of recombinant human GH for 7 days. The osmotic minipump, an Alzet 1007, implanted into the back of the mice,was designed to release

its contents at a rate of 0.5 μl/hr. Verification of the surgery was confirmed by a lack of weight gain and the absence of pituitary fragments during necropsy at the end of the study. GH replacement was checked using body weight gain. Animals were housed in the Laboratory Animal Center of the University of Toyama under the supervision of certified laboratory veterinarians and treated according to a research protocol approved by the University's Institutional Animal Care and Use Committee. Animals were allowed to take food and water *ad libitum* and subjected to a 12-h light/dark cycle. The liver was excised immediately after death and used for the preparation of total RNA and ChIP assay.

Construction of Plasmids. For the construction of *Cyp3a41* (Accession No., AC114427) reporter genes, a mouse *Cyp3a41* gene fragment (-3680/+191) containing a part of the first exon and 5'-flanking region was amplified by PCR with, 5'-GAACCCTCATGATGCCTTCCTCCAC-3' (-3680/-3656) and 5'-ACATGGTCTGGGAAGGAACTGGGAAG-3' (+166/+191) as the sense and antisense primers, respectively. Genomic DNA prepared from the liver of a C57BL/6NCrj male mouse (Clea Japan, Tokyo, Japan) served as a template. The DNA fragment was subcloned into a pGEM T-easy vector (Promega, Madison, WI). The resultant plasmid was used as a template for the amplification of a series of progressive 5'-deleted fragments. Sense primers used for this 2nd amplification were as follows; 5'-TCACACAGATCGGTACCTTAAAG-3' (-177/-155), 5'-CAGCAGTGAGTGGCGGTACCTGTC-3' (-613/-590), 5'-AGACTTTGGTACCACGTGTGTGAC-3' (-680/-657), 5'-TCACTCAGTCCTATCCTTCACGCGTCCACA-3' (-863/-834), 5'-TGGTTGGTTCACGCGTGACAGTTATC-3' (-1643/-1618),

5'-CTTAGAGGGACGCGTGCAGGTTGTTTGTTA-3' (-2405/-2376), 5'-AACCCTCATGACGCGTTCCTCCACA TT-3' (-3679/-3653). The common antisense primer was 5'-GTCTGTCAGCAAGCTTACTCCTCA GT-3' (+52/+72). All primers used in this step included a restriction enzyme recognition site (underlined); namely a *Kpn* I or *Mlu* I site in sense primers and a *Hin*d III site in the common antisense primer. DNA fragments amplified with the respective sense primer and the common antisense primer were digested with the appropriate restriction enzymes, and then ligated into the *Mlu* I */Hin*d III site or *Kpn* I/*Hin*d III site of the pGL3-basic vector (Promega, Madison, WI). The resultant constructs were named based on the region they included (-3669/+61-Luc, -2396/+61-Luc, -1633/+61-Luc, -844/+61-Luc, -670/+61-Luc, -596/+61-Luc, and -163/+61-Luc). The HNF4α-binding site-mutated reporter construct (-163/+61-HNF4α mut-Luc) was produced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primers were designed to carry a two-base pair CC to AA mutation. The primer sequences were as follows: sense: 5'-GGGAAAGAGA**CGACCA**A**AGTAAA**GGTGATGCAGG-3'; and antisense: 5'-CCTGCATCACC**TTTACTTTGGTCG**TCTCTTTCCC-3'. The putative response element is indicated in bold, and the mutated bases are underlined. The HNF4α expression plasmid was generated by replacing the DNA fragment between the *Hin*d III and *Xba* I sites containing the coding sequence of *Renilla* luciferase of phRL-SV40 (Promega, Madison, WI) with a 1,425-bp cDNA fragment including the entire coding region of mouse HNF4α (pMHNF-4 (Accession No., D29015), Hata S et al., 1995). The DNA sequences of all plasmids were determined using a dye terminator cycle sequencing FS ready reaction kit with an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Preparation of Primary Hepatocyte Cultures. The livers of ddY mice were perfused with collagenase-containing Hanks'solution, and viable hepatocytes were isolated by Percoll isodensity centrifugation as described (Nemoto and Sakurai, 1995). The cells were dispersed in Waymouth MB 752/1 medium containing bovine serum albumin (2 g/l), transferring (0.5 mg/l), and selenium (0.5 μg/l), and seeded in dishes at a density of $5x10^5$ cells/ml/35-mm dish. The Waymouth medium did not contain phenol red, a pH indicator, to exclude estrogen-like action. The culture dishes were maintained at 37°C in a CO_2-humidified incubator. The medium was renewed 24 h after seeding.

Transfection of the Reporter Construct and HNF4α Expression Plasmid into Hepatocytes in Culture. Mouse hepatocytes were cultured for 24 h in Waymouth medium and then transfected using Transpass D1 Transfection Reagent (New England Biolabs, Hercules, CA). The transfection mixure consisted of Waymouth medium, the reporter construct, phRL-SV40, empty plasmid or the HNF4α expression plasmid if required and Transpass D1 at 0.6 ml, 1.8 μg, 0.3 μg, 5.0 μg, and 1.5 μl, respectively. Transfection continued for 3 h, and then the medium was changed. After a further 48 h of incubation, a luciferase assay was performed. For the analysis of the effects of GH treatment on CYP3A41 mRNA expression in hepatocytes, GH was added 3 h after transfection of the HNF4α expression plasmid at a final concentration of 71 ng/ml, and the cells cultured a further 24 h. The cells were harvested and total RNA was prepared.

Hydrodynamic Infusion of the Reporter Construct. Six-week-old ddY mice were given a rapid (5 s) tail vein injection of the pGL3-basic vector (10 μg), *Cyp3a41* reporter construct (-163/+61-Luc, 10 μg), or *Cyp3a41* HNF4α mutated reporter construct (-163/+61-HNF4α mut-Luc, 10 μg) and pRL-SV40 vector (1.5 μg), an internal standard, dissolved in transfection reagent in a volume equal to 10% of body weight.

After 24 h, the mice were sacrificed, and the livers were homogenized in a 5-fold volume of the lysis buffer (0.1 M Tris-HCl, 2 mM EDTA, and 0.1% Triton X-100). The homogenates were then centrifuged (15,000 x g, 4°C, 10 min). An aliquot of the supernatant was diluted 60 fold with 1M HEPES (pH 7.5), and luciferase activity was determined.

Luciferase Assay. The luciferase assay was performed with the dual-luciferase reporter assay system (Promega, Madison, WI) as recommended, and luminescence was determined with a TD-20/20 luminometer (Promega, Madison, WI). Values of firefly luciferase activity were normalized to those of *Renilla* luciferase activity in individual experiments.

Western Blot Analysis. Amounts of HNF4α protein in nuclear extracts prepared from the livers of male and female mice were examined by Western blotting. The same preparations were also analyzed by EMSA. Nuclear extracts were prepared from the livers of male and female ddY mice as reported (Gorski et al., 1986). Twenty micrograms of protein from the nuclear extracts was separated on an 8% SDS-polyacrylamide gel, transferred to a PVDF membrane, and incubated for 1 h with a polyclonal rabbit antibody against mouse HNF4α at a dilution 1: 1000. After incubation with a biotinylated anti-rabbit IgG secondary antibody, and a streptavidin-biotinylated Horseradish peroxidase complex, bands were detected by chemiluminescence (ECL Plus; GE Healthcare Biosciences, Buckinghamshire, UK) and visualized using LAS-1000 plus (Fujifilm, Tokyo, Japan).

Electrophoretic Mobility Shift Assay. *In vitro* transcribed/translated mouse HNF4α protein synthesized using the TNT®T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI) or the nuclear extract prepared from liver of male and female ddY mice

according to a previously reported method (Gorski et al., 1986) was used. The control lysate for *in vitro* transcribed/translated mouse HNF4α protein was prepared using nuclease-free water as a template. Double-stranded oligonucleotides were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase and purified with MicroSpin™ G-25 Columns (GE Healthcare BioSciences, Buckinghamshire, UK). The binding reaction was carried out in a 20-µl volume containing 50 mM Tris-HCl (pH7.5), 250 mM NaCl, 2.5 mM DTT, 2.5 mM EDTA, 5 mM $MgCl_2$, 20% glycerol, 0.25 mg/ml poly(dI-dC), and 5 µl of synthesized HNF4α, or 5 µg of the nuclear extract prepared from mouse liver. Reaction mixtures were preincubated at 25°C for 10 min before the addition of the [γ-^{32}P] ATP-labeled probe. A 50- or 100-fold excess of unlabeled oligonucleotide-containing reaction mixture was also preincubated before adding the radiolabeled probe. Samples were kept at 25°C for an additional 20 min. In supershift experiments, 2 µg of the anti-HNF4α polyclonal antibody was added to the binding reaction mixure at 25°C before addition of the probe and then all samples were separated on a 4% polyacrylamide gel in 0.5X Tris/borate/EDTA buffer at 200 V for 90 min. The gel was dried and exposed to an imaging plate to detect DNA-protein complexes with a Fujifilm Bio-imaging analyzer-5000 (Fuji Film, Tokyo, Japan).

