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# Regulation of Cytochrome *b*<sub>5</sub> Expression by miR-223 in Human Liver: Effects on Cytochrome P450 Activities

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Short title: miR-223 Regulates Human Cytochrome *b*<sub>5</sub> Expression

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**Abbreviations:** Ad, Adenovirus; *b*<sub>5</sub>, cytochrome *b*<sub>5</sub>; CYP, cytochrome P450; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; miRNA, microRNA; MOI, multiplicity of infection; MRE, miRNA recognition element; NPR, NADPH-cytochrome P450 reductase; P450, cytochrome P450; PAGE, polyacrylamide gel electrophoresis; pre-miRNA, precursor miRNA; RT-PCR, reverse transcription-polymerase chain reaction; siRNA, small interfering RNA.

## ABSTRACT

**Purpose** Cytochrome  $b_5$  ( $b_5$ ) is a hemoprotein that transfers electrons to several enzymes to fulfill functions in fatty acid desaturation, methemoglobin reduction, steroidogenesis, and drug metabolism. Despite the importance of  $b_5$ , the regulation of  $b_5$  expression in human liver remains largely unknown. We investigated whether microRNA (miRNA) might be involved in the regulation of human  $b_5$ .

**Methods** Twenty-four human liver specimens were used for correlation analysis. In silico analysis and luciferase assay were performed to determine whether the predicted miRNAs functionally target to  $b_5$ . The miR-223 was overexpressed into HepG2 cells infected with adenovirus expressing human cytochrome P450.

**Results** In human livers, the  $b_5$  protein levels were not positively correlated with the  $b_5$  mRNA levels, and miR-223 levels were inversely correlated with the  $b_5$  mRNA levels or the translational efficiencies. The luciferase assay showed that miR-223 functionally binds to the element in the 3'-untranslated region of  $b_5$  mRNA. The overexpression of miR-223 significantly reduced the endogenous  $b_5$  protein level and the mRNA stability in HepG2 cells. Moreover, the overexpression of miR-223 significantly reduced CYP3A4-catalyzed testosterone 6 $\beta$ -hydroxylation activity and CYP2E1-catalyzed chlorzoxazone 6-hydroxylase activity but not CYP1A2-catalyzed 7-ethoxyresorufin *O*-deethylase activity.

**Conclusions** miR-223 down-regulates  $b_5$  expression in the human liver, modulating P450 activities.

**Keywords:** Post-transcriptional regulation, drug-metabolizing enzymes, interindividual variability

## INTRODUCTION

Cytochrome  $b_5$  ( $b_5$ ) is a hemoprotein that is localized to the endoplasmic reticulum membrane with its obligate electron donor, NADH-cytochrome  $b_5$  reductase.  $b_5$  acts as an electron transfer component in a number of oxidative reactions in biological tissues, such as fatty acid desaturation (1), steroidogenesis (2), and the reduction of methemoglobin (3) and metmyoglobin (4). In addition,  $b_5$  is involved in cytochrome P450 (CYP, P450)-catalyzed drug metabolism (5). Much evidence exists that supports the essentiality of  $b_5$  activity in mammals. Hereditary  $b_5$  deficiencies cause congenital methemoglobinemia in human (6). Knocking out  $b_5$  expression in mice dramatically reduced P450-catalyzed steroid hormone biosynthesis, with low neonatal body weights, dry skin, and coat aberration (7). The conditional  $b_5$  knockout mouse showed the significant reduction of a wide range of P450-dependent metabolic activities in the liver (8).

P450s are responsible for the metabolism of a wide variety of exogenous compounds, such as drugs and carcinogens, and endogenous compounds (9). In humans, three families, CYP1, CYP2, and CYP3, contribute to the oxidative metabolism of more than 70% of clinical drugs. P450 reactions require two electrons. The first electron is derived from NADPH-cytochrome P450 reductase (NPR), and the second electron is derived from NPR or  $b_5$  (10). The requirement of  $b_5$  is P450 isoform-dependent, as previous *in vitro* studies have revealed that  $b_5$  stimulates CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2E1, and CYP3A4 activities (5,11). In addition to the catalytic role of  $b_5$ , non-catalytic role has been proposed. That is, apo- $b_5$  stimulates CYP3A4 and CYP17 activities, suggesting that the interaction of  $b_5$  with P450 causes structural changes that impact P450 activities (12).

Previous studies have reported that hepatic  $b_5$  expression is influenced through several factors. The administration of ethanol reduced  $b_5$  levels in the hamster liver, while the administration of carbon tetrachloride, *p*-nitroanisole, malotilate, and griseofulvin increased  $b_5$  levels in the rat liver (1). However, the mechanisms underlying these changes remain unknown. It has been reported that some transcription factors, including Sp3, GATA-6, and

steroidogenic factor 1, are involved in the transcription of  $b_5$  in human adrenal NCI-H295A cells (13). Experimental information demonstrating the regulatory mechanisms for  $b_5$  is rather limited.

MicroRNAs (miRNAs) are a large family of evolutionarily conserved non-coding RNAs of 21~23 nucleotides in length. miRNAs play an important role in gene regulation via translational repression or mRNA degradation by pairing with the 3'-untranslated region (3'-UTR) of target mRNAs (14). Currently, more than 2,000 miRNAs have been identified in humans (miRBase ver. 19). It has been predicted that over 60% of human mRNAs are regulated through miRNAs (15,16). Accumulating evidence reveals that miRNAs are involved in diverse biological processes, including development, cell proliferation, differentiation, apoptosis, and cancer initiation or progression (17-19). Recently, we reported a role for miRNAs in the metabolism of xenobiotics or endobiotics (20) in humans. For example, miR-27b regulates human CYP1B1 (21), miR-378 regulates human CYP2E1 (22), and miR-125b regulates human CYP24A1 (23). To expand the current understanding of the role of miRNAs in drug metabolism, we investigated whether miRNAs might be involved in the regulation of  $b_5$  in the human liver.

## **MATERIALS AND METHODS**

### **Chemicals and Reagents**

$\alpha$ -Amanitin was purchased from Calbiochem (San Diego, CA). 7-Ethoxyresorufin, resorufin, chlorzoxazone, and 6-hydroxychlorzoxazone were purchased from Sigma-Aldrich (St. Louis, MO). Testosterone was obtained from Wako Pure Chemicals (Osaka, Japan).

6 $\beta$ -Hydroxytestosterone was obtained from Steraloids (Wilton, NH). NADH was purchased from Oriental Yeast (Tokyo, Japan). The pGL3-promoter (pGL3) vector, phRL-TK, and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). RNAiso, random hexamer, SYBR pre-mix Ex Taq, Adenovirus Expression Vector kit (Dual Version), and QuickTiter Adenovirus Titer Immunoassay kit were purchased from Takara

(Shiga, Japan). Rox was purchased from Stratagene (La Jolla, CA). ReverTra Ace was obtained from Toyobo (Osaka, Japan). Pre-miR miRNA Precursors for miR-223 and negative control #2, TaqMan MicroRNA Reverse Transcription Kit, TaqMan microRNA Assays, TaqMan Universal Master Mix (No AmpErase UNG), Stealth Select RNAi for human CYB5A (HSS175741) (*b*<sub>5</sub> siRNA) and negative control low GC duplex #2 (control siRNA), Lipofectamine 2000, and Lipofectamine RNAiMAX were purchased from Life Technologies (Carlsbad, CA). All primers and oligonucleotides were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Rabbit anti-human GAPDH polyclonal antibodies were obtained from IMGENEX (San Diego, CA). Rabbit anti-human CYP2E1 polyclonal antibodies (H-21) and rabbit anti-human cytochrome *b*<sub>5</sub> polyclonal antibodies (H-114) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human CYP3A4 polyclonal antibodies and rabbit anti-human CYP1A2 polyclonal antibodies were purchased from BD Gentest (Worburn, MA) and Nosan (Yokohama, Japan), respectively. Rabbit anti-human/rat NPR polyclonal antibodies were obtained from Millipore (Billerica, MA). IRDye 680 goat anti-rabbit IgG was purchased from LI-COR Biosciences (Lincoln, NE). All other chemicals and solvents were of the highest grade commercially available.

### **Human Livers and Preparation of Homogenates, Microsomes, and Total RNA**

Human liver samples from 15 donors were obtained from the Human and Animal Bridging Research Organization (Chiba, Japan) that is in partnership with the National Disease Research Interchange (NDRI, Philadelphia, PA), and the human livers from 9 donors were obtained from autopsy materials discarded after pathological investigation (Supplemental Table 1). The use of the human livers was approved through the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan). Total cell homogenates were prepared through homogenization with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1% Nonidet P-40) containing protease inhibitors (0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride, 2 µg/ml aprotinin, and 2 µg/ml leupeptin). Microsomes were prepared using a previously described method (24). The

protein concentration was determined using Bradford protein assay reagent (Bio-Rad, Hercules, CA) with  $\gamma$ -globulin as a standard. Total RNA was prepared using RNAiso according to the manufacturer's protocols, and the integrity was assessed by estimating the ratio of the band density of 28S and 18S rRNA.

### **Determination of $b_5$ Level in Human Liver Microsomes**

The  $b_5$  levels in human liver microsomes were determined from difference spectrum reduced minus oxidized, taking  $\Delta\epsilon$  (424-409) = 185 mM<sup>-1</sup>cm<sup>-1</sup> according to the method of Omura and Sato (25).

### **Cell Culture**

Human cervical carcinoma-derived HeLa cells were obtained from American Type Culture Collection (Rockville, MD). Human liver-derived HepG2 and HuH-7 cells were obtained from the Riken Gene Bank (Tsukuba, Japan). Human liver-derived HLE cells were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). HepG2 cell lines stably expressing CYP3A4 (Hepc/3A4.2-30), CYP2E1 (Hepc/2E1.3-8), and CYP1A2 (Hepc/1A2.9) established by Yoshitomi et al (26) were kindly provided. HeLa and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.1 mM non-essential amino acids (Life Technologies) and 10% fetal bovine serum (FBS) (Life Technologies). HuH-7 and HLE cells were cultured in DMEM supplemented with 10% FBS. Hepc/3A4.2-30, Hepc/2E1.3-8, and Hepc/1A2.9 cells were cultured in DMEM supplemented with 200  $\mu$ g/ml G418 (Gibco BLR, Grand Island, NY). These cells were maintained at 37°C under an atmosphere of 5% CO<sub>2</sub> in 95% air.

