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Regulation of UDP-Glucuronosyltransferase 2B15 by miR-331-5p in prostate cancer cells involves canonical and non-canonical target sites

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Running Title: Regulation of UGT2B15 by miR-331-5p.

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ABSTRACT

UGT2B15 is an important androgen metabolizing UGT and the mechanisms controlling its expression are of considerable interest. Recent studies showed that miR-376c regulates *UGT2B15* in prostate cancer cells via a canonical target site in the 3'untranslated region *(3'UTR).* The *UGT2B15 3'UTR* also contains a canonical miR-331-5p target site; previous work indicated that deleting this site reduced, but did not abolish, the ability of miR-331-5p to repress a luciferase reporter carrying the *UGT2B15 3'UTR*. We report here the discovery and characterization of a second, non-canonical miR-331-5p target site in the *UGT2B15 3'UTR*. miR-331-5p-mediated repression of a *UGT2B15 3'UTR*-reporter was partly inhibited by mutating either of the two miR-331-5p target sites separately, but completely abolished by mutating the two sites simultaneously, indicating that the two sites act cooperatively. miR-331-5p mimics significantly reduced both UGT2B15 mRNA levels and glucuronidation activity in prostate cancer cells, confirming that the native transcript is a miR-331-5p target. Transfection of either miR-331-5p or miR-376c mimics repressed the activity of the *UGT2B15 3'UTR*-reporter; however, co-transfection of both miRNAs further reduced activity, indicating cooperative regulation by these two miRNAs. A significant negative correlation between miR-331 and UGT2B15 mRNA levels was observed in a tissue RNA panel, and analysis of the TCGA Hepatocellular Carcinoma dataset provided further evidence that miR-331 may play an important role in regulation of *UGT2B15 in vivo*. There was no significant correlation between miR-331 and UGT2B15 mRNA levels in the TCGA Prostate Adenocarcinoma cohort, which may reflect the complexity of androgen-mediated regulation in determining *UGT2B15* levels in prostate cancer. Finally we show that miR-331-5p does not regulate UGT2B17, providing the first evidence for a post-transcriptional mechanism that differentially regulates these two important androgen-metabolising UGTs.

Introduction

Human UDP-glucuronosyltransferases (UGTs) are a superfamily of enzymes that catalyze glucuronidation, a conjugation reaction that is critical for detoxification and clearance of a large number of endogenous and exogenous small lipophilic compounds including carcinogens, drugs, and biological active signalling molecules (e.g. steroid hormones) (Mackenzie et al., 2005). Human UGTs are categorized into four subfamilies according to their sequence similarity, namely UGT1, UGT2, UGT3, and UGT8 (Mackenzie et al., 2005; Mackenzie et al., 1997). In humans, glucuronidation of androgens (e.g. testosterone and dihydrotestosterone) and their metabolites (e.g. androsterone and androstane-3α,17β-diol) is carried out by three UGTs, namely UGT2B7, UGT2B15 and UGT2B17 (Turgeon et al., 2001). The androgen/androgen receptor signaling pathway plays a pivotal role in human prostate growth and function; however, excessive androgen signaling within the prostate contributes to prostate cancer development and progression (Kaarbo et al., 2007). UGT2B15 and UGT2B17 are expressed in the prostate and their glucuronidation of the active androgens (e.g. testosterone and dihydrotesterone) renders them inactive as ligands for the androgen receptor (AR), thus representing the end of androgen signaling within the prostate (Chouinard et al., 2008). These two UGTs are also expressed in liver where they conjugate and promote elimination of androgens, thus reducing circulating androgen levels. Consistent with their important functions in androgen signaling, altered UGT2B15 and UGT2B17 activity is related to the risk of developing androgen-dependent prostate diseases such as prostate cancer (Chouinard et al., 2007; Heinlein and Chang, 2004; Turgeon et al., 2001).

Mechanisms that control the intraprostatic and hepatic levels of UGT2B15 and UGT2B17 are still not well defined. The transcriptional regulation of *UGT2B15* and *UGT2B17* in the prostate has been the subject of many studies using androgen receptor (AR)-positive prostate cancer cell lines (e.g. LNCaP). These studies have shown that *UGT2B15* and *UGT2B17* are downregulated by a number of endogenous and exogenous compounds including androgens (e.g. testosterone and dihydrotestosterone), farnesoid X receptor activators (e.g. chenodeoxycholic acid), and alcitriol (1α,25-dihydroxyvitamin D_3) (Bao et al., 2008; Chouinard et al., 2007; Hu et al., 2014; Kaeding et al., 2008a; Kaeding et al., 2008b).

MicroRNAs, 21-25-nucleotide long, non-coding small RNAs, control gene expression at the post transcriptional level through translational repression and/or mRNA degradation (He and Hannon, 2004; Shukla et al., 2011). Each miRNA has a 6-nt 'seed sequence' that is considered to be important for recognition of its target mRNA. The corresponding 6-nt region in the mRNA target which makes direct contact with the miRNA seed sequence is called the 'seed site'. The miRNA seed sequence is located between nucleotides 2 and 7 from the 5' terminus of a miRNA. The seed site (in the mRNA) is located between nucleotides 2 and 7 from the 3′ terminus of the miRNA recognition site in the target mRNA.

Canonical miRNA target sites in mRNAs have a seed site that has a perfect Watson-Crick base pairing to its cognate miRNA seed sequence. Noncanonical miRNA target sites contain a seed site that mis-pairs to the seed sequence of its cognate miRNA by at least 1 nucleotide. At canonical sites, the miRNA/mRNA duplex formation mainly relies on Watson-Crick base-pairing via the seed sequence (5' pairing) whereas at noncanonical sites, the miRNA/mRNA duplex formation primarily occurs via an extensive base-pairing at the miRNA centre (middle pairing) or 3' end (3'pairing). Recent studies have shown that about 50% of miRNA target sites are noncanonical sites (Helwak et al., 2013; Loeb et al., 2012;

Martin et al., 2014; Shin et al., 2010). Post-transcriptional regulation of several *UGT* genes by miRNAs via canonical miRNA target sites in various cellular contexts have been recently reported (Dluzen et al., 2014; Dluzen et al., 2016; Margaillan et al., 2016; Wijayakumara et al., 2015; Wijayakumara et al., 2017). For example, we and others recently reported post-transcriptional regulation of UGT2B15 and UGT2B17 by miR-376c in prostate cancer LNCaP cells via a canonical target site that is conserved in their 3'UTRs (Margaillan et al., 2016; Wijayakumara et al., 2015). Margaillan et al also reported a putative canonical miR-331-5p target site at the UGT2B15 3'UTR and showed that deleting the seed site of this canonical miR-331-5p site reduced the ability of miR-331-5p to repress the activity of a luciferase reporter carrying the UGT2B15 3'UTR (Margaillan et al., 2016). In the present study, we discovered a second, non-canonical miR-331-5p site in the UGT2B15 3'UTR and showed that the two sites mediate cooperative regulation of UGT2B15. miR-331-5p was also shown to target endogenous UGT2B15 expression and thus activity levels; however it did not alter UGT2B17 mRNA levels. UGT2B15 and miR-331-5p levels are inversely correlated in RNA samples from multiple tissues, and in a liver cancer dataset, but not in a prostate cancer dataset. Overall, our data suggest that miR-331-5p and miR-376c are important regulators of UGT2B15 levels, but may play varying roles in different tissues; moreover, miR-331-5p mediates differential regulation of the UGT2B15 and UGT2B17 mRNAs.

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Materials and Methods

miRNA Mimics and Human Tissue Total RNAs. Synthetic mimics corresponding to hsa-miR-331-5p, hsa-miR-376c, and a negative control miRNA (miR-neg) were purchased from Shanghai GenePharma (Shanghai, China). Total RNA samples representing a human multi-tissue panel were purchased from Ambion (FirstChoice® Human Total RNA Survey Panel).

Cell Transfection, RNA Extraction, and Reverse-Transcriptase Quantitative Real-Time Polymerase Chain Reaction. The prostate cancer LNCaP cells were maintained in RPMI 1640 medium (Gibco/ Life Technologies, Grand Island, NY) containing 5% (v/v) fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere. Prior to transfection, cells were cultured in phenol-red free RPMI 1640 medium (Invitrogen) supplemented with 5% dextran-coated charcoal-stripped FBS for 32 hours and plated in 6-well plates at approximately $1x10^6$ cells per well. Following overnight culture (~16 hours), cells at ~70% confluence were transfected with miR-331-5p mimics or miR-neg in triplicate at 30 nM using 8 µl Lipofectamine 2000 (Invitrogen). Total RNA was extracted 24 h post-transfection using TRIzol (Invitrogen) and the target genes were quantified using reverse-transcriptase quantitative real-time polymerase chain reactions (RT-qPCRs) in a RotorGene 3000 instrument (Corbett Research, NSW, Australia) as previously reported (Hu et al., 2010; Hu and Mackenzie, 2009; Hu and Mackenzie, 2010). Quantification of miR-331-5p and miR-U6 small nuclear-2 RNA (termed RNU6-2) was performed as previously reported (Balcells et al., 2011; Wijayakumara et al., 2015). The mRNA levels of *UGT2B15* and *GAPDH* relative to 18S rRNA and the miR-331-5p levels relative to that of RNU6-2 levels were quantified using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The miRNA-specific primers used are listed

in Table 1.

Generation of pGL3 Reporter Constructs and Mutagenesis. The pGL3 reporter constructs containing the UGT2B15 3'UTR (pGL3/2B15/UTR) or UGT2B17 3'UTR (pGL3/2B17/UTR) cloned downstream of the luciferase gene were recently reported (Wijayakumara et al., 2015). Using the pGL3/2B15/UTR construct as the template and the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), the seed site of the canonical miR-331-5p target site (5'-AUACCUA-3') was either mutated to 5'-CGCAACA-3' or deleted. The mutated (pGL3/2B15/UTR/miR-331-5p/MT) and deleted (pGL3/2B15/UTR/miR331-5p/delMT) reporter constructs are shown in Fig. 1B.