Chromatin Immunoprecipitation (ChIP) Assay. The ChIP assay was carried out using the kit purchased from Upstate Biotechnology (Charlottesville, VA) according to the manufacturer's protocol with some modifications. Briefly, frozen liver tissues were fixed with 1% formaldehyde in phosphate-buffered saline at room temperature for 15 min and quenched with 0.125 M glycine for another 5 min. Liver tissues were washed twice with ice-cold PBS, and then homogenized by a Dounce homogenizer. After centrifugation, the cell pelletes were resuspended in cell lysis buffer (5 mM PIPES (pH

8.0), 85 mM KCl, 0.5% NP-40, and protease inhibitors), and incubated for 15 min. After further centrifugation, nuclei were resuspended in SDS lysis buffer supplemented in the kit and incubated for 20 min, and the lysates were sonicated to obtain DNA fragments 600 bp in average length. Samples were then treated as indicated by the manufacturer's protocol. The amount of antibodies used in the experiment was 3 μg each. After reversion of DNA-protein cross-linking by incubation with NaCl, the remaining proteins were digested by adding proteinase K (final concentration, 36 μg/ml) and incubation at 45 °C for 2 h. The DNA was recovered using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) Two microliters of product was used as a template for quantitative PCR. The primers for amplification of the *Cyp3a41* gene -207/+18 fragment were 5'-GCCTTACTCACATGGCACTGTT-3' (sense) and 5'-AAGGGCTAACCTTTCTGCCTACTC-3' (antisense). *ApoCIII* was used as a negative control gene which is regulated by HNF4α but shows no sex-related difference in mRNA expression. The primers for amplification of the *ApoCIII* gene -336/+34 fragment were 5'-AGCAGGCCTGAGGTCCAAG-3' (sense) and 5'-GTAGCTAGCTGCTTCTAGG-3' (antisense). Chromosomal DNA fragments of both genes were amplified by Prime STAR HS DNA polymerase (TaKaRa Shuzo, Kyoto, Japan) and detected with SYBR Green reagent. PCR conditions for the two genes were as follows: 40 cycles of denaturation at 98°C for 10 s, annealing/extension at 68°C for 30 s. The amount of immunoprecipitated chromosomal DNA of the respective gene was normalized to that inputed and expressed as a percentage of input. Amplification and detection were performed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with ABI Prism® 7000 SDS software. A dissociation curve was obtained after PCR to verify the specificity of the amplification.

The specificity of the amplification was also confirmed by direct sequencing of the PCR products of some samples.

Total RNA preparation and Quantitative RT-PCR Analysis. Total RNA was prepared from primary mouse hepatocytes or hepatic tissues using guanidine thiocyanate as described previously (Nemoto and Sakurai, 1995). Quantitative real-time RT-PCR was performed using a TaKaRa RNA PCR Kit (AMV), version 3.0 in combination with a gene-specific TaqMan MGB Gene Expression Detection Kit or SYBR Green reagent. The forward primer, reverse primer, and TaqMan MGB probe of the TaqMan MGB Gene Expression Detection Kit for CYP3A41, designed by ourselves with assistance from Primer Express software, were 5'-GCCAAAGGGATTTTAAGAGTTGACT-3', 5'-GGTGTCAGGAATGGAAAAAGTACA-3', and 5'-FAM-ATCCTTTGGTCTTCTCAG-MGB-3', respectively. GAPDH cDNA was detected with the SYBR Green reagent and a gene-specific primer set. The forward and reverse primers were 5'-TCCACTCACGGCAAATTCAACG-3'and 5'-TAGACTCCACGACATACTCAGC-3', respectively. PCR conditions were as follows: initial denaturation at 95°C for 4 min, then for CY3A41, 50 cycles of denaturation at 95°C for 15 s, and annealing /extension at 60°C for 1 min, and for GAPDH, 40 cycles of denaturation at 95°C for 15 s, annealing at 64°C for 15 s, and extension at 72°C for 1 min. The mRNA level of CYP3A41 was normalized to that of GAPDH and expressed as the fold-change with the control mRNA level as 1. Amplification and detection were performed using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) with ABI Prism® 7000 SDS software.

Statistical Analysis. Data are presented as the mean ± S.D. Statistically significant differences among groups were identified by one-way analysis of variance (Tukey post hoc test). Significance was established at $p < 0.05$.

Results

Using a series of *Cyp3a41* luciferase reporter constructs, -3669/+61-Luc, -2396/+61-Luc, -1633/+61-Luc, -844/+61-Luc, -670/+61-Luc, -596/+61-Luc, and -163/+61-Luc, designed based on their deletion end points relative to the *Cyp3a41* transcription start site, reporter gene assays were performed in primary cultures of hepatocytes from female mice (Fig. 1). -163/+61-Luc showed significant luciferase activity, the highest level among the constructs examined. Luciferase activity was decreased to approximately 60% of that of -163/+61-Luc by extension to-596. A further decrease was observed by extension from -1633 to -844. This decrease was partially recovered by extension up to -3669. These results suggest the existence of regions involved in transcriptional activation of the *Cyp3a41* gene between -163 and +61, and between -3669 and -2396, and negative regulatory elements between -596 and -163, and between -1633 and -844.

Searching the sequence between -163 and +61 using the web-based program TFSEARCH revealed a binding site similar to the sequences containing direct repeats of the hexamer AGGTCA separated by one base (DR1), reported as the HNF4α-binding element. HNF4α, known as a member of hepatocyte-enriched nuclear factors, has been reported to act as a regulating factor in the hepatic expression of sex-specific genes in the liver (Wiwi and Waxman, 2004). We termed the possible regulatory element a putative HNF4α-binding site (Fig. 2A). To identify this putative HNF4α-binding site necessary for the expression of *Cyp3a41*, and to investigate the role of HNF4α,we performed a luciferase assay using a construct with a mutated HNF4α-binding site and mouse HNF4α expression plasmid in primary cultured hepatocytes from female mice. As shown in Fig. 2B, mutation of the putative HNF4α-binding site significantly

decreased the transcriptional activity to nearly that of the pGL3 basic vector. Transcriptional activity of the wild-type construct was significantly increased by transfection of the HNF4α expression plasmid. In contrast, transfection of the HNF4α expression plasmid had no effect on the reporter gene activity of the construct containing the mutated HNF4α-binding site. This result suggests HNF4α to be involved in the transcriptional activation of *Cyp3a41* at the putative HNF4α-binding site.

To examine the ability of the putative HNF4α-binding site to bind HNF4α protein *in vitro*, an EMSA was carried out with HNF4α protein synthesized *in vitro* (Fig. 3). A shift of the probe-protein complex was observed. The specificity of the binding was investigated with competition assays. The complex was completely competed out by 100-fold excess of unlabeled HNF4α probe, but not by the mutated HNF4α probe or non-specific SP1 probe (Fig.3B). The result of this EMSA indicates that the putative-HNF4α binding site of *Cyp3a41* can bind to HNF4α *in vitro*.

Considering that HNF4α is essential for a large number of liver-expressed genes including sex-specific genes, we wondered whether it participates in the sex-specific expression of *Cyp3a41*. We anticipated that, if this was the case, one possible mechanism might be a difference of cellular HNF4α activity. An EMSA using nuclear extracts prepared from the livers of male and female mice was then performed (Fig. 4A). When nuclear extracts were incubated with the radiolabeled putative HNF4α-binding site probe, two shifted bands were observed. The lower band was efficiently competed out by an unlabeled putative HNF4α-binding site probe, but not by an unlabeled mutated HNF4α-binding site probe and an unlabeled non-specific SP1 probe. Supershift of the lower complex by the addition of anti-HNF4α antibodies was observed. These results indicate that the lower complex corresponds to that containing

HNF4α. Unexpectedly, the shifts of the lower complexes observed with nuclear extracts were not significantly different between males and females. When the radiolabeled mutated HNF4α site probe was used, no shifted band was observed. This result indicates that the two-nucleotide substitution, which is the same substitution introduced into the reporter construct "-163/+61-HNF4α mut-Luc" (Fig. 2), caused the loss of HNF4α binding and that there were no new, artifactual specific DNA/protein interactions with the mutated probe. A Western blot analysis revealed similar levels of HNF4α protein in males and females (Fig. 4B). These results suggest cellular HNF4α activity is not a key determinant of female-specific *Cyp3a41* expression.

To further explore whether HNF4α participated in the sex-related difference in *Cyp3a41* gene expression, we next performed a ChIP assay. With this system, one can detect HNF4α bound to the HNF4α-binding site of the *Cyp3a41* gene in the chromatin structure *in situ*. As shown in Fig. 5A, about four-fold more HNF4α was detected on the *Cyp3a41* gene in hepatic tissues of female mice than male mice. On the other hand, *ApoCIII*, known to be regulated by HNF4α but shows no sex-related difference in mRNA expreession (Wiwi et al., 2004), bound to HNF4α at similar levels in both sexes (Fig. 5B). This finding suggests that differences in the histone modification of *Cyp3a41* between female and male mice resulted in the different levels of HNF4α, present at the HNF4α-binding site of the *Cyp3a41* gene.

To examine whether the amount of HNF4α on *Cyp3a41* is linked with the level of CYP3A41 mRNA, the amounts of HNF4α bound to the gene and mRNA expression were compared in two sets of hepatic tissue samples. CYP3A41 mRNA expression was similar between male and female livers at 3 weeks of age, whereas females specifically expressed the gene at 7 weeks of age (Fig. 6B). Consistent with the mRNA levels,

amounts of HNF4α bound to the putative HNF4α element were similar between males and females at 3 weeks of age, but higher in females at 7 weeks of age (Fig. 6A). Fig. 6C and 6D show the results for hypophysectomized mice. *Cyp3a41* expression is under the control of GH, the female-type secretion of which is the key determinant (Sakuma et al., 2002). Expression of CYP3A41 mRNA completely disappeared after hypophysectomy and was partially recovered by continuous administration of GH by an osmotic infusion pump which mimics female-type secretion (Fig. 6D). In accordance with mRNA levels, amounts of HNF4α binding decreased to 40% of the control after hypophysectomy (Fig. 6C). These results indicating that the direction of the effect is consistent with HNF4α binding to the putative element is consistent with a role in regulating *Cyp3a41* sex-specific expression.

The epigenetic code (e.g., DNA methylation and histone modification) is implicated in the regulation of gene expression. Methylation at histone-3-lysine-4 (H3K4) is linked to activation of gene transcription, whereas methylation at histone-3-lysine-27 (H3K7) is associated with repression of gene transcription and the maintenance of heterochromatin (Heintzman et al., 2007; Wang et al., 2008). Therefore, the degree of histone-3-lysine-4 dimethylation (H3K4me2) and histone-3-lysine-27 trimethylation (H3K7me3) around the HNF4α-binding site of the *Cyp3a41* gene was investigated using a ChIP assay. As shown in Fig. 7, more H3K4me2 was found in adult females than adult males. The opposite was the case for H3K27me3. Histone acetylation results in chromatin relaxation (higher fluidity) allowing for greater accessibility of transcription factors to the recognition sites in nucleosomal DNA. The degree of histone H4 acetylation in the *Cyp3a41* promoter was much higher in females than males.