### **Prediction of Potential miRNAs that Bind to Human $b_5$**

Six computational programs, PicTar (<http://pictar.mdc-berlin.de/>), TargetScan (<http://www.targetscan.org/>), Microcosm (<http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/>), microRNA.org (<http://www.microrna.org/microrna/get-MirnaForm.do>),

TargetSpy (<http://www.targetspy.org/>), and Target Rank (<http://hollywood.mit.edu/targetrank/>) were used to predict potential miRNAs that bind to human *b<sub>5</sub>*. A miRNA, miR-223, which was commonly predicted, was investigated further.

### **Construction of Reporter Plasmids**

To construct the luciferase reporter plasmids, fragments were inserted into the *Xba* I site downstream of the luciferase gene in the pGL3 promoter vector. The sequence from +591 to +628 in the 3'-UTR of human *b<sub>5</sub>* mRNA was identified as the miRNA recognition element (MRE) for miR-223. A fragment containing the MRE, 5'- CTA GAT GGA CAC *GGG AGA AAA GAA GCC ATT GCT AAC TAC TTC AAC TGA CAG AAA CCT T* -3' (the MRE is italicized), was cloned into the pGL3-promoter vector (pGL3/MRE). A fragment containing the perfectly matching sequence with the mature miR-223, 5'- CTA GAT *GGG GTA TTT GAC AAA CTG ACA T* -3' (the matching sequence of miR-223 is italicized) was also cloned (pGL3/c-miR-223). DNA sequencing analyses were performed to confirm the nucleotide sequences of these plasmids using a Long-Read Tower DNA sequencer (GE Healthcare Bio-Sciences, Piscataway, NJ).

### **Luciferase Assay**

Various pGL3 luciferase reporter plasmids were transiently transfected with the phRL-TK plasmid into HeLa cells. Briefly, the day before transfection, the cells were seeded into 24-well plates. After 24 hr, 190 ng of pGL3p plasmid, 10 ng of phRL-TK plasmid and 50 nM of precursor for miR-223 or control were transfected into HeLa cells using Lipofectamine 2000. After incubation for 48 hr, the cells were resuspended in passive lysis buffer, and the luciferase activity was measured with a luminometer using the Dual-Luciferase Reporter Assay System.



## **Transfection of Precursor for miR-223 into HepG2 Cells and Preparation of Cell Homogenates and Total RNA**

The HepG2 cells were seeded into 12-well plates and transfected with 50 nM of precursor for miR-223 or control using Lipofectamine RNAiMAX. After 72 hr, the cells were harvested and resuspended in a small amount of TGE buffer [10 mM Tris-HCl, 20% glycerol, 1 mM EDTA (pH 7.4)], disrupted through freeze-thawing three times and homogenization. Total RNA was prepared as described above.

### **Real-time RT-PCR for $b_5$**

The cDNA was synthesized from total RNA using ReverTra Ace. The  $b_5$  mRNA level was determined using quantitative real-time RT-PCR. The forward and reverse primers were 5'-GGC CGT GAA GTA CTA CAC CCT AG-3' and 5'-CTG TAG AGT GCC CGA CAT CCT C-3', respectively. A 1- $\mu$ l portion of the reverse-transcribed mixture was added to a PCR mixture containing 8 pmol of each primer, 10  $\mu$ l of SYBR Premix Ex Taq solution and 75 nM ROX in a final volume of 20  $\mu$ l. The following PCR conditions were used: after an initial denaturation at 95°C for 30 sec, the amplification was performed through denaturation at 94°C for 4 sec, annealing and extension at 64°C for 20 sec for 45 cycles. Real-time RT-PCR was performed using Mx3000P (Stratagene, La Jolla, CA) with MxPro QPCR software. The mRNA levels were normalized to those of GAPDH mRNA as previously described (27).

### **Real-time RT-PCR for Mature miR-223**

The expression of miR-223 was determined through quantitative real-time PCR using TaqMan MicroRNA Assays. All procedures were performed according to the manufacturer's instructions. The cDNAs were synthesized from 5 ng of total RNA using a TaqMan MicroRNA Reverse Transcription Kit and an RT primer. The TaqMan Universal PCR Master Mix (No AmpErase UNG) and TaqMan MicroRNA Assay were added to the cDNA sample, and real-time PCR was performed using Mx3000P with MxPro QPCR software. Since the PCR was performed 40 cycles, the data are presented as 40-Ct values to facilitate

understanding that the higher values represent higher expression levels.

### **Assessment of $b_5$ mRNA Stability**

HepG2 cells were transfected with the precursor for miR-223 as described above and simultaneously treated with 10  $\mu\text{g/ml}$  of  $\alpha$ -amanitin. Total RNA was prepared at 3, 6, 9, 12, and 24 hr later. The  $b_5$  mRNA level was determined using real-time RT-PCR as described above.

### **Construction of Recombinant Adenovirus**

The recombinant adenoviruses expressing CYP3A4 (AdCYP3A4) and CYP2E1 (AdCYP2E1) have been previously described (28,29). Recombinant adenoviruses expressing CYP1A2 (AdCYP1A2) were also generated using the following procedure. A fragment containing the full-length coding region of the human CYP1A2 cDNA was amplified by PCR using appropriate primer pairs and human liver cDNA as a template. The forward and reverse primers were 5'-TCT ACA GTT GGT ACA GAT GG-3' and 5'-TCA GTT GAT GGA GAA GCG CA-3', respectively. The fragments were subcloned into the pAxCawtit vector at the *Swa* I site. These vectors and the adenovirus genome DNA-terminal protein complex were co-transfected into HEK293 cells using Lipofectamine 2000. The recombinant adenovirus was isolated and propagated. Viral titers were determined using the QuickTiter Adenovirus Titer Immunoassay kit. The multiplicity of infection (MOI) was defined as the ratio of infectious units divided by the number of cells.

### **Infection of Recombinant Adenovirus to HepG2 Cells**

The day before infection, HepG2 cells were seeded at  $7.5 \times 10^5$  cells/well into a 12-well plate and grown to confluency. The cells were infected at various MOIs (0.05, 0.1, 0.2, 0.5, 1, and 2) of AdCYP. After 24 hr, the cultured medium was replaced with fresh medium without adenovirus. After 48 hr, total cell homogenates were prepared through homogenization with TGE buffer. The protein concentration was determined using Bradford protein assay reagent

with  $\gamma$ -globulin as a standard.

### **SDS-PAGE and Western Blot Analyses**

Total cell homogenates of HepG2 cells (30  $\mu\text{g}$ ) or human livers (5  $\mu\text{g}$ ) were separated using 15% SDS-PAGE for the detection of  $b_5$ . Total cell homogenates of HepG2 cells infected with AdCYPs (30  $\mu\text{g}$ ) or human liver microsomes (20  $\mu\text{g}$ ) were separated using 7.5% SDS-PAGE for the detection of P450 and NPR. The separated proteins were transferred to an Immobilon-P transfer membrane (Millipore). The membranes were probed with rabbit anti-human  $b_5$ , rabbit anti-human CYP3A4, rabbit anti-human CYP2E1, rabbit anti-human CYP1A2, or rabbit anti-human/rat NPR antibodies and the corresponding fluorescent dye-conjugated secondary antibodies. To determine the relative expression levels, the protein levels were normalized to the GAPDH protein level. To determine the absolute expression levels of each CYP isoform in human liver microsomes, recombinant CYP3A4, CYP2E1, and CYP1A2 were expressed in human lymphoblast cells (BD Gentest) and used to generate standard curves. Since the  $b_5$  level in HepG2 cells was too low to be measured spectrophotometrically, it was determined by Western blotting. The spectrophotometrical analysis quantifies holo protein while Western blotting measure total protein of holo and apo proteins. To determine the absolute  $b_5$  levels in microsomes from 11 human livers a human liver microsomal sample, whose  $b_5$  level was spectrophotometrically determined as described above, was used to make a standard curve. This enabled to compare the  $b_5$  protein levels in HepG2 cells and human liver microsomes, although an assumption underlies that the ratio of holo/apo proteins would be not largely different between HepG2 and each human liver microsomal sample. The band intensities were quantified using the Odyssey Infrared Imaging system (LI-COR Biosciences).

### **Assessment of CYP3A4, CYP2E1, and CYP1A2 Activities in HepG2 Cells Infected with AdCYPs**

The day before infection with AdCYP, the HepG2 cells were seeded at  $7.5 \times 10^5$  cells/well into a 12-well plate with 50 nM of precursor for miR-223 or 20 nM *b<sub>5</sub>* siRNA using Lipofectamine RNAiMAX. Subsequently, the HepG2 cells were infected with AdCYP3A4 (MOI 2) or AdCYP2E1 (MOI 0.5) or CYP1A2 (MOI 0.05) as described above. After 48 hr, the cultured medium was replaced with 1 ml of fresh medium containing 100  $\mu$ M of testosterone, 100  $\mu$ M of chlorzoxazone, and 1  $\mu$ M of 7-ethoxyresorufin, without FBS. After incubation for 5, 15, and 60 min, an aliquot of the medium was removed to measure the formed metabolite, and the cells were collected to prepare total cell homogenates.

