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CYP2A7 pseudogene transcript affects CYP2A6 expression in human liver by acting as a decoy for miR-126*

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Running title: CYP2A7 affects down-regulation of CYP2A6 by miRNA

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ABBREVIATIONS: AsO, Antisense oligonucleotides; CYP, cytochrome P450; miRNA, microRNA; MRE, microRNA recognition element; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PTEN, phosphatase and tensin homolog deleted from chromosome 10; 3'-UTR, 3'-untranlated region.

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Abstract

Human CYP2A6 is responsible for the metabolic activation of tobacco-related nitrosamines, as well as the metabolism of nicotine and some pharmaceutical drugs. There are large interindividual differences in CYP2A6 activity and expression, largely attributed to genetic polymorphisms. However, the variability was observed within homozygotes of the wild type *CYP2A6* gene. In this study, we investigated the possibility that CYP2A6 might be regulated by microRNA. A luciferase assay revealed that a microRNA recognition element (MRE) of miR-126* found in the 3'-untranlated region (UTR) of CYP2A6 mRNA is functional. We established two HEK293 cell lines stably expressing CYP2A6, with one including and the other excluding the full-length 3'-UTR (HEK/2A6+UTR and HEK/2A6 cells, respectively). Overexpression of miR-126* markedly decreased CYP2A6 protein levels, enzyme activity and mRNA level in HEK/2A6+UTR cells, whereas it marginally decreased those in HEK/2A6 cells, indicating that the 3'-UTR including the MRE is functional for the down-regulation of CYP2A6 by miR-126*. The inhibition of miR-126* increased CYP2A6 protein levels in primary human hepatocytes, suggesting that miR-126* downregulates endogenous CYP2A6 expression. In 20 human liver samples, the expression ratios of CYP2A6 and a pseudogene transcript CYP2A7 mRNA were highly variable (CYP2A7/CYP2A6: 0.1 to 12). Interestingly, we found that CYP2A7 was another target of miR-126* and restored the miR-126*-dependent down-regulation of CYP2A6 by acting as a decoy for miR-126*. In conclusion, this study demonstrates that human CYP2A6 is post-transcriptionally regulated by miR-126* and that CYP2A7 affects CYP2A6 expression by competing for miR-126* binding.

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Introduction

Human CYP2A6 is responsible for the metabolic activation of tobacco-related nitrosamines, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Tiano et al., 1993) and the metabolism of nicotine (Nakajima et al., 1996). In addition, CYP2A6 catalyzes the metabolism of coumarin and several pharmaceutical agents such as tegafur, losigamone, and letrozole (Nakajima et al., 2002). There is a large interindividual variability in CYP2A6 activity and expression, mainly due to genetic polymorphisms. Many studies have revealed that the interindividual variability in CYP2A6 activity affects smoking behavior or cancer susceptibility (Nakajima, 2007; Strasser et al., 2007; Fujieda et al., 2004). In addition to polymorphisms, large interindividual variability was observed within the homozygotes of the wild type *CYP2A6* gene (Nakajima et al., 2006). Non-genetic factors contributing to this variability have been proposed, including administration of drugs, exposure to environmental chemicals, diet, and sex hormone have been proposed (Koudsi et al., 2010). These can be partly explained by the activation of transcriptional factors such as pregnane X receptor (Itoh et al., 2006), glucocorticoid receptor (Onica et al., 2010), nuclear factor-erythroid 2 related factor 2 (Yokota et al., 2011), and estrogen receptor (Higashi et al., 2007).

To expand the understanding of the regulation of CYP2A6 expression, we sought to investigate the possibility of its regulation through a microRNA (miRNA). miRNAs, an evolutionarily conserved class of endogenous ~ 22-nucleotide noncoding RNAs, bind to the 3'-untranslated region (3'-UTR) of target mRNAs causing translational repression or mRNA degradation (Bartel, 2004). To date, over 2,500 miRNAs have been identified in humans, and it has been estimated that 60% of human mRNA could be targeted by miRNAs (Bartel, 2004; Friedman et al., 2009). Previously, we found that miRNAs regulate human xenobiotic-metabolizing enzymes such as CYP1B1 (Tsuchiya et al., 2006) and CYP2E1 (Mohri et al., 2010). Another research group reported that human CYP2C8 is also regulated by miRNAs (Zhang et al., 2012). Kalscheuer et al. (2008) reported that rat CYP2A3, showing high sequence similarity to human CYP2A6, is regulated by miR-126*, whose expression in the lung was decreased by administration of NNK for 20 weeks. This report prompted us to

examine whether human CYP2A6 might also be regulated by miR-126* because the miRNA recognition element (MRE) of miR-126* is highly homologous to the MRE of CYP2A3 mRNA.

CYP2A7, another member of the human CYP2A subfamily, shows 96.5% nucleotide identity with CYP2A6. CYP2A7 mRNA is substantially expressed in the human liver (Koskela et al., 1999), but the protein product is functionally inactive because it cannot incorporate heme (Ding et al., 1995; Yamano et al., 1990). Because CYP2A7 mRNA has an MRE for miR-126* and is perfectly matched to that of the CYP2A6 mRNA, we investigated the possibility that CYP2A7 mRNA may act as a decoy for binding of miR-126* to modulate CYP2A6 expression.

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Materials and Methods

Chemicals and reagents

Coumarin and 7-hydroxycoumarin were purchased from Sigma-Aldrich (St. Louis, MO). $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). The pGL3-promoter vector, the phRL-TK plasmid, the pTARGET vector and the dual-luciferase reporter assay system were purchased from Promega (Madison, WI). Lipofectamine 2000 and Lipofectamine RNAiMAX were purchased from Invitrogen (Carlsbad, CA). Pre-miR miRNA Precursors for miR-126*, negative control #1, Silencer Select siRNA for human CYP2A7 (s194373) (siCYP2A7), and negative control #1 were purchased from Ambion (Austin, TX). Antisense locked nucleic acid (LNA)/DNA oligonucleotides (AsO) for miR-126* (5′-CGC GTA CCA AAA GTA ATA ATG -3′; LNA is indicated by the underline) and for negative control (5′-AGA CUA GCG GUA UCU UAA ACC-3′) were commercially synthesized by Gene Design (Osaka, Japan). RNAiso, DNase I, random hexamer and SYBR Premix Ex Taq were obtained from Takara (Shiga, Japan). ROX was purchased from Stratagene (La Jolla, CA). ReverTra Ace was purchased from Toyobo (Osaka, Japan). All primers and oligonucleotides were commercially synthesized by Hokkaido System Sciences (Sapporo, Japan). G418 was obtained from Wako Pure Chemicals (Osaka, Japan). α-Amanitin was purchased from Calbiochem (San Diego, CA). The mouse anti-human CYP2A6 monoclonal antibody, the rabbit anti-human β-actin polyclonal antibody and the anti-human GAPDH polyclonal antibody were purchased from BD Gentest (Woburn, MA), Bio Vision (Mountain view, CA) and IMGENEX (San Diego, CA), respectively. IRDye 680 goat anti-rabbit IgG and goat anti-mouse IgG were obtained from LI-COR Biosciences (Lincoln, NE). Restriction enzymes were obtained from New England Biolabs (Ioswich, MA). All other chemicals and solvents were of the highest grade commercially available.

Construction of plasmids

To construct the luciferase reporter plasmids, various fragments were inserted into the *XbaI*

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site, downstream of the luciferase gene in the pGL3-promoter vector. The sequence from +1718 to +1738 of the human CYP2A6 mRNA (5'-ATG CTA TGA AGA GTA GTA ATA-3') was termed the MRE. A fragment containing the sequence from +1691 to +1744 of the CYP2A6 mRNA containing the MRE was cloned into the pGL-3 promoter vector, resulting in a forward insertion (pGL3/MRE) and a reverse insertion (pGL3/MRE-Rev). A fragment containing the perfectly matching sequence with the mature miR-126*, 5'-CTA GA*C GCG TAC CAA AAG TAA TAA TG*T-3' (the matching sequence of miR-126* is italicized), was cloned into the pGL3-promoter vector (pGL3/c-miR-126*).