We further examined the role of the different chromatin structures by

conducting an *in vivo* reporter gene assay using the hydrodynamic method. In this assay, reporter gene activity does not reflect chromatin structure because a naked plasmid was transfected. We hypothesized that if the sex-related expression of *Cyp3a41* is regulated by the difference in HNF4α activity between males and females, a sex-difference in *Cyp3a41* gene transcription would be observed. Meanwhile, if the sex-related expression of *Cyp3a41* is regulated by the chromatin structure, no difference would be seen. As shown in Fig. 8, significant *Cyp3a41* transcriptional activity in the liver was observed not only in females but also in males when the mice were transfected with the wild-type construct. The transcriptional activity was decreased approximately 50% by mutation of the putative HNF4α-binding site in both sexes. This result supports our hypothesis, and also suggests that HNF4α activity in hepatocytes might not differ drastically between the sexes. This possibility is consistent with the results of EMSA using liver nuclear extracts and western blotting of HNF4α protein shown in Fig. 4 With regard to the findings above, we next examined the effect of the HNF4α expression plasmid on the mRNA expression of *Cyp3a41* in hepatocytes of female and male mice. We hypothesized that if causes other than the cellular amount of HNF4α, such as chromatin structure, determine the sex-specific expression of *Cyp3a41*, the response of the *Cyp3a41* gene in chromosomal DNA to transfection of the same amount of the HNF4α expression plasmid might differ between the sexes. As shown in Fig. 9, exogenous HNF4α induced *Cyp3a41* gene expression dominantly in hepatocytes of female mice. Taken together with the results from the ChIP assay and *in vivo* reporter assay, we predict the chromatin structure including modification of histones around the *Cyp3a41* gene to differ between females and males, which may contribute to the sex-specific expression of *Cyp3a41* in the liver of female mice. Next, we examined the

effect of GH-treatment on HNF4α-induced CYP3A41 mRNA expression to see whether these factors cooperate in the regulation of the *Cyp3a41* gene. As shown in Fig. 9, the response observed in female hepatocytes with both GH and HNF4α was additive.

Discussion

The expression of some hepatic *CYP* genes in both rodents and humans shows sex differences. The regulation of sex-specific *Cyp* genes may reflect the coordinated actions of multiple hepatocyte-enriched nuclear factors. Here, we investigated the mechanism whereby HNF4α regulates female-specific *Cyp3a41* expression. HNF4α increased the transcriptional activity of *Cyp3a41* through direct binding to the putative HNF4α-binding site located in the -99/-87 region of the *Cyp3a41* gene promoter. Moreover, our findings suggest that the difference in chromatin structure between males and females contributes to the sex-specific expression of the *Cyp3a41* gene.

Experiments with HNF4α-knocked out mouse liver have revealed the important role of HNF4α in the regulation of sex-specific *Cyp3a41* gene expression (Wiwi et al., 2004). The existence of two putative HNF4α-binding sites in the 5'-flanking region of the *Cyp3a41* gene at -4096/-4084 and -2570/-2557 was proposed by means of the web-based program Cluster Buster and the TransFac database, and the substantial decrease in expression of the *Cyp3a41* gene seen in HNF4α-knocked out mice was considered evidence of the functioning of these sites (Wiwi et al., 2004). In the present study, we identified a novel HNF4α regulatory sequence in another region (-99/-87). This region acts to increase the transcriptional activity of the *Cyp3a41* promoter. In relation to the female-specific expression dependent on different chromatin structures, another mechanism is considered to be the inaccessibility of repressive transcription factors to silencer elements due to chromatin condensation in the liver of females but not males. With regard to this possibility, we found two suppressive regions at -596/-163 and -1633/-844 in the 5'-flanking sequence of the *Cyp3a41* gene (Fig.1). We are planning to examine these areas further.

Methylation at histone 3 lysine 4 (H3K4me2) normally results in an open chromatin configuration, whereas methylation at histone 3 lysine 27 is associated with an inactivation of gene transcription and the maintenance of heterochromatin. Methylation at H3K27 and H3K4 can act as bivalent switches to turn on/off associated gene (Lan et al., 2007; Swigut and Wysocka, 2007). Considering these findings, higher H3K4me2 and lower H3K27me3 levels within the *Cyp3a41* gene promoter in females might result in more appropriate chromatin configuration to activate gene transcription than in males, and therefore, be associated with the higher binding of HNF4α to the HNF4α-binding site in *Cyp3a41,* resulting in an enhanced expression. The opposite profiles of the methylation of histone 3 at the *Cyp3a41* gene promoter in males and females are consistent with the proposed role of this modification.

Another well established mechanism for the sex-related differences in the expression of *CYP* genes is the sex-dependent secretion of GH (MacGeoch et al., 1985; Kato and Yamazoe, 1993; Waxman and O'Connor, 2006). Mice show sexually dimorphic GH-secretory patterns, with more frequent GH pulses and a shorter GH-free interpulse interval in females than males (MacLeod et al., 1991). Our previous observations indicated the sex difference in GH secretion to be a determinant of the female-specific expression of *Cyp3a41* in mouse liver (Sakuma et al., 2002; Jarukamjorn et al., 2006). Recent studies identified STAT5b as a key mediator of the sex-dependent actions of GH in the liver of males (Holloway et al., 2006). Although STAT5b is considered to be more abundant in males, several lines of evidence demonstrated a role for this factor in the regulation of female-specific genes (Holloway et al., 2006; Sasaki et al., 1999; Hashita et al., 2008). It was suggested that STAT5b acts directly or indirectly to suppress female-specific genes such as the mouse *Cyp2b9* in

male mice, as well as modulate a subset of female-specific genes such as the rat *CYP2C12* resulting in the activation of gene expression. However, the possibility that STAT5b participates in the expression of *Cyp3a41* is negligible, since the mouse *Cyp3a41* gene was not expressed in the liver of either hypophysectomized or neonatally monosodium glutamate-treated male mice (Sakuma et al., 2002; Jarukamjorn et al., 2006), and the loss of STAT5b in mouse liver had no effect on the expression of *Cyp3a41* (Holloway et al., 2006).

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Authorship Contributions

Participated in research design: Bhadhprasit, Sakuma, Kawasaki, and Nemoto.

Conducted experiments: Bhadhprasit, Sakuma, Kawasaki, and Nemoto.

Contributed new reagents or analytic tools: Bhadhprasit, Sakuma, and Kawasaki.

Performed data analysis: Bhadhprasit and Sakuma.

Wrote or contributed to the writing of the manuscript: Bhadhprasit, Sakuma, Kawasaki, and Nemoto.

Other: Sakuma and Nemoto acquired funding for the research.

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Footnotes

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Figure legends

Fig. 1 Transcriptional activity of the 5'-flanking region of the *Cyp3a41* gene in primary cultured hepatocytes from female mice.

A series of *Cyp3a41* luciferase reporter gene constructs were prepared as described under Materials and Methods and are shown on the left. Numbers indicate the positions relative to the transcription start site. These reporter constructs were introduced into primary cultured hepatocytes of female mice at 24 h after perfusion. After a further 48 h of incubation, the cells were harvested. Cell extracts were assayed for firefly luciferase activity, which was normalized to *Renilla* luciferase activity. Each bar represents the mean ± S.D. of six determinations from a single experiment. Values are shown relative to that for -163/+61-Luc-transfected hepatocytes (=100%). The data are representative of two independent experiments. *, $p<0.01$, significantly different from cells transfected with the pGL3 basic vector; #, $p<0.05$, significantly different from cells transfected with -163/+61-Luc; $, $p<0.01$, significantly different from cells transfected with -844/+61-Luc; &, $p<0.01$, significantly different from cells transfected with the -2396/+61-Luc reporter construct. Significance was examined using one-way ANOVA followed by the Tukey test.

Fig. 2 Functional analyses of the putative HNF4α-binding site of the *Cyp3a41* gene in primary cultured hepatocytes from female mice.

A. The nucleotide sequence of the putative HNF4α-binding site of the *Cyp3a41* gene is compared with that of the consensus HNF4α-binding site (DR1).

B. Influence of mutation of the putative HNF4α-binding site and transfection of the mouse HNF4α expression plasmid on *Cyp3a41* transcriptional activity. Left, schematic

representations of the wild-type (-163/+61-Luc) and mutated (-163/+61-HNF4α mut-Luc) reporter gene constructs are shown. Primary cultured hepatocytes from a female were transfected with -163/+61-Luc or -163/+61-HNF4α mut-Luc containing two nucleotide substitutions at the putative HNF4α-binding site. An expression plasmid for HNF4α or an empty plasmid was co-transfected into the cells. Luciferase activity was determined as described in the legend of Figure 1. Significance was examined using the one-way ANOVA followed by the Tukey Test: *, $p<0.01$, significantly different from the cells transfected with the pGL3 basic vector; #, $p<0.01$, significantly different from the cells transfected with -163/+61-Luc; $, $p<0.01$, significantly different from the cells co-transfected with -163/+61-Luc and the HNF4α expression plasmid.

Fig. 3 Specific binding of HNF4α to the putative HNF4α-binding site of *Cyp3a41 in vitro*.

A. Nucleotide sequences of the oligonucleotides used for the electrophoretic mobility shift assay (EMSA) are shown. The putative HNF4α-binding site of the *Cyp3a41* gene is shown in uppercase. Mutated nucleotides are underlined.
B. EMSAs were performed with a ^{32}P-radiolabeled *Cyp3a41* probe containing the putative HNF4α-binding site. Incubation was carried out with HNF4α synthesized *in vitro* as described under Materials and Methods. *; the second lane from the left was loaded with a sample consisting of radiolabeled probe and *in vitro* transcription/translation reaction mixture incubated with nuclease-free water as the template (negative control). Competition assays were performed with a 100-fold excess of unlabeled *Cyp3a41* probe containing the putative HNF4α-binding site, or *Cyp3a41* mutated competitor containing mutated HNF4α-binding site, or a 100-fold

excess of non-specific SP1 probe. The HNF4α-probe complex, non-specific bands, and free probe are indicated.