The following HPLC conditions were used to measure the metabolites: an L-2130 pump (Hitachi, Tokyo, Japan), an L-2400 UV detector or L-7485 fluorescence detector (Hitachi), an L-2200 autosampler (Hitachi), an L-2500 integrator (Hitachi), and L-2350 column oven (Hitachi) were used. The flow rate was 1.0 ml/min and the column temperature was 35°C. To determine the 6 $\beta$ -hydroxytestosterone formation, a mobile phase of 50% methanol containing 10 mM potassium phosphate buffer (pH 7.4) was used, and the eluent was monitored at 240 nm using a COSMOSIL Cholester column (4.6  $\times$  150 mm; 5  $\mu$ m; Nacalai Tesque, Kyoto, Japan). To determine the 6-hydroxychlorzoxazone formation, a mobile phase of 25% methanol containing 50 mM potassium dihydrogenphosphate was used, and the eluent was monitored at 295 nm using Wakopak Handy-ODS column (4.6  $\times$  150 mm; 6  $\mu$ m; Wako). To determine the resorufin formation a mobile phase of 37% methanol containing 20 mM sodium perchlorate was used, and the eluent was monitored at 574 nm for excitation and 596 nm for emission using a Wakopak Handy-ODS column (4.6  $\times$  150 mm; 6  $\mu$ m). The quantification of the metabolites was performed by comparing the HPLC peak heights with those of authentic standards with reference to an internal standard.

### **Statistical analysis**

The statistical significance was evaluated using an unpaired, two-tailed Student's *t* test. The correlation analyses were performed using Spearman's rank method. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### **The $b_5$ mRNA and Protein Levels in Human Livers**

The  $b_5$  protein levels in a panel of 24 human livers were determined by Western blotting using total cell homogenates and normalized to the GAPDH protein levels (Fig. 1A). The  $b_5$  mRNA levels were determined by real-time RT-PCR analysis and normalized to the GAPDH mRNA levels (Fig. 1B). The  $b_5$  protein levels showing 15-fold interindividual variability were not positively correlated ( $R_s = 0.18$ ) with the  $b_5$  mRNA levels, showing 17-fold interindividual variability (Fig. 1C). The results suggested the post-transcriptional regulation in  $b_5$  expression in human liver.

### **A miR-223 Complementary Sequence on the 3'-UTR of Human $b_5$ mRNA**

As a mechanism for post-transcriptional regulation, we assessed regulation through miRNA. By using six computational programs, many different miRNAs were predicted for binding to the 3'-UTR of human  $b_5$ : 3 miRNAs were predicted using PicTar, 84 miRNAs were predicted using TargetScan, 51 miRNAs were predicted using Microcosm Targets, 14 miRNAs were predicted using microRNA.org, 9 miRNAs were predicted using TargetSpy, and 230 miRNAs were predicted using Target Rank, although the length of 3'-UTR is only 258 bp (Fig. 2A). Among these predictions, only miR-223 was common, and it was reported that the miR-223 is substantially expressed in human liver (30). As shown in Fig. 2A, the predicted MRE in the 3'-UTR of human  $b_5$  showed a perfectly matched the seed sequences (nucleotides 2-8 at the 5'-end of miRNA) of miR-223. Accordingly, we investigated the possibility that miR-223 might regulate human  $b_5$ .

### **Luciferase Assay to Determine Whether the MRE is Functional**

To determine whether the MRE is functional in miR-223-mediated regulation, a luciferase assay was performed using HeLa cells in which the miR-223 was barely expressed (data not

shown). The miR-223 levels were strikingly increased (40-Ct value: 21.0) after the transfection of the precursor for miR-223 into the HeLa cells. When the cells were transfected with the precursor for miR-223 and the pGL3/c-miR-223 plasmid, the luciferase activity was significantly ( $P < 0.001$ ) reduced (9% of control), suggesting that the overexpressed miR-223 was functional. When the pGL3/MRE plasmid was transfected into cells, the luciferase activity was also significantly ( $P < 0.001$ ) reduced (41% of control) through the overexpression of miR-223. These results suggested that miR-223 functionally recognized the MRE on human  $b_5$  mRNA.

### **Effects of miR-223 Overexpression on Endogenous $b_5$ Levels in HepG2 Cells**

We examined whether the miR-223 down-regulates endogenous  $b_5$  expression in a human liver-derived cell line. To select an appropriate cell line, we determined the  $b_5$  protein and mRNA levels in HepG2, HuH7, and HLE cells (Fig. 3). Among the three cell lines, HepG2 cells showed the highest  $b_5$  protein and mRNA levels. Therefore, HepG2 cells were selected for use in subsequent studies. After the precursor for miR-223 was transfected into HepG2 cells, the miR-223 levels were strikingly increased (40-Ct value: 21.1), similar to the levels observed with HeLa cells, as described above; thereby, the  $b_5$  protein level was significantly ( $P < 0.05$ ) reduced (49% of control) (Fig. 4A). The  $b_5$  mRNA level was also significantly ( $P < 0.01$ ) reduced after the overexpression of miR-223 (34% of control) (Fig. 4B). These results suggest that the miR-223 down-regulates  $b_5$  expression through reductions in the mRNA level. To determine whether miR-223 facilitates the degradation of the  $b_5$  mRNA, we investigated the  $b_5$  mRNA levels after treatment with  $\alpha$ -amanitin, an inhibitor of transcription. The overexpression of miR-223 reduced the  $b_5$  mRNA level compared with that of the control (Fig. 4C). These results suggested that miR-223 facilitates the degradation of  $b_5$  mRNA.

### **The Expression of miR-223 in Human Livers and Inverse Association with mRNA Levels and Translational Efficiencies of $b_5$**

To determine whether the miR-223-dependent regulation of  $b_5$  actually occurs in the human liver, we examined the relationship between the miR-223 and  $b_5$  levels using a panel of 24 human livers. The miR-223 levels showed a 4-fold interindividual variability, with 40-Ct values ranging from 11.2 – 15.1. Interestingly, the miR-223 levels were inversely correlated with the  $b_5$  mRNA levels ( $R_s = -0.50$ ,  $P < 0.05$ ) and translational efficiencies of  $b_5$  ( $R_s = -0.43$ ,  $P < 0.01$ ) (Fig. 5). One sample showed high translational efficiencies of  $b_5$  (13.2) (Fig. 5B); however, this exception did not change the overall findings ( $R_s = -0.47$ ,  $P < 0.05$ ). These results suggested that miR-223-dependent regulation greatly impacts constitutive  $b_5$  expression in the human liver through mRNA degradation or translational repression.

### **Effects of miR-223-dependent $b_5$ Regulation on P450 Activities in HepG2 Transformants**

We investigated the effects of the miR-223-dependent down-regulation of  $b_5$  on P450 activities. CYP3A4 and CYP2E1 were selected as the  $b_5$ -dependent human isoforms, whereas CYP1A2 was selected as the  $b_5$ -independent human isoform (31). HepG2 transformants expressing these P450s (26) were used. When the miR-223 was overexpressed in Hepc/3A4.2-30 cells, the  $b_5$  protein level was significantly ( $P < 0.05$ ) reduced (45% of control) (Fig. 6A), similar to the reduction observed in the intact HepG2 cells (Fig. 4A). Testosterone 6 $\beta$ -hydroxylation activity, catalyzed through CYP3A4, was significantly ( $P < 0.001$ ) reduced (64% of control) after the overexpression of miR-223 (Fig. 6B). To determine whether the reduced CYP3A4 activity reflected reduced  $b_5$  expression, we transfected  $b_5$  siRNA into the cells as a positive control. Unexpectedly, testosterone 6 $\beta$ -hydroxylation activity was not affected, although the  $b_5$  protein level was significantly ( $P < 0.05$ ) reduced (23% of control). The results suggested that the  $b_5$  expression, relative to the CYP3A4 expression in this system, might be too high to determine the effects of reduced  $b_5$  expression on CYP3A4 activity. Notably, the testosterone 6 $\beta$ -hydroxylation activity was reduced after the overexpression of miR-223. Moreover, we observed that the overexpression of miR-223 reduced CYP3A4 protein (38% of control) and mRNA (67% of control) levels (Figs. 6C and D). Reduced P450 protein and mRNA levels, after miR-223 overexpression, were also

observed in Hepc/2E1.3-8 and Hepc/1A2.9 cells (Suppl Fig. 1). The miR-223 has been predicted to target CYP2E1, but not CYP3A4 and CYP1A2. Taken together, the results indicate that the promoter or 3'-UTR of pcDNA3.1(+) vector might be affected through miR-223 expression. The unsuitability of these transformants for these experiments was suggested; therefore, we sought established other P450 expression systems with appropriate expression ratios of  $b_5$  and P450.

### **Establishment of A P450 Expression System in HepG2 Cells Using Adenovirus**

We applied an adenovirus expression system because this method generates higher expression levels than the plasmid system, and the expression levels can easily be modulated. Upon the overexpression of P450 in HepG2 cells, we compared the molar ratios of  $b_5$ /P450 in these cells to those in the human liver. For this purpose, we determined the  $b_5$ , CYP3A4, CYP2E1, and CYP1A2 protein levels in 11 human liver microsomes (Table I). Accordingly, the molar ratios of  $b_5$ /CYP3A4,  $b_5$ /CYP2E1, and  $b_5$ /CYP1A2 were calculated as 0.6 to 4.5, 1.3 to 5.8, and 2.1 to 158.2, respectively.

When HepG2 cells were infected with recombinant adenovirus, P450 was expressed in a MOI-dependent manner (Table II). The molar ratios of  $b_5$ /CYP3A4,  $b_5$ /CYP2E1, and  $b_5$ /CYP1A2 were also calculated. For subsequent studies, we set the MOI values for AdCYP3A4, AdCYP2E1, and AdCYP1A2 at 2, 0.5, and 0.05, respectively, to ensure that the  $b_5$ /P450 molar ratios would be in the ranges observed in human liver microsomes.

### **Effects of miR-223-dependent $b_5$ Regulation on CYP3A4 Activity in HepG2 Cells Infected with AdCYP3A4**

To investigate the effects of the miR-223-dependent down-regulation of  $b_5$  on CYP3A4 activity, miR-223 was overexpressed in HepG2 cells infected with AdCYP3A4. The  $b_5$  siRNA was transfected as a positive control. The  $b_5$  protein levels were significantly reduced after the overexpression of miR-223 ( $P < 0.05$ , 60% of control) and  $b_5$  siRNA ( $P < 0.01$ , 37% of control) (Fig. 7A). The testosterone 6 $\beta$ -hydroxylation activity was significantly reduced



after the overexpression of miR-223 ( $P < 0.001$ , 64% of control) and  $b_5$  siRNA ( $P < 0.05$ , 64% of control) (Fig. 7B). However, the CYP3A4 protein level was not altered through the overexpression of miR-223 or  $b_5$  siRNA (Fig. 7C). In addition, the protein level of NPR, another electron transfer enzyme, was also not altered after miR-223 or  $b_5$  siRNA overexpression (Fig. 7D). These results suggest that the down-regulation of  $b_5$  through miR-223 reduces CYP3A4 activity.