Human CYP2A6 cDNA was amplified by PCR using a reverse-transcribed product from RNA of a human liver sample with the forward primer 5′-CCA CCA TGC TGG CCT CAG GG-3′ and the reverse primer 5′-GCT CAG CGG GGC AGG AAG-3′. This cDNA fragment was cloned into the pTARGET vector, resulting in the pTARGET/CYP2A6 plasmid. The 3'-UTR of CYP2A6 was amplified by PCR using human genome DNA with the forward primer 5′-AAA ATG GGC ATG AAC GCC C-3′ and the reverse primer 5′-CAA GTG TAC CTG GCA GGA AA-3′. This fragment was cloned into the pTARGET vector (pTARGET/3'-UTR). The CYP2A6 coding region digested from pTARGET/CYP2A6 was cloned into the pTARGET/3'-UTR, resulting in the pTARGET/CYP2A6+UTR.

Human CYP2A7 cDNA including the 3′-UTR was amplified by PCR using a reverse-transcribed product from RNA of a human liver sample with the forward primer 5′-CCA CCA TGC TGG CCT CAG GG-3′ and the reverse primer 5′-TCA GGA AAT AAG AGC TGC T-3′. The amplified fragment was cloned into the pTARGET vector, resulting in the pTARGET/CYP2A7 plasmid.

Cell culture and luciferase assay

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.1 mM nonessential amino acids (Invitrogen) and 10% fetal bovine serum (FBS; Invitrogen). HEK293 cells were cultured in DMEM containing 4.5 g/liter glucose, 10 mM HEPES, and 10% FBS. Human cryopreserved

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hepatocytes (lot HC1-30: African American female, 47 years) (Xenotech, Lenexa, KS) were maintained in HCM hepatocytes culture medium (Cambrex, East Rutherford, NJ) on collagen-coated plates at 37°C under 5% CO_2.

Various luciferase reporter plasmids (pGL3) were transiently transfected with phRL-TK plasmid into MCF-7 cells. Briefly, the day before transfection, cells were seeded onto 24-well plates at a density of 5×10^4 cells/well. After 24 h, 490 ng of pGL3 plasmid, 10 ng of phRL-TK plasmid and the precursors for miR-126* or control were co-transfected using Lipofectamine 2000. After incubation for 48 h, the cells were lysed with a passive lysis buffer and then the luciferase activity was measured with a luminometer (Wallac, Turku, Finland) using the dual-luciferase reporter assay system.

Establishment of two HEK293 cell lines stably expressing human CYP2A6 including or excluding the 3'-UTR

HEK293 cells were seeded onto 24-well plates, and 500 ng of pTARGET/CYP2A6+UTR or pTARGET/CYP2A6 plasmids were transfected using Lipofectamine 2000 according to the manufacturer's protocols. Forty-eight hours after transfection, the cells were passaged and subsequently grown in medium containing 400 mg/ml G418, and diluted from 1:10 to 1:1000. The selective medium was replaced every 3-4 days and individual G418-tolerable colonies were selected after 2 weeks in culture. Tolerable clones were screened by Western blot analysis and coumarin 7-hydroxylase activity. We confirmed that the CYP2A6 protein levels and enzyme activity were sustained regardless of the repeated subculture.

Transfection of precursor for miR-126* and CYP2A7 expression plasmid into HEK/2A6+UTR or HEK/2A6 cells and preparation of cell homogenates and total RNA

The precursor miRNA was transfected into HEK/2A6+UTR or HEK/2A6 cells as follows: the day before transfection, the cells were seeded onto 6-well plates at a density of 5×10^5 cells/well. After 24 h, 50 nM precursor for miR-126* or control was transfected into HEK/2A6+UTR cells and HEK/2A6 cells using Lipofectamine RNAiMAX. When 100, 500 or

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1000 ng pTARGET/CYP2A7 and precursor miRNA were transiently co-transfected, Lipofectamine2000 was used instead of Lipofectamine RNAiMAX. After incubation for 48 h, the cells were harvested and suspended in a small amount of TGE buffer [10 mM Tris-HCl, 20% glycerol, 1 mM EDTA (pH 7.4)] and disrupted by freeze-thawing three times. Total RNA was prepared using RNAiso and then treated with 5 U/μg RNA DNase I to remove plasmid DNA as needed according to the manufacturer's protocol.

Transfection of precursor for miR-126*, AsO for miR-126* and siRNA for CYP2A7 into human hepatocytes

The precursor miRNA, AsO and siRNA were transfected into primary human hepatocytes as follows: after 6 h of seeding human hepatocytes onto 6-well plates at a density of 5×10^5 cells/well, 50 nM precursor for miR-126* or control, 10 nM AsO for miR-126* or control and 5 nM siRNA for CYP2A7 or control were transfected into the cells using Lipofectamine RNAiMAX. After 24 h incubation, the cells were harvested and total cell homogenates and total RNA were prepared as described above.

Determination of the half-life of CYP2A6 mRNA

The HEK/2A6+UTR cells and HEK/2A6 cells were transfected with precursor for miR-126* or control as described above and were simultaneously treated with 2 μg/mL α-amanitin. Total RNA was prepared 2, 4, 6 and 8 h later. The CYP2A6 mRNA levels were determined by real time RT-PCR as described below.

Preparation of homogenates, total RNA and genomic DNA from 20 human liver samples

Human liver samples (Supplemental Table 1) from 13 donors were obtained from the Human and Animal Bridging (HAB) Research Organization (Chiba, Japan), which is in partnership with the National Disease Research Interchange (NDRI, Philadelphia, PA). The other 7 samples were obtained from autopsy materials that were discarded after pathological investigation with approval by the Ethics Committees of Kanazawa University (Kanazawa, Japan) (No. 170) and

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Iwate Medical University (Morioka, Japan) (HG H20-4). Homogenates were prepared from the human liver samples by homogenization with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) containing protease inhibitors (0.5 mM (*p*-amidinophenyl) methanesulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin). The protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad, Hercules, CA) with γ-globulin as a standard. Total RNA was prepared using RNAiso according to the manufacturer's protocols, and the integrity was confirmed by estimating the ratio of the band density of 28S and 18S rRNA. Genomic DNA was extracted using the Puregene DNA isolation kit (Qiagen, Tokyo, Japan).

SDS-PAGE and western blot analyses of CYP2A6

Total cell homogenates from HEK/2A6+UTR cells or HEK/2A6 cells (20-30 μg) and human liver homogenates (5-50 μg) were separated by 7.5% SDS-PAGE and transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA). The membranes were probed with mouse anti-human CYP2A6, rabbit anti-human β-actin or rabbit anti-human GAPDH primary antibodies and the corresponding fluorescent dye-conjugated secondary antibodies. The band densities were quantified with the Odyssey Infrared Imaging system (LI-COR Biosciences). The CYP2A6 protein levels were normalized to the β-actin or GAPDH protein levels.

Coumarin 7-hydroxylase activity

Coumarin 7-hydroxylase activity was determined as follows: a typical incubation mixture (final volume of 0.2 mL) contained 50 mM potassium phosphate buffer (pH 7.4), 1 μM coumarin, and 40 μg/mL total cell homogenates from HEK/2A6+UTR cells or HEK/2A6 cells. The reaction was initiated by the addition of NADPH-generating systems (0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, 5 mM $MgCl_2$, and 1 U/mL glucose-6-phosphate dehydrogenase) after a 2-min preincubation at 37°C. After a 15 min incubation at 37°C, the reaction was terminated by the addition of 20 μL of ice-cold 10% perchloric acid. After removal of the protein by centrifugation at 15,000 rpm for 5 min, a portion of the supernatant was subjected to

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high-performance liquid chromatography (HPLC). The HPLC analyses were performed using an L-2100 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), an L-2485 fluorescence detector (Hitachi), and a D-2500 integrator (Hitachi) equipped with a Mightysil RP-18 (4.6 × 150 mm; 5 μm) column (Kanto Chemical, Tokyo, Japan). The eluate was monitored with the excitation wavelength set at 320 nm and emission at 415 nm. The mobile phase was 15% acetonitrile containing 20 mM perchloric acid. The flow rate was 1.0 mL/min. The column temperature was 35°C. The quantification of the metabolites was performed by comparing the HPLC peak height with that of authentic standards.

