Molecular Pharmacology Fast Forward. Published on August 6, 2010 as doi:10.1124/mol.110.066258

MOL #66258

Title Page:

Lyn Kinase-dependent Regulation of miR181 and Mcl-1 Expression: Implications for Drug Resistance in Myelogenous Leukemia

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Molecular Pharmacology Fast Forward. Published on August 6, 2010 as DOI: 10.1124/mol.110.066258
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Running Title Page:

Running title: MiR181 regulates Mcl-1 in Lyn-dependent imatinib-resistant CML.

Non-standard Abbreviations: None

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Number of pages: 27

Number of tables: 0

Number of figures: 6

Number of references: 42

Numbers of words:

Abstract – 246

Introduction – 631

Discussion – 804

Molecular Pharmacology Fast Forward. Published on August 6, 2010 as DOI: 10.1124/mol.110.066258
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Abstract:

Imatinib, a BCR-Abl inhibitor, is a successful front-line treatment for chronic myelogenous leukemia (CML). Despite the success of imatinib, multiple mechanisms of resistance remain a problem, including over-expression of Lyn kinase (Lyn) and Bcl-2 family anti-apoptotic proteins. Profiling miRNA expression in a model of Lyn-mediated imatