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Aryl hydrocarbon receptor nuclear translocator in human liver is regulated by miR-24

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Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CA IX, carbonic anhydrase IX; CYP, cytochrome P450; DFOM, deferoxamine mesylate; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H_2O_2, hydrogen peroxide; HIF, hypoxia-inducible factor; 3-MC, 3-methylcholanthrene; miRNA, microRNA; MRE, microRNA recognition element; ROS, reactive oxygen species; RT, reverse transcription; siRNA, small interfering RNA; UTR, untranslated region.

ABSTRACT

Aryl hydrocarbon receptor nuclear translocator (ARNT) forms a heterodimer with aryl hydrocarbon receptor or hypoxia inducible factor 1α to mediate biological responses to xenobiotic exposure and hypoxia. Although the regulation mechanism of the ARNT expression is largely unknown, earlier studies reported that the human ARNT protein level was decreased by hydrogen peroxide or reactive oxygen species. These stimuli increase the miR-24 level in various human cell lines. In silico analysis predicts that some microRNAs including miR-16 and miR-23b may bind to ARNT mRNA. This background prompted us to investigate whether human ARNT is regulated by microRNAs. Overexpression of miR-24 into HuH-7 and HepG2 cells significantly decreased the ARNT protein level, but not the ARNT mRNA level, indicating translational repression. However, overexpression of miR-16 or miR-23b caused no change in the ARNT expression. The miR-24-dependent down-regulation of ARNT decreased the expression of its downstream genes such as CYP1A1 and carbonic anhydrase IX. Luciferase assay was performed to determine the element on the ARNT mRNA to which miR-24 binds. Finally, it was demonstrated that the miR-24 levels in a panel of 26 human livers were inversely correlated with the protein levels or the translational efficiency of ARNT. Taken together, we found that miR-24 negatively regulates ARNT expression in human liver, affecting the expression of its downstream genes. miR-24 would be one of the factors underlying the mechanisms by which ARNT protein is decreased by reactive oxygen species.

Keyword: microRNA, ARNT, HIF1, AhR, CYP1A1, post-transcriptional regulation

Introduction

MicroRNAs (miRNAs), an evolutionarily conserved class of endogenous ~22-nucleotide noncoding RNAs, bind to target mRNAs causing translational repression or mRNA degradation (Bartel et al., 2004). In animals, miRNA target sites are located mainly in the 3'-untranslated region (3'-UTR) of target mRNAs (Chekulaeva and Filipowicz, 2009), although there are examples of target sites in the coding region (Qin et al., 2010). Until now, over 1400 miRNAs have been identified in humans. One miRNA has the potential to target a large number (on average about 500) of genes. It has been estimated that 60% of human mRNAs could be targets of miRNAs (Betel et al., 2008; Friedman et al., 2009). However, the miRNA:mRNA pairs largely remain to be identified. Recently, we reported that miRNAs were involved in the regulation of human xenobiotics-metabolizing enzymes such as CYP1B1 (Tsuchiya et al., 2006) and CYP2E1 (Mohri et al., 2010) and human nuclear receptors such as pregnane X receptor (Takagi et al., 2008), vitamin D receptor (Mohri et al., 2009), and hepatocyte nuclear factor 4α (Takagi et al., 2010). As a sequel study, we focused on human aryl hydrocarbon receptor nuclear translocator (ARNT).

ARNT, which is known as hypoxia-inducible factor 1β (HIF1β), is a member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors. It is ubiquitously and constitutively expressed in human tissues (Gradin et al., 1996), and the levels are particularly high in ovary, lung, spleen, testis and pancreas (Yamamoto et al., 2004). ARNT forms heterodimers with other bHLH-PAS proteins including aryl hydrocarbon receptor (AhR) and HIF1α. In response to xenobiotics, the heterodimer of ARNT/AhR binds to xenobiotic response element of the target genes such as cytochrome P450 and UDP-glucuronosyltransferases, which are important enzymes for the detoxification or metabolic activation, to increase their expression (Köhle and Bock, 2007; Ramadoss et al., 2005). In addition, under a hypoxia condition, ARNT dimerizes with HIF1α to upregulate the expression of genes involved in angiogenesis and tumorigenesis (Ke and Costa, 2006). Furthermore, ARNT homodimer binds to palindromic enhancer box (E-box) sequence to upregulate the expression of some genes such as mouse *Cyp2a5* (Arpiainen et al., 2007). In the human genome, more than 13,000 putative ARNT binding sites have been estimated to exist by in silico analysis (Gunton et al., 2005). Collectively, ARNT regulates the expression of a vast number of genes.

Despite the physiological importance of ARNT, it is largely unknown how the human ARNT expression is regulated. Earlier studies reported that the ARNT protein level was decreased by hydrogen peroxide (H_2O_2) or reactive oxygen species (ROS) in human cell lines (Choi et al., 2006, 2008), although the underlying molecular mechanism remains to be clarified. In our previous study, it was demonstrated that the treatment of cells with H_2O_2 increased the level of miR-24 (Takagi et al., 2010), which was supported by the finding that the ROS generation by hemin (Lal et al., 2009b) or arsenic trioxide (Meng et al., 2011) resulted in an increase of the miR-24 level. In this study, we investigated the possibility that the human ARNT expression might be regulated by miR-24. In addition, two other miRNAs, miR-16 and miR-23b, which were predicted by in silico analysis, were also investigated.

Materials and Methods

Chemicals and reagents. 3-Methylcholanthrene (3-MC) was obtained from Wako Pure Chemicals (Osaka, Japan). Deferoxamine mesylate (DFOM) was purchased from Sigma-Aldrich (St. Louis, MO). The pGL3-promoter (pGL3p) vector, phRL-TK and Dual-Luciferase Reporter Assay System were purchased from Promega (Madison, WI). Pre-miR miRNA Precursor Molecules for miR-24, miR-16, miR-23b and Negative Control #1 (Control) were from Ambion (Austin, TX). Lipofectamine RNAiMAX, Stealth Select RNAi for human ARNT (HSS100700) (siARNT) and Negative Control Medium GC Duplex #2 (siControl) were from Invitrogen (Carlsbad, CA). RNAiso, random hexamer, and SYBR Premix Ex Taq were from Takara (Shiga, Japan). ROX was purchased from Stratagene (La Jolla, CA). ReverTra Ace was obtained from Toyobo (Osaka, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Goat anti-human ARNT polyclonal antibodies (N-19), rabbit anti-human AhR polyclonal antibodies (H-211) and rabbit anti-human HIF1α polyclonal antibodies (H-206) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human GAPDH polyclonal antibodies and rabbit anti-human β-actin polyclonal antibodies were from IMGENEX (San Diego, CA) and BioVision (Mountain view, CA), respectively. Alexa Fluor 680 donkey anti-goat IgG was from Invitrogen. IRDye 680 goat anti-rabbit IgG was from LI-COR Biosciences (Lincoln, NE). All other chemicals and solvents were of the highest grade commercially available.