Genotyping of *CYP2A6* alleles

The genotyping of *CYP2A6*4* (Fukami et al., 2007) and *CYP2A6*9* (Nakajima et al., 2006) for 20 human liver samples was performed as described previously.

Real-time RT-PCR for CYP2A6

The cDNA was synthesized from total RNA prepared from cells or human liver samples. The primers for human CYP2A6 (Nakajima et al., 2006), human β-actin (Oda et al., 2012) and GAPDH (Tsuchiya et al., 2004) were described previously. A 1-μL portion of the reverse-transcribed mixture was added to a PCR mixture containing 10 pmol of each primer, 12.5 μL of SYBR Premix Ex Taq solution and 75 nM Rox in a final volume of 25 μL. The PCR condition as follows: after an initial denaturation at 95°C for 30 s, the amplification was performed by denaturation at 94°C for 4 s, annealing and extension at 64°C for 20 s for 40 cycles. The real-time RT-PCR was performed using Mx3000P (Stratagene, La Jolla, CA) with the MxPro QPCR software. Copy numbers were determined using a standard curve made with the quantified PCR product. The CYP2A6 and CYP2A7 mRNA levels were normalized to β-actin (HEK/2A6+UTR, HEK/2A6 cells and human liver samples) or GAPDH (human primary hepatocytes) mRNA levels.

Real-Time RT-PCR for mature miR-126*

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The expression levels of mature miR-126* were determined by using the TaqMan microRNA assay (Applied Biosystems, Foster City, CA). The cDNA templates were prepared using the TaqMan microRNA Reverse Transcription kit, which utilizes the stem-loop reverse primers according to the manufacturer's protocols. After the reverse transcription reaction, the product was mixed with TaqMan Universal PCR Master Mix and TaqMan MicroRNA assay containing the forward and reverse primers as well as the TaqMan probe for miR-126*. The PCR conditions were as follows: after an initial denaturation at 95 °C for 10 min, the amplification was performed by denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 60 s for 40 cycles. The expression levels of U6 small nuclear RNA (U6 snRNA) were also determined by using the TaqMan microRNA assay and were used to normalize the miR-126* levels.

Statistical analysis

Statistical significance was determined by analysis of variance followed by Dunnett's multiple comparisons test. Comparison of two groups was made with an unpaired, two-tailed Student's t test. Correlation analyses were performed by Pearson's product-moment method. When the *P* value was less than 0.05, the differences were considered to be statistically significant.

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Results

Luciferase assay to investigate whether the MRE on the CYP2A6 3'-UTR is functional

By computational search, we found an MRE of miR-126* on the CYP2A6 3'-UTR (+1670 to +1723), the sequence of which is highly homologous to the MRE of CYP2A3 mRNA (Fig. 1A). To investigate whether the MRE is functional, a luciferase assay using reporter plasmids containing a fragment including the MRE was performed with MCF-7 cells. Since the miR-126* level in MCF-7 cells was marginal, the cell line was selected to examine the effects of overexpression of the miR-126*. When the pGL3/c-miR-126* plasmid containing the miR-126* complementary sequence was assayed, luciferase activity was significantly ($P <$ 0.001) decreased (18% of control) by the co-transfection of the precursor for miR-126*, suggesting that the overexpressed miR-126* was functional. The luciferase activity of the pGL3/MRE plasmid was significantly ($P < 0.01$) decreased (66% of control), whereas that of the pGL3/MRE-Rev plasmid was not (Fig. 1B). These results suggest that miR-126* functionally recognizes the MRE in the human CYP2A6 3'-UTR.

Effects of overexpression of miR-126* on CYP2A6 protein level, enzyme activity and mRNA level in HEK293 expression systems

To examine whether miR-126* downregulates CYP2A6 expression, HEK293 cell lines stably expressing human CYP2A6 including (Fig. 2A) or excluding the 3'-UTR (Fig. 2B) (HEK/2A6+UTR and HEK/2A6 cells, respectively) were established. In these cell lines, the expression level of miR-126* was marginal (Ct value: 29.2). When the precursor for miR-126* was transfected into HEK/2A6 +UTR cells, the CYP2A6 protein level was significantly ($P <$ 0.05) decreased (38% of NT) (Fig. 2C). In contrast, in the HEK/2A6 cells, CYP2A6 protein levels tended to be decreased, although the difference was statistically insignificant (Fig. 2D). The coumarin 7-hydroxylase activity was significantly decreased by the overexpression of miR-126* in HEK/2A6+UTR cells (59% of NT and 57% of control, $P < 0.05$) (Fig. 2E) and in HEK/2A6 cells (64% of NT, $P < 0.01$) (Fig. 2F). When the miR-126* was overexpressed in

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HEK/2A6+UTR cells, the CYP2A6 mRNA level was significantly ($P < 0.001$) decreased (51% of NT and 47% of control) (Fig. 2G), whereas it was marginally decreased in HEK/2A6 cells (Fig. 2H). These results suggest that the 3'-UTR including the MRE plays an important role in the miR-126*-dependent down-regulation of CYP2A6.

Effects of overexpression of miR-126* on CYP2A6 mRNA stability

To investigate whether miR-126* facilitates the degradation of the CYP2A6 mRNA, cells transfected with the precursor for miR-126* were treated with α-amanitin, and CYP2A6 mRNA levels were determined at different time points. The half-life of CYP2A6 mRNA in the HEK/2A6+UTR cells (Fig. 3A) and HEK/2A6 cells (Fig. 3B) was calculated to be 4.3 h and 8.9 h, respectively, and it was not affected by the overexpression of miR-126*. Thus, the finding that the half-life of CYP2A6 mRNA in HEK/2A6+UTR cells was shorter than in HEK/2A6 cells is interesting, but the miR-126* appeared to have no effect on CYP2A6 mRNA stability.

Effects of overexpression or inhibition of miR-126* on CYP2A6 expression levels in primary human hepatocytes

We investigated whether miR-126* downregulates endogenous CYP2A6 expression in human hepatocytes. As shown in Fig. 4A and B, the overexpression and inhibition of miR-126* significantly ($P < 0.01$) decreased (74% of control) and increased (2.0-fold) CYP2A6 protein levels, respectively. The CYP2A6 mRNA levels were not affected by either treatment (Figs. 4C and D). These results suggest that the endogenous CYP2A6 in human hepatocytes is regulated by miR-126* through translational repression.

Relationship between the expression levels of CYP2A6 mRNA, CYP2A6 protein, CYP2A7 mRNA and miR-126* in the human liver

To evaluate the significance of the miR-126*-dependent downregulation of CYP2A6 in human livers, we examined the relationship between the expression levels of CYP2A6 mRNA, CYP2A6 protein, and miR-126* in 20 individual human liver specimens. First, the CYP2A6

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genotypes in the liver samples were determined for the CYP2A6**4* (entire deletion) and *CYP2A6*9* (-1013A > G; -48T > C) alleles because these alleles result in no or decreased expression. Among 20 liver sample genotypes, one sample was *CYP2A6*1/CYP2A6*4*, two samples were *CYP2A6*1/CYP2A6*9*, one sample was *CYP2A6*4/CYP2A6*9*, and two sample were *CYP2A6*9/CYP2A6*9*. Overall variability of the CYP2A6 mRNA and protein levels in the 20 samples were 660-fold and 130-fold, respectively, and those in samples with *CYP2A6*4*/*CYP2A6*9* and *CYP2A6*9*/*CYP2A6*9* tended to be lower than the other groups having one or no defective alleles (Figs. 5A and B). The CYP2A6 protein levels were significantly correlated with the CYP2A6 mRNA levels (Fig. 5C). The miR-126* levels showed a relatively small interindividual variability (12-fold variability) and did not show any correlation with the CYP2A6 mRNA levels (Fig. 5D), protein levels (Fig. 5E), or translational efficiency (CYP2A6 protein/mRNA ratio) (Fig. 5F). Even when the haplotypes were taken into consideration, the results did not change. Thus, the miR-126*-dependent downregulation of the CYP2A6 expression might be masked by the large variability of transcriptional activity.