### **Effects of miR-223-dependent $b_5$ Regulation on CYP2E1 Activity in HepG2 Cells Infected with AdCYP2E1**

To investigate the effects of the miR-223-dependent down-regulation of  $b_5$  on CYP2E1 activity, miR-223 was overexpressed in HepG2 cells infected with AdCYP2E1. The  $b_5$  protein level was significantly reduced after the overexpression of miR-223 ( $P < 0.05$ , 47% of control) and  $b_5$  siRNA ( $P < 0.05$ , 30% of control) (Fig. 8A). Chlorzoxazone 6-hydroxylation activity was significantly reduced after the overexpression of miR-223 ( $P < 0.001$ , 44% of control) and  $b_5$  siRNA ( $P < 0.01$ , 80% of control) (Fig. 8B). However, CYP2E1 and NPR protein levels were not altered through miR-223 or  $b_5$  siRNA overexpression (Figs. 8C and 8D). These results suggest that the down-regulation of  $b_5$  through miR-223 reduces CYP2E1 activity.

### **Effects of miR-223-dependent $b_5$ Regulation on CYP1A2 Activity in HepG2 Cells Infected with AdCYP1A2**

To characterize the effects of the miR-223-dependent down-regulation of  $b_5$  on CYP1A2 activity, miR-223 was overexpressed in HepG2 cells infected with AdCYP1A2. The  $b_5$  protein level was significantly reduced after the overexpression of miR-223 ( $P < 0.01$ , 60% of control) and  $b_5$  siRNA ( $P < 0.05$ , 51% of control) (Fig. 9A). 7-Ethoxyresorufin *O*-deethylation activity was not altered through miR-223 and  $b_5$  siRNA overexpression (Fig. 9B). Moreover, CYP1A2 and NPR proteins were not altered through miR-223 and  $b_5$  siRNA overexpression

(Figs. 9C and D). These results suggest that the down-regulation of  $b_5$  through miR-223 does not affect CYP1A2 activity.

## DISCUSSION

The  $b_5$  protein plays an important role in the modulation of P450 catalysis through electron donation and/or allosteric modification (1). Thus,  $b_5$  is nearly obligatory for P450-mediated catalysis. However, the regulation of human  $b_5$  is not fully understood. We observed that there was no positive correlation between  $b_5$  mRNA and protein levels in human livers, suggesting the involvement of post-transcriptional regulation. Accordingly, we investigated whether miRNAs might be involved in the regulation of human  $b_5$ . Many miRNAs were predicted through in silico analysis using six programs. The false positive rates of this prediction were as high as 30%-50% (32) and the identification of the potential miRNAs was usually based on commonalities beyond the prediction programs. miR-223 was the only miRNA commonly predicted in all six programs and substantially expressed in human liver; therefore, we investigated whether miR-223 regulates human  $b_5$ .

The luciferase assay revealed that the potential MRE in the 3'-UTR of human  $b_5$  mRNA was functionally recognized by miR-223 (Fig. 2B). The overexpression of miR-223 in HepG2 cells significantly reduced endogenous  $b_5$  expression at the protein and mRNA levels (Figs. 4A and B), and facilitated the degradation of  $b_5$  mRNA (Fig. 4C). Thus, it was clearly demonstrated that the miR-223 down-regulates  $b_5$  expression through mRNA degradation. To determine whether this regulatory mechanism occurs in the human liver, we investigated the relationship between the miR-223 and  $b_5$  levels using a panel of 24 human livers. The miR-223 levels were inversely correlated with the mRNA levels and translational efficiencies of  $b_5$  (Fig. 5), suggesting that the miR-223-dependent negative regulation for  $b_5$  is actually functional in the human liver, and not only mRNA degradation but also translational repression might be potential mechanisms.

We characterized the effects of the miR-223-dependent down-regulation of  $b_5$  on P450 activities. CYP3A4 and CYP2E1 were selected as the  $b_5$ -dependent human isoforms, whereas CYP1A2 was selected as the  $b_5$ -independent human isoform (31). When we used a HepG2 transformant, stably expressing CYP3A4 (Hepc/3A4.2-30 cells), the overexpression of miR-223 reduced CYP3A4 activity through reduced levels of CYP3A4 *per se* rather than the down-regulation of  $b_5$  (Fig. 6). This phenomenon was observed in HepG2 transformants stably expressing CYP2E1 or CYP1A2. Therefore, we propose that miR-223 might affect the promoter or 3'-UTR of the used plasmid, pcDNA3.1 (+) vector. The reduction of  $b_5$  protein levels through the overexpression of  $b_5$  siRNA did not affect CYP3A4 activity, supporting that the decrease of CYP3A4 activity by miR-223 was not due to the down-regulation of  $b_5$ . In addition, the negative results implied that CYP3A4 expression might be too low to be altered through changes in the  $b_5$  expression levels. The quantification of CYP3A4 and  $b_5$  protein levels showed that the molar ratio of  $b_5$ /CYP3A4 in the Hepc/3A4.2-30 cells was 23 (data not shown), and the ratio was markedly higher than that observed in the human liver (0.6 - 4.5). Using reconstituted systems, we showed that a 1 molar equivalent of  $b_5$  is sufficient to enhance human P450 activity (11, 31), which explains why the overexpression of miR-223 did not affect CYP3A4 activity. Because the unsuitability of these cells lines for this experiment was revealed, we established other P450 expression systems, considering the optimum molar ratios of  $b_5$ /P450, i.e., ratios similar to those observed in human livers.

Modulating the MOI in the infection of HepG2 cells with adenovirus expressing P450 facilitated the establishment of a P450 expression system with a molar ratio of  $b_5$ /P450, which is similar to that observed in human livers. Moreover, we observed that the overexpression of miR-223 reduced  $b_5$  protein levels, and CYP3A4 and CYP2E1 activities, without affecting CYP3A4, CYP2E1, and NPR protein levels. In addition, we confirmed that NADH-cytochrome  $b_5$  reductase activity was not affected through miR-223 overexpression (data not shown). These results clearly demonstrated that the miR-223-dependent regulation would be a modulating factor of CYP3A4 and CYP2E1 activities. In contrast, CYP1A2 activity was not affected through the overexpression of miR-223. These results supported that

CYP3A4 and CYP2E1 are  $b_5$ -dependent P450 isoforms, whereas, CYP1A2 is a  $b_5$ -independent P450 isoform.

The role of  $b_5$  in P450 monooxygenase reactions has been studied for nearly 40 years (33). However, almost all studies on  $b_5$  have been performed *in vitro*. This study is the first to demonstrate the role of  $b_5$  in P450 activities *in cellulo*. Recently, *in vivo* studies demonstrating the importance of  $b_5$  in drug metabolism were performed using knockout mice (7,8). The miR-223 sequences in human and mouse are identical, and the MRE in the 3'-UTR of  $b_5$  mRNA in human and mouse is highly conserved (89.5%). Therefore, the mouse might be a good model to investigate the significance of the miR-223-dependent regulation of  $b_5$  *in vivo*. The  $b_5$  protein has versatile functions, including fatty acid desaturation (1), steroidogenesis (2), and methemoglobin reduction (3). Although the present study focused on the role of  $b_5$  in drug metabolism in the liver, it would be interesting to investigate whether miR-223-mediated  $b_5$  regulation might occur in other tissues and determine the significance of this mechanism in above functions.

miR-223 is a hematopoietic-specific miRNA, with crucial functions in myeloid lineage development (34). miR-223 plays an essential role in promoting granulocytic differentiation, while also suppressing erythrocytic differentiation. miR-223 is repressed in leukemia (35). The increased expression of miRNA-223 has been associated with extranodal marginal-zone lymphoma of mucosa-associated lymphoid tissue of the stomach (36) and recurrent ovarian cancer (37). Beside the roles of miR-223 in bone marrow, the present study revealed a new function for miR-223 in the human liver. It has been reported that human miR-223 expression is regulated through transcription factors, CCAAT/enhancer binding protein, nuclear factor I-A, and PU.1 in acute promyelocytic leukemia-NB4 cells (38, 39). In addition, mouse miR-223 expression in lymphocytes was up-regulated through estrogen treatment (40). Although this study demonstrated that the interindividual variability in miR-223 expression in normal human livers was slight, it would be interesting to determine which stimuli might alter the miR-223 expression in the human liver to understand the biological significance of the

miR-223-dependent regulation of  $b_5$  in the liver for the potential modulation of the pharmacokinetics or toxicokinetics.

In conclusion, we showed that  $b_5$  is post-transcriptionally regulated through miR-223, thus affecting P450 activities in the human liver. This regulatory mechanism might be an additional factor for the inter- and intraindividual variability in the pharmacokinetics of drugs.

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## Figure legends

**Fig. 1.** Expression levels of  $b_5$  protein (A) and mRNA levels (B) in a panel of 24 human livers and their relationship between the  $b_5$  protein and mRNA levels (C). The  $b_5$  protein and mRNA levels were determined using Western blot analysis and real-time RT-PCR, respectively, and normalized to GAPDH levels. The values represent the levels relative to that of the lowest sample. Each column represents the mean  $\pm$  SD of three independent experiments.

**Fig. 2.** Predicted sequence of MRE for miR-223 in the 3'-UTR of  $b_5$  mRNA and luciferase assay. Schematic representation of human  $b_5$  mRNA and the predicted sequence of MRE for miR-223 (A). The numbering refers to 5' end of mRNA as 1, and the coding region is from +142 to +546. The MRE (from +591 to +628) is located on the 3'-UTR of  $b_5$  mRNA. Luciferase assay using the plasmids containing the MRE for miR-223 in the 3'-UTR of  $b_5$  mRNA (B). The reporter plasmids (190 ng) were transiently transfected with phRL-TK plasmid (10 ng) and 50 nM precursors for miR-223 or negative control #2 (control) into HeLa cells. The firefly luciferase activity for each construct was normalized with the *Renilla* luciferase activities. The values are expressed as percentages of the relative luciferase activity of pGL3-p plasmid. Each column represents the mean  $\pm$  SD of three independent experiments. \*\*\* $P < 0.001$ , compared with control.