Deletion analysis was used to discover the non-canonical miR-331-5p target site. Eight different segments of the UGT2B15 3'UTR were amplified using pGL3/2B15/UTR as template and Phusion hot-start high-fidelity DNA polymerase (Themo Fisher Scientific, Pittsburgh, PA). The segments of the UGT2B15 3' UTR were numbered relative to the nucleotide G (set as position "0") of the stop codon TAG (NM_001076.3). The amplicons were cloned into the XbaI site of the pGL3 promoter vector downstream of the luciferase gene, generating eight pGL3 reporter constructs: pGL3/2B15/1-75, pGL3/2B15/1-150, pGL3/2B15/1-225, pGL3/2B15/1-314, pGL3/2B15/1-384, pGL3/2B15/244-314, pGL3/2B15/244-384, and pGL3/2B15/244-454 (Fig. 4A).

To further define the noncanonical miR-331-5p target site predicted in the 75-150 bp-region of the UGT2B15 3' UTR, we created mutation at site A (MT1, MT2) and site B (MT3) which were predicted by RNAhybrid as potential miR-331-5p target sites and also at three other positions as negative controls (MT4, MT5, MT6) using the pGL3/2B15/1-150 construct as template and the QuikChange site-directed mutagenesis kit. The sequence of each mutation is shown in Fig. 4B.

To investigate the cooperativity of the two miR-331-5p sites, the canonical (site 1)

and non-canonical (site 2) miR-331-5p target sites were mutated separately (Fig. 5A, MT A, MT B, MT D) and simultaneously (Fig. 5A, MT C, MT E) using the pGL3/2B15/UTR reporter construct as the template.

The identities of all constructs above were confirmed by DNA sequencing. Primers used for cloning and mutagenesis are listed in Table 1.

Luciferase reporter assays. LNCaP cells were plated in 96-well plates at approximately $1.25x10^5$cells per well, cultured overnight, and then transfected with 100 ng of plasmid, 0.8 ng of pRL-null vector and 4.5 pmol of either miR-331-5p mimics or miR-neg. 24 hours post-transfection, cells were lysed in passive lysis buffer, and subjected to Dual-Luciferase Reporter Assay according to the manufacturer's instructions (Promega, Madison, WI). Firefly and Renilla luciferase activities were measured on a Packard TopCount luminescence and scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA). The firefly luciferase activity was normalized to the renilla luciferase activity and presented relative to that of the empty pGL3-promoter vector (set as a value of 1).

4-Methylumbelliferone Glucuronidation Assays. 4-Methylumbelliferone (4-MU) glucuronidation assays were performed using whole cell lysates of LNCaP cells transfected with miR-331-5p mimics or miR-neg as previously reported (Wijayakumara et al., 2015). Briefly, the 200-µl glucuronidation assay mixture that contained 100 mM potassium phosphate pH 7.4, 4 mM $MgCl_2$, 400 µM 4-MU, 225 µg lysate and 5 mM UDP-glucuronic acid was incubated for 2 hours at 37°C in a shaking water bath. Previous studies indicate that the assay remains linear at this time point. After the addition of 2 µl of 70% perchloric acid, samples were kept on ice for a minimum of 10 minutes and centrifuged at 5000g for 10 minutes at 4°C. 60 µl of the supernatant fraction was analysed by high-performance liquid

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chromatography using an Agilent 1100 series instrument (Agilent Technologies, Sydney, Australia) as previously described (Uchaipichat et al., 2004). Concentrations of 4-MU-glucuronide in samples were quantified using a standard curve of 4-MU glucuronide prepared over the concentration ranges of 0.5 to 10 µM. Human UGT2B15 supersomes (In Vitro Technologies) were included as controls in these assays.

Analyses of Prostate Adenocarcinoma and Hepatocellular Carcinoma Datasets. Transcriptome profiling RNA sequencing (RNAseq) and miRNA sequencing (miRNAseq) data from hepatocellular carcinomas (LIHC) and prostate adenocarcinomas (PRAD) were downloaded from The Cancer Genome Atlas (TCGA) data portal (https://gdc-portal.nci.nih.gov/) and analysed as previously reported (Wijayakumara et al., 2017). Briefly, the RNAseq expression data from 371 LIHC samples and 498 PRAD samples were represented in the form of high-throughput sequencing counts. Genes (protein coding and noncoding) with a mean of less than 10 counts were discarded; the counts of the remaining genes were normalized using the upper quantile normalization method. Correlation analyses between the expression levels of UGT and miRNA gene sets were conducted using the Spearman rank method and plots were drawn using the R statistical package (https://cran.r-project.org/; R Foundation for Statistical Computing, Vienna, Austria).

Statistical Analysis. Statistical analysis of all data was performed using GraphPad Prism 6 software (La Jolla, CA, USA), with a two-tailed Student's independent t-test. Correlation between UGT2B15 mRNA and miR-331-5p levels in the human tissue RNA panel was analysed using Spearman's correlation. P-values <0.05 were considered statistically significant.

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Results

A Canonical miR-331-5p Target Site Is Present in the UGT2B15 3'UTR. TargetScan (release 7.0: August 2015) predicted a miR-331-5p target site at nucleotides 224-249 of the UGT2B15 3'UTR (Fig. 1A). This site pairs perfectly to miR-331-5p at nucleotides 2-7 (seed pairing) and nucleotides 13-15 (3'-pairing) and hence is considered a canonical miR-331-5p target site (Grimson et al., 2007; Lewis et al., 2005). Although this site has been previously reported, whether miR-331-5p is a regulator of endogenous UGT2B15 mRNA and enzymatic activity has not yet been assessed.

To investigate regulation of UGT2B15 by miR-331-5p we chose prostate cancer LNCaP cells because they express high levels of UGT2B15 mRNA (Wijayakumara et al., 2015) and extremely low levels of miR-331-5p (data not shown). Transfection of miR-331-5p mimics into LNCaP cells significantly reduced UGT2B15 mRNA levels as compared with those in miR-neg–transfected cells (Fig. 2A). Control GAPDH mRNA levels were not significantly altered by miR-331-5p mimics (Fig. 2B). Next we transfected LNCaP cells with miR-331-5p mimics or the miR-neg control and assessed changes in 4-MU glucuronidation activity as previously reported (Wijayakumara et al., 2015). As shown in Fig. 2C, miR-331-5p mimics significantly reduced 4-MU glucuronidation activity as compared to miR-neg mimics. Thus miR-331-5p reduces both UGT2B15 mRNA levels and activity. To assess the role of the predicted canonical miR-331-5p target site in regulation of UGT2B15, we generated luciferase reporters carrying the wild-type UGT2B15 3'-UTR (pGL3/2B15/UTR), and reporters with either mutation or deletion at the seed site of the miR-331-5p target site within the 3'UTR (pGL3/2B15/UTR/miR331-5p/MT or pGL3/2B15/UTR/miR331-5p/delMT). These reporters were transfected with miR-331-5p mimics or miR-neg into LNCaP cells. As shown in Figs. 3A and 3B, miR-331-5p mimics significantly repressed the

activity of the reporter carrying the wild-type UGT2B15 3'UTR by ~70%. Interestingly, this repression was only partially abrogated by mutation (Fig. 3A) or deletion (Fig. 3B) at the seed site of the miR-331-5p target site. While these results confirmed that miR-331-5p can repress the UGT2B15 3'UTR at least partially through the canonical miR-331-5p site as reported by (Margaillan et al., 2016); it also suggested that there are other regions of the 3'UTR involved in miR-331-5p activity.

A Non-canonical miR-331-5p Target Site Is Present in the UGT2B15 3'UTR. Our observation (Fig. 3A and 3B) that miR-331-5p mimics repressed pGL3/2B15/UTR reporters in which the canonical miR-331-5p target site was deleted/mutated strongly suggested that the 3'UTR might contain additional miR-331-5p target site(s). Unfortunately, such site(s) could not be readily identified using bioinformatic approaches as most miRNA target site prediction programs including TargetScan rely on a seed sequence match between a miRNA and its target mRNAs for target site prediction and hence are unable to predict non-canonical target sites with a mismatched seed site or without a seed site (Cloonan, 2015; Grimson et al., 2007; Lewis et al., 2005; Loeb et al., 2012; Shin et al., 2010). To identify additional non-canonical miR-331-5p target site(s), we used an unbiased deletion analysis approach. A series of pGL3 reporter constructs carrying different segments of the UGT2B15 3'UTR (i.e. nt1-75, nt1-150, nt1-225, nt1-314, nt1-384, nt1-454, nt244-318, nt244-384, or nt244-454) were transfected with either miR-331-5p mimics or miR-neg into LNCaP cells (Fig. 4A). As expected, miR-331-5p mimics significantly reduced the activity of two constructs carrying the canonical miR-331-5p target site (nt1-314 and nt1-384) and had no impact on the activity of another three constructs lacking the canonical miR-331-5p targe site (nt244-318, nt244-384, or nt244-454). The miR-331-5p mimics significantly reduced the activities of two other constructs (nt1-150, nt1-225) lacking the canonical miR-331-5p target site and did not affect

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the activity of the construct (nt1-75), thus suggesting the existence of a miR-331-5p target site within nt75-150.

Analysis of the 75-150-bp region of the UGT2B15 3'UTR by RNAhybrid, a miRNA target site prediction program previously shown to be able to predict non-canonical target sites (Kruger and Rehmsmeier, 2006), predicted two energetically favourable duplex formation sites between miR-331-5p and UGT2B15 mRNA at nucleotides 76-122 (termed site A: -18.8 kcal/mol) and at nucleotides 141-150 (termed site B: -15.7 kcal/mol) (Fig. 4B). To assess whether miR-331-5p could target these two sites, we created mutations in the nt1-150 construct that either disrupted (MT1, MT2, and MT3) or did not affect (MT4, MT5, MT6) the predicted duplex formation sites (Fig. 4B). Transfection of these mutated constructs with either miR-331-5p mimics or miR-neg into LNCaP cells showed that only MT3 completely abolished the ability of miR-331-5p mimics to repress reporter activity (Fig. 4C), thus indicating that site B is an active miR-331-5p target site. Site B consists of 10 nucleotides, 8 of which pair with miR-331-5p at its 3' end, suggesting extensive 3' pairing between the miRNA and target site (Fig. 4B). Analysis of the 454-bp full-length UGT2B15 3'UTR by RNAhybrid predicted a 22-nt miR-331-5p target site containing the 10-nt site B at nucleotides 141-162 (-22.1 kcal/mol) (Fig. 4B). RNAhybrid predicted Watson-Crick pairing at 16 nucleotides between this site and miR-331-5p, including pairing in the seed site but with a 1-bp mismatch; hence we define this as a non-canonical miR-331-5p target site. For simplicity, we termed this non-canonical miR-331 site as miR-331-5p site 2 and the canonical seed-matched miR-331-5p site as miR-331-5p site 1 (Fig. 1A).