Fig. 4 Capability of nuclear HNF4α to bind the putative HNF4α-binding site of *Cyp3a41 in vitro*.

A. Nuclear extracts prepared from the liver of male and female mice were subjected to EMSA. A ^{32}P-radiolabeled *Cyp3a41* probe containing the putative HNF4α-binding site (HNF4α wild) or two-nucleotide substituted HNF4α-binding site (HNF4α mutated) was incubated with nuclear extracts and electrophoresed on a 4% polyacrylamide gel as described under Materials and Methods. Competition experiments comprised a radiolabeled probe incubated with nuclear extracts and a 100-fold excess of unlabeled HNF4α wild probe (W), or 100-fold excess of unlabeled HNF4α mutated probe (M), or 100-fold excess of non-specific SP1 probe (N). Supershift analyses were carried out with 2 µg of antibody against HNF4α protein. *; *in vitro* translated mouse HNF4α protein was added as a positive control, NE; nuclear extracts. The HNF4α-probe complex, supershifted HNF4α-probe complex, non-specific bands, and free probe are indicated.

B. Western blot analyses of HNF4α expression in nuclear extracts prepared from the liver of male and female mice. Western blotting was performed in duplicate using antibodies against HNF4α as described under Materials and Methods.

Fig. 5 Quantitative ChIP analysis of HNF4α binding to the putative HNF4α-binding site of the *Cyp3a41* gene in the livers of adult mice.

Chromatin was extracted from the hepatic tissues of mice at 7 weeks of age. ChIP

assays were performed using an antibody against HNF4α or control rabbit IgG. PCR was performed on chromatin templates with primer pairs which amplify -207/+18 of the *Cyp3a41* gene (A) or -336/+34 of the *ApoCIII* gene (B). The region amplified by the primer pair for *Cyp3a41* covers one HNF4α-binding site (-99/-87), whereas that for *ApoCIII* covers two HNF4α-binding sites (-139/-127, -336/-318). PCR products of HNF4α-enriched chromatin were quantified and shown as a percentage of input. Bars represent the mean $\pm$ S.D. from 3 individual samples. Significance was examined using the one-way ANOVA followed by the Tukey Test: *, $p<0.05$, significantly different from male mice. Open and closed columns indicate males and females, respectively.

Fig. 6 Association between HNF4α binding on the promoter and transcription levels of the *Cyp3a41* gene. Levels of HNF4α binding and mRNA expression were analyzed in hepatic tissue sets prepared from both sexes of mice at 3 and 7 weeks of age (A and B) or from sham-operated, hypophysectomized,or human GH-infused hypophysectomized female mice at 7 weeks of age (C and D). A and C, chromatin was extracted from the hepatic tissues. ChIP assays were performed using an antibody against HNF4α or control rabbit IgG. PCR was performed on chromatin templates with a primer pair which amplifies -207/+18 of the *Cyp3a41* gene. PCR products were quantified and shown as a percentage of input. B and D, mRNA expression of the *Cyp3a41* gene was quantified by TaqMan real-time RT-PCR and then normalized to the level of GAPDH and shown relative to that in the 7-week-old female (B) or sham-operated (D) group. Bars represent the mean $\pm$ S.D. from 3 individual samples. Significance was examined using the one-way ANOVA followed by the Tukey Test: *, $p<0.05$; **, $p<0.01$. Open and closed columns indicate males and females, respectively.

Abbreviations: sham, sham-operated; hypox, hypophysectomized; GH(i), continuous infusion of human GH using an osmotic minipump.

Fig. 7 Different chromatin modifications of the mouse *Cyp3a41* gene (-207/+18) between males and females examined by the ChIP assay.

Chromatin was extracted from the hepatic tissues of mice at 7 weeks of age. ChIP assays were performed using antibodies against H3K4me2, H3K27me3, and AcH4K8 or control rabbit IgG. PCR was performed on chromatin templates with a primer pair which amplifies -207/+18 of the *Cyp3a41* gene. PCR products were quantified and shown as a percentage of input. Bars represent the mean $\pm$ S.D. from 3 individual samples. Significance was examined using the one-way ANOVA followed by the Tukey Test: *, $p<0.05$, significantly different from male mice; **, $p<0.01$, significantly different from male mice.

Fig. 8 Transcriptional activity of the 5'-flanking region of the *Cyp3a41* gene and functional analysis of the putative HNF4α-binding site in male and female mice determined by the *in vivo* reporter assay.

Schematic representations of the wild-type (-163/+61-Luc) and mutated (-163/+61-HNF4α mut-Luc) reporter gene constructs are shown on the left. These constructs were introduced into mice by i.v. administration as described under Materials and Methods. After 24 h, the mice were sacrified and luciferase activity was determined. Relative luciferase activity was expressed as the firefly luciferase activity normalized to *Renilla* luciferase activity and is shown relative to that of -163/+61-Luc in female livers (=100%). Data are expressed as the mean $\pm$ S.D. of three to five determinations from a

single experiment. The data are representative of two independent experiments. Significance was examined using a one-way ANOVA followed by the Tukey Test: *, $p<0.05$, significantly different from the pGL3-transfected hepatocytes of female mice; #, $p<0.05$, significantly different from the pGL3-transfected hepatocytes of male mice.

Fig. 9 Effects of transfection of the HNF4α expression plasmid and GH treatment on the expression of CYP3A41 mRNA in primary cultured hepatocytes of male and female mice.

Hepatocytes isolated from male and female ddY mice were cultured for 48 h and transfected with empty plasmid or the mouse HNF4α expression plasmid. Three hours after transfection, GH was added at a final concentration of 71 ng/ml, and the cells cultured a further 24 h. The cells were harvested and CYP3A41 mRNA expression was evaluated by quantitative real-time RT-PCR. The expression of each mRNA was normalized to the level of GAPDH mRNA and is shown relative to that in the empty plasmid-transfected female hepatocytes. Each column represents the mean $\pm$ S.D. of four determinations from a single experiment. The data are representative of two independent experiments. *, $p<0.01$, significance was examined by one-way ANOVA followed by the Tukey Test.

Fig. 1

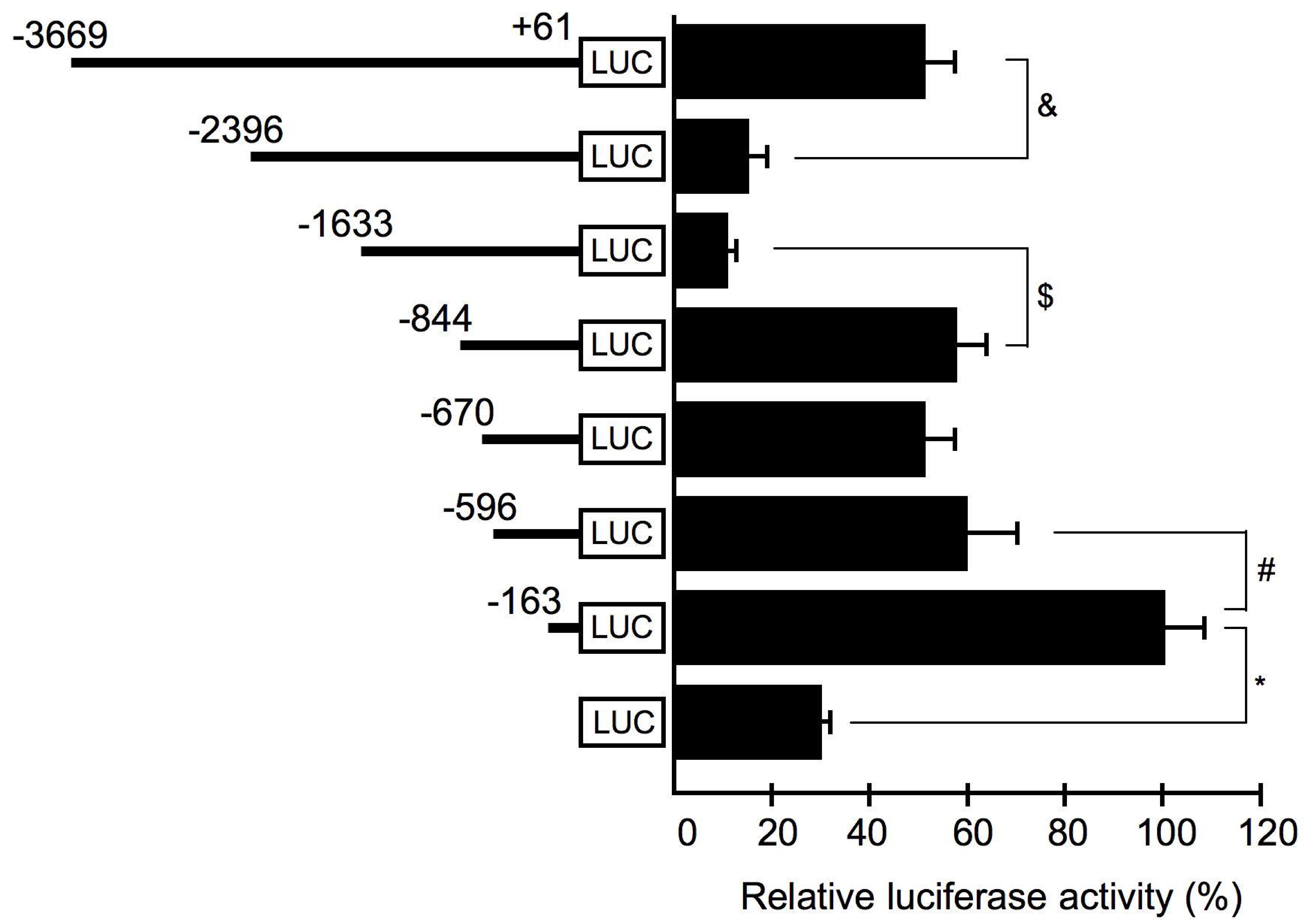


Fig. 2

A

-102 -84

putative HNF4α-binding site of *Cyp3a41*: aga**CGACCA**a**AGTCCA**ggt

consensus HNF4α-binding site (DR1) : nnn**AGGTCA**n**AGGTCA**nnn

B

empty HNF4α

-163 HNF4α +61 LUC

CGACCAaAGTAAA

-163 HNF4α +61 LUC

CGACCAaAGTCCA

LUC

$ # # *

0 20 40 60 80 100 120 140 160

Relative luciferase activity (%)