Human liver samples and preparation of homogenates and total RNA. Human liver samples from 16 donors were supplied by National Disease Research Interchange (Philadelphia, PA) through the Human and Animal Bridging Research Organization (Chiba, Japan) and those from 10 donors were obtained from autopsy materials that were discarded after pathological investigation in Iwate Medical University (Morioka, Japan). The use of the human livers was approved by the Ethics Committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University. Total cell homogenates were prepared 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 concentration was determined using 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 assessed by estimating the ratio of the band density of 28S and 18S rRNA.

Cell culture. Human hepatocellular carcinoma cell lines HuH-7 and HepG2 were obtained from Riken Gene Bank (Tsukuba, Japan). HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). HepG2 cells were cultured in DMEM supplemented with 0.1 mM non-essential amino acid (Invitrogen) and 10% FBS. These cells were maintained at 37°C under an atmosphere of 5% CO_2-95% air.

Transfection of pre-miRNAs or siRNA and preparation of cell homogenates and total RNA. The HuH-7 or HepG2 cells were seeded into 6-well plates and transfected with 50 nM pre-miRNA or 20 nM siRNA using Lipofectamine RNAiMAX. After 72 hr, 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)], disrupted by freeze-thawing three times and homogenized. Total RNA was also prepared as described above. In some cases, the cells were treated with 10 μM 3-MC (or 0.1% DMSO) for 24 hr or 200 μM DFOM for 12 hr, and cell homogenate or total RNA were prepared.

SDS-PAGE and Western blot analyses. The cell homogenates from the HuH-7 and HepG2 cells (20-30 μg) or human liver samples (40 μg) were separated with 7.5% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P transfer membrane (Millipore, Bedford, MA). The membranes were probed with goat anti-human ARNT, rabbit

anti-human AhR, rabbit anti-human HIF1α, rabbit anti-human GAPDH or rabbit anti-human β-actin polyclonal antibodies and the corresponding fluorescent dye-conjugated second antibodies. The band densities were quantified with Odyssey Infrared Imaging system (LI-COR Biosciences). The ARNT and AhR protein levels were normalized with the GAPDH or β-actin protein levels.

Real-time RT-PCR for ARNT and its downstream genes. The cDNAs were synthesized from total RNA using ReverTra Ace. The sequences of the primers are shown in Table 1. 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 conditions were 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 60°C (ARNT) or 64°C (carbonic anhydrase IX (CA IX), GAPDH and β-actin) for 20 s for 40 cycles. For CYP1A1, after an initial denaturation at 95°C for 1 min, the amplification was performed by denaturation at 94°C for 15 s, annealing at 60°C for 30 s and extension at 72°C for 30 s for 40 cycles. The real-time RT-PCR was performed using Mx3000P (Stratagene, La Jolla, CA) with the MxPro QPCR software. The ARNT and CYP1A1 mRNA levels were normalized with the GAPDH mRNA level, and the CA IX mRNA level was normalized with the β-actin mRNA level.

Real-time RT-PCR for mature miR-24. The expression levels of mature miR-24 were determined by using the TaqMan microRNA assay (Applied Biosystems, Foster City, CA). The cDNA templates were prepared with the TaqMan microRNA Reverse Transcription kit which utilized 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-24. The PCR condition was 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-24 levels.

Enzymatic activity. The enzymatic activity of CYP1A1 in HuH-7 cells was determined using

a P450-Glo Assay kit (Promega). After the transfection with pre-miRNA or siRNA, the cells seeded into 24-well plates were treated with 10 μM 3-MC (or 0.1% DMSO) for the last 24 hr, and then the medium was replaced with medium containing 50 μM Luciferin 6'-chloroethyl ether. After the incubation at 37°C for 8 hr under an atmosphere of 5% CO_2-95% air, 100 μL of the medium were added to 100 μL of Luciferin Detection Reagent. After incubation for 20 min at room temperature, the luminescence was measured with a luminometer (Wallac, Turku, Finland). The cells were resuspended in passive lysis buffer, and the protein concentrations were determined as described above. The enzymatic activity was normalized with the protein content.

Construction of plasmids. The fragments +2933 to +3023, +3941 to +4118, and +844 to +1262 of the human ARNT mRNA were amplified by PCR and subcloned into the pGL3p vector at the *Xba* I site, downstream of the *luciferase* gene. These products were termed pGL3/MRE24_1, pGL3/MRE24_2, and pGL3/MRE24_3, respectively. A fragment containing the perfectly matching sequence with the mature miR-24, 5'- CTA G*UG GCU CAG UUC AGC AGG AAC AG*-3' (the matching sequence of miR-24 is italicized), was also cloned (pGL3/c-24). DNA sequencing analyses confirmed the nucleotide sequences of these plasmids using Long-Read Tower DNA sequencer (GE Healthcare Bio-Sciences, Piscataway, NJ).

Luciferase assay. Various pGL3 luciferase reporter plasmids were transiently transfected with phRL-TK plasmid into HepG2 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 Pre-miRNA were transfected into HepG2 cells using Tfx-20 reagent (Promega). After incubation for 48 hr, the cells were resuspended in passive lysis buffer, and then the luciferase activity was measured with a luminometer using the Dual-Luciferase Reporter Assay System.

Statistical analysis. Statistical significance was determined by analysis of variance followed by Dunnett multiple comparisons test or Tukey method test. Comparison of two groups was made with an unpaired, two-tailed Student's *t* test. Correlation analyses were performed by Spearman's rank method. A value of $P < 0.05$ was considered statistically significant.

Results

Effects of overexpression of miRNAs on ARNT expression in HuH-7 or HepG2 cells.

When the precursor for miR-24 was transfected into HuH-7 cells, the ARNT protein level was significantly ($P < 0.01$) decreased (27% of control), but the ARNT mRNA level was not (Fig. 1A), indicating that the miR-24 negatively regulates the human ARNT expression through translational repression. Similar results (decreased 33% of control) were obtained with HepG2 cells (Fig. 1B). On this occasion, it was confirmed that the mature miR-24 level was significantly increased in both cell lines, although the extent in HuH7 cells was larger than that in HepG2 cells probably owing to the difference in transfection efficiency or processing of pre-miRNA to mature miRNA. The miR-24-dependent down-regulation of ARNT was also observed in colon carcinoma cell lines LS180 (40% of control) and breast adenocarcinoma cell lines MCF-7 (60% of control) (data not shown), suggesting that the phenomenon was common in the human cell lines.

Next, we investigated whether other miRNAs might affect the ARNT expression. By a computational prediction using miRanda (http://www.microrna.org/microrna/home.do), PicTar (http://pictar.mdc-berlin.de/), and TargetScan (http://www.targetscan.org/), a number of miRNAs were predicted to bind to human ARNT. Among them, we chose miR-16 and miR-23b, because they are substantially expressed in human liver, and two out of three programs predicted them. When the precursor for miR-16 or miR-23b was transfected into HuH-7 or HepG2 cells, the ARNT protein level was not affected (Fig. 1C). Accordingly, it appeared that human ARNT is specifically regulated by miR-24.