Effects of CYP2A7 mRNA on miR-126*-dependent downregulation of CYP2A6

Recently, it was reported that pseudogene-derived RNA transcripts compete with their cognate genes for miRNA-binding (Poliseno et al., 2010). Because the pseudogene transcript CYP2A7 has an MRE of miR-126* that perfectly matches the one found in CYP2A6 mRNA (Fig. 6), we sought to examine whether CYP2A7 may function as a miRNA decoy. First, we determined the CYP2A7 mRNA expression levels in 20 human livers and compared them to CYP2A6 mRNA levels (Fig. 7). Substantial CYP2A7 mRNA expression was observed in all samples, and the CYP2A7 mRNA/CYP2A6 mRNA ratios varied from 0.3 to 12. The liver samples with *CYP2A6*4* or *CYP2A6*9* had relatively higher CYP2A7 mRNA/CYP2A6 mRNA ratios. Although some liver samples genotyped as *CYP2A6*1/CYP2A6*1* showed relatively lower CYP2A7 and CYP2A6 mRNA levels, the expression levels of housekeeping genes in these samples were not too different than those in the other samples (data not shown), supporting the integrity of our RNA samples.

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To investigate whether CYP2A7 mRNA inhibits the downregulation of CYP2A6 via miR-126*, pTARGET/CYP2A7 and miR-126* were co-transfected into HEK/2A6+UTR cells which hardly express endogenous CYP2A7. When 100, 500, and 1000 ng pTARGET/CYP2A7 was transfected into HEK/2A6+UTR cells, the CYP2A7 mRNA copy numbers resulted in approximately 0.4, 2.9, and 8.7 times higher expression than that of CYP2A6 mRNA, respectively (Fig. 8A). These CYP2A7/CYP2A6 mRNA ratios were within the range of those in human livers. The CYP2A7 mRNA levels in any condition were significantly ($P < 0.01$) decreased (53-62% of control) by overexpression of miR-126*, suggesting that CYP2A7 is also regulated by miR-126*. Interestingly, the expressed CYP2A7 restored the miR-126*-dependent downregulation of CYP2A6 protein (Fig. 8B) and mRNA (Fig. 8C) levels when 1000 ng pTARGET/CYP2A7 was transfected, although 100 and 500 ng pTARGET/CYP2A7 had no effects. These results suggest that CYP2A7 mRNA affects CYP2A6 expression by acting as a decoy for binding of miR-126* when the CYP2A7 mRNA expression level is much higher than that of CYP2A6.

To examine whether endogenous CYP2A7 affects CYP2A6 expression level as a miRNA decoy, siCYP2A7 was transfected into primary human hepatocytes. CYP2A7 mRNA was remarkably decreased to 26%, but the knockdown of CYP2A7 mRNA (Fig. 9A) did not affect CYP2A6 mRNA (Fig. 9B) or protein levels (Fig. 9C). CYP2A7 mRNA levels may be too low to inhibit miR-126*-dependent down-regulation of CYP2A6 in these human hepatocytes as the CYP2A7/CYP2A6 mRNA ratio was 1.0. Although we could not expand this study, CYP2A7 would function as a decoy for miR-126* in hepatocytes in which CYP2A7 mRNA expression levels are much higher than those of CYP2A6.

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Discussion

There are large interindividual differences in CYP2A6 activity and expression that can affect smoking behavior or cancer susceptibility. In this study, we investigated the possibility that miRNA-dependent post-transcriptional regulation is involved in the variability of CYP2A6 expression. It had been reported that miR-126* regulates rat CYP2A3 (Kalscheuer et al., 2008). We found bioinformatically that CYP2A6 mRNA also has the miR-126* recognition element. The free energy of the hybrid from the CYP2A6 3'-UTR MRE and miR-126* (-20.5 kcal/mol) was lower than that of CYP2A3 (-12.4 kcal/mol) (RNAhybrid, http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/). Hence, we sought to examine the role of miR-126* on CYP2A6 regulation.

The luciferase assay revealed that the MRE in the 3'-UTR of human CYP2A6 mRNA was functionally recognized by miR-126* (Fig. 1B). The CYP2A6 protein levels and enzyme activity in HEK/2A6+UTR cells were markedly decreased by the overexpression of miR-126* (Fig. 2C and E), whereas those in HEK/2A6 cells were slightly decreased (Fig. 2D and F), suggesting that the 3'-UTR including the MRE of miR-126* plays an important role in regulation, and the possibility that the MRE of miR-126* may also exist in the coding region is implied. However, a computer-based search could not find the sequence similar to the MRE for miR-126* in the coding region. The overexpressed miR-126* decreased the CYP2A6 mRNA levels in HEK/2A6+UTR cells (Fig. 2G), but did not affect the CYP2A6 mRNA stability, suggesting miR-126* does not have the ability to facilitate degradation of CYP2A6 mRNA (Figs. 3A and B). Although there was a possibility that miR-126* may affect the CMV promoter activity of the pTARGET vector, this possibility was rejected because overexpressed miR-126* did not affect the expression of CYP2E1 (data not shown) which has no MRE for the miR-126* in the same vector/host cells system (pTARGET/HEK293) established in our previous study (Mohri et al., 2010). Because several studies have provided evidence that translational repression is often coupled to or precedes mRNA deadenylation and decay (Hu and Coller., 2012), this theory may apply to the case of CYP2A6 in HEK/2A6+UTR cells. In human hepatocytes, the overexpressed miR-126* did not affect CYP2A6 mRNA levels (Fig.

4C), but decreased protein levels (Fig. 4A). Although the reason of the inconsistency in the change in CYP2A6 mRNA levels in HEK293 cells stably expressing systems and primary human hepatocytes is unclear, translational repression would be the predominant mechanism in the regulation of CYP2A6 by miR-126*.

Using a panel of 20 human liver samples, we found that CYP2A6 protein levels were correlated with the CYP2A6 mRNA levels and that miR-126* levels showed no correlation with CYP2A6 mRNA levels, protein levels or translational efficiency of CYP2A6 (Figs. 5D-F). Because the samples with two defective alleles had lower CYP2A6 mRNA (Fig. 5A) and protein (Fig. 5B) levels compared with samples with no defective alleles, transcriptional regulation might be more predominant than post-transcriptional regulation by miR-126* for CYP2A6 expression in the human liver. However, the inhibition of miR-126* increased CYP2A6 protein levels (Fig. 3B), suggesting that miR-126* actually affects CYP2A6 expression. Although genetic polymorphisms substantially contribute to the interindividual differences in CYP2A6 expression, miR-126*-dependent regulation is an additional factor contributing to the intraindividual variability of CYP2A6 levels because the miRNA expression is readily changed by factors such as diseases, stress, or exposure to chemicals (Nakajima and Yokoi., 2011).

Previously, it was reported that the regulation of a tumor suppressor gene PTEN (phosphatase and tensin homolog deleted from chromosome 10), by miRNA is inhibited by its pseudogene, PTENP1 (Poliseno et al., 2010). In that report, the knockdown of PTENP1 resulted in the decrease of PTEN expression. Referring the report, we examined whether CYP2A7 is also regulated by miR-126* and function as a decoy for miR-126*. Using expression systems, we found that CYP2A7 mRNA has the ability to regulate CYP2A6 expression by restoring the miR-126*-dependent downregulation when CYP2A7 mRNA levels are higher than CYP2A6 mRNA levels (Fig. 8). However, the knockdown of CYP2A7 did not decrease CYP2A6 in primary human hepatocytes because the CYP2A7 mRNA levels in the lot of hepatocytes was too low to inhibit miR-126*-dependent down-regulation of CYP2A6. Actually, 120-fold interindividual differences were observed in CYP2A7 mRNA/CYP2A6

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mRNA ratios in a panel of 20 human livers (Fig. 7). Although untested, studies using multiple lots of human primary hepatocytes would strengthen the presumption that CYP2A7 interferes with the miR-126*-dependent downregulation of CYP2A6 in human hepatocytes.

The *miR-126** gene is within the intron 7 of the *epidermal growth factor-like domain 7* gene on human chromosome 9. The miR-126* has been reported to be decreased in lung cancer (Yanaihara et al., 2009). Decreased miR-126* may increase CYP2A6 in lung cancer, which would result in the augmentation of metabolic activation of tobacco-related nitrosamines. It would be of interest to compare miR-126* levels as well as CYP2A6 expression in lung cancerous tissue versus in non-cancerous tissues. CYP2A13, which is expressed in the lung, has a similar MRE as the one for miR-126*. We performed a luciferase assay using a reporter construct containing the MRE of the CYP2A13 mRNA. Luciferase activity was decreased by miR-126* (data not shown), suggesting that CYP2A13 is also regulated by miR-126*. Previously, we reported that CYP2A13 expression was higher in non-small cell lung carcinomas than in adjacent noncancerous tissue (Fukami et al., 2010). Although we did not measure the miR-126* levels in these samples, it would be interesting to determine whether the difference in CYP2A13 expression levels might be due to differences in miR-126* expression.

The current study revealed that the half-life of the CYP2A6 mRNA in HEK/2A6+UTR cells was shorter than that in HEK/2A6 cells (Figs. 3A and B), suggesting that CYP2A6 3'-UTR plays an important role in CYP2A6 mRNA stability. Previously, it has been reported that the half-life of the mRNA derived from *CYP2A6*1B*, a CYP2A6 variant allele in which a part of sequence in the 3'-UTR (+1533 to +1589) is converted to a corresponding sequence of the *CYP2A7* gene, is longer than that of the mRNA derived from the wild-type gene, *CYP2A6*1A* (Wang et al., 2006). We surmised that the difference in the half-life might be due to the difference in the miRNA-mediated regulation. Because the MRE of miR-126* (+1699 to 1719) is not included in the converted region, miR-126* is not likely to be involved in this difference. By computer analysis, miR-22, miR-150 and miR-328 were predicted to bind to the sequence unique to CYP2A6 mRNA of the *CYP2A6*1A* allele. When we transfected the precursor of these miRNAs into HEK2A6+UTR cells, CYP2A6 expression was not affected (data not

shown). Thus, further evaluation is needed to determine whether some miRNAs are involved in the difference in the stability of mRNAs derived from *CYP2A6*1A* and *CYP2A6*1B* genes.

In conclusion, we found that miR-126* negatively regulates the expression of human CYP2A6, and this regulatory mechanism is inhibited by CYP2A7 mRNA in the human liver. This mechanism would contribute to the interindividual variability of CYP2A6 expression.

Authorship Contributions

Participated in research design: Nakano, Fukami, Yokoi, and Nakajima

Conducted experiments: Nakano

Contributed new reagents or analytic tools: Nakano, Fukushima, Yokota, Takamiya, and Aoki

Performed data analysis: Nakano and Nakajima

Wrote or contributed to the writing of the manuscript: Nakano and Nakajima

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Footnotes

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Figure Legends

Fig. 1. Luciferase assay using plasmids containing the MRE in the 3'-UTR of human CYP2A6 mRNA. (A) Schematic representation of human CYP2A6 mRNA, rat CYP2A3 mRNA and the predicted target sequences of miR-126*. The numbering refers to the ATG in translation starting with A as 1. MRE is located in the 3'-UTR of human CYP2A6 mRNA (from +1699 to +1719) and rat CYP2A3 (from +1631 to +1650). *Bold letter*, seed sequence. (B) Luciferase assay using reporter plasmids containing various fragments downstream of the firefly luciferase gene. The reporter plasmids (490 ng) were transiently transfected with phRL-TK plasmid (10 ng) and 50 nM precursors for miR-126* or negative control #1 (control) into MCF-7 cells. Firefly luciferase activity for each construct was normalized to *Renilla* luciferase activity. The values represent the values relative to the pGL3-p plasmid. Each column represents the mean ± SD of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$.

Fig. 2. Effects of overexpression of miR-126* on CYP2A6 protein levels, enzyme activity and mRNA levels. Schematic representation of the CYP2A6 expression plasmids including (A) or excluding (B) the 3'-UTR of CYP2A6 (pTARGET/2A6+UTR or pTARGET/2A6). CYP2A6 protein levels in HEK/2A6+UTR (C) and HEK/2A6 (D) cells 48 hr after the transfection of 50 nM precursor for miR-126* or the negative control #1 (control) were determined by Western blot analysis and were normalized to the levels of β-actin. The values represent the levels relative to no-transfection controls (NT). Coumarin 7-hydroxylase activity was measured using the cell homogenates from HEK/2A6+UTR (E) and HEK/2A6 (F) cells. The substrate concentration was 1 μM. The CYP2A6 mRNA levels in HEK/2A6+UTR (G) and HEK/2A6 (H) cells 48 hr after the transfection of 50 nM precursor for miR-126* or the negative control #1 (control) were determined by real-time RT-PCR and normalized to β-actin mRNA levels. The values represent the levels relative to NT. Each column represents the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. NT: No transfection.

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Fig. 3. Effects of overexpression of miR-126* on CYP2A6 mRNA stability. The CYP2A6 mRNA levels in HEK/2A6+UTR (A) and HEK/2A6 (B) cells transfected with 50 nM precursor for miR-126* or the negative control #1 (control) and treated with 2 μg/mL α-amanitin were determined by real-time RT-PCR and normalized to β-actin mRNA. The amounts of mRNA at time 0 (the time of addition of α-amanitin) in each treatment were assigned 100%. Data are shown as the mean ± SD of three independent experiments.

Fig. 4. Effects of overexpression or inhibition of miR-126* on the CYP2A6 protein and mRNA levels in primary human hepatocytes. CYP2A6 protein levels 24 hr after the transfection of 50 nM precursor (A) or 5 nM AsO for miR-126* (B) or control were determined by Western blot analysis and were normalized to GAPDH protein levels. CYP2A6 mRNA levels 24 hr after the transfection of 50 nM precursor (C) or 10 nM AsO (D) for miR-126* or the control were determined by real-time RT-PCR and were normalized to GAPDH mRNA levels. The values represent the levels relative to NT (A and C) or control (B and D). Each column represents the mean ± SD of three independent experiments. *$P < 0.05$ and **$P < 0.01$. NT: No transfection.

Fig. 5. Expression levels of CYP2A6 and miR-126* in a panel of 20 human liver samples and their relationships. CYP2A6 mRNA (A) and protein levels (B) with *CYP2A6* genotypes. Relationship between CYP2A6 mRNA and protein levels (C), miR-126* and CYP2A6 mRNA levels (D), protein levels (E) or translational efficiency (CYP2A6 protein/mRNA) (F). Data are the mean of two independent experiments. The values of miR-126* and CYP2A6 protein levels represent the levels relative to those of the lowest one. The values of CYP2A6 mRNA represent the copy numbers normalized to β-actin mRNA.

Fig. 6. Comparison of MREs for miR-126* between CYP2A6 and CYP2A7. The numbering refers to the ATG in translation starting with A as 1. MRE is located in the 3'-UTR of human CYP2A6 (from +1699 to 1719) and CYP2A7 (from +1698 to 1718). Boxes represent the coding region. *Bold letter*, seed sequence.

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Fig. 7. CYP2A6 and CYP2A7 mRNA levels, CYP2A7/CYP2A6 mRNA level ratio and CYP2A6 genotypes in a panel of 20 human liver samples. The values of CYP2A6 and CYP2A7 represent the copy numbers normalized to β-actin mRNA. Each column represents the mean of two independent experiments.

Fig. 8. Effects of overexpression of CYP2A7 on miR-126*-dependent downregulation of CYP2A6. HEK/2A6+UTR cells were transfected with 10 nM precursor of the miR-126* (+) or negative control #1 (control) (-) and 100, 500, 1000 ng pTARGET/CYP2A7 (+), or empty plasmid (-). The CYP2A7 mRNA levels and CYP2A7/CYP2A6 mRNA ratio (A), the CYP2A6 protein levels (B) and the CYP2A6 mRNA levels (C) were determined by real-time RT-PCR or Western blot analysis and normalized to β-actin levels. Each column represents the mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Fig. 9. Effects of knockdown of CYP2A7 on CYP2A6 protein and mRNA level in primary human hepatocytes. Primary human hepatocytes were transfected with 10 nM siRNA for CYP2A7 or negative control #1.The CYP2A7 mRNA levels (A), the CYP2A6 protein levels (B) and the CYP2A6 mRNA levels (C) were determined by real-time RT-PCR or Western blot analysis and normalized to GAPDH levels. Each column represents the mean ± SD of three independent experiments. $***P < 0.001$.