Fig. 3

A

Cyp3a41 probe containing HNF4α-binding site : gggaaagagaCGACCAaAGTCCAggtgatgcagg (-109 … -76)

Cyp3a41 mutation competitor containing mutated HNF4α-binding site : gggaaagagaCGACCAaAGTAAAggtgatgcagg (-109 … -76)

SP1(non-specific competitor) : attcgatcggggcggggcgagc

B

				HNF4α wild	HNF4α mut	SP1
Competitor (X100)	-	-	-	+	+	+
HNF4α	-	-*	+	+	+	+

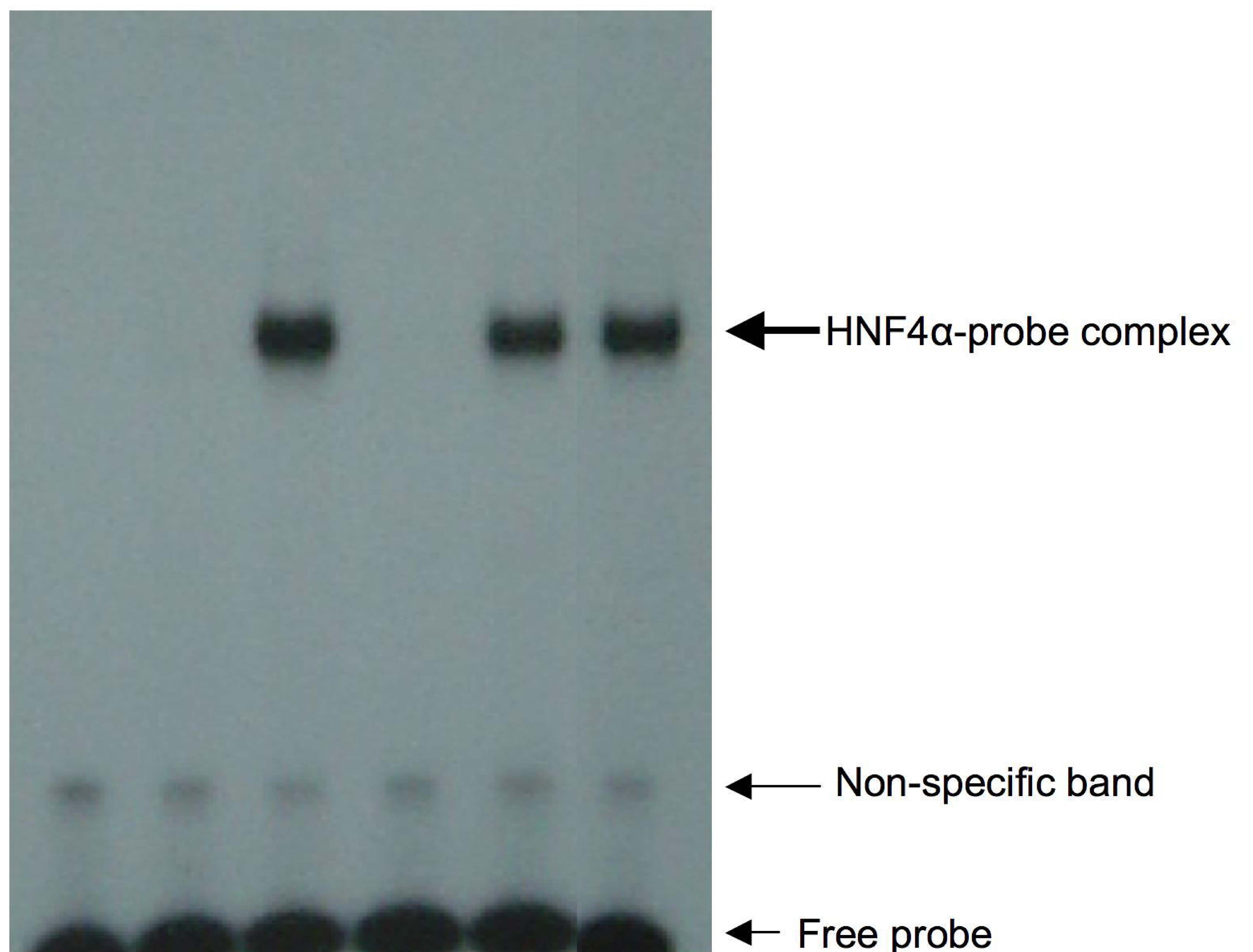
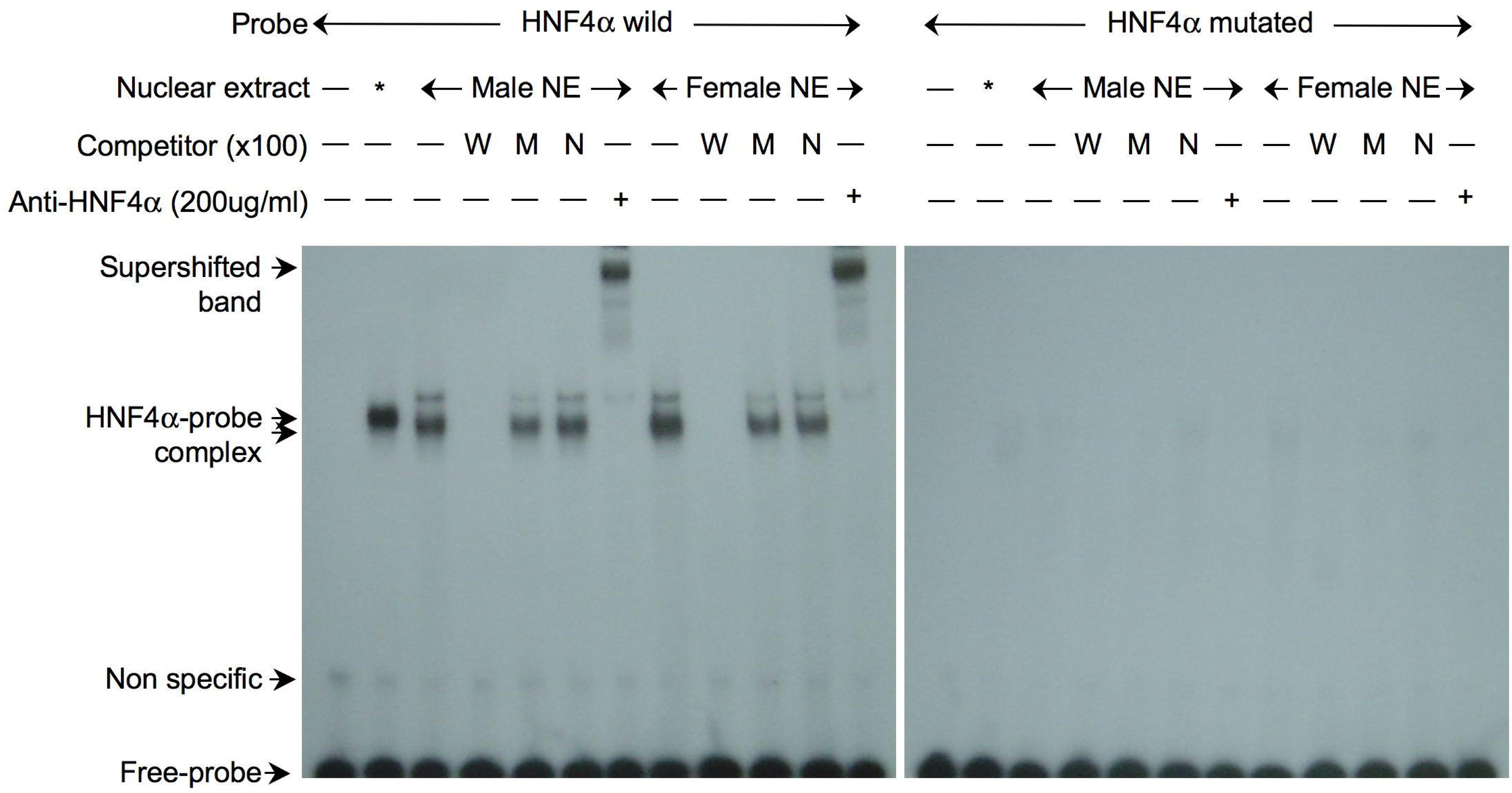