Effects of overexpression of miR-24 on induction of ARNT downstream genes.

We investigated whether the miR-24-dependent down-regulation of ARNT might affect the induction of the downstream genes of ARNT. The treatment of HuH-7 cells with 3-MC, a potent ligand of AhR, significantly increased the CYP1A1 mRNA level (4.9-fold) (Fig. 2A). Interestingly, the induction was completely abrogated by the overexpression of miR-24. In addition, the overexpression of miR-24 significantly decreased the basal expression level of CYP1A1. It was confirmed that the ARNT protein was decreased by the overexpression of miR-24 in the presence of 3-MC (29% of control) (Fig. 2B), although the ARNT levels in the 3-MC-treated cells were significantly lower than those in control cells. To investigate whether the decrease of CYP1A1 was due to the miR-24-dependent decrease of ARNT protein, we

performed a knocked down assay of ARNT. When the siARNT was transfected into HuH-7 cells, the ARNT protein levels were decreased either in the absence or presence of 3-MC (38% and 40% of control, respectively) (Fig. 2B), which was similar to the result with miR-24. Under this condition, the induction of CYP1A1 was completely abrogated (Fig. 2A). Next, we investigated whether the miR-24 may affect the expression of AhR. It was demonstrated that the AhR protein level was also decreased by the overexpression of miR-24 either in the absence or presence of 3-MC, and that the AhR protein levels in the 3-MC-treated cells were significantly lower than those in control cells (Fig. 2C). The siARNT hardly affected the AhR protein levels. From these results, it was suggested that, although the decrease of AhR by miR-24 may also contribute, the miR-24-dependent down-regulation of ARNT would be a causal force of the repressed induction and the decreased basal expression of CYP1A1. Finally, we determined whether the CYP1A1 protein level was also affected. Since the CYP1A1 protein level in HuH-7 cells was too low to be detected by Western blot analysis, we evaluated the enzymatic activity of CYP1A1 using P450-Glo assay (Fig. 2D). The CYP1A1 activity was significantly ($P < 0.001$) increased by the treatment with 3-MC (3.1-fold), but the activity was diminished to under the detection limit by the overexpression of miR-24, and no induction by 3-MC was observed. In addition, the effects of siARNT on the CYP1A1 activity were similar to those of miR-24. These results suggest that the miR-24-dependent down-regulation of ARNT decreased the induction or basal expression of CYP1A1 protein.

We also investigated the effects of miR-24 on CYP1A1 mRNA expression in HepG2 cells (Fig. 2E-G). Although the basal level of CYP1A1 mRNA in HepG2 cells was similar to that in HuH-7 cells (data not shown), the induction by 3-MC (23.1-fold) was more potent than that in HuH-7 cells. When the miR-24 was overexpressed, the expression levels of CYP1A1 mRNA in the presence of 3-MC were significantly ($P < 0.001$) decreased, although the induction was still observed (Fig. 2E). This might be related to the fact that the ARNT protein level in HepG2 cells was ~2 fold higher than that in HuH-7 cells (Fig. 2F). Like in HuH-7 cells, the ARNT protein levels in HepG2 cells were decreased by the overexpression of miR-24 either in the absence or presence of 3-MC. In addition, the ARNT protein levels were decreased by siARNT in the absence or presence of 3-MC (~40% of control), which was similar to the result with miR-24. The effects of siARNT on the CYP1A1 induction or expression were similar to those of miR-24. In contrast to HuH-7 cells, the AhR protein level in HepG2 cells was hardly decreased by 3-MC treatment, but that in the 3-MC-treated cells was decreased by overexpression of miR-24, whereas that in the control cells was increased

by overexpression of miR-24 (Fig. 2G). The siARNT hardly affected the AhR protein level like in the HuH-7 cells. These results suggest that the miR-24-dependent down-regulation of ARNT would be a determinant of the decreased the induction of CYP1A1 irrespective of the cell line.

Next, we investigated the effects of miR-24 on the expression of a target gene of HIF1α, another heterodimer partner of ARNT (Fig. 3A-C). When the HuH-7 cells were treated with DFOM, which is an inhibitor of the degradation of HIF1α that makes it accumulate like hypoxia, the CA IX mRNA level was significantly ($P < 0.001$) increased (4.0-fold) (Fig. 3A). When the miR-24 was overexpressed, the basal and DFOM-induced levels of CA IX mRNA were significantly decreased, although the induction was observed. It was confirmed that the ARNT protein was decreased by the overexpression of miR-24 in the presence of DFOM (32% of control) (Fig. 3B). When the siARNT was transfected into HuH-7 cells, the induction of CA IX by DFOM was repressed (Fig. 3A). We investigated whether the miR-24 may affect the expression of HIF1α protein. Although we did not quantify because of the interfering non-specific bands, we found no effect of miR-24 on the band density of HIF1α protein (Fig. 3C). Thus, it was suggested that the miR-24-dependent down-regulation of ARNT decreased the expression and induction of the target genes of HIF1α. Since the CA IX mRNA level was not induced by DFOM in HepG2 cells (data not shown), we did not investigate further.

Search of functional miRNA recognition element (MRE) in the ARNT mRNA.

We sought to identify the MRE for miR-24 (MRE24) in the ARNT mRNA. We used three programs, miRanda, PicTar, and TargetScans, to search possible elements in 3'-UTR based on complementarity to the seed sequence (nucleotides 2–8 at the 5'-end) of miRNA, free energy of the binding of miRNA to its target, or evolutionary conservation of MRE of the target gene. These programs did not raise MRE24, but raised the MRE for miR-16 and miR-23b (MRE16 and MRE23b). As shown in Fig. 4A, the predicted MRE16 and MRE23b showed a perfect match with the seed sequences. However, as described, the miR-16 or miR-23b did not function in the regulation of ARNT. Next, we sought to find the MRE24s using a software RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/), which calculates the free energy of the mRNA:miRNA hybrid without the restriction to 3'-UTR of the target gene. In the results, two MRE24s in the 3'-UTR and an MRE24 in the coding region of ARNT mRNA were predicted (Fig. 4A) and termed MRE24_1, MRE24_2, and

MRE24_3. Their free energies (-22.1 kcal/mol, -18.7 kcal/mol, and -28.6 kcal/mol, respectively) were comparable with those of MRE16 (-24.3 kcal/mol) or MRE23b (-16.6 kcal/mol).

To investigate whether these MRE24s are functional, luciferase assay using reporter plasmids containing a fragment including each MRE24 in 100 ~ 400-bp length was performed with HepG2 cells (Fig. 4B). When the pGL3/c-24 plasmid containing the miR-24 complementary sequence downstream of the *luciferase* gene was transfected into the cells, the luciferase activity was significantly ($P < 0.001$) decreased by the co-transfection with the precursor for miR-24, suggesting that the overexpressed miR-24 was functional. When the pGL/MRE24_1, pGL/MRE24_2, or pGL/MRE24_3 plasmids were transfected, the luciferase activity was slightly decreased by the overexpression of miR-24, although the difference was statistically insignificant. Unfortunately, the luciferase assay could not identify the functional MRE for miR-24.

Relationship between the expression levels of ARNT protein, ARNT mRNA, and miR-24 in human livers.

To evaluate the significance of the miR-24-dependent regulation of the ARNT in human livers, we examined the relationship between the expression levels of ARNT protein, ARNT mRNA, and miR-24 using a panel of 26 human livers. The ARNT protein levels showing 22-fold variability (except a sample No.24) (Fig. 5A) were not positively correlated with the ARNT mRNA levels (9-fold variability), indicating the involvement of post-transcriptional regulation (Fig. 5B). Interestingly, the miR-24 levels (25-fold variability) inversely correlated with the ARNT protein levels ($r = -0.40$, $P < 0.05$, Fig. 5C) as well as the translational efficiency calculated as the ratio of ARNT protein/mRNA ($r = -0.38$, $P = 0.057$, Fig. 5D). These results suggested miR-24 would contribute to the constitutive expression of ARNT in human livers.

Discussion

ARNT mediates various biological actions such as hypoxia reaction, xenobiotic metabolism, teratogenesis, and immunosuppression through gene regulation by forming a heterodimeric complex with AhR, HIF1α and its homologous factors (HIF2α and HIF3α), or

with single minded homolog 2 (Liang et al., in press). A previous study (Maltepe et al., 1997) reported that systemic ARNT-null mice were embryonic lethal due to defects of angiogenesis and placental development, indicating the important role of ARNT in development. In spite of the indispensability of ARNT in mammals, it is largely unknown how the ARNT expression is regulated. In this study, we investigated the possibility that human ARNT might be regulated by miRNA.

It was clearly demonstrated that the ARNT expression in human liver-derived cells is negatively regulated by miR-24. The miR-24-dependent down-regulation of ARNT affected the induction and expression of CYP1A1, a downstream gene of ARNT with a heterodimer partner AhR. As an unexpected result, we found that miR-24 decreased the AhR protein level. An MRE for miR-24 was predicted in the 3'-UTR of AhR by the TargetScan program supporting our result, although we did not investigate further. Thus, the decrease of AhR by miR-24 may also contribute, but the miR-24-dependent down-regulation of ARNT would be a determinant of the decrease of induction and expression of CYP1A1, since the effects of miR-24 and siARNT were similar. The AhR protein level was decreased by 3-MC treatment, in accordance with previous studies reporting the accelerated degradation through the ubiquitin-proteasome pathway after ligand binding (Davarinos and Pollenz, 2000; Ma and Baldwin, 2000). Although the mechanism of the decrease of ARNT by 3-MC treatment is unknown, we confirmed that it was not due to the increase of miR-24 level by 3-MC (data not shown). Anyway, the miR-24-dependent down-regulation of ARNT or AhR was irrespective of the presence of absence of ligand, the mechanism are independent of the mechanism of degradation by 3-MC. Next, we found that the miR-24-dependent down-regulation of ARNT affected the expression or induction of CA IX, a downstream gene of ARNT with a heterodimer partner HIF1α. Since the miR-24 did not affect the HIF1α protein, we can suggest that the change of CA IX expression was due to the decrease of ARNT. In contrast to CYP1A1, the effects of miR-24 and siARNT on the induction of CA IX were inconsistent. For this matter, it was speculated that miR-24 might be involved in another pathway(s) for CA IX induction that is independent of ARNT. Collectively, it was uncovered that the miR-24-dependent down-regulation of ARNT affects the expression or induction of its downstream genes whether the heterodimer is AhR or HIF1α.

It has been reported that the ARNT protein level was decreased by H_2O_2 or ROS in human cell lines (Choi et al., 2006, 2008). In contrast, ARNT mRNA was not affected by these treatments (Choi et al., 2006), indicating the possibility of post-transcriptional regulation.

Previous evidence showed that ROS generated by H_2O_2 (Takagi et al., 2010), hemin (Lal et al., 2009b) or arsenic trioxide (Meng et al., 2011) increased the miR-24 level in human cell lines. Since the present study uncovered the repression of ARNT by miR-24, it is suggested that the increase of the miR-24 by ROS and the decrease of ARNT by ROS could be causally related.

The present study could not specify the MRE(s) on the ARNT mRNA that is functional in the miR-24-dependent regulation. It is possible that three predicted MRE24s showing faint down-regulation might function synergistically, because it has been reported that a combination of weak sites can effectively target an mRNA for silencing (Li et al., 2008). Another possibility is that the other unidentified MRE(s) might be functional. Although we searched MRE(s) based on the matching with the seed sequence, it is possible that the other seedless MRE(s) might be functional, because recent studies have shown that some miRNAs including miR-24 could regulate the target gene by recognizing seedless MREs (Lal et al., 2009a, 2009b; Azzouzi et al., 2011). The identification of seedless MREs would be more difficult than canonical MREs. A luciferase assay using plasmids containing a sequential fragment of ARNT mRNA might be useful to identify the MREs, but we did not perform such an assay. Since we could not identify the functional MREs, one may surmise that the miR-24 might indirectly affect the ARNT expression through down-regulation of some transcriptional factors. However, this possibility could be excluded, because the ARNT mRNA levels were not affected by the overexpression of miR-24 (Fig. 1A).

During the process of preparing this report, an independent study reported that human ARNT in a colon carcinoma cell line HCT116 was down-regulated by miR-107 (Yamakuchi et al., 2010). It was reported that the miR-107-mediated regulation of ARNT affected the colon cancer tumor growth through the regulation of vascular endothelial growth factor, a target gene of HIF. The miR-107 was actually predicted to be a potential regulator of ARNT by in silico analysis, but we did not focus on miR-107 because the miRanda did not raise miR-107 as liver-enriched miRNAs. Supporting these data, we obtained by TaqMan MicroRNA Assay that miR-107 was not detectable in human hepatocytes (data not shown). However, it is known that miR-107 is overexpressed in various cancer tissues including colon, pancreas, stomach, and breast cancers (Volinia et al., 2006, Chen et al., 2011). Thus, the regulation of ARNT by miR-107 might be specific to cancer cells or tissues other than liver. By contrast, the miR-24 is the sixth most abundant miRNA in normal human liver. Thus, the regulation of ARNT in human liver by miR-24 would be very feasible. The inverse correlation between the ARNT protein and miR-24 levels in human liver samples supported the significance of the miR-24-dependent regulation of ARNT in liver (Fig. 5).

A previous study reported that miR-24 down-regulates E2F2 and Myc expression, playing a role in the inhibition of cell proliferation (Lal et al., 2009a). In addition, our previous study (Takagi et al., 2010) found that miR-24 down-regulated HNF4α in human liver, affecting bile acid synthesis. The present study could provide new evidence that miR-24 has pharmacological and toxicological relevance.