Cooperative Regulation of UGT2B15 3'UTR by miR-331-5p via the two miR-331-5p Target Sites. To assess any cooperativity between the two miR-331-5p target sites, we mutated the two sites separately or in combination (MT C) in the pGL3/UTR/UGT2B15

nt1-454 luciferase reporter (Fig. 5A); the mutants were co-transfected with either miR-331-5p mimics or miR-neg into LNCaP cells. The mutation in site 1 was located in the seed site (MT A); whereas two mutations were generated in site 2: a mutation disrupting the 3'pairing (MT B, C), and a mutation in the seed site (MT D, E) (see Fig 5A). The ability of miRNA-331-5p mimics to repress reporter activity was partially but significantly reduced by mutation of the two sites separately; however it was completely abrogated when the two sites were mutated simultaneously (Fig. 5B). This demonstrates a synergistic regulation of UGT2B15 by the two miR-331-5p sites. Of note, this cooperativity was not seen when the miR-331-5p site was mutated at the 1-bp mismatched seed site (MT E) but only when mutated at the 3' sequence (MT C), suggesting that the 3' pairing plays a critical role at miRNA/mRNA duplex formation at the UGT2B15 miR-331-5p site 2.

Cooperative Regulation of UGT2B15 3'UTR by miR-331-5p and miR-376c. The recently reported miR-376c target site is located between the two miR-331-5p sites characterized in Figure 5 (Margaillan et al., 2016; Wijayakumara et al., 2015) (Fig. 1A). To examine whether miR-376c and miR-331-5p cooperatively regulate the UGT2B15 3'UTR, we transfected the pGL3/UTR/UGT2B15 nt1-454 luciferase reporter with miR-331-5p or miR-376c mimics alone or in combination into LNCaP cells. Both miR-331-5p and miR-376c mimics significantly reduced reporter activity when transfected alone (by 55-65%); however, co-transfection of these two miRNA mimics further decreased the reporter activity (by ~80%) (Fig. 6), indicating synergistic regulation of UGT2B15 3'UTR by miR-331-5p and miR-376c.

UGT2B17 3' UTR Is Not Regulated by miR-331-5p. The sequences of the UGT2B15 and UGT2B17 3' UTRs are highly conserved at their 5'-ends (approximately 210

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nt after the TAG codon) but diverge at their 3'-ends (Fig. 1A). The canonical miR-331-5p target site (site 1) lies in the divergent region; hence the UGT2B17 3' UTR does not contain a site that is equivalent to UGT2B15 miR-331-5p site 1 (Fig. 1A). The non-canonical miR-331-5p site (site 2) resides in the region that is conserved between the UGT2B15 and UGT2B17 3' UTRs (Fig. 1A) and a sequence similar to miR-331-5p site 2 can be identified in the UGT2B17 3' UTR. However, transfection of miR-331-5p mimics into LNCaP cells had no impact on UGT2B17 mRNA levels (Fig. 7A), nor on the activity of a luciferase reporter carrying the wild-type UGT2B17 3' UTR (Fig. 7B). This suggests that the putative 'UGT2B17 miR-331-5p site 2' is not functional. In support of this observation, RNAhybrid predicted the formation of a miR-331-5p/mRNA duplex (-14.9 kcal/mol) at this putative site that was much less energetically favourable compared to that (-22.1 kcal/mol) formed at the UGT2B15 miR-331-5p site 2 (data not shown). Collectively, these data indicate that the UGT2B17 3' UTR lacks a functional miR-331-5p target site and is hence not regulated by miR-331-5p.

miR-331-5p and UGT2B15 RNA Levels are Negatively Correlated in Normal Human Tissues and Hepatocellular Carcinoma. We measured miR-331-5p levels in a panel of RNAs from 17 normal human tissues using RT-qPCR (Fig. 8A); UGT2B15 mRNA levels in the same tissue panel were recently reported (Wijayakumara et al., 2015). Spearman's correlation analysis showed that miR-331-5p and UGT2B15 mRNA levels were significantly inversely correlated across the tissues (rho = -0.4952; $p<0.05$) (Fig. 8B), suggesting that miR-331-5p may be involved in repression of UGT2B15 in a number of tissues in vivo. Liver had the highest UGT2B15 mRNA levels (Wijayakumara et al., 2015) and the lowest miR-331-5p levels (Fig. 8A); moreover, analysis of the TCGA hepatocellular carcinoma RNA- and miRNA-seq datasets (TCGA-LIHC, 371 tissues) showed a significant

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inverse correlation between miR-331 and UGT2B15 levels (rho = -0.171, p =0.0009) (Fig. 8C). Similar analysis of the TCGA prostate adenocarcinoma (TCGA-PRAD) cohort (498 tissues) also suggested inverse correlation between levels of miR-331 and UGT2B15, but this correlation was not statistically significant (rho = -0.043, p = 0.335) (Supplemental Figure 1).

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Discussion

This study identifies the novel mechanism by which miR-331-5p regulates UGT2B15. Specifically, we find that repression by this miRNA requires two target sites, one of which mediates canonical seed-based interactions (site 1) and the other mediating non-canonical 3' pairing (site 2). Deletion of the seed sequence of site 1 was recently shown to reduce, but not abolish, the ability of miR-331-5p to repress a reporter construct carrying the UGT2B15 3' UTR in LNCaP cells (Margaillan et al., 2016). Our discovery and characterization of the neighbouring non-canonical miR-331-5p site (site 2) provides an explanation for the continued ability of miR-331-5p to repress the UGT2B15 3'UTR in the absence of site 1. Moreover, our study provides a clear example of cooperative regulation by two miRNA target sites.

Canonical miRNA sites contain four types of seed-matched sites (6mer site, 7mer-m8 site, 7mer-A1 site, 8mer site) (Brennecke et al., 2005; Friedman et al., 2009; Lewis et al., 2005). An 8mer site is defined by a perfect match at the 6-nt miRNA seed supplemented by an A at miRNA nucleotide 1 and Watson-Crick match to miRNA nucleotide 8. According to this definition, the UGT2B15 miR-331-5p site 1 is a 8mer site (Fig. 1A). Additional Watson-Crick pairing at miRNA nucleotides 12–17 is believed to enhance miRNA targeting of 7mer and 8mer sites (Grimson et al., 2007); these types of 7mer and 8mer sites are termed 3' supplementary seed sites (Friedman et al., 2009; Grimson et al., 2007). The 8mer UGT2B15 miR-331-5p site 1 pairs to miR-331-5p at nucleotides 13-15 and hence corresponds to a typical 3' supplementary 8mer site. Non-canonical miRNA target sites generally contain centered sites and 3' compensatory sites (Shin et al., 2010). A 3' compensatory site is defined by extensive 3' pairing in the context of an imperfect seed match (Friedman et al., 2009). The UGT2B15 miR-331-5p target site 2 shows a 1-bp-mismatched seed site and an extensive 3'

pairing to miR-331-5p and hence corresponds to a typical 3’ compensatory site (Fig. 1A). Recent genome-wide crosslinking immunoprecipitation (CLIP)/RNA-sequencing studies have shown that around 40-60% miRNA binding sites are non-canonical and include centered sites and 3' compensatory sites (Helwak et al., 2013; Loeb et al., 2012; Martin et al., 2014; Shin et al., 2010). For 3' compensatory sites, the 3’ pairing is believed to help augment the imperfect seed-mediated weak mRNA/miRNA hybridization. In the case of UGT2B15 miR-331-5p site 2, we showed by mutagenesis that disrupting the 3’ pairing, rather than the seed pairing, effectively abolished regulation of the UGT2B15 3’UTR by miR-331-5p, suggesting that the 3’ pairing plays the pivotal role in mRNA/miRNA duplex formation at this non-canonical miRNA-331-5p site. Collectively, these data indicate that post-transcriptional regulation of UGT2B15 3’UTR by miR-331-5p in prostate cancer LNCaP cells is achieved through 2 target sites: a 3’ supplementary 8mer site and a 3’ compensatory site.

The human *miR-331* gene (12q22), an intergenic microRNA gene, gives rise to two mature miRNAs: miR-331-3p and miR-331-5p. miR-331-5p is considered as a biomarker for Parkinson’s disease (Cardo et al., 2013) and Facioscapulohumeral muscular dystrophy (FSHD) (Portilho et al., 2015). miR-331-5p is also implicated in a number of cancers including colorectal adenomas (Verma et al., 2015), leukaemia (Feng et al., 2011), and lung cancer (Zhan et al., 2017). In this study, miR-331-5p mimics dramatically repressed endogenous UGT2B15 in LNCaP cells. Moreover, miR-331 and UGT2B15 levels were inversely correlated in a tissue panel and hepatocellular carcinoma (TCGA/LIHC) cohort, suggesting that miR-331-5p plays an important role in defining the basal level of UGT2B15 expression in a range of tissues and in liver cancer.