**Fig. 3.** Expression levels of  $b_5$  protein and mRNA in human liver-derived cell lines. The levels of  $b_5$  protein (A) and mRNA (B) in HepG2, HuH-7, and HLE cells were determined using Western blot analysis and real-time RT-PCR, respectively, and these levels were normalized to the GAPDH levels. The values represent the levels relative to that of the lowest sample. Each column represents the value of single experiment.

**Fig. 4.** Effects of the overexpression of miR-223 on the  $b_5$  level and the stability of  $b_5$  mRNA in HepG2 cells. The cells were transfected with 50 nM pre-miR-223 or control #2 (control) and incubated for 72 hr. The  $b_5$  protein (A) and mRNA (B) levels were determined using Western blot analysis and real-time RT-PCR, respectively, and were normalized to the GAPDH levels. Each column represents the mean  $\pm$  SD (n = 3). \* $P$  < 0.05 and \*\* $P$  < 0.01, compared with the control using Student's  $t$ -test. The values represent the levels relative to that of the control. Stability of the  $b_5$  mRNA in the HepG2 cells (C). The cells transfected with 50 nM pre-miR-223 or control #2 (control) were simultaneously treated with 10  $\mu$ g/ml of  $\alpha$ -amanitin. Total RNA was prepared at 3, 6, 9, 12 and 24 hr later. The  $b_5$  mRNA levels were determined using real-time RT-PCR and normalized to the GAPDH mRNA levels. The values are expressed as percentages relative to the values at 3 hr. Each point represents the mean  $\pm$  SD (n = 3). \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001, compared with the control. NT: No transfection.

**Fig. 5.** Relationship between the miR-223 levels and  $b_5$  mRNA levels (A) or translational efficiencies (B) in a panel of 24 human livers. The values represent the levels relative to that of the lowest sample. The data are presented as the means of three independent experiments.

**Fig. 6.** Effects of the overexpression of miR-223 on  $b_5$  level and CYP3A4 activity and expression in Hepc/3A4.2-30 cells. The cells were transfected with 50 nM pre-miR-223 or 20 nM  $b_5$  siRNA and incubated for 72 hr. The  $b_5$  protein level (A), CYP3A4 protein (C), and mRNA (D) levels were determined using Western blot analysis and real-time RT-PCR, respectively, and normalized to the GAPDH levels. The values represent the levels relative to that of the precursors for the control. The testosterone 6 $\beta$ -hydroxylation activity was measured as described in the Materials and Methods (B). Each column represents the mean  $\pm$  SD (n = 3). \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001, compared with the control. NT: No transfection.

**Fig. 7.** Effects of the overexpression of miR-223 on  $b_5$  level and CYP3A4 activity and expression in HepG2 cells infected with AdCYP3A4. HepG2 cells were transfected with 50 nM of pre-miR-223 or 20 nM of  $b_5$  siRNA, incubated for 24 hr, and subsequently infected with AdCYP3A4 at an MOI of 2 for 48 hr. The protein levels of  $b_5$  (A), CYP3A4 (C), and NPR (D) were determined using Western blot analysis and normalized to the GAPDH levels. The values represent the levels relative to that of the precursors for the control. The testosterone 6 $\beta$ -hydroxylation activity was measured as described in the Materials and Methods (B). Each column represents the mean  $\pm$  SD (n = 3). \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001, compared with the control. NT: No transfection.

**Fig. 8.** Effects of the overexpression of miR-223 on  $b_5$  level and CYP2E1 activity and expression in HepG2 cells infected with AdCYP2E1. HepG2 cells were transfected with 50 nM pre-miR-223 or 20 nM  $b_5$  siRNA, incubated for 24 hr, and subsequently infected with AdCYP2E1 at an MOI of 0.5 for 48 hr. The protein levels of  $b_5$  (A), CYP2E1 (C), and NPR (D) were determined using Western blot analysis and normalized to the GAPDH levels. The values represent the levels relative to that of the precursors for the control. The chlorzoxazone 6-hydroxylation activity was measured as described in the Materials and Methods (B). Each column represents the mean  $\pm$  SD (n = 3). \* $P$  < 0.05, \*\* $P$  < 0.01 and \*\*\* $P$  < 0.001, compared with the control. NT: No transfection.

**Fig. 9.** Effects of the overexpression of miR-223 on  $b_5$  level and CYP1A2 activity and expression in HepG2 cells infected with AdCYP1A2. HepG2 cells were transfected with 50 nM of pre-miR-223 or 20 nM of  $b_5$  siRNA, incubated for 24 hr, and subsequently infected with AdCYP1A2 at an MOI of 0.05 for 48 hr. The protein levels of  $b_5$  (A), CYP1A2 (C), and NPR (D) were determined using Western blot analysis and normalized to the GAPDH levels. The values represent the levels relative to that of the precursors for the control. The 7-Ethoxyresorufin *O*-deethylase activity was measured as described in the Materials and Methods (B). Each column represents the mean  $\pm$  SD (n = 3). \* $P$  < 0.05 and \*\* $P$  < 0.01,

compared with the control. NT: No transfection.

**Table I** Expression of  $b_5$ , CYP3A4, CYP2E1, CYP1A2, and the molar ratios of  $b_5$  to P450 in human liver microsomes.

The data are presented as the means of replicate measurements.

Sample	Expression level (pmol/mg)				Molar ratio		
	$b_5$	CYP3A4	CYP2E1	CYP1A2	$b_5$ /CYP3A4	$b_5$ /CYP2E1	$b_5$ /CYP1A2
1	168.3	242.3	29.3	4.8	0.7	5.7	35.2
2	241.0	131.3	56.4	9.5	1.8	4.3	25.3
3	148.8	165.7	57.2	0.9	0.9	2.6	158.2
4	127.6	208.0	29.1	3.2	0.6	4.4	39.9
5	199.3	148.9	55.4	93.7	1.3	3.6	2.1
6	246.5	124.7	93.2	79.1	2.0	2.6	3.1
7	195.9	227.6	149.8	1.8	0.9	1.3	106.2
8	131.4	29.7	61.0	11.1	4.4	2.2	11.8
9	201.8	96.2	37.7	11.8	2.1	5.4	17.1
10	82.8	18.4	34.7	18.7	4.5	2.4	4.4
11	213.0	280.2	36.7	18.7	0.8	5.8	11.4
Mean $\pm$ SD	177.9 $\pm$ 50.9	152.1 $\pm$ 84.1	58.2 $\pm$ 35.7	23.0 $\pm$ 32.1	1.8 $\pm$ 1.4	3.7 $\pm$ 1.6	37.7 $\pm$ 49.7
Range	82.3 - 246.5	18.4 - 242.3	29.1 - 149.8	0.9 - 93.7	0.6 - 4.5	1.3 - 5.8	2.1 - 158.2

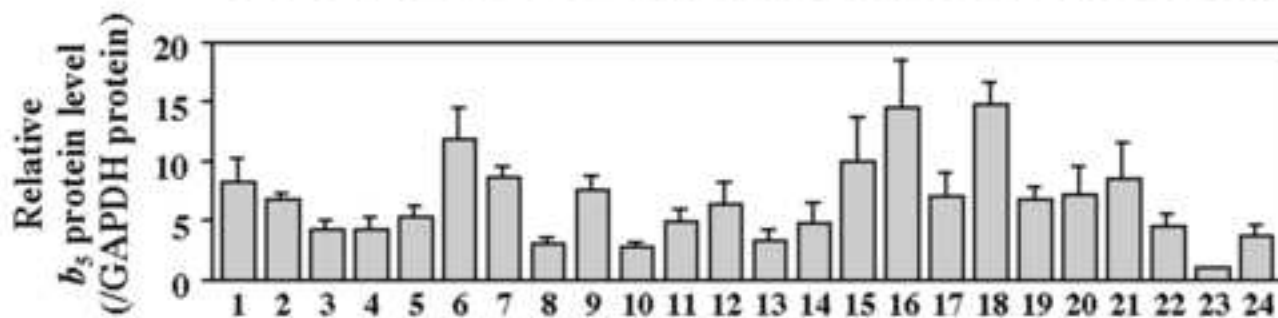
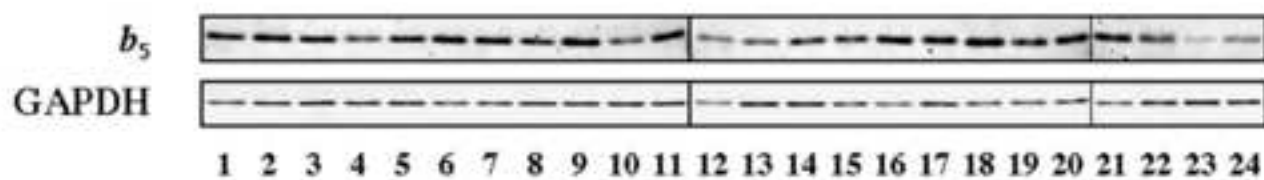
**Table II** Expression of  $b_5$ , CYP3A4, CYP2E1, CYP1A2, and the molar ratios of  $b_5$  to P450 in HepG2 cells infected with AdCYPs.

Total cell homogenates were used for the determination. The data are presented as the means of replicate measurements. MOI: Multiplicity of infection, ND: Not detected, -: Not calculated.

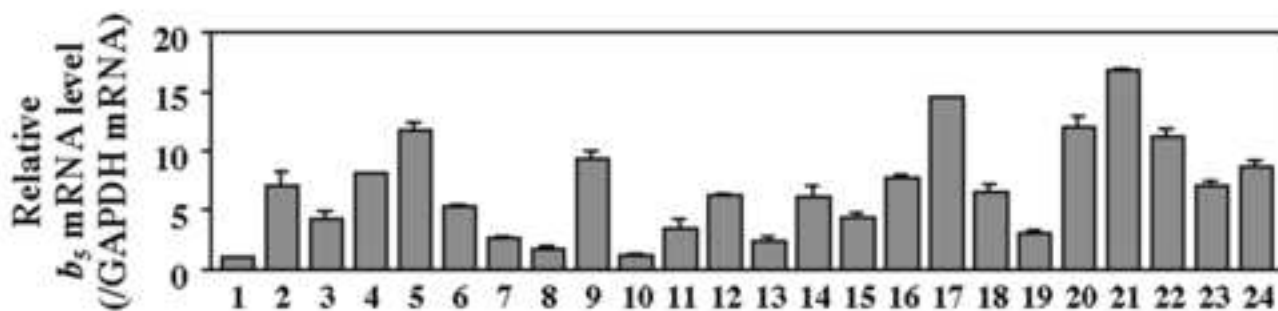
MOI	Expression level (pmol/mg)				Molar ratio		
	$b_5$	CYP3A4	CYP2E1	CYP1A2	$b_5$ /CYP3A4	$b_5$ /CYP2E1	$b_5$ /CYP1A2
0.05	9.2	0.8	ND	1.4	11.5	-	6.6
0.1	9.2	1.1	ND	3.7	8.8	-	2.5
0.2	9.2	1.9	ND	6.8	5.0	-	1.4
0.5	9.2	2.8	5.2	13.8	3.3	1.8	0.7
1.0	9.2	7.7	14.2	16.7	1.2	0.6	0.6
2.0	9.2	11.9	39.4	21.4	0.8	0.2	0.4

Fig. 1

A



B



C

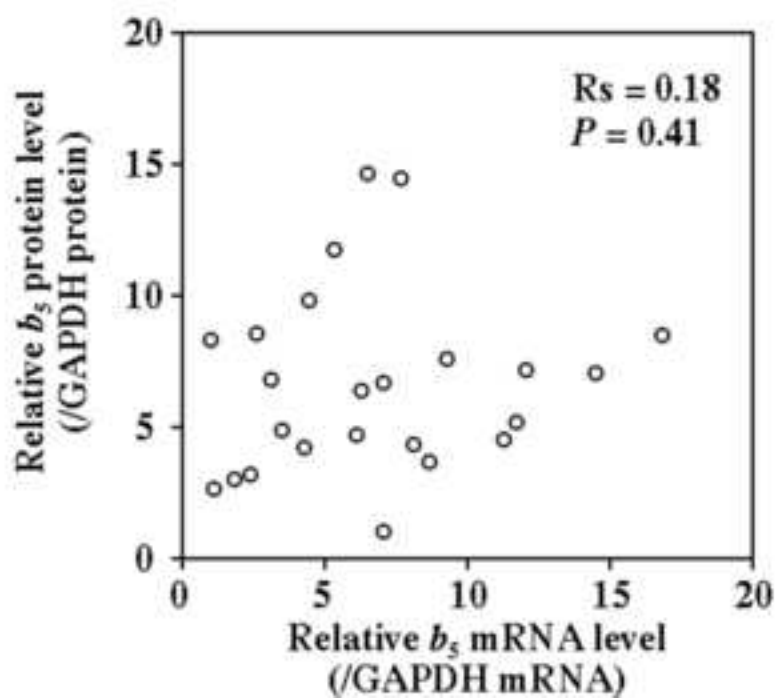
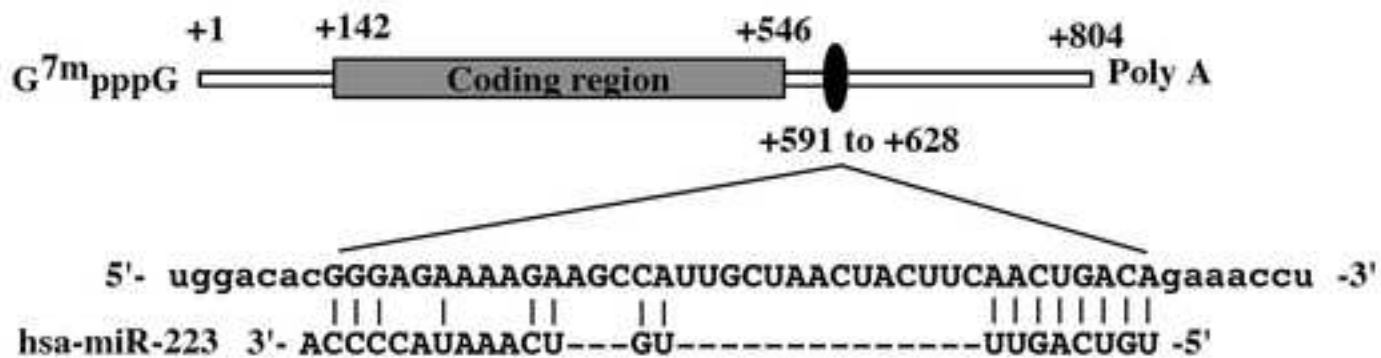




Fig. 2

A

Human cytochrome *b<sub>5</sub>* mRNA (NM\_148923)



B

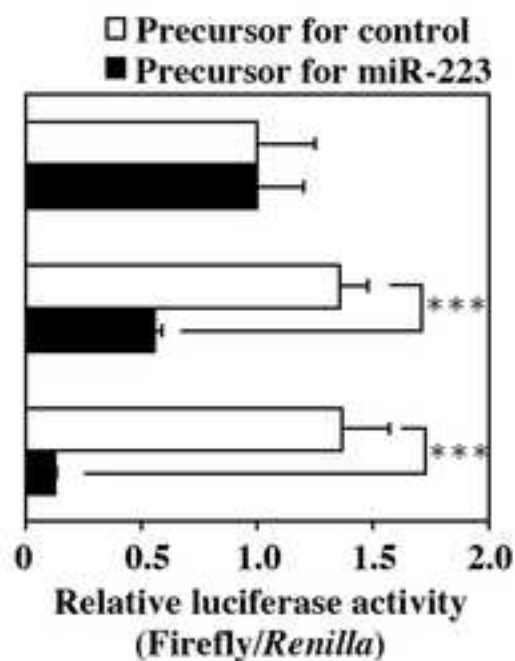
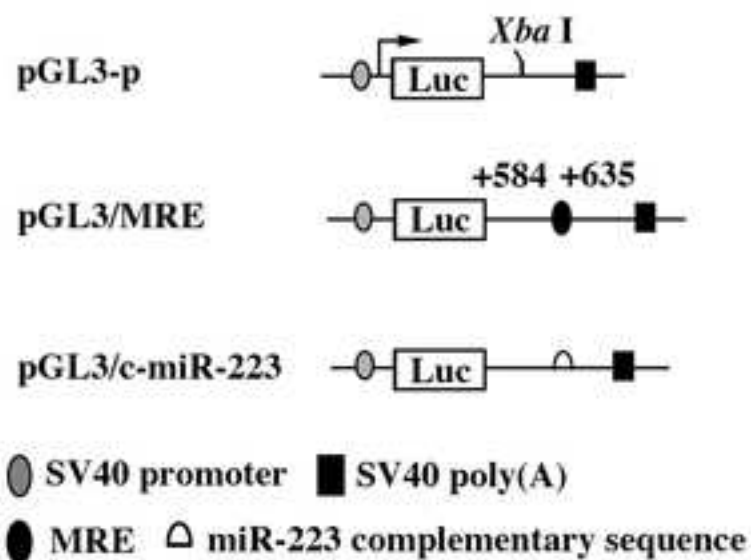