In conclusion, we found that miR-24 negatively regulated the expression of human ARNT, affecting the expression of its downstream genes. Since miR-24 is under the control of cellular stress, elucidation of the regulation mechanism of the miR-24 expression would be valuable for understanding the change of ARNT expression in adaptation to environmental stimuli.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Figure 1. Effects of overexpression of miRNAs on ARNT protein and mRNA level. (A, B) The ARNT protein levels in HuH-7 and HepG2 cells 72 hr after the transfection of precursors for miR-24 or negative control #1 (control) (50 nM) were determined by Western blot analysis and normalized with the GAPDH protein levels. The ARNT mRNA and miR-24 levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA and U6 snRNA levels, respectively. (C) The ARNT protein levels in HuH-7 and HepG2 cells 72 hr after the transfection of precursors for miR-16 or miR-23b were also determined. The values represent the levels relative to no transfection (NT). Each column represents the mean ± SD of three independent experiments. $^{**}P < 0.01$. M: Marker.

Figure 2. Effects of overexpression of miR-24 on the induction of CYP1A1. The precursors or siRNAs were transfected into HuH-7 and HepG2 cells. After 72 hr, the cells were treated with 10 μM 3-MC (or 0.1% DMSO for control) for 24 hr. (A, E) The CYP1A1 mRNA levels were determined by real-time RT-PCR and normalized with the GAPDH mRNA levels. The values represent the levels relative to that of control or siControl with DMSO treatment. (B, C, F, G) The ARNT and AhR protein levels were determined by Western blot analysis and normalized with the GAPDH protein levels. The values represent the levels relative to that of control or siControl of HuH-7 cells with DMSO treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with DMSO treatment. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, compared with the precursor for control with DMSO treatment. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$, compared with the precursor for control with 3-MC treatment. M: Marker. (D) The enzymatic activities were determined by P450-Glo assay. $^{***}P < 0.001$, compared with DMSO treatment; ND: Not detected. Each column represents the mean ± SD of three independent experiments.

Figure 3. Effects of overexpression of miR-24 on the induction of CA IX in HuH-7 cells. The precursors or siRNAs were transfected into HuH-7 cells. After 72 hr, the cells were treated with 200 μM DFOM for 12 hr. (A) The CA IX mRNA levels were determined by real-time RT-PCR and normalized with the β-actin mRNA levels. (B, C) The ARNT and HIF1α protein levels were determined by Western blot analysis and normalized with the β-actin protein levels; M: Marker. $^{***}P < 0.001$, compared with vehicle. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$,

compared with the precursor for control or siControl with vehicle. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$, compared with the precursor for control or siControl with DFOM treatment. The values represent the levels relative to that of control or siControl with vehicle. Each column represents the mean ± SD of three independent experiments.

Figure 4. Luciferase assay using the plasmids containing the MRE24 in the 3'-UTR and coding region of human ARNT mRNA. (A) Schematic representation of human ARNT mRNA and the predicted target sequences of miR-24. The numbering refers to the 5' end of mRNA as 1. MRE24_1 (from +2984 to +3007), and MRE24_2 (from +4012 to +4030) are located on the 3'-UTR of human ARNT mRNA (A). MRE24_3 (from +971 to +994) was located on the coding region of human ARNT mRNA. The predicted target sequences of miR-16 and miR-23b are shown in dotted line. Mfe, minimum free energy. *Bold letters*, seed sequence. The aglinment of each miRNA with the ARNT mRNA was drawn using RNAhybrid. (B) Luciferase assays using the reporter plasmids containing various fragments downstream of the firefly luciferase gene. The reporter plasmids (190 ng) were transiently transfected with phRL-TK plasmid (10 ng) and 50 nM precursor or control into HepG2 cells. The firefly luciferase activity for each construct was normalized with the *Renilla* luciferase activities. Values are expressed as percentage of the relative luciferase activity of pGL3p plasmid. Each column represents the mean ± SD of three independent experiments. $^{***}P < 0.001$.

Figure 5. Relationship between the ARNT protein, ARNT mRNA, and miR-24 levels in human livers. (A) The ARNT protein levels in a panel of 26 human livers were determined by Western blot analysis and normalized with the GAPDH protein levels. The values represent the levels relative to that of the lowest sample among 25 detectable ones. M: Marker, ND: not detectable (B) Relationship between the ARNT mRNA and protein levels, (C, D) relationship between the miR-24 and ARNT protein levels or translational efficiency of ARNT (ARNT protein/mRNA ratio) in human livers. The expression levels of miR-24 and ARNT mRNA were determined by real-time RT-PCR and normalized with the U6 snRNA levels and GAPDH mRNA levels, respectively. The values represent the levels relative to that of the lowest sample. Data are the mean of two independent experiments.

Table 1. Sequence of primers used for real-time RT-PCR analysis.

Primer	Sequence
ARNT forward[a]	5' - GGAACAAGATGACAGCCTAC - 3'
ARNT reverse[a]	5' - CAGAAAGCCATCTGCTGCC - 3'
CYP1A1 forward[a]	5' - TCTTTCTCTTCCTGGCTATC - 3'
CYP1A1 reverse[a]	5' - CTGTCTCTTCCCTTCACTCT - 3'
CA IX forward	5' - CAGTTGCTGTCTCGCTTGGAAG - 3'
CA IX reverse	5' - TCGCTCGGAAGTTCAGCTGTAG - 3'
GAPDH forward[b]	5' - CCAGGGCTGCTTTTAACTC - 3'
GAPDH reverse[b]	5' - GCTCCCCCCTGCAAATGA - 3'
β-actin forward	5' - TCACCCTGAAGTACCCCATC - 3'
β-actin reverse	5' - GATAGCACAGCCTGGATAGC - 3'

[a]Iwanari et al (2002); [b]Tsuchiya et al (2004).

Figure 1

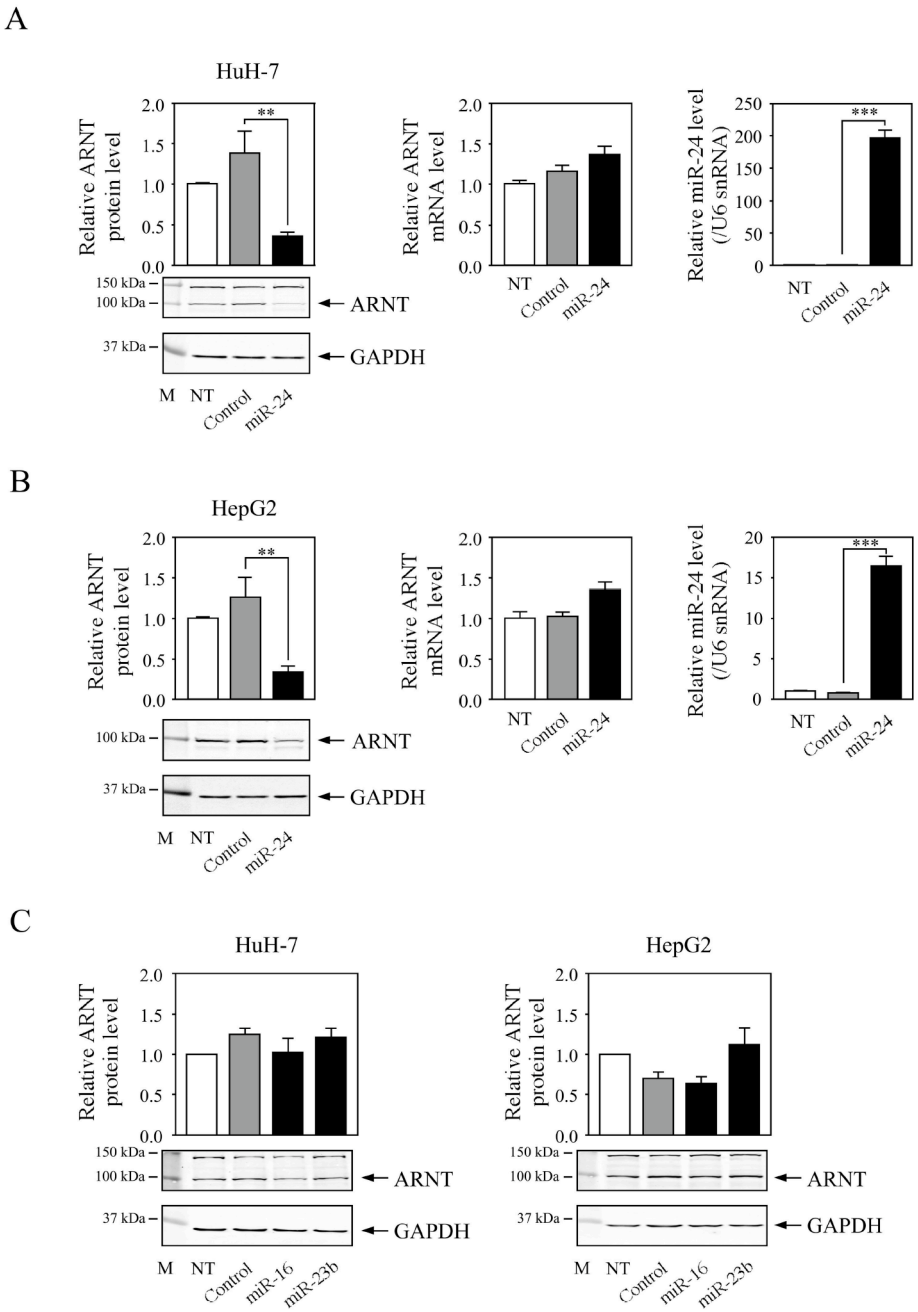

A
HuH-7
Relative ARNT protein level
2.0
1.5
1.0
0.5
0.0
**
150 kDa
100 kDa
ARNT
37 kDa
GAPDH
M NT Control miR-24
Relative ARNT mRNA level
NT Control miR-24
Relative miR-24 level (/U6 snRNA)
250
200
150
100
50
0
***
NT Control miR-24
B
HepG2
Relative ARNT protein level
100 kDa
ARNT
37 kDa
GAPDH
M NT Control miR-24
Relative ARNT mRNA level
Relative miR-24 level (/U6 snRNA)
20
15
10
5
C
HuH-7
HepG2
Relative ARNT protein level
M NT Control miR-16 miR-23b

Figure 2

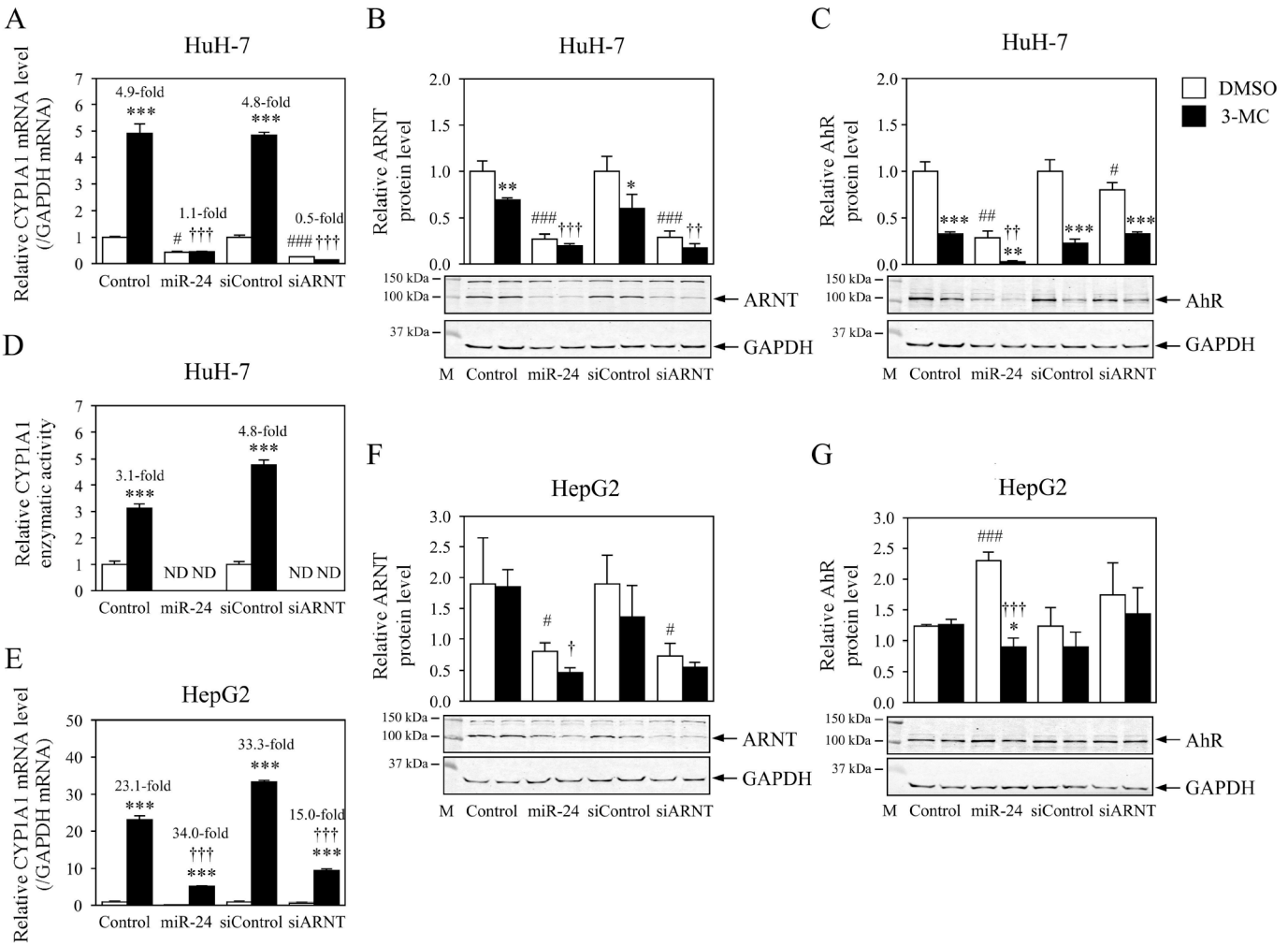
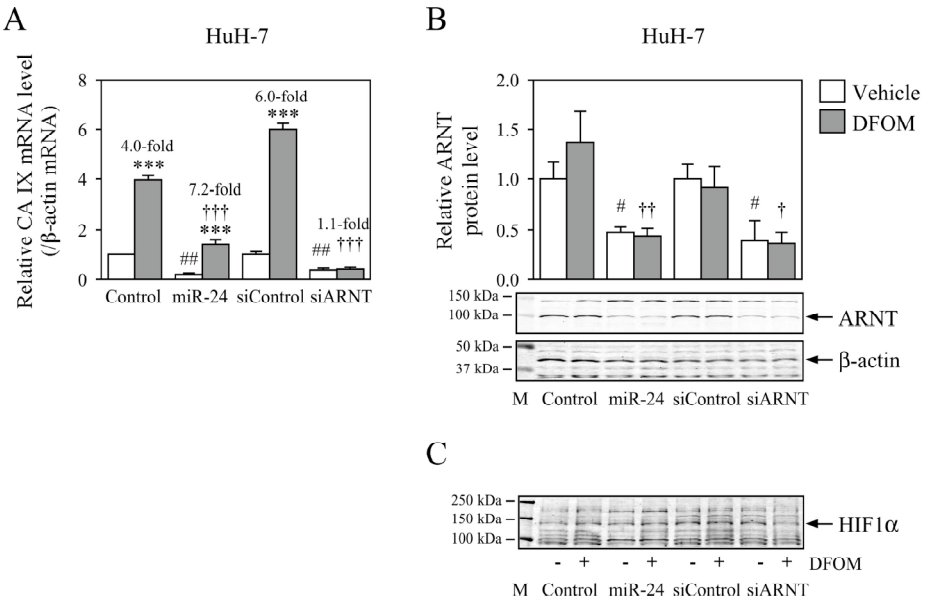