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Figure 1

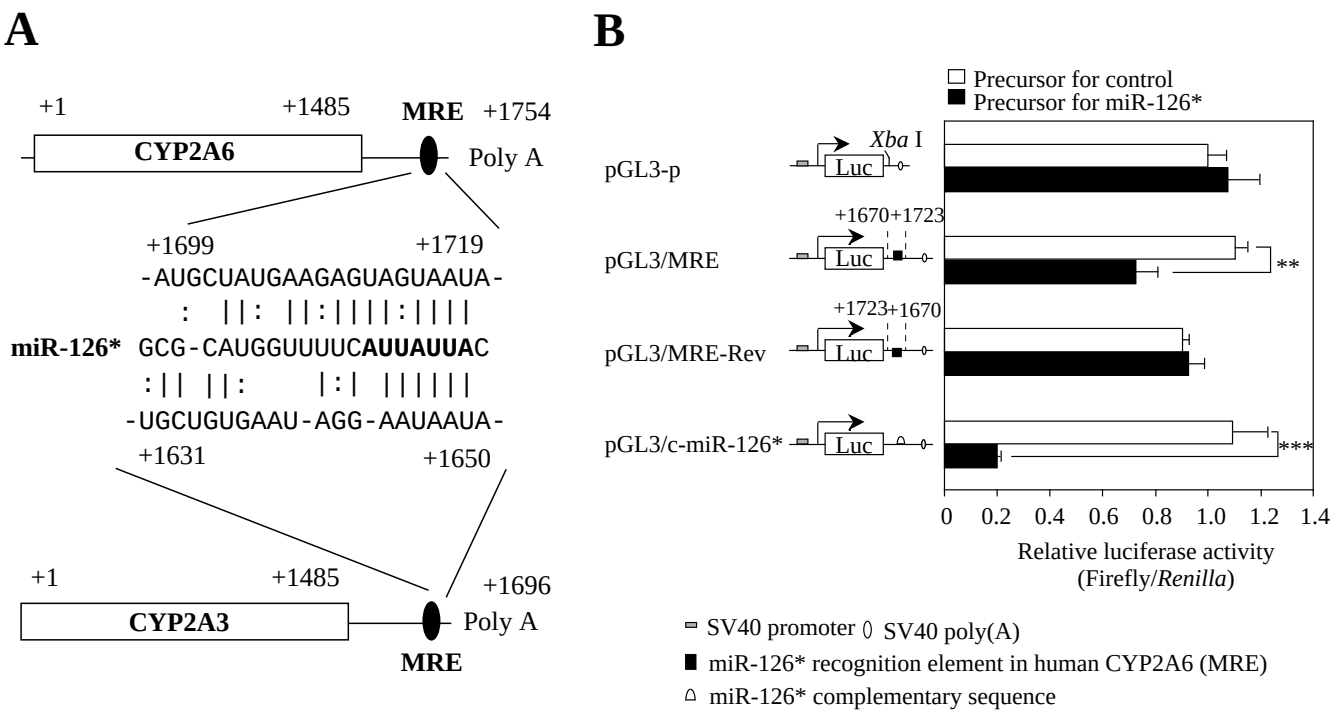

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Figure 2

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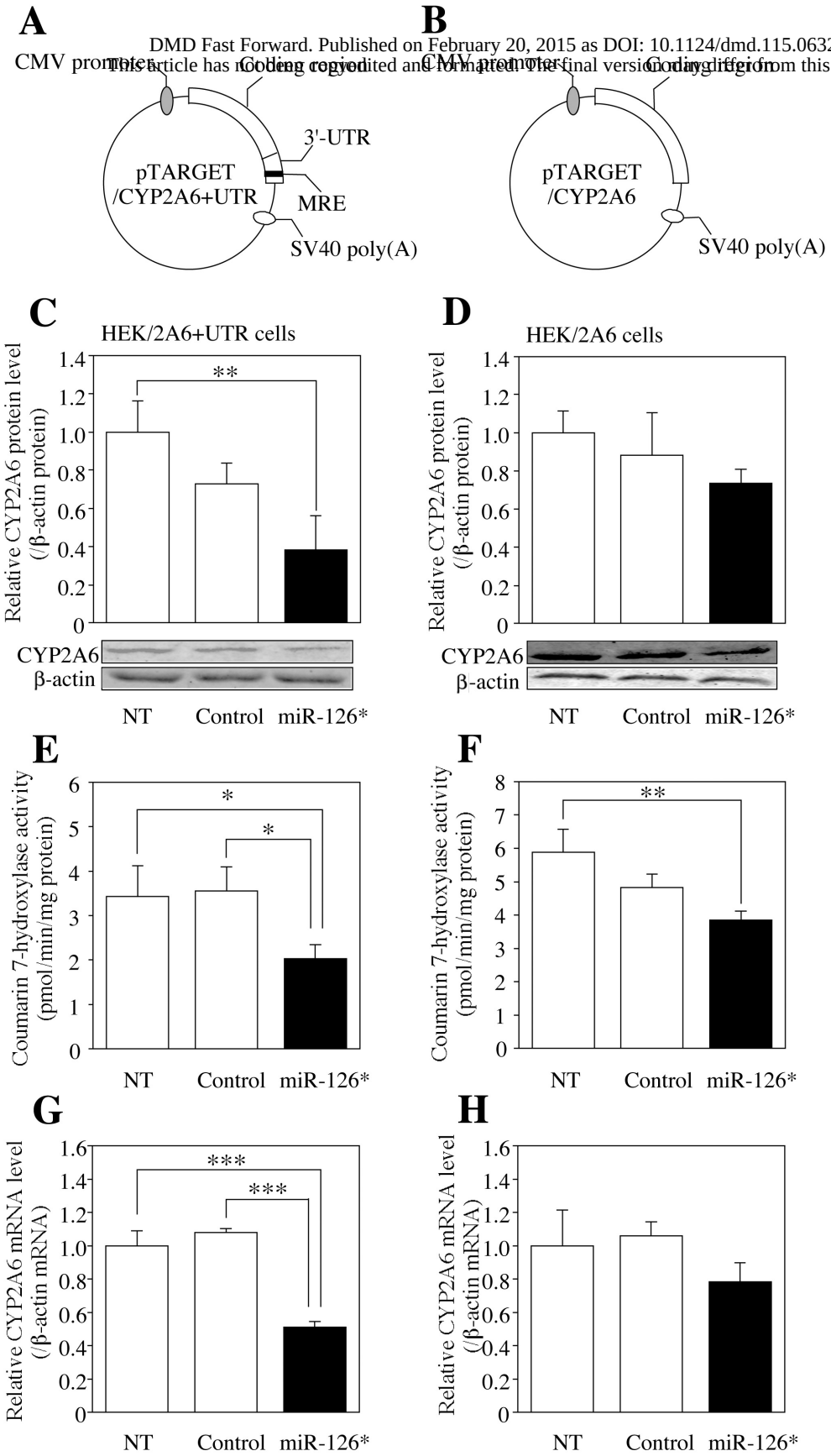