Fig. 4

A



B

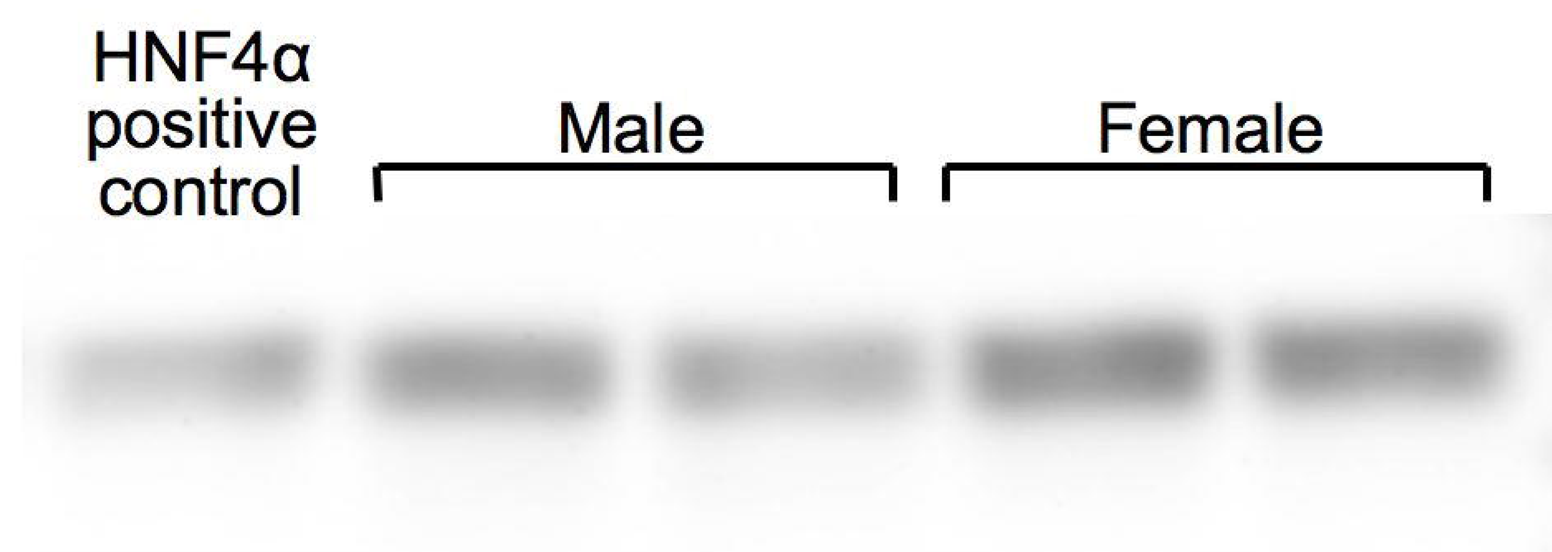
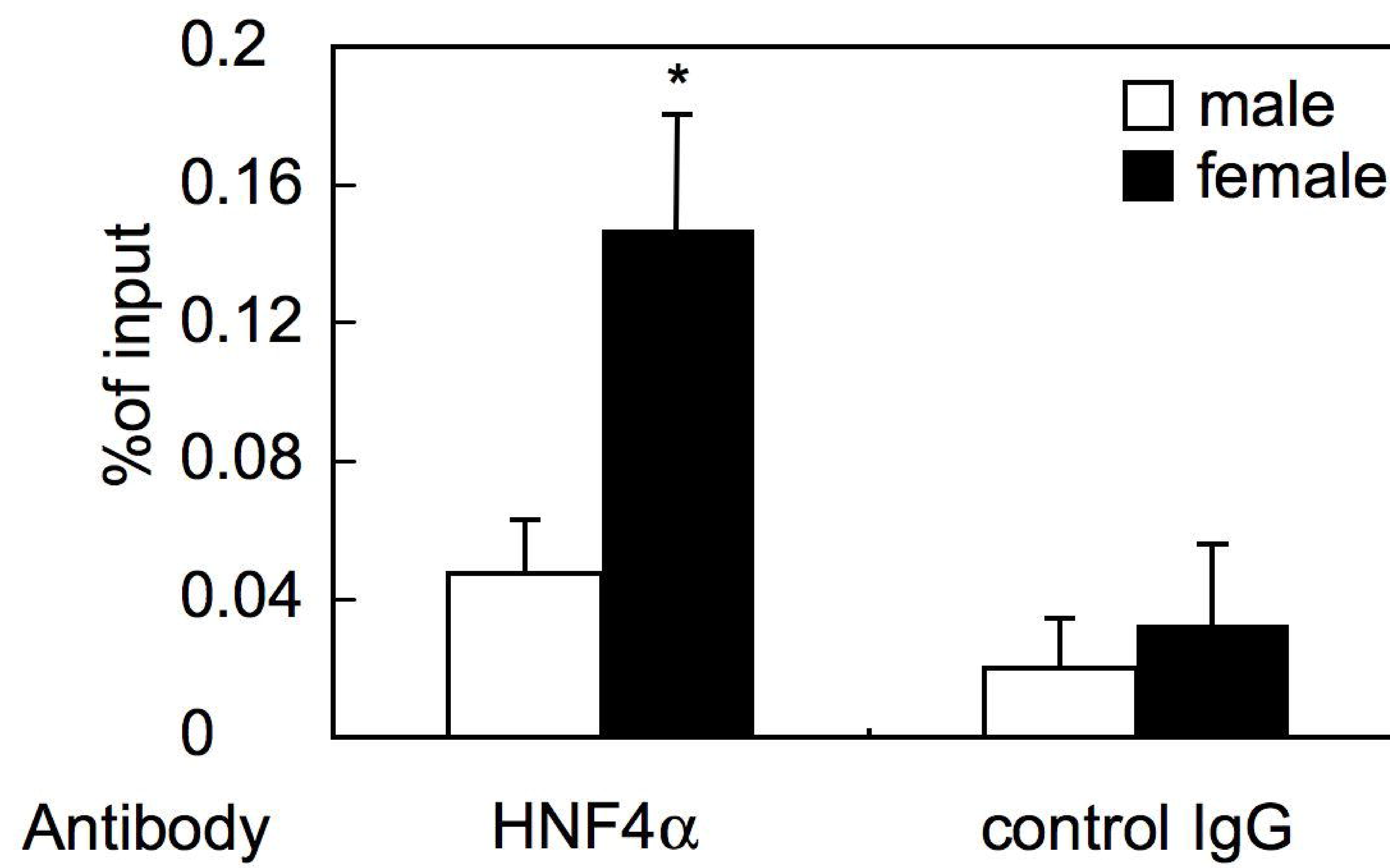