A
HuH-7
Relative CYP1A1 mRNA level (/GAPDH mRNA)
4.9-fold
1.1-fold
4.8-fold
0.5-fold
Control miR-24 siControl siARNT
B
HuH-7
Relative ARNT protein level
150 kDa
100 kDa
37 kDa
ARNT
GAPDH
M Control miR-24 siControl siARNT
C
HuH-7
Relative AhR protein level
DMSO
3-MC
AhR
GAPDH
D
HuH-7
Relative CYP1A1 enzymatic activity
3.1-fold
4.8-fold
ND ND
E
HepG2
Relative CYP1A1 mRNA level (/GAPDH mRNA)
23.1-fold
34.0-fold
33.3-fold
15.0-fold
F
HepG2
G
HepG2

Figure 3


A
HuH-7
Relative CA IX mRNA level (/β-actin mRNA)
8
6
4
2
0
4.0-fold
***
7.2-fold
†††
***
6.0-fold
***
1.1-fold
##
†††
##
Control
miR-24
siControl
siARNT
B
HuH-7
Relative ARNT protein level
2.0
1.5
1.0
0.5
0.0
Vehicle
DFOM
#
††
#
†
150 kDa
100 kDa
ARNT
50 kDa
37 kDa
β-actin
M
Control
miR-24
siControl
siARNT
C
250 kDa
150 kDa
100 kDa
HIF1α
- + - + - + - +
DFOM
M
Control
miR-24
siControl
siARNT