Although, miR-331-5p and UGT2B15 levels appeared to be inversely correlated in LNCaP cells (miR-331-5p levels are low and UGT2B15 levels are high), we did not see significant correlation between miR-331 and UGT2B15 levels in a large prostate cancer cohort. UGT2B15 (and UGT2B17) expression is strongly downregulated by androgens (Bao et al., 2008; Chouinard et al., 2007; Guillemette et al., 1996; Guillemette et al., 1997) in prostate cancer and AR-signalling is likely to maintain tight transcriptional control over the UGT2B15 gene. In this context, direct miRNA-mediated regulation might not be expected to play a central role in defining overall UGT2B15 levels. However, adding further complexity to the regulatory model, the miR-331 gene generates both miR-331-5p and miR-331-3p; miR-331-3p was reported to repress the AR-signalling pathway through targeting the human epidermal growth factor receptor 2 (ERBB-2) and PI3k/AKT signalling in prostate cancer cells (Epis et al., 2009). This ability of miR-331-3p to repress AR-signalling suggests that it might also indirectly modulate UGT2B15 expression at the transcriptional level; moreover repression of AR activity could potentially increase UGT2B15 transcription. It is tempting to suggest that the lack of a significant correlation between UGT2B15 and miR-331 levels in prostate cancer tissues might reflect the opposing effects of miR-331-5p and miR-331-3p. However understanding the complex network of direct and indirect effects of miR-331-derived miRNAs on androgen signalling and UGT2B15 expression will require further study.

Recent studies defined a miR-376c target site at the UGT2B15 3'UTR (Margaillan et al., 2016; Wijayakumara et al., 2015). This miR-376c site is located between the two miR-331-5p sites: 62 bp upstream of the miR-331-5p site 2 and 15 bp downstream of the miR-331-5p site 1 (Fig. 1A). We demonstrated cooperative regulation of UGT2B15 3'UTR by miR-331-5p and miR-376c through these closely located target sites in prostate cancer LNCaP cells. Similar cooperative regulation of UGT2B4 3'UTR by several miRNAs in liver cancer cell lines via adjacent target sites has been recently reported (Wijayakumara et al.,

2017). It remains to be determined whether other *UGT* genes are also cooperatively regulated by multiple miRNAs.

The proximal UGT2B15 and UGT2B17 promoters have similar sequences and contain highly conserved response elements for the androgen receptor (AR) and estrogen receptor (ER), and thus they are both downregulated by androgens in prostate cancer cells (Bao et al., 2008; Chouinard et al., 2007) and upregulated by estrogens in breast cancer cells (Hu and Mackenzie, 2009; Hu et al., 2016). The proximal 3'UTRs of UGT2B15 and UGT2B17 also have similar sequences including the highly conserved miR-376c target site and thus they are both regulated by miR-376c in prostate cancer cells (Margaillan et al., 2016; Wijayakumara et al., 2015). In this study, we demonstrated negative regulation of UGT2B15 by miR-331-5p via two target sites that are not conserved in the UGT2B17 3'UTR. The UGT2B15 miR-331-5p site 1 is located in the distal part of the UGT2B15 3'UTR that is highly divergent from that of UGT2B17 (Fig. 1A). In contrast, the UGT2B15 miR-331-5p site 2 is located in the proximal part of the UGT2B15 3'UTR that is more highly conserved with UGT2B17. However, the region of the UGT2B17 3'UTR that is putatively analogous to UGT2B15 miR-331-5p site 2, shows two nucleotide mismatches when compared with the UGT2B15 sequence; a G/A mismatch in the 3' pairing sequence of the miR-331-5p target site and a C/T mismatch in the seed site (Fig. 1A). We postulate that these nucleotide differences prohibit interactions between the UGT2B17 3'UTR and miR331-5p at both the 3' end and the seed site. Thus the UGT2B17 3'UTR lacks a functional miR-331-5p target site and is not regulated by miR-331-5p. This provides the first evidence for a post-transcriptional mechanism that can differentially regulate these two important androgen metabolizing UGTs.

In summary, we report the discovery and characterization of a non-canonical miR-331-5p target site in the UGT2B15 3'UTR and demonstrate that this site cooperates with a

neighbouring canonical target site (Margaillan et al., 2016; Wijayakumara et al., 2015) to mediate regulation of UGT2B15 by miR-331-5p. Furthermore, we show that miR-331-5p and miR-376c synergistically regulate the UGT2B15 3'UTR, and that UGT2B17 3'UTR cannot be regulated by miR-331-5p. UGT2B15 and UGT2B17 are major determinants of the androgen response in prostate cancer cells (Chouinard et al., 2007) and also control systemic androgen levels via hepatic metabolism and clearance; defining miRNAs that regulate their expression provides new insights into the mechanisms controlling androgen-signalling and may direct novel approaches for prostate cancer therapy.

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Authorship Contributions

Participated in research design: Wijayakumara, Mackenzie, Hu, Meech.

Conducted experiments: Wijayakumara, and Hu.

Performed data analysis: Wijayakumara, Mackenzie, Hu, Meech.

Wrote or contributed to the writing of the manuscript: Hu, Meech, Wijayakumara, Mackenzie, McKinnon

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Footnote

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Legends of Figures

Fig. 1. Predicted miRNA target sites in the UGT2B15 and UGT2B17 3'UTRs. (A) Sequence alignment of UGT2B15 (NM_001076.3) and UGT2B17 (NM_001077.3) 3'UTR regions. Boxes indicate the UGT2B15 miR-331-5p target sites 1 and 2, and the miR-376c target site that is conserved and functional in both UGT2B15 and UGT2B17 3'UTRs. (B) Schematic representation of firefly luciferase reporter constructs containing the full-length UGT2B15 3'UTR bearing a wild-type miR-331-5p site 1 (pGL3/2B15/UTR), a seed-site mutated miR-331-5p site 1 (pGL3/2B15/UTR/miR-331-5p/MT), or a seed-site deleted miR-331-5p site 1 (pGL3/2B15/UTR/miR-331-5p/delMT).

Fig. 2. miR-331-5p reduces both UGT2B15 mRNA levels and enzymatic activity in LNCaP cells. (A, B) LNCaP Cells were transfected with miR-331-5p or miR-neg at 30 nM in triplicate; RNA was extracted and subjected to qRT-PCR to measure target mRNA levels. After normalization to β-actin mRNA levels, the mRNA levels of UGT2B15 (A) or GAPDH (B) in cells transfected with miR-331-5p are presented relative to those in miR-neg–transfected cells (set at a value of 100%). (C) The 4-Methylumbelliferone (4-MU) glucuronidation activity in LNCaP cells transfected with miR-331-5p mimics is presented relative to those in miR-neg–transfected cells (set as a value of 100%). Data shown are mean ± S.E.M. from at least two independent experiments performed in triplicate. ***$P < 0.001$.

Fig. 3. miR-331-5p mimics reduce the activity of a pGL3 firefly luciferase reporter construct carrying the UGT2B15 3'UTR via a canonical target site (site 1) in LNCaP cells. Reporter constructs containing the full-length UGT2B15 3'UTR with wildtype, seed site-mutated (A), or seed site-deleted (B) miR-331-5p site 1 were transfected with miR-neg or miR-331-5p into

LNCaP cells. Luciferase reporter assays were performed as described in *Materials and Methods* section. The activity of the reporter constructs was normalized to the activity of the pRL-null reference vector and is presented relative to the activities of the empty pGL3-promoter vector (set at a value of 100%). Data shown are mean ± S.E.M. from at least two independent experiments performed in quadruplicate. ***P < 0.0001.

Fig. 4. Discovery of a noncanonical miR-331-5p target site (site 2) in the UGT2B15 3'UTR. (A) A series of 8 firefly luciferase reporter constructs containing eight different regions of the UGT2B15 3'UTR (nt1-75, nt1-150, nt1-225, nt1-314, nt1-384, nt1-454, nt244-318, nt244-384) (left diagram) were cotransfected into LNCaP cells with either miR-neg or miR-331-5p mimics, and their activity measured using luciferase assays as described in *Materials and Methods* section. (B) RNAhybrid predicted two energetically favorable UGT2B15 3'UTR/miR-331-5p duplex formation sites (site A and site B) within nucleotides 75-150 of the UGT2B15 3'UTR. Six mutations were created in the pGL3/2B15/3UTR(1-150) reporter construct: mutations MT1, MT2, and MT3 disrupt predicted duplex formation sites; mutations MT4, MT5, and MT6 are in control non-duplex sites. Mutated sites are boxed with the mutated sequences shown above the wild type sequences. (C) The reporter constructs were cotransfected into LNCaP cells with either miR-neg or miR-331-5p mimics; reporter activities were assessed using luciferase assays as described in *Materials and Methods* section. The activity of the reporter constructs (A and C) was first normalized to the activity of the pRL-null vector and then presented relative to that of pGL3-promoter vector activity (set at a value of 100%). Data shown were from two independent experiments performed in quadruplicate, the error bar representing 1 S.E.M. ***P < 0.001. ns, not statistically significant.

Fig. 5. Cooperative regulation of UGT2B15 3'UTR by miR-331-5p via two target sites. (A) Using reporter vectors containing the full-length UGT2B15 3'UTR, the two miR-331-5p target sites were mutated [mutation at seed site of site 1 (MT A) or mutation at either the 3'pairing site (MT B) or seed site (MT D) of site 2] separately or in different combinations (MT C, MT D). (B) Wildtype (WT) or mutated reporter constructs were transfected with either miR-331-5p mimics or neg-miR into LNCaP cells; reporter activities were assessed using luciferase assays as described in *Materials and Methods* section. The activity of the reporter constructs was first normalized to the activity of the pRL-null vector and then presented relative to that of pGL3-promoter vector activity (set at a value of 100%). Data shown were from at least two independent experiment performed in quadruplicate, the error bar representing 1 S.E.M, ***P < 0.001. ns, not statistically significant.

Fig. 6. Cooperative regulation of UGT2B15 3'UTR by miR-331-5p and miR-376c. The firefly luciferase reporter carrying the full-length UGT2B15 3'UTR was transfected into LNCaP cells with miR-neg, miR-331-5p, or miR-376c mimics alone or in combination; reporter activities were assessed using luciferase assays as described in *Materials and Methods* section. The activity of the reporter constructs was first normalized to the activity of the pRL-null vector and then presented relative to those of the empty pGL3-promoter vector (set at a value of 100%). Data shown represent mean ± S.E.M. from two independent experiments performed in triplicate. ***P <0.0001. ns, not statistically significant.

Fig. 7. UGT2B17 3' UTR is not regulated by miR-331-5p. (A) LNCaP cells were transfected with miR-331-5p or miR-neg at 30 nM in triplicate and RNA was extracted from the cells and then subjected to qRT-PCR to measure UGT2B17 mRNA levels. After normalization to

the β-actin mRNA levels, the UGT2B17 mRNA levels in cells transfected with miR-331-5p are presented relative to those in miR-neg–transfected cells (set at a value of 100%). (B) The firefly luciferase reporter carrying the full-length UGT2B17 3'UTR was transfected into LNCaP cells with miR-neg or miR-331-5p; reporter activities were assessed using luciferase assays as described in the *Materials and Methods* section. The activity of the reporter constructs was first normalized to the activity of the pRL-null vector and then presented relative to those of the empty pGL3-promoter vector (set at a value of 100%). Data shown represent mean ± S.E.M. from at least two independent experiments performed in triplicate. ns, not statistically significant.

Fig. 8. The miR-331-5p and UGT2B15 levels are significantly inversely correlated in a human tissue panel and the TCGA hepatocellular carcinoma cohort. (A) qRT-PCR analysis of miR-331-5p was conducted using total RNAs from a human tissue panel as described in *Materials and Methods* section. Levels of miR-331-5p were normalized to RNU6-2 and then presented relative to that in liver (set at a value of 1). Data shown are from a representative experiment performed in triplicate, the error bar representing ± SD. (B) Spearman rank correlation analysis between UGT2B15 mRNA and miR-331-5p levels in the human tissue panel using GraphPad Prism 6 software. (C) Transcriptome profiling data (RNAseq and miRNAseq) from the TCGA hepatocellular carcinoma cohort (371 samples) were downloaded from the TCGA data portal. Data were normalized and correlation analysis between miR-331 and UGT2B15 mRNA levels was conducted using the Spearman rank method; the graph was drawn using the R statistical package as described in the *Materials and Methods* section.

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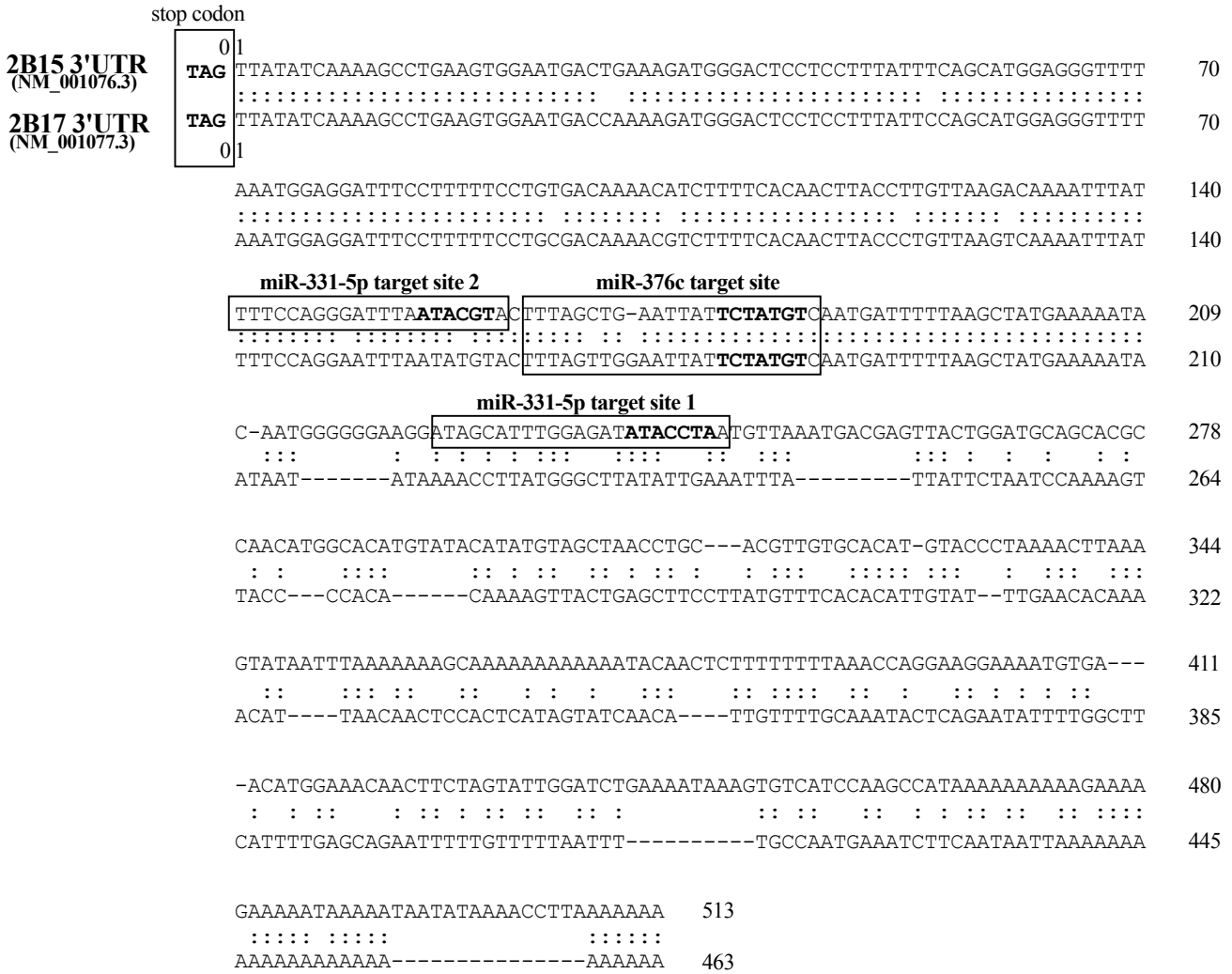
Table 1

Primers used in this study for mutagenesis, cloning and RT-qPCR (5'–3')

Primer	Sequence (5' - 3')
Site directed mutagenesis	
miR331-5p_F	GATAGCATTTGGAGATCGCAACAATGTTAAATGACGA
miR331-5p_R	TCGTCATTTAACATTGTTGCGATCTCCAAATGCTATC
miR-331-5p/delMT_F	GGGGAAGGATAGCATTTGGAGATATGTTAAATGACGAGT
miR-331-5p/delMT_R	ACTCGTCATTTAACATATCTCCAAATGCTATCCTTCCCC
MT B_F and MT C_F	GACAAAATTTATTTTAGCGAGATTTAATACGTAC
MT B_R and MT C_R	GTACGTATTAAATCTCGCTAAAATAAATTTTGTC
MT D_F and MT E_F	TTTTCCAGGGATTTACGCAGTACTTTAGCTGGAA
MT D_R and MT E_R	TTCCAGCTAAAGTACTGCGTAAATCCCTGGAAAA
MT1_F	GAGGATTTCCTTTTAGACTTGACAAAACATCTTTTC
MT1_R	GAAAAGATGTTTTGTCAAGTCTAAAAGGAAATCCTC
MT2_F	CATCTTTTCACAACTGTAGCTGTTAAGACAAAATT
MT2_R	AATTTTGTCTTAACAGCTACAGTTGTGAAAAGATG
MT3_F	GACAAAATTTATTTTAGCTAGATCTAGAGTCGGGGC
MT3_R	GCCCCGACTCTAGATCTCGCTAAAATAAATTTTGTC
MT4_F	CCTTGTTAAGACAAACGAGCTTTTCCAGGGATCTAG
MT4_R	CTAGATCCCTGGAAAAGCTCGTTTGTCTTAACAAGG
MT5_F	TCCTTTTTCCTGTGAAGCGCCATCTTTTCACAAC
MT5_R	GTTGTGAAAAGATGGCGCTTCACAGGAAAAAGGA
MT6_F	TGTGACAAAACATCTCAGAGCAACTTACCTTGTTAAG
MT6_R	CTTAACAAGGTAAGTTGCTCTGAGATGTTTTGTCACA
Cloning	
2B15/UTR_F1	CCGCTCTAGATTATATCAAAAGCCTGAAGT
2B15/UTR (1-75)_R	CCGCTCTAGACATTTAAAACCCTCCATGCT
2B15/UTR (1-150)_R	CCGCTCTAGATCCCTGGAAAATAAATTTTG
2B15/UTR (1-225)_R	CCGCTCTAGAATCCTTCCCCCCATTGTATT
2B15/UTR (1-314)_R	CCGCTCTAGAGCAGGTTAGCTACATATGTA
2B15/UTR (1-384)_R	CCGCTCTAGAAAGAGTTGTATTTTTTTTTT
3UTR_F2	CCGCTCTAGAAATGTTAAATGACGAGTTAC
2B15/UTR (244-318)_R	CCGCTCTAGAGCAGGTTAGCTACATATGTA
2B15/UTR (244-384)_R	CCGCTCTAGAAAGAGTTGTATTTTTTTTTT
2B15/UTR (244-454)_R	CCGGTCTAGAGACACTTTATTTTCAGATCC
RT-qPCR	
miR331-5p_qPCR_F	GCAGCTAGGTATGGTCCCA
miR-331-5p_qPC_R	CCAGTTTTTTTTTTTTTTTGGATCCC

Figure 1

A


stop codon
2B15 3'UTR
(NM_001076.3)
2B17 3'UTR
(NM_001077.3)
TAG
TAG
miR-331-5p target site 2
miR-376c target site
miR-331-5p target site 1

B

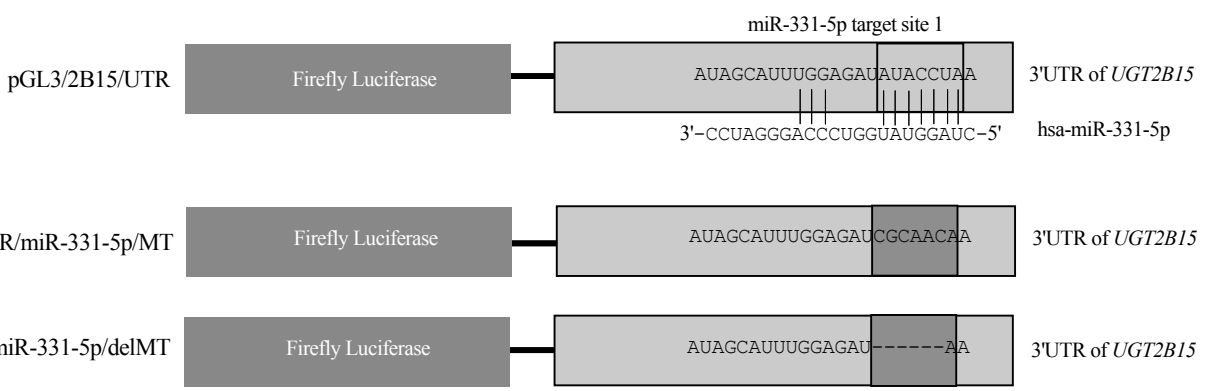

miR-331-5p target site 1
pGL3/2B15/UTR
Firefly Luciferase
AUAGCAUUUGGAGAUAUACCUAA
3'UTR of UGT2B15
3'-CCUAGGGACCCUGGUAUGGAUC-5'
hsa-miR-331-5p
pGL3/2B15/UTR/miR-331-5p/MT
Firefly Luciferase
AUAGCAUUUGGAGAUCGCAACAA
3'UTR of UGT2B15
pGL3/2B15/UTR/miR-331-5p/delMT
Firefly Luciferase
AUAGCAUUUGGAGAU------AA
3'UTR of UGT2B15

Figure 2

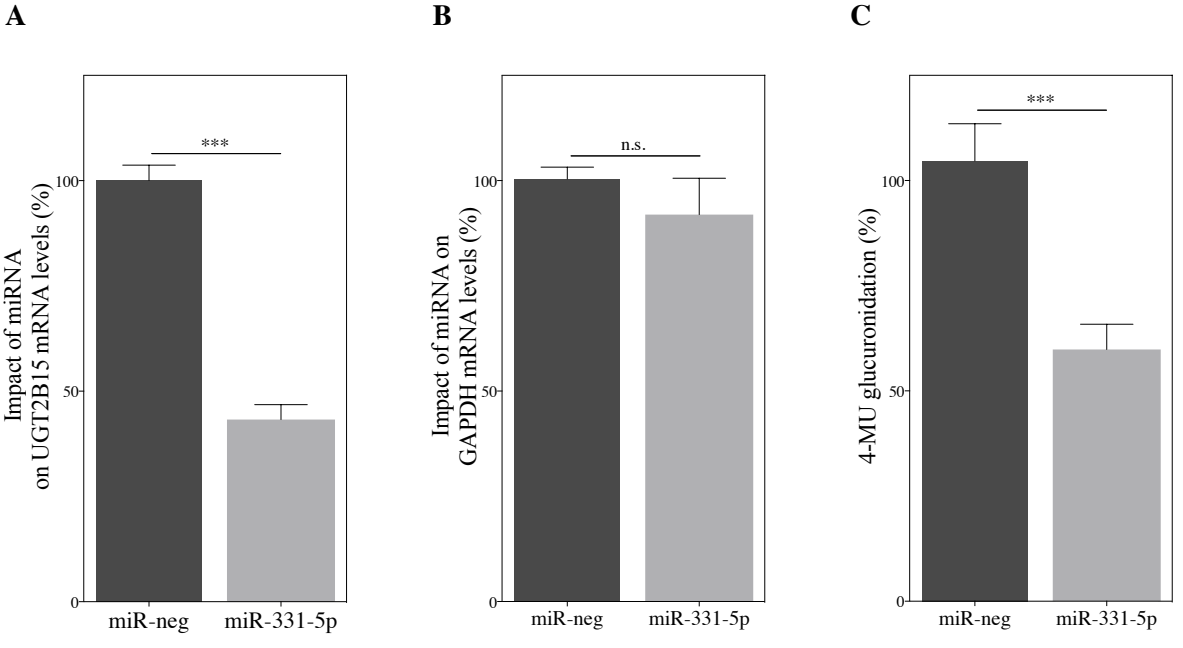

A
B
C
Impact of miRNA on UGT2B15 mRNA levels (%)
Impact of miRNA on GAPDH mRNA levels (%)
4-MU glucuronidation (%)
***
n.s.
***
100
50
0
miR-neg
miR-331-5p

Figure 3

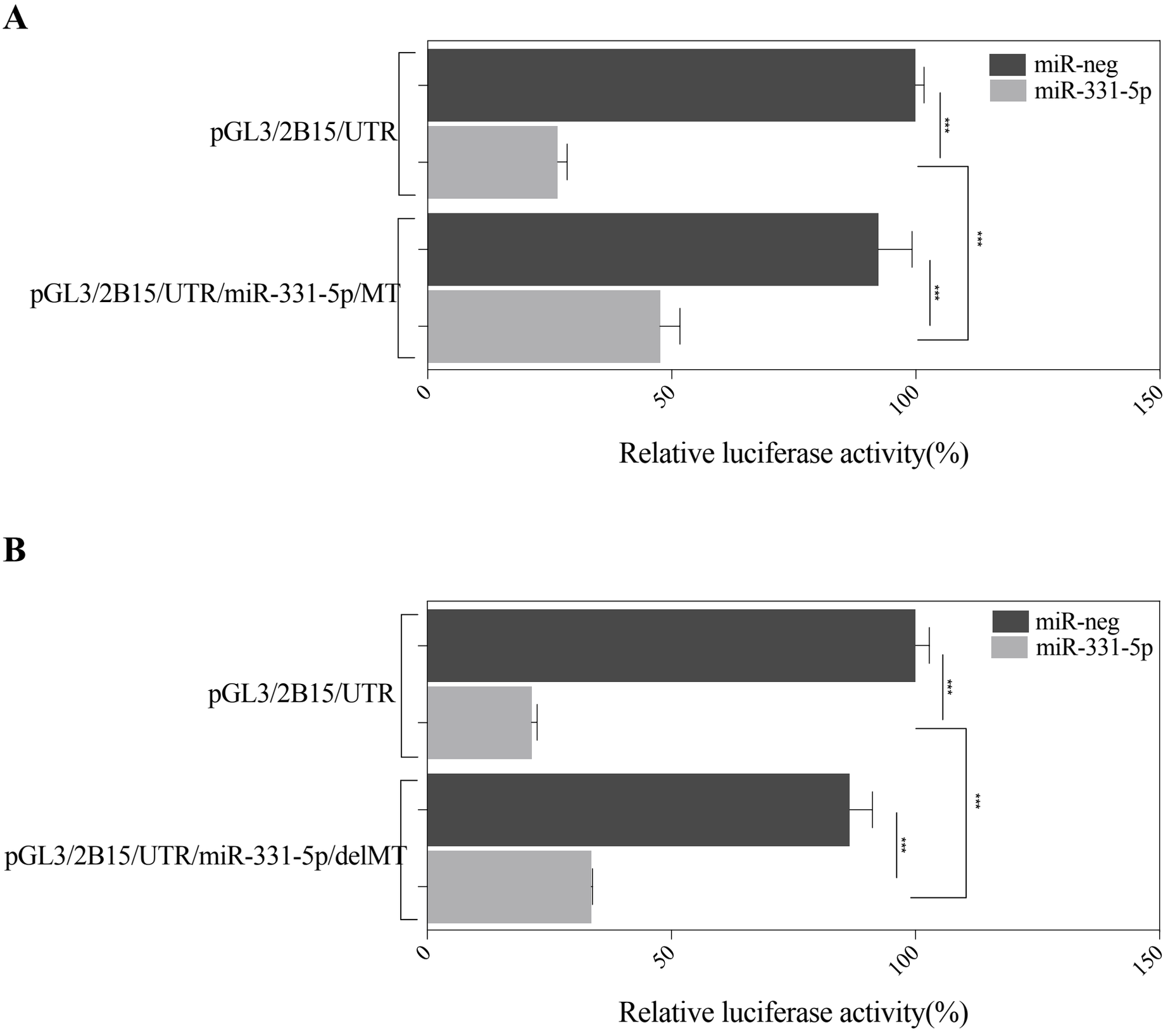

A
miR-neg
miR-331-5p
pGL3/2B15/UTR
pGL3/2B15/UTR/miR-331-5p/MT
***
***
***
0
50
100
150
Relative luciferase activity(%)
B
miR-neg
miR-331-5p
pGL3/2B15/UTR
pGL3/2B15/UTR/miR-331-5p/delMT
***
***
***
0
50
100
150
Relative luciferase activity(%)