Fig. 3

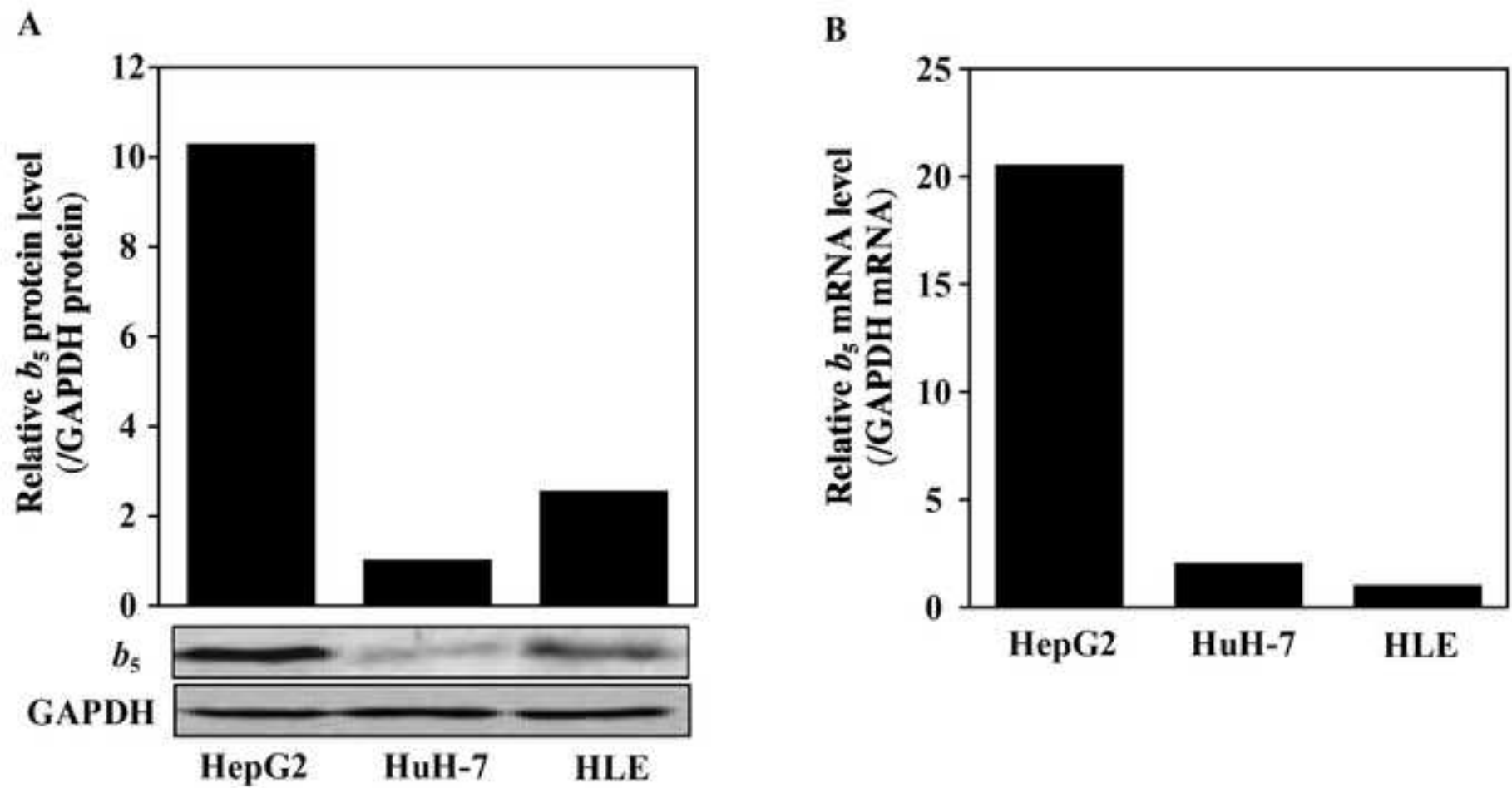


Fig. 4

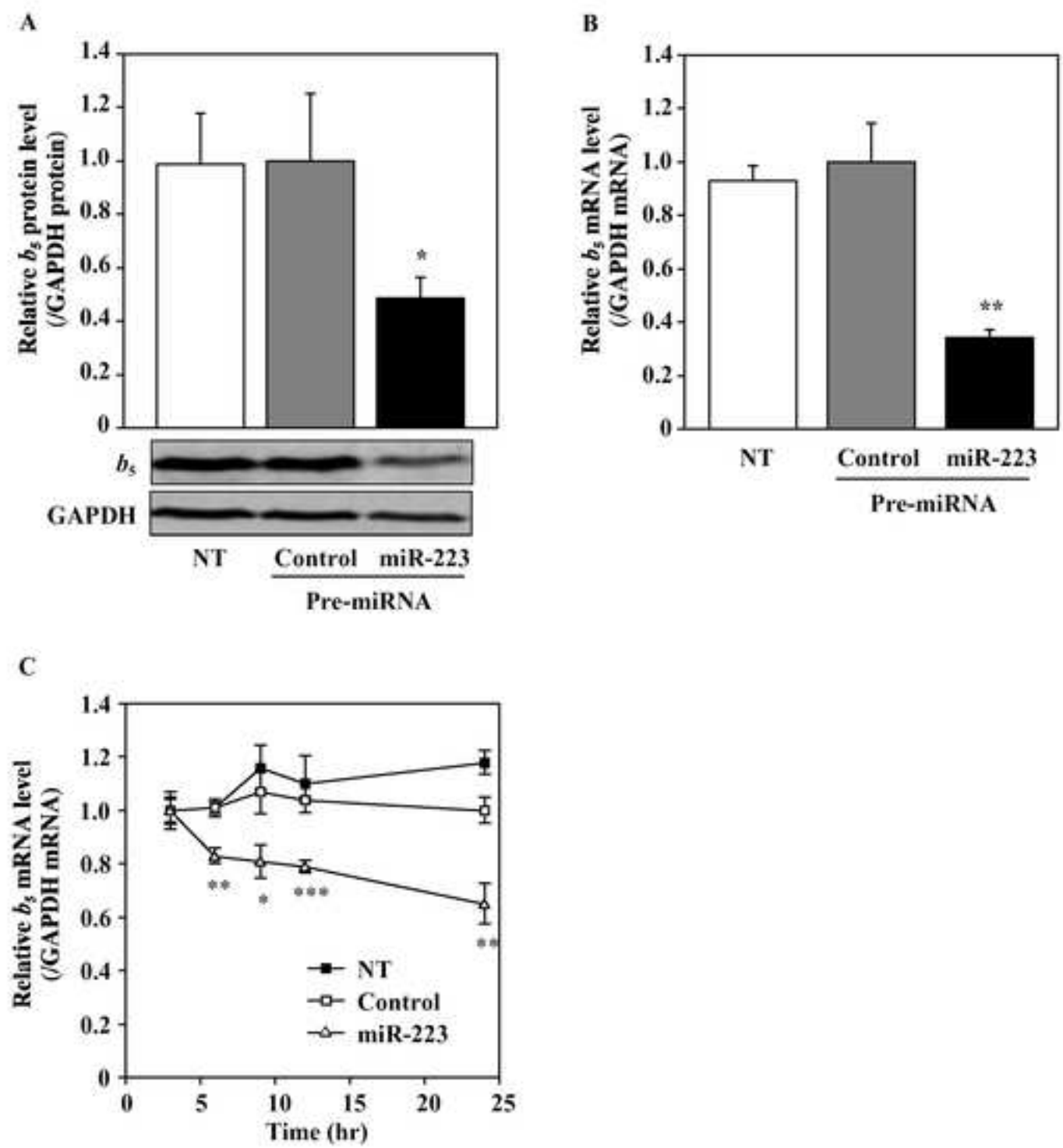


Fig. 5

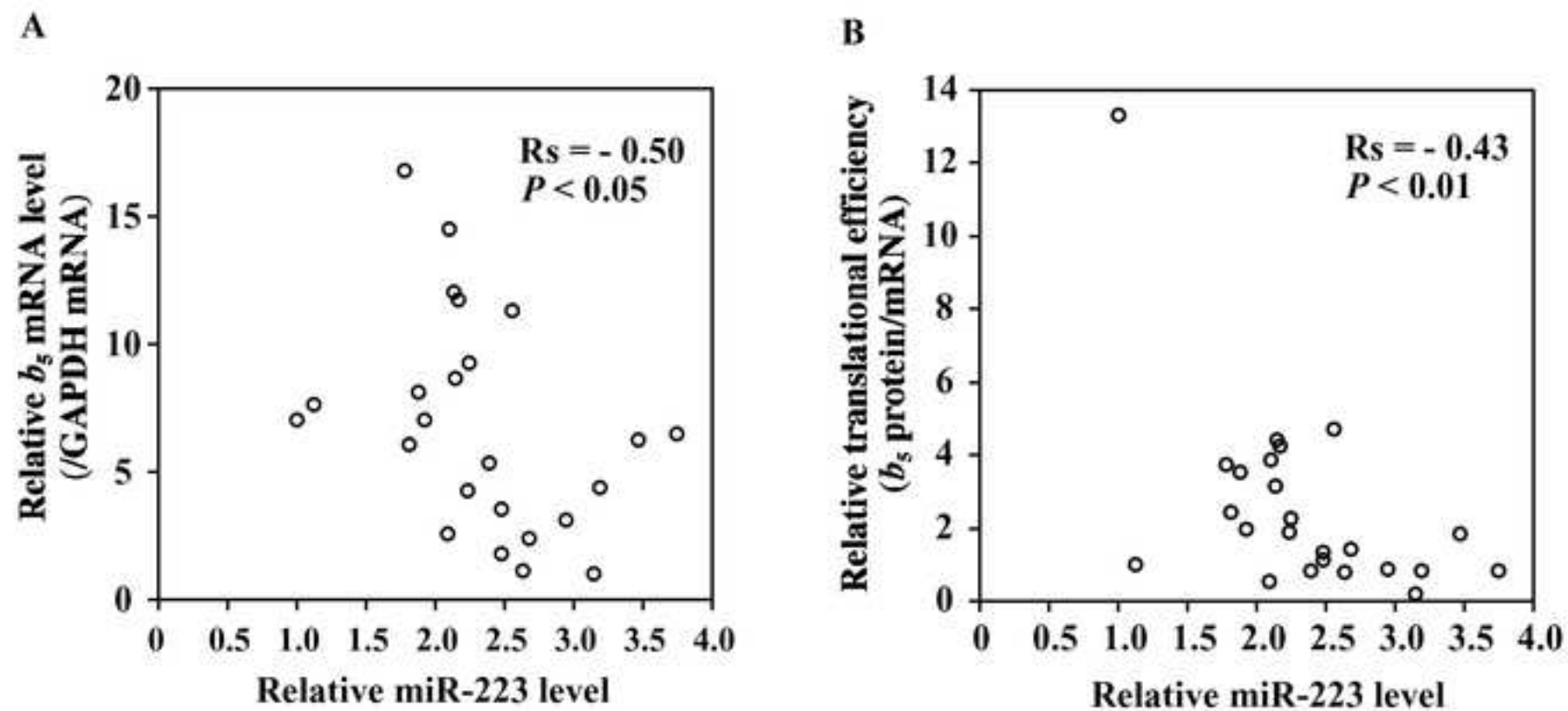