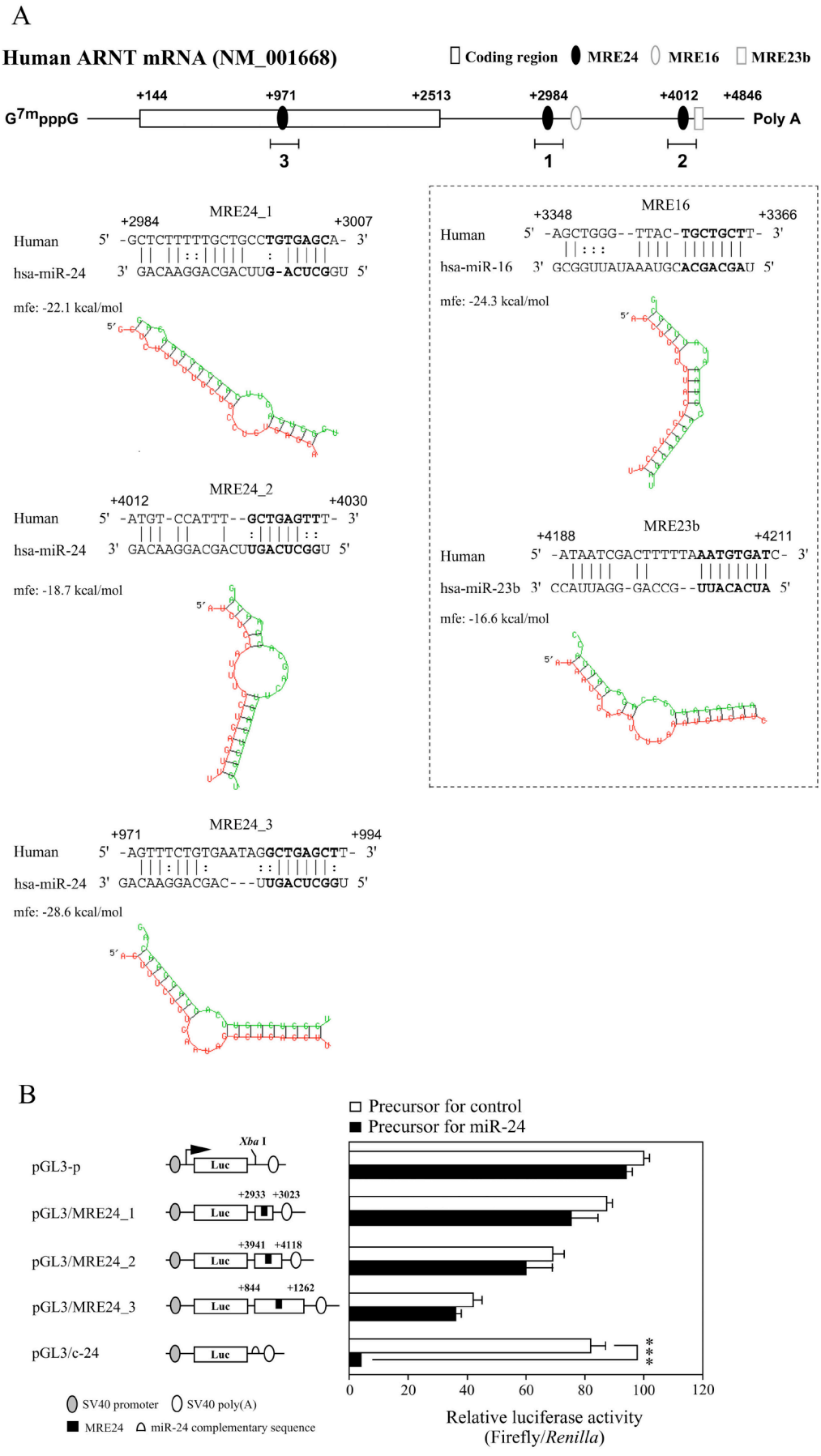
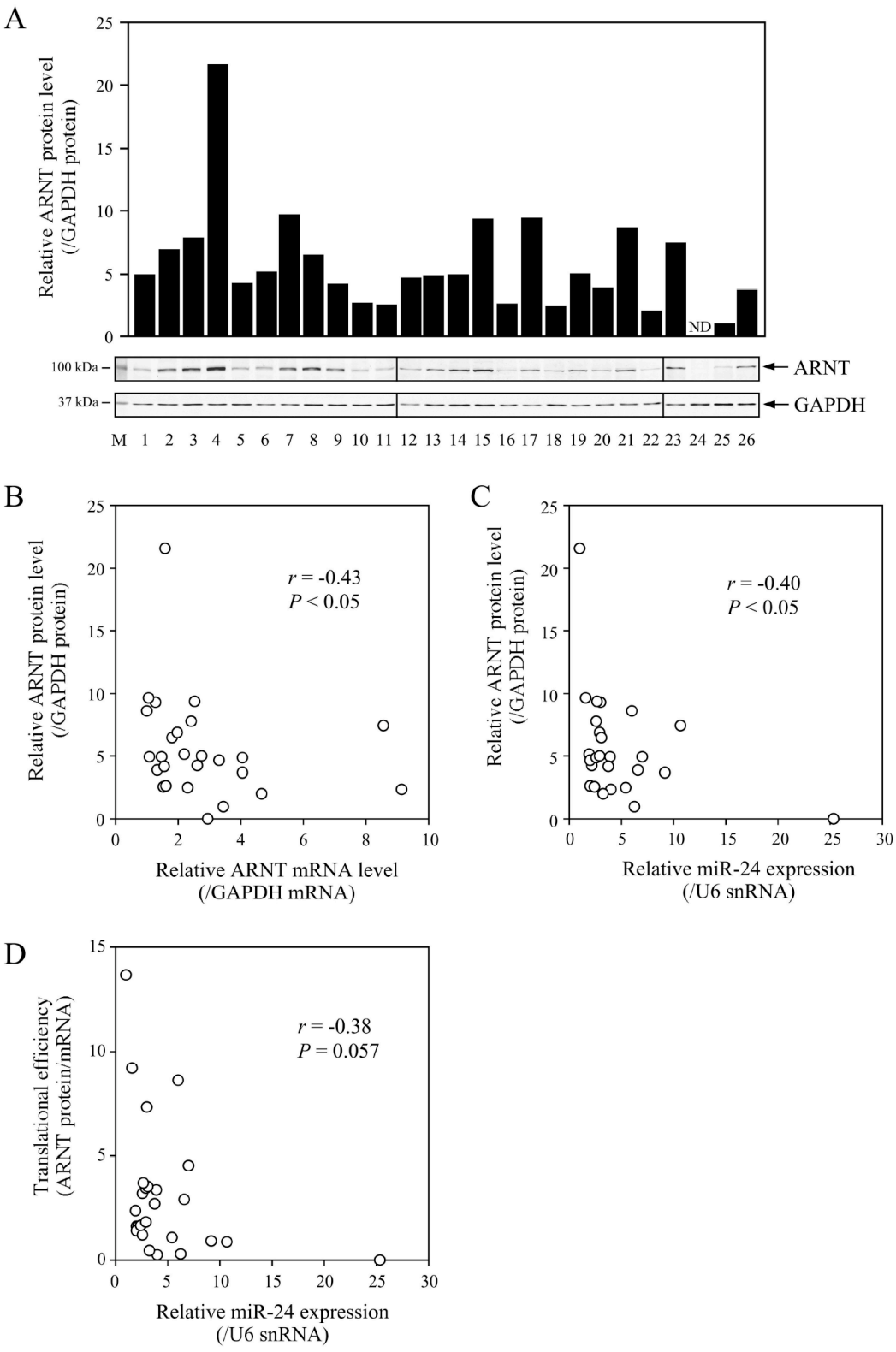

A
Human ARNT mRNA (NM_001668)
Coding region
MRE24
MRE16
MRE23b
+144
+971
+2513
+2984
+4012
+4846
G7mpppG
Poly A
3
1
2
MRE24_1
+2984
+3007
Human 5' -GCTCTTTTTGCTGCCTGTGAGCA- 3'
hsa-miR-24 3' GACAAGGACGACUUG-ACUCGGU 5'
mfe: -22.1 kcal/mol
MRE24_2
+4012
+4030
Human 5' -ATGT-CCATTT--GCTGAGTTT- 3'
hsa-miR-24 3' GACAAGGACGACUUGACUCGGU 5'
mfe: -18.7 kcal/mol
MRE24_3
+971
+994
Human 5' -AGTTTCTGTGAATAGGCTGAGCTT- 3'
hsa-miR-24 3' GACAAGGACGAC---UUGACUCGGU 5'
mfe: -28.6 kcal/mol
MRE16
+3348
+3366
Human 5' -AGCTGGG--TTAC-TGCTGCTT- 3'
hsa-miR-16 3' GCGGUUAUAAAUGCACGACGAU 5'
mfe: -24.3 kcal/mol
MRE23b
+4188
+4211
Human 5' -ATAATCGACTTTTTAAATGTGATC- 3'
hsa-miR-23b 3' CCAUUAGG-GACCG--UUACACUA 5'
mfe: -16.6 kcal/mol
B
Precursor for control
Precursor for miR-24
pGL3-p
pGL3/MRE24_1
pGL3/MRE24_2
pGL3/MRE24_3
pGL3/c-24
Xba I
Luc
+2933 +3023
+3941 +4118
+844 +1262
SV40 promoter
SV40 poly(A)
MRE24
miR-24 complementary sequence
0
20
40
60
80
100
120
**
Relative luciferase activity
(Firefly/Renilla)

Figure 5


A
Relative ARNT protein level (/GAPDH protein)
ND
100 kDa
37 kDa
ARNT
GAPDH
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26
B
Relative ARNT protein level (/GAPDH protein)
r = -0.43
P < 0.05
Relative ARNT mRNA level (/GAPDH mRNA)
C
Relative ARNT protein level (/GAPDH protein)
r = -0.40
P < 0.05
Relative miR-24 expression (/U6 snRNA)
D
Translational efficiency (ARNT protein/mRNA)
r = -0.38
P = 0.057
Relative miR-24 expression (/U6 snRNA)