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Figure 3

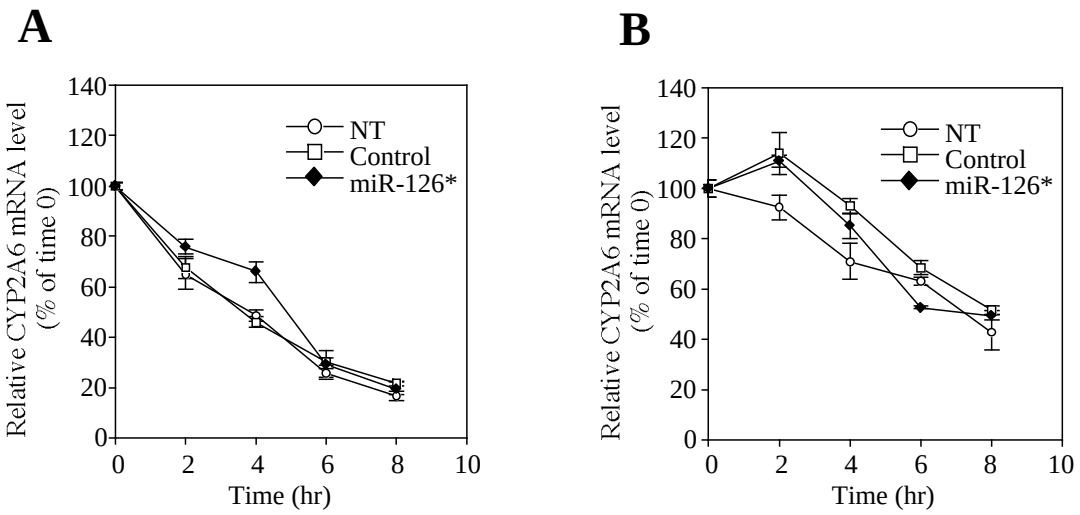

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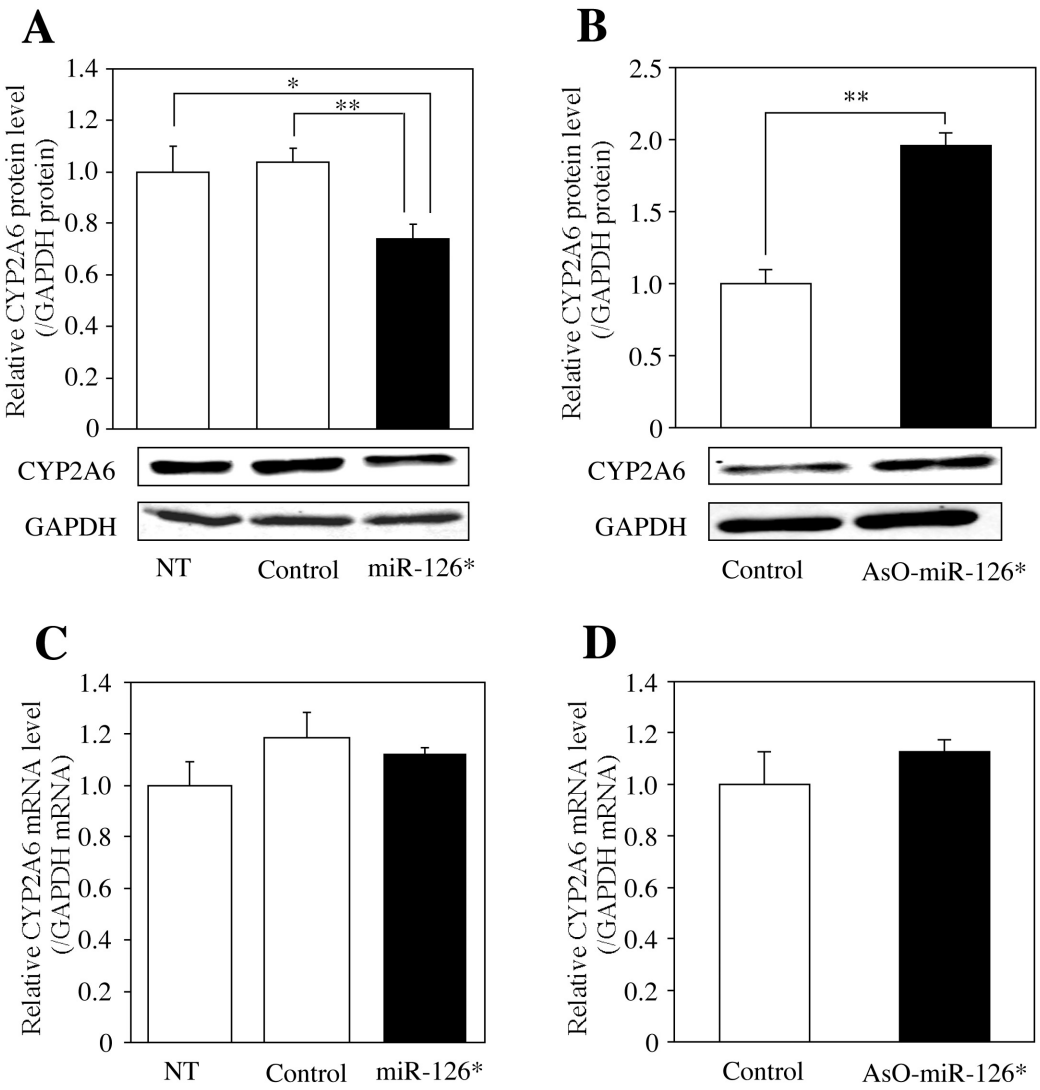
Figure 4



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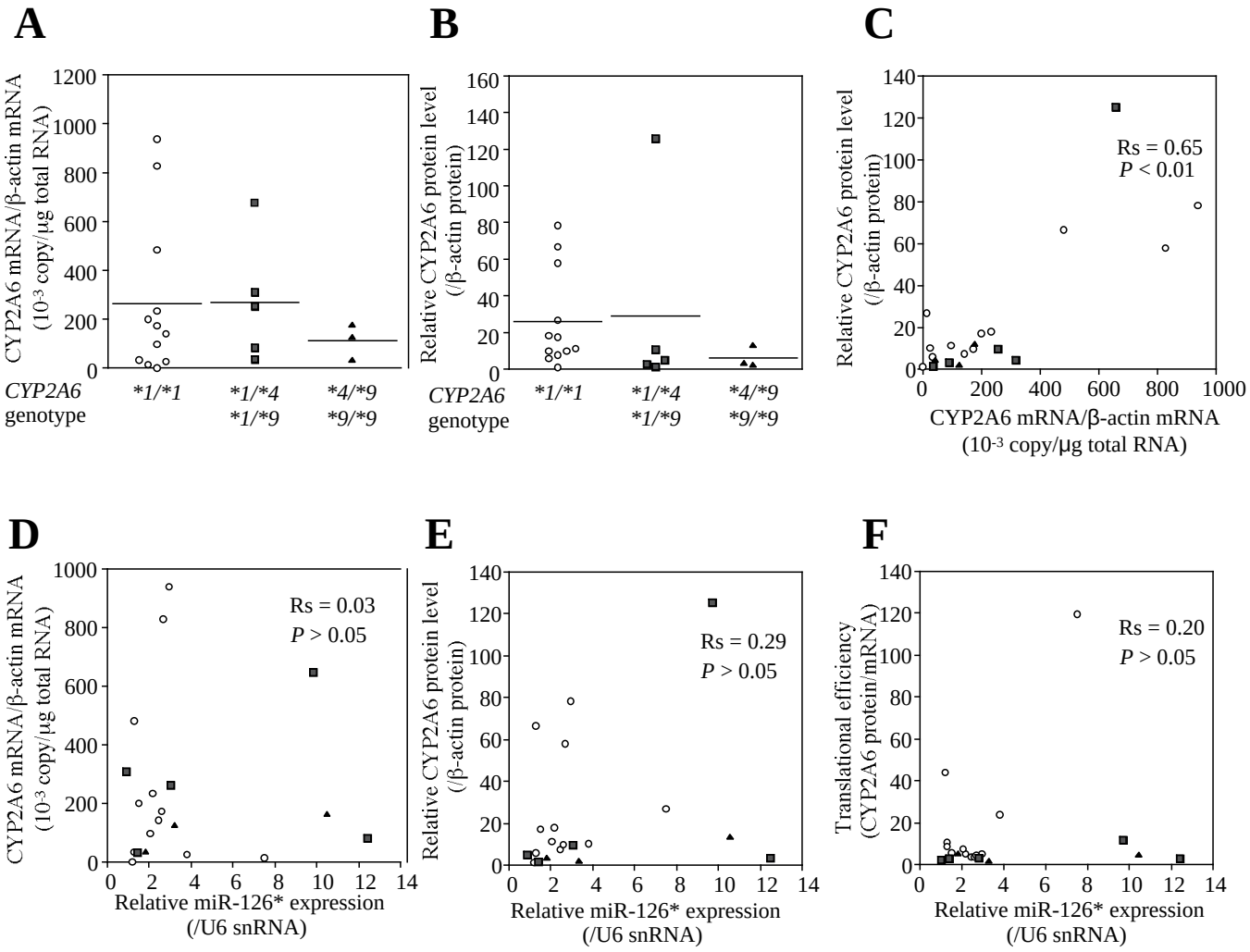