Fig. 5



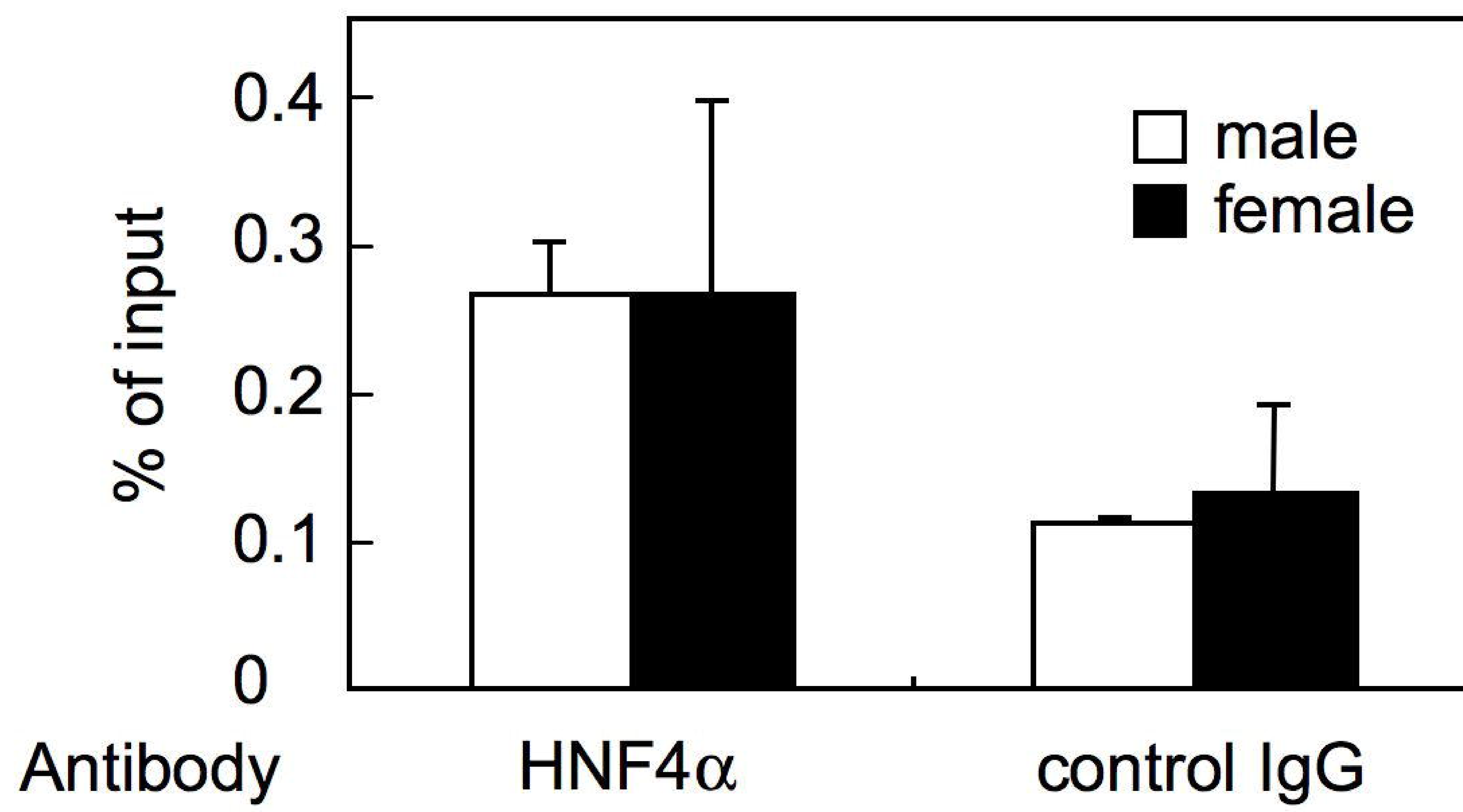
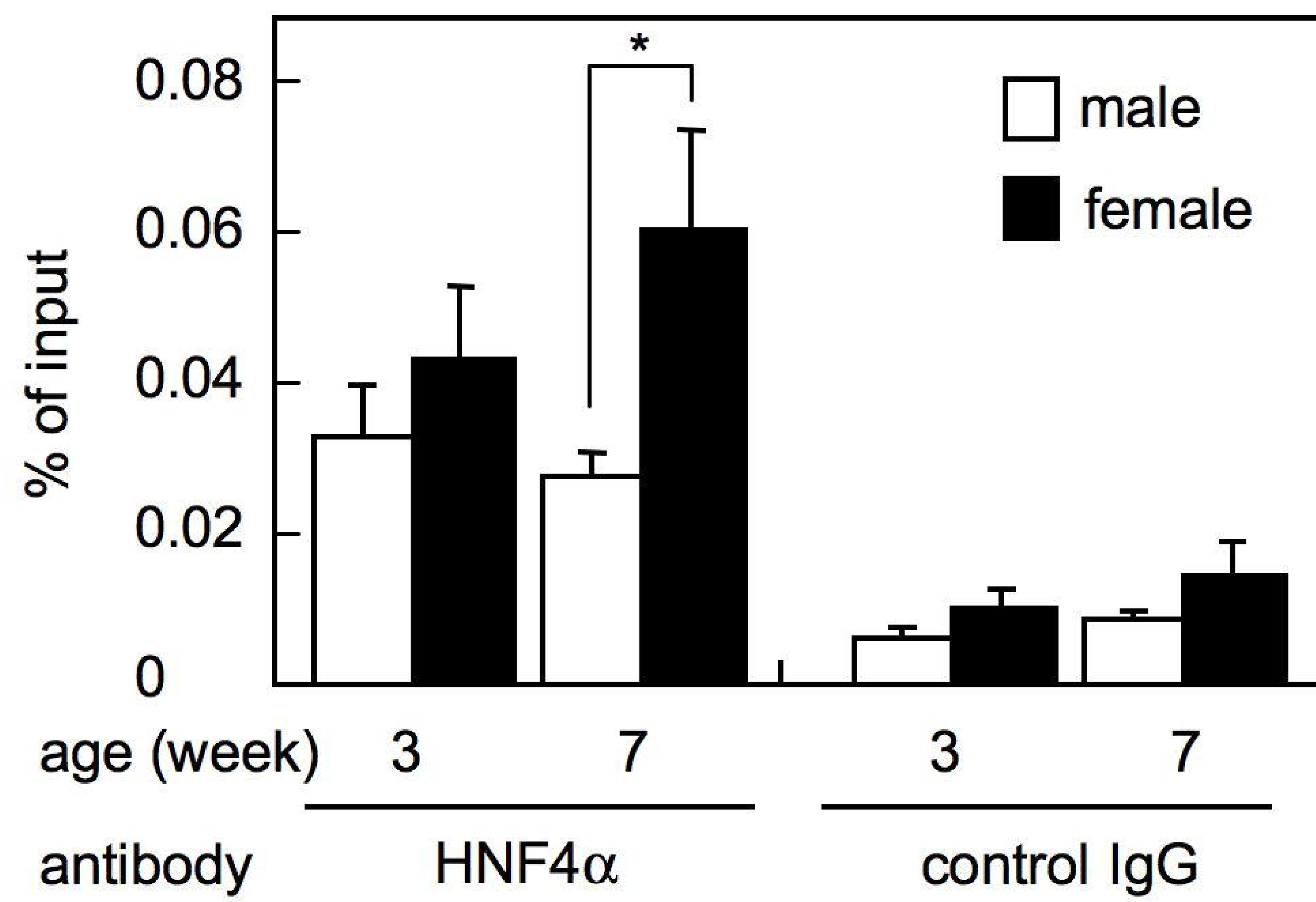
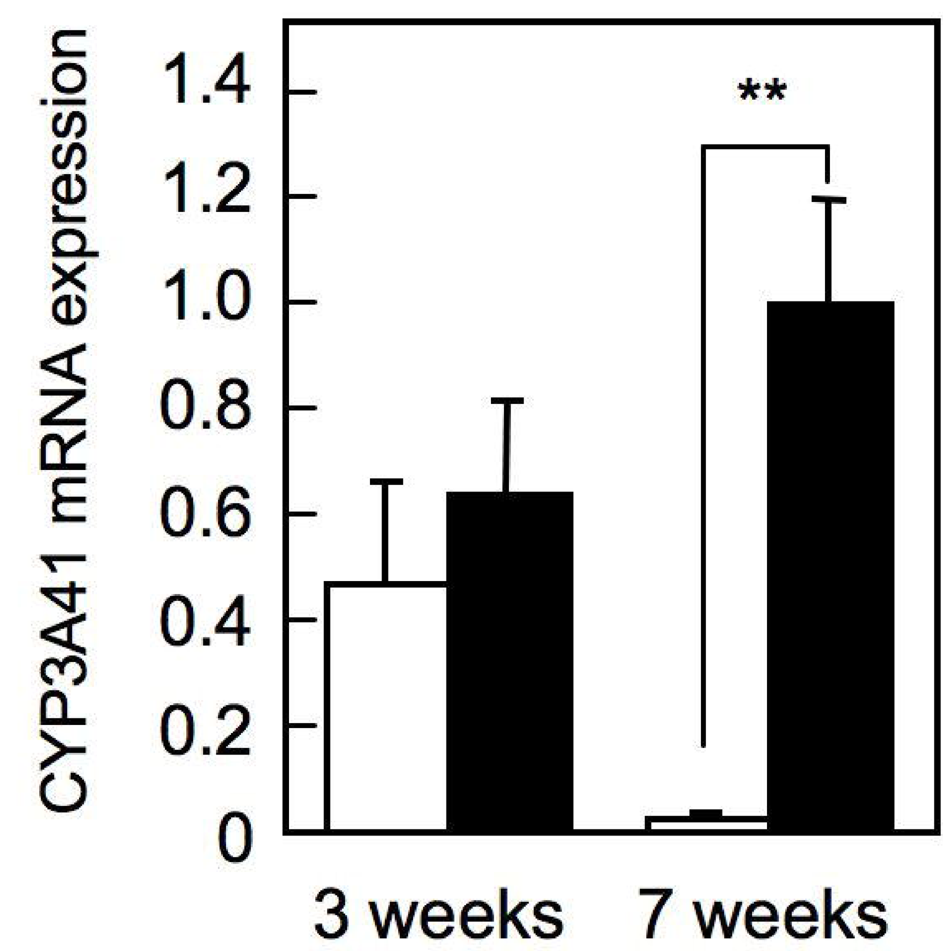
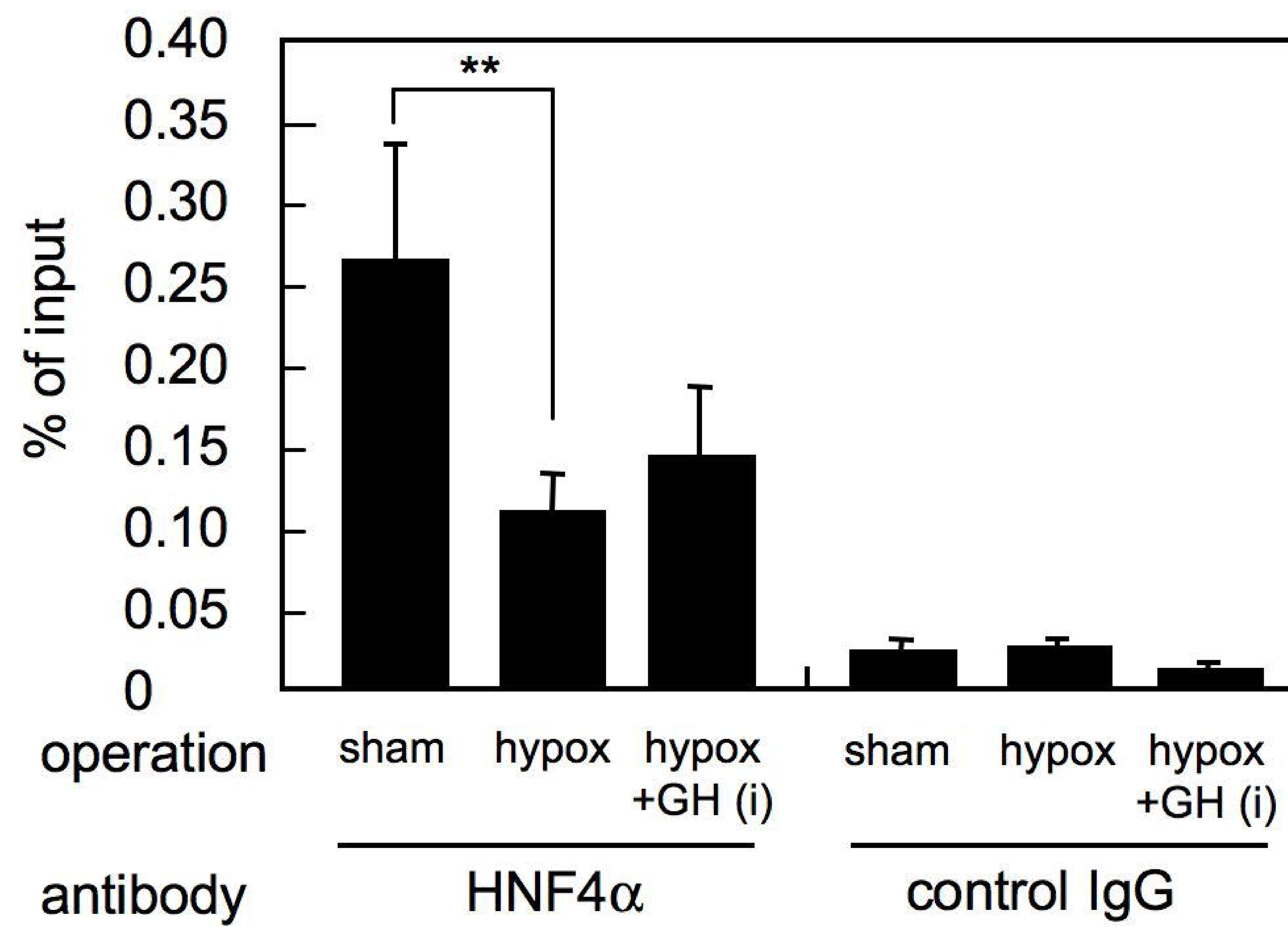