Fig. 6

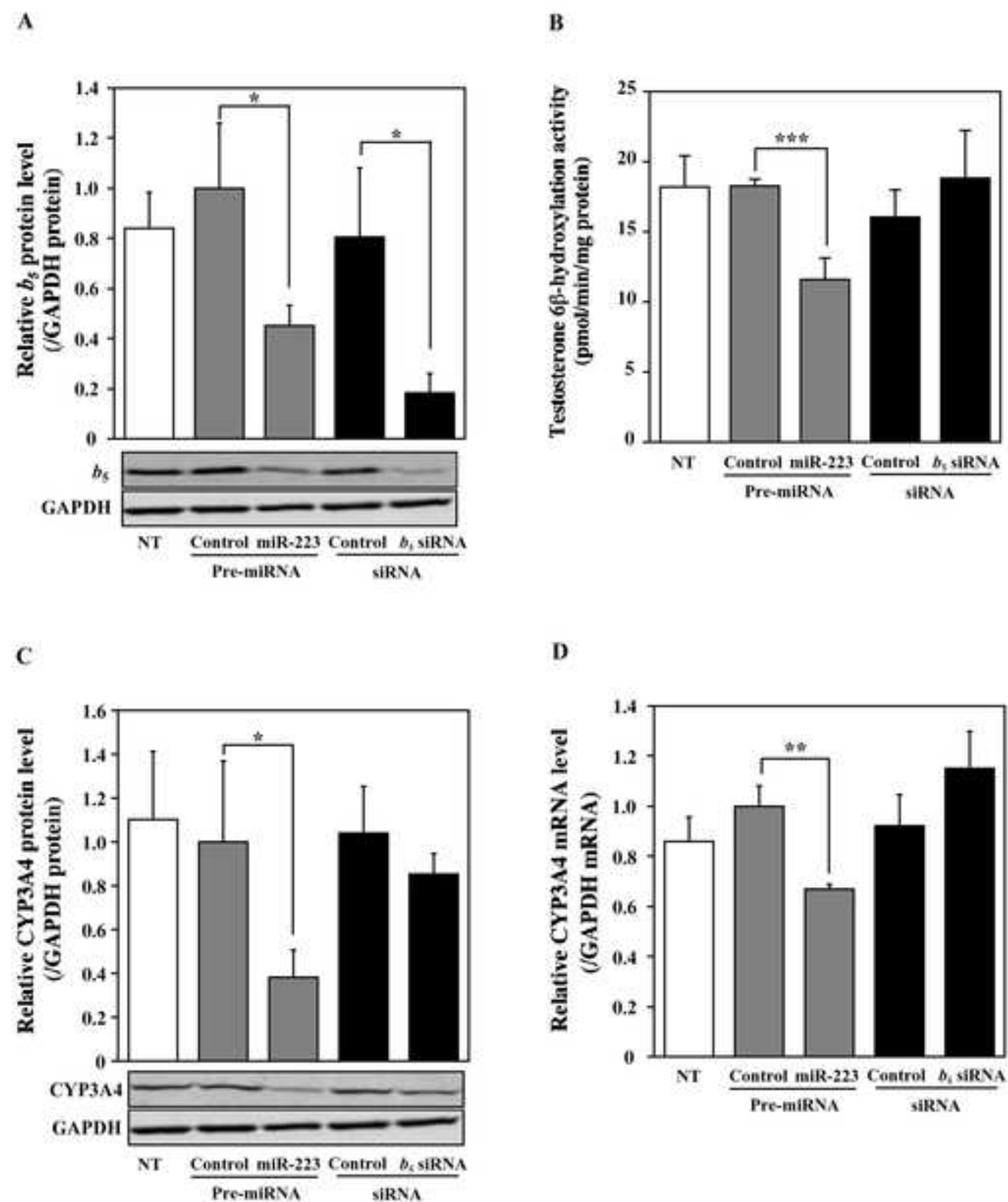


Fig. 7

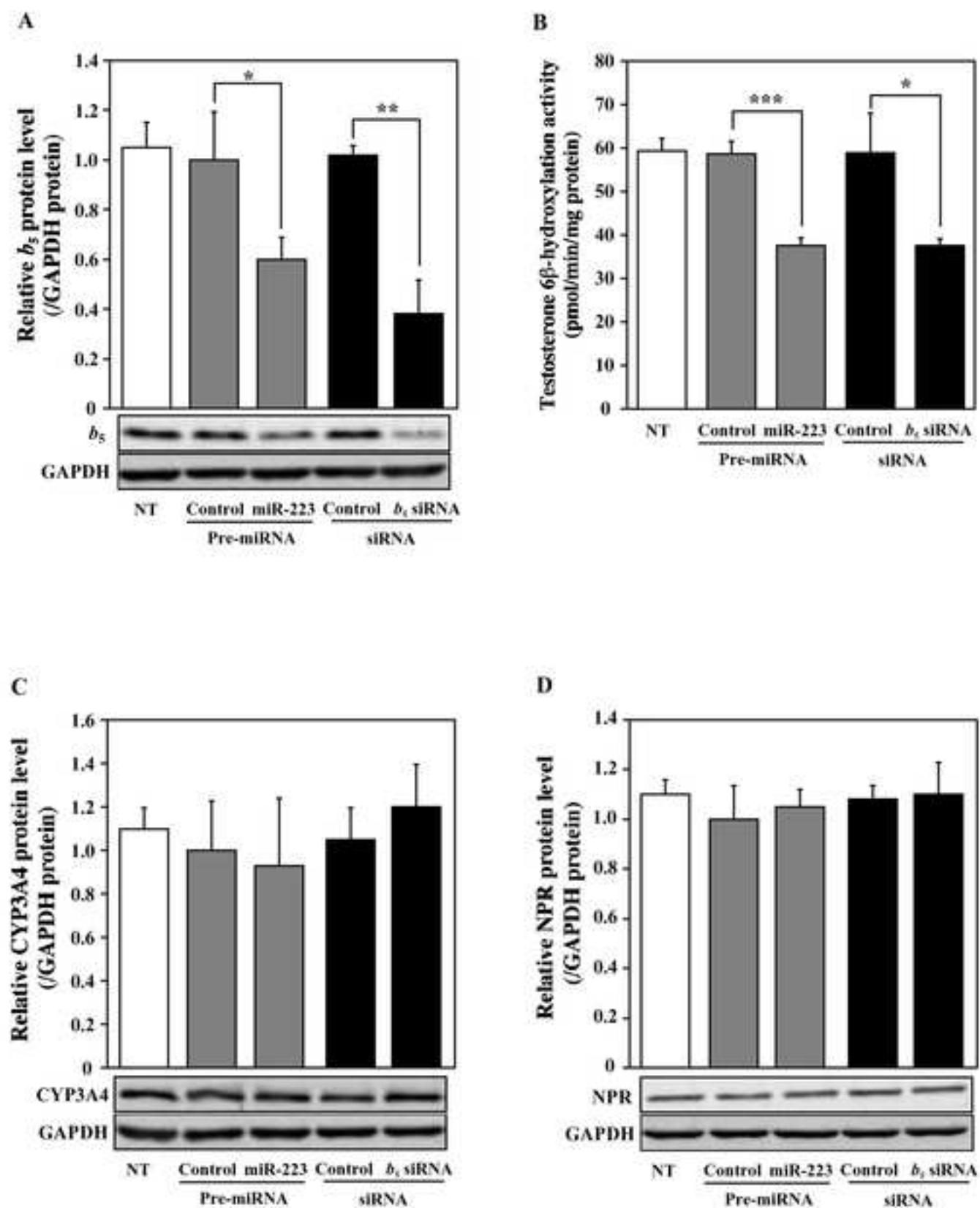


Fig. 8

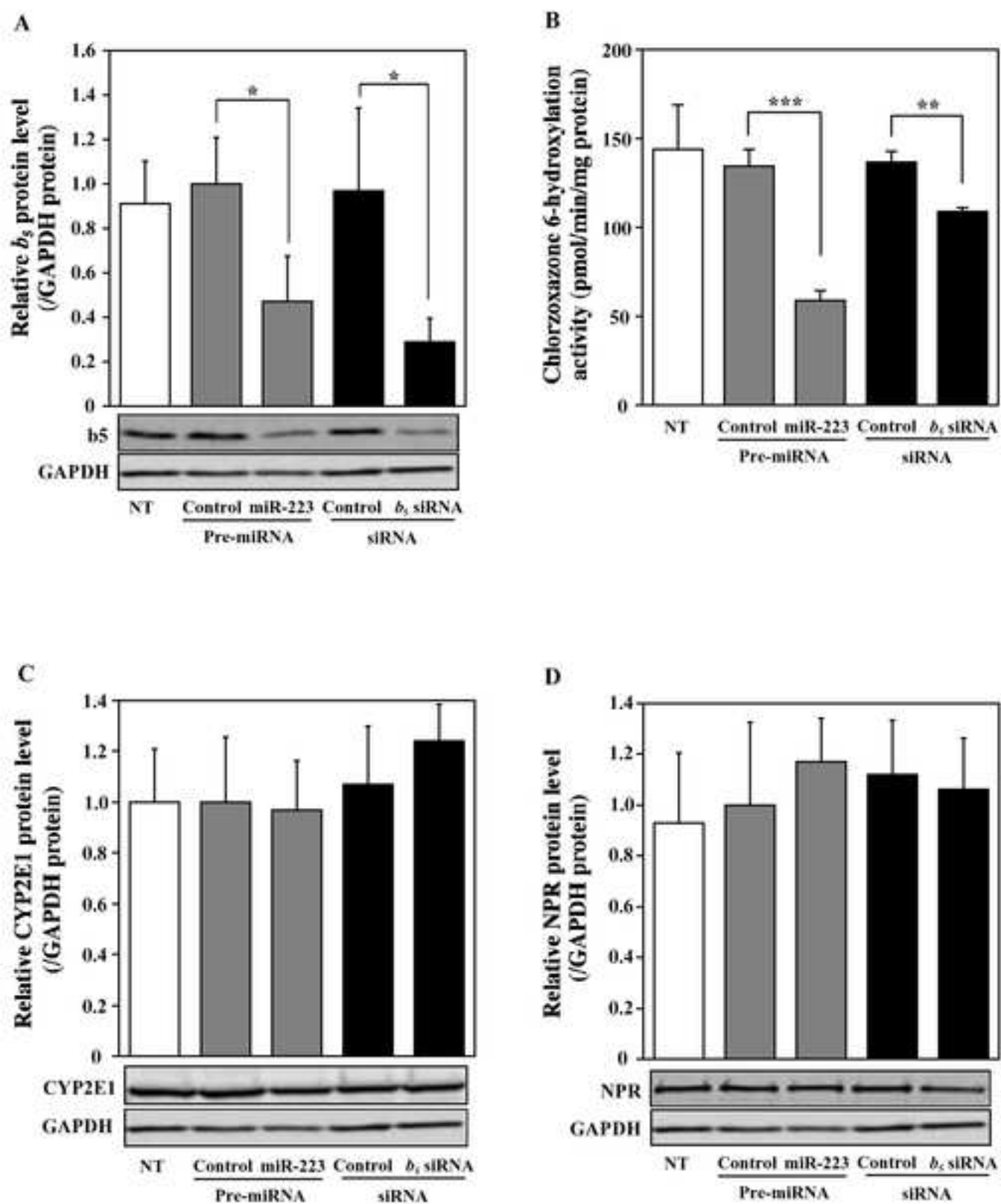


Fig. 9