Figure 6

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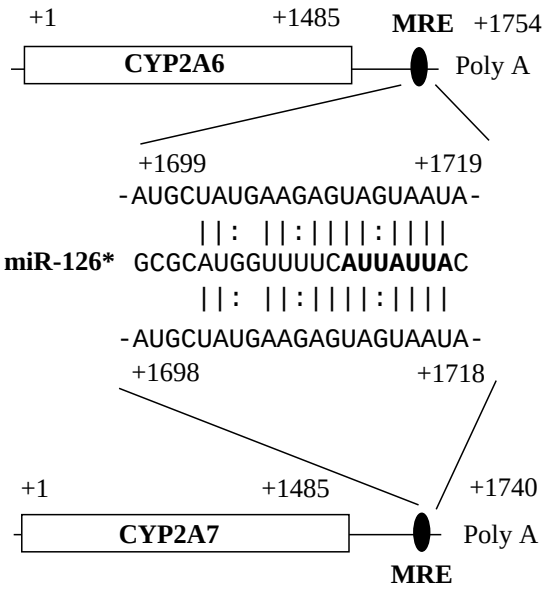

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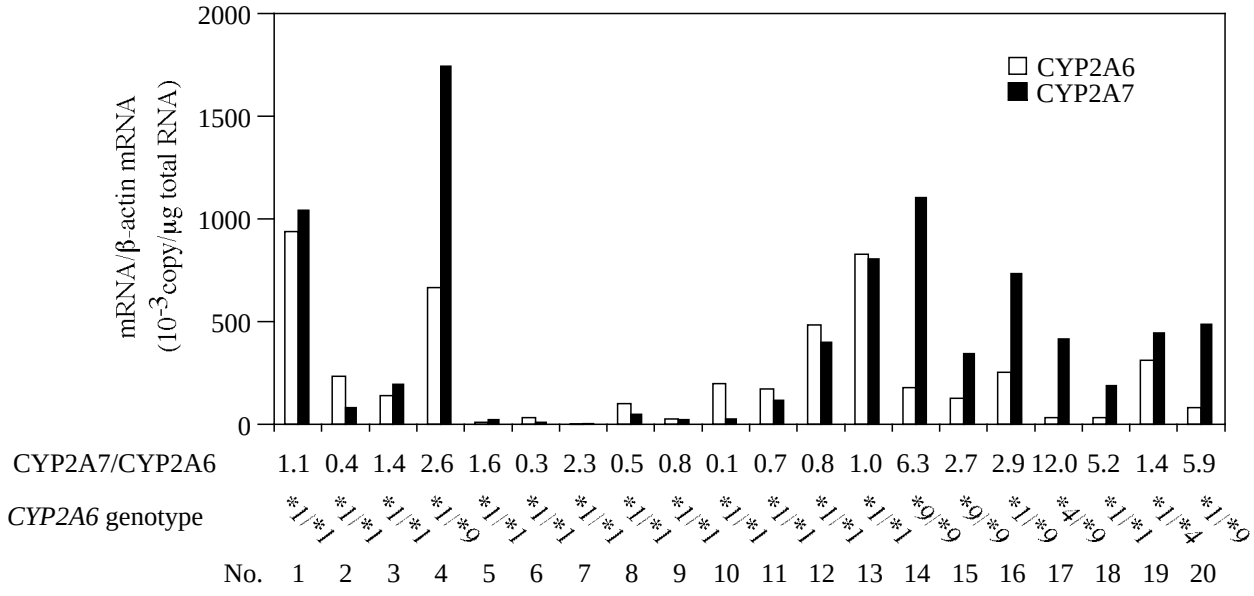
Figure 7



CYP2A7/CYP2A6	1.1	0.4	1.4	2.6	1.6	0.3	2.3	0.5	0.8	0.1	0.7	0.8	1.0	6.3	2.7	2.9	12.0	5.2	1.4	5.9
CYP2A6 genotype	*1/*1	*1/*1	*1/*1	*1/*9	*1/*1	*1/*1	*1/*1	*1/*1	*1/*1	*1/*1	*1/*1	*1/*1	*1/*1	*9/*9	*9/*9	*1/*9	*4/*9	*1/*1	*1/*4	*1/*9
No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Figure 8

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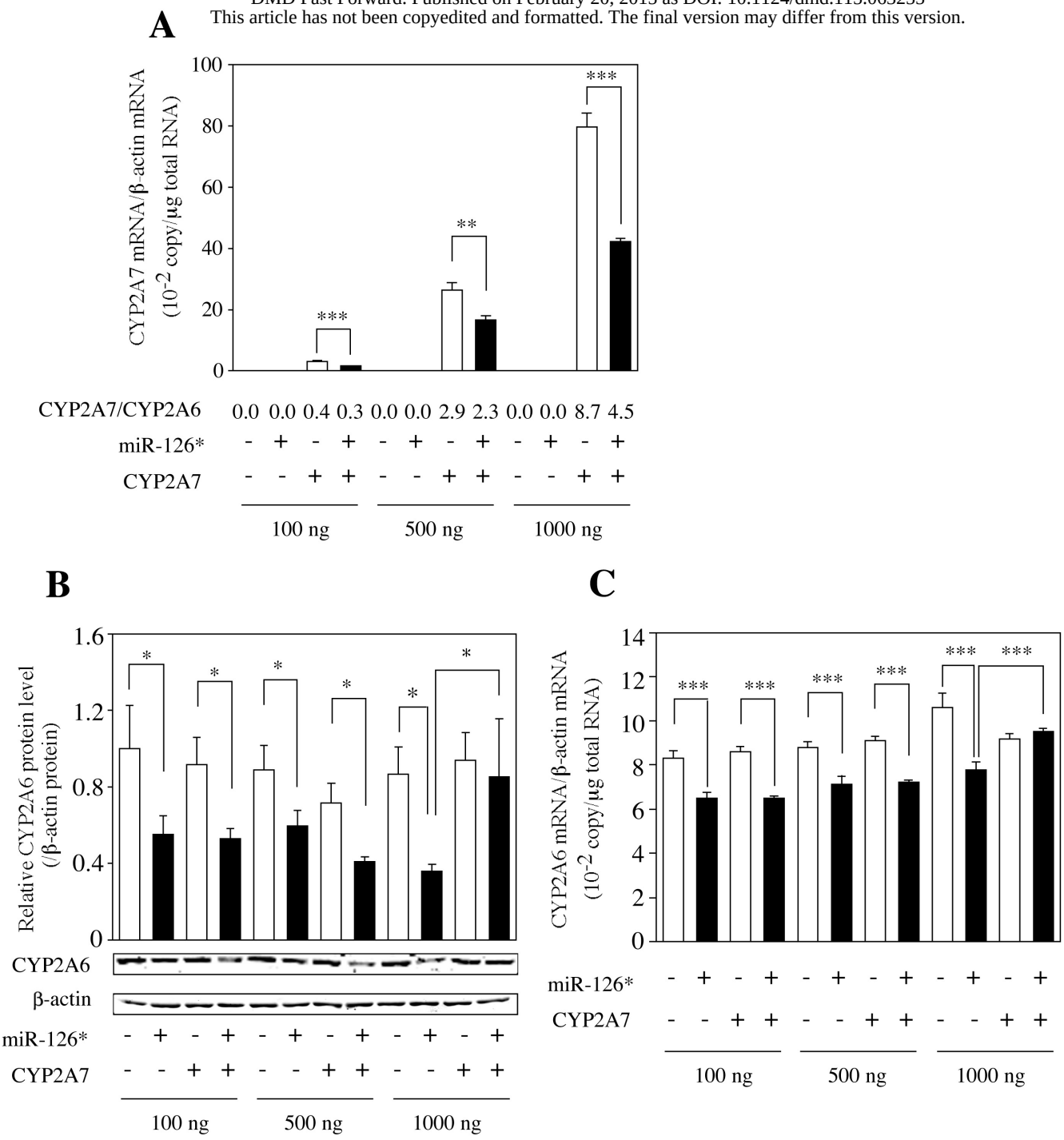

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Figure 9