Fig. 6







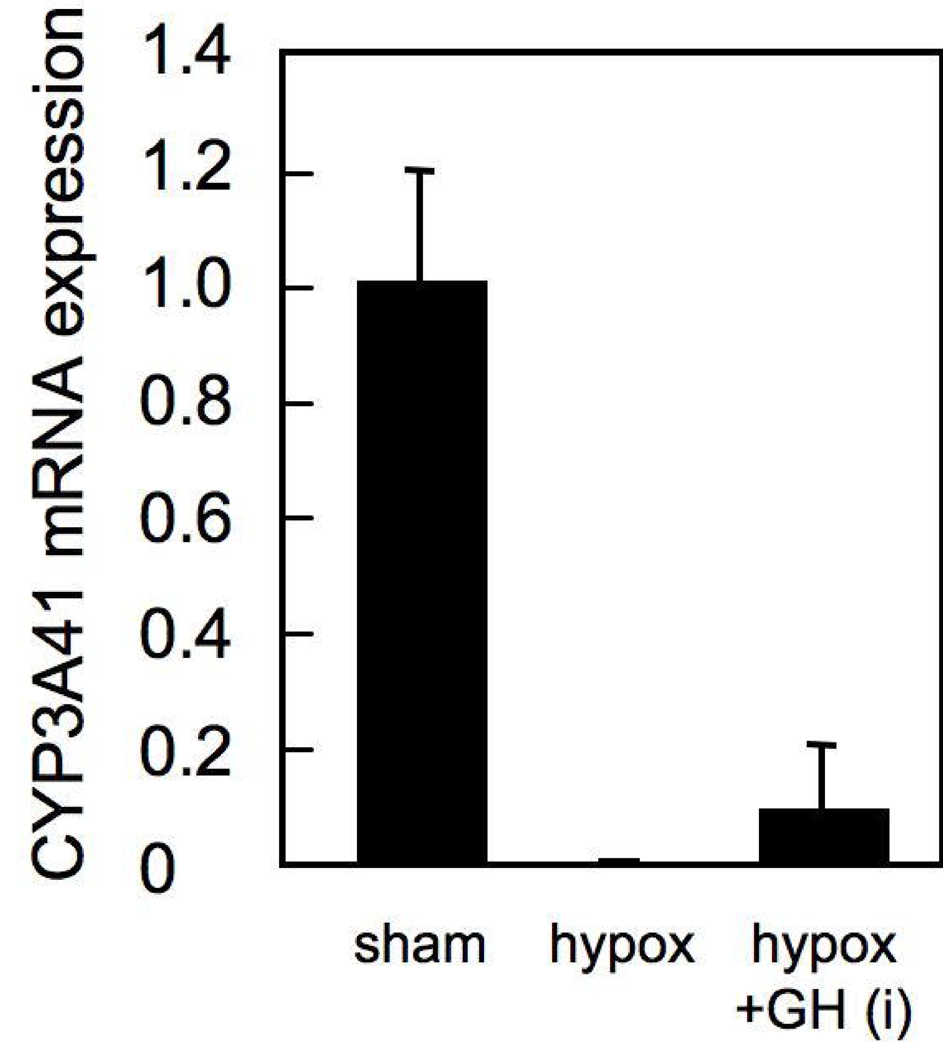


Fig. 7

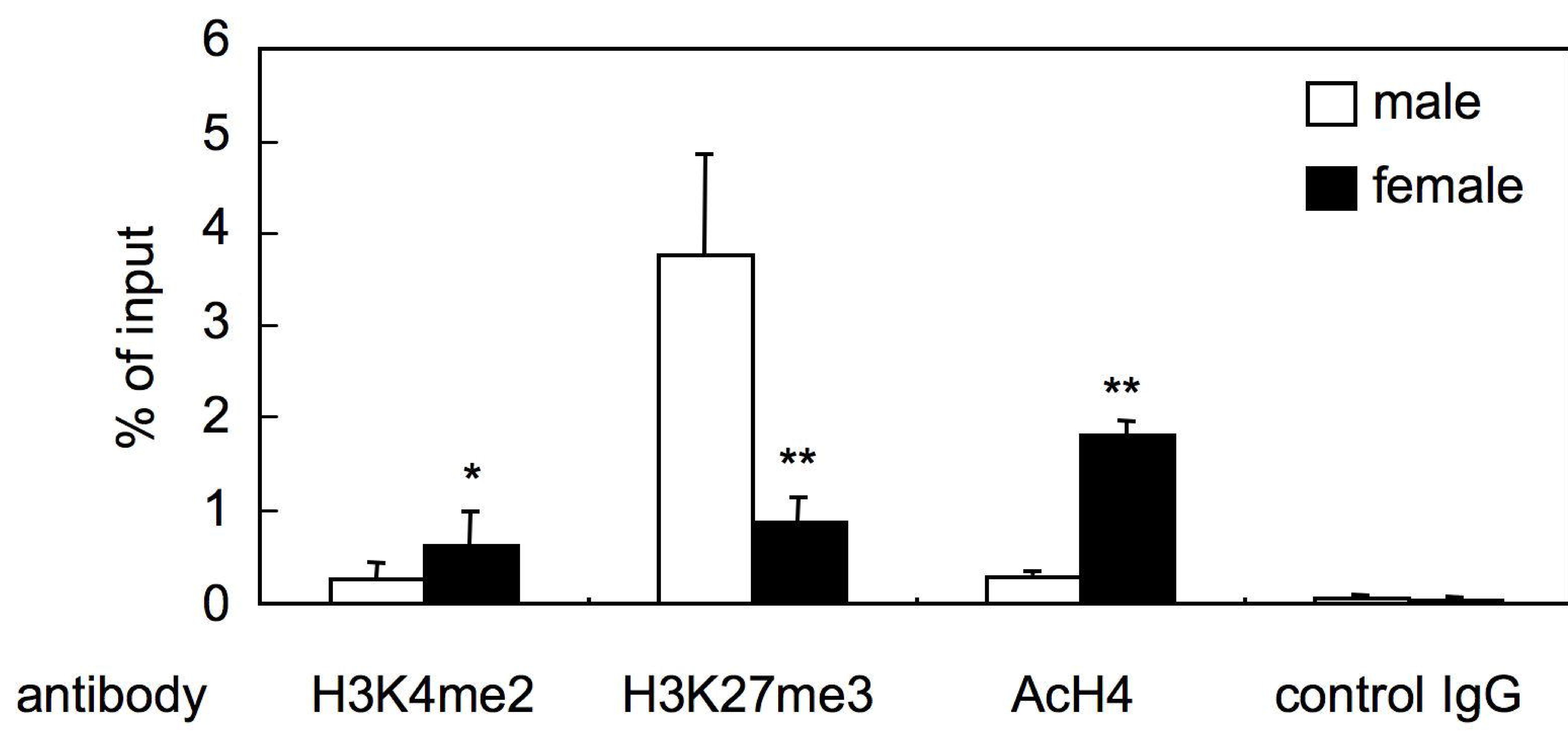


Fig. 8

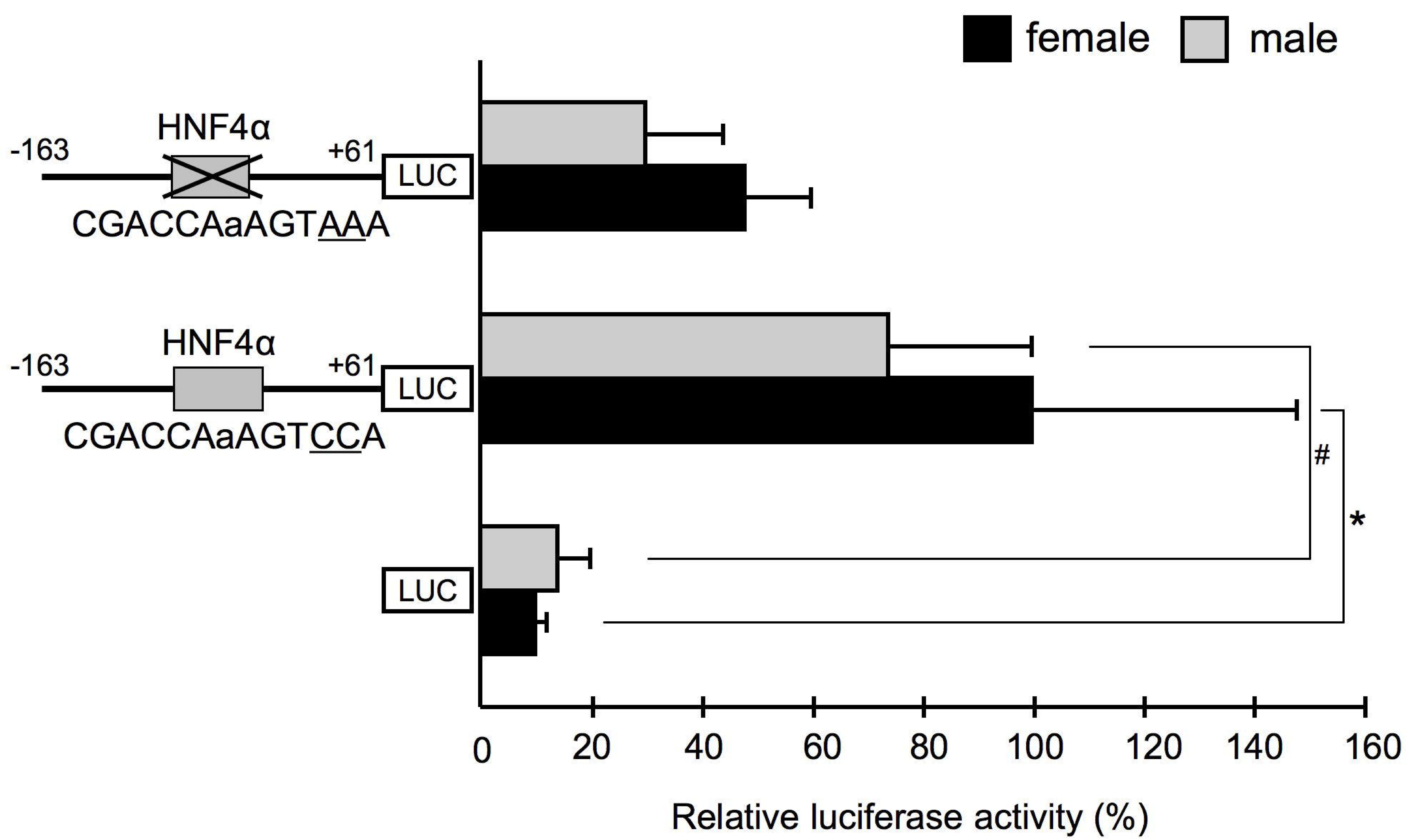


Fig. 9