Figure 4

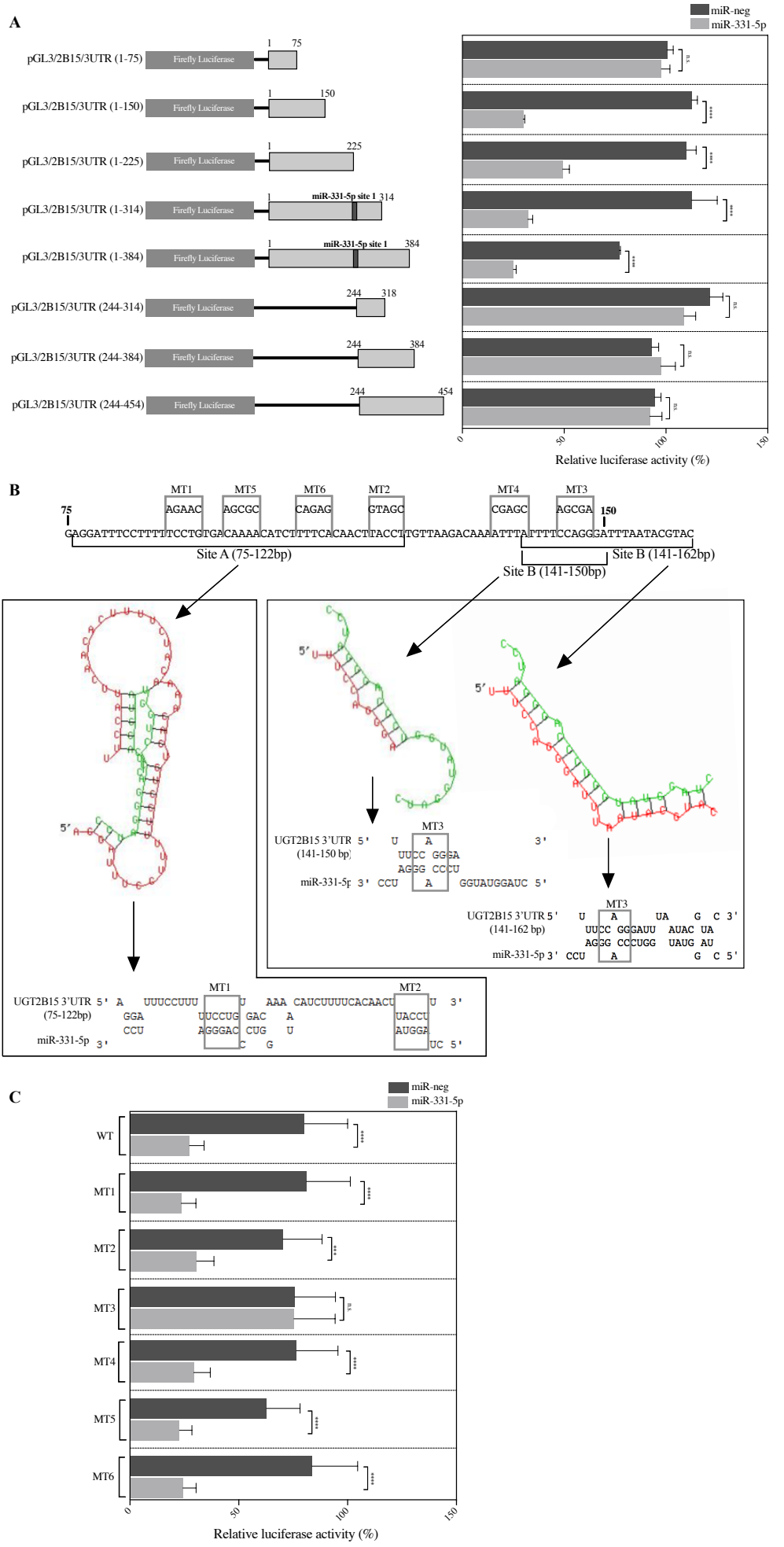

A
miR-neg
miR-331-5p
pGL3/2B15/3UTR (1-75)
pGL3/2B15/3UTR (1-150)
pGL3/2B15/3UTR (1-225)
pGL3/2B15/3UTR (1-314)
pGL3/2B15/3UTR (1-384)
pGL3/2B15/3UTR (244-314)
pGL3/2B15/3UTR (244-384)
pGL3/2B15/3UTR (244-454)
Firefly Luciferase
miR-331-5p site 1
Relative luciferase activity (%)
B
MT1
MT5
MT6
MT2
MT4
MT3
GAGGATTTCCTTTTTCCTGTGACAAAACATCTTTTCACAACTTACCTTGTTAAGACAAAATTTATTTTCCAGGGATTTAATACGTAC
Site A (75-122bp)
Site B (141-150bp)
Site B (141-162bp)
UGT2B15 3'UTR (141-150 bp)
miR-331-5p
UGT2B15 3'UTR (141-162 bp)
UGT2B15 3'UTR (75-122bp)
C
WT
MT1
MT2
MT3
MT4
MT5
MT6
Relative luciferase activity (%)

Figure 5

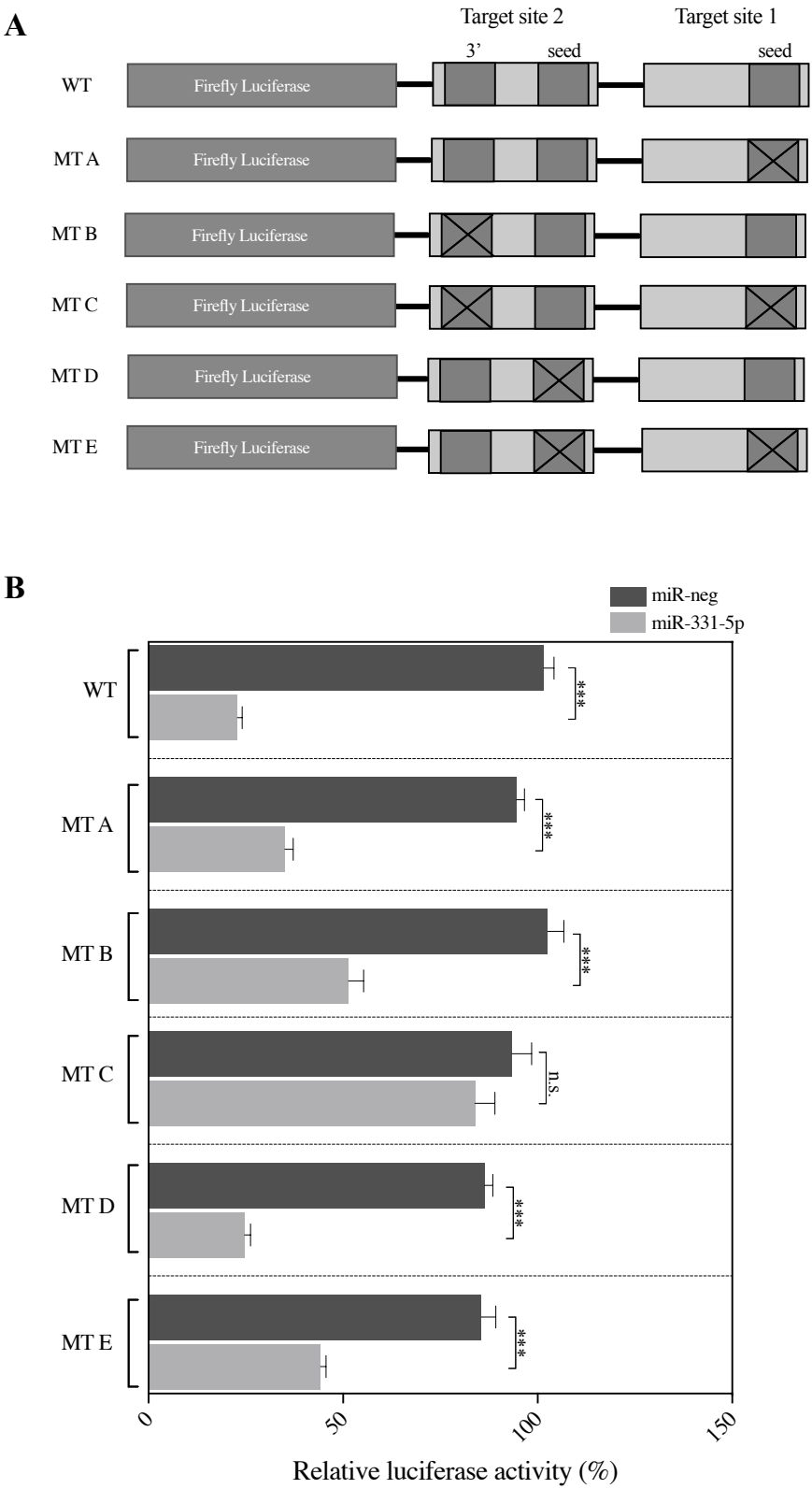

A
Target site 2
Target site 1
3'
seed
seed
WT
MT A
MT B
MT C
MT D
MT E
Firefly Luciferase
B
miR-neg
miR-331-5p
WT
MT A
MT B
MT C
MT D
MT E
***
***
***
n.s.
***
***
0
50
100
150
Relative luciferase activity (%)

Figure 6

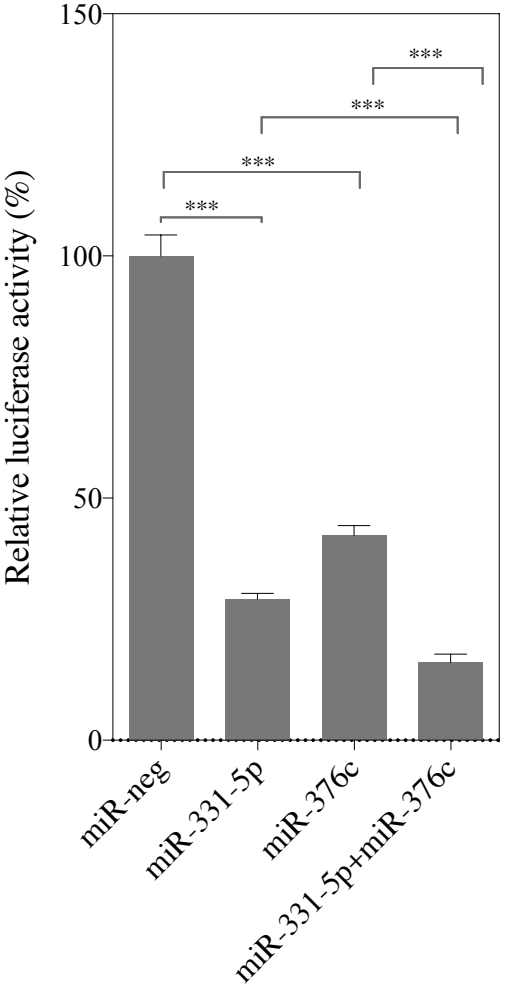
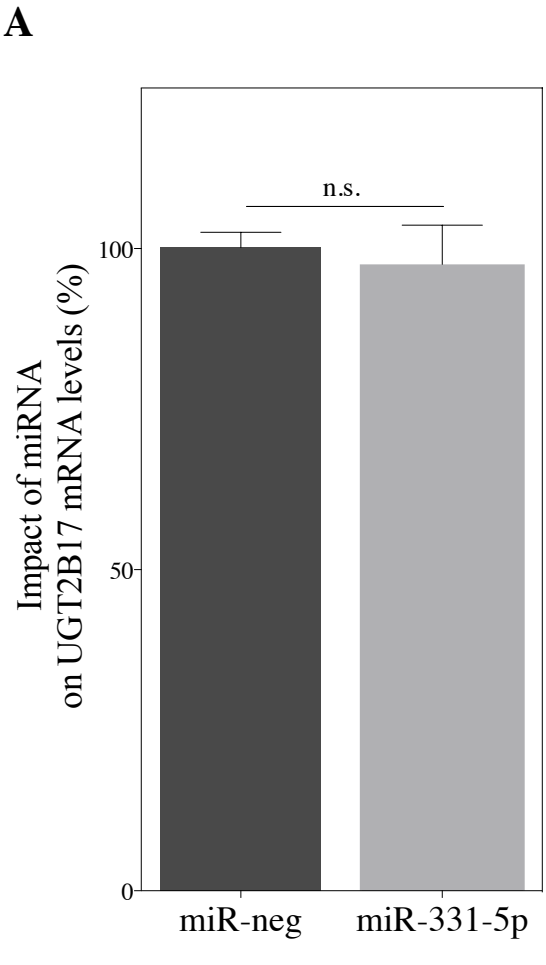

150
100
50
0
Relative luciferase activity (%)
***
***
***
***
miR-neg
miR-331-5p
miR-376c
miR-331-5p+miR-376c

Figure 7


A
n.s.
100
50
0
Impact of miRNA
on UGT2B17 mRNA levels (%)
miR-neg
miR-331-5p

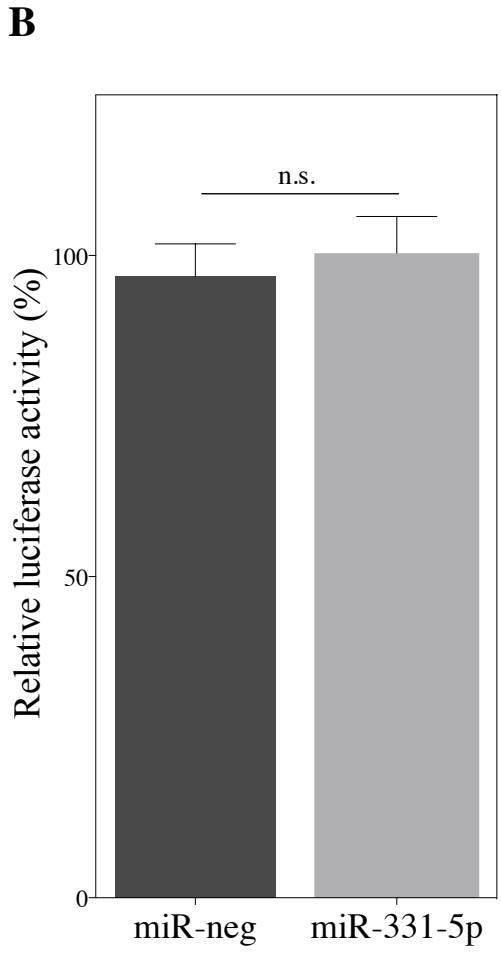
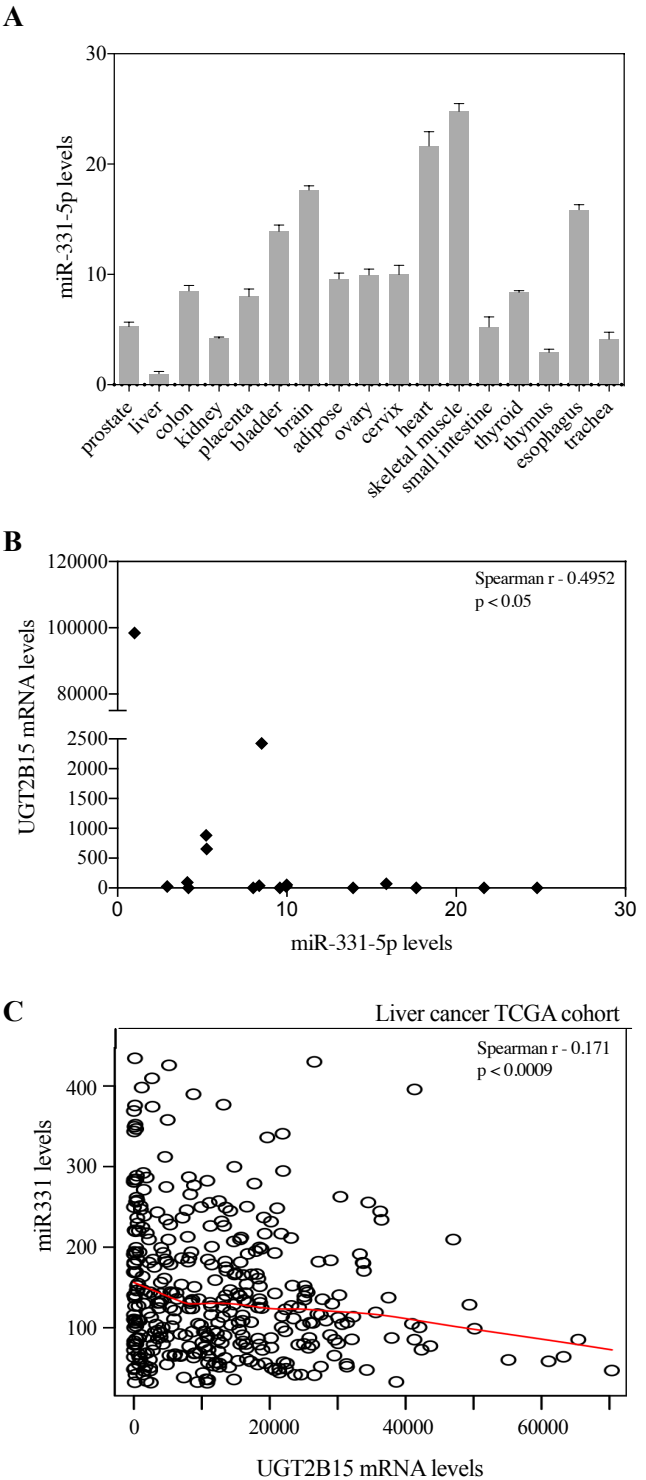

B
n.s.
100
50
0
Relative luciferase activity (%)
miR-neg
miR-331-5p

Figure 8


A
miR-331-5p levels
30
20
10
0
prostate
liver
colon
kidney
placenta
bladder
brain
adipose
ovary
cervix
heart
skeletal muscle
small intestine
thyroid
thymus
esophagus
trachea
B
UGT2B15 mRNA levels
120000
100000
80000
2500
2000
1500
1000
500
0
Spearman r - 0.4952
p < 0.05
0
10
20
30
miR-331-5p levels
C
Liver cancer TCGA cohort
Spearman r - 0.171
p < 0.0009
miR331 levels
400
300
200
100
0
20000
40000
60000
UGT2B15 mRNA levels

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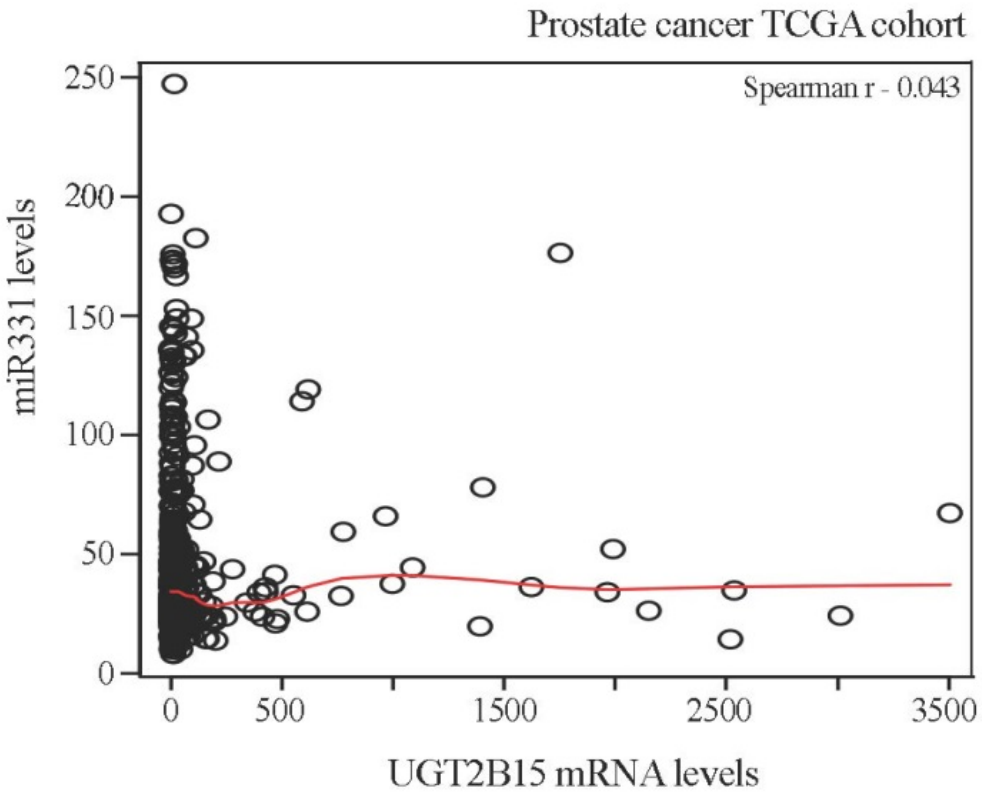
Regulation of UDP-Glucuronosyltransferase 2B15 by miR-331-5p in prostate cancer cells involves canonical and non-canonical target sites

Dhilushi D. Wijayakumara, Peter I. Mackenzie, Ross A. McKinnon, Dong Gui Hu, and Robyn Meech

Supplemental Figure 1.



Supplemental Figure 1. The miR-331 and UGT2B15 levels are not significantly correlated in the TCGA prostate adenocarcinoma (TCGA-PRAD) cohort (rho = -0.043, p = 0.335). Transcriptome profiling data (RNAseq and miRNAseq) from the TCGA-PRAD cohort (498 samples) were downloaded from the TCGA data portal. Data were normalized and correlation analysis between miR-331 and UGT2B15 mRNA levels was conducted using the Spearman rank method; the graph was drawn using the R statistical package as described in the *Materials and Methods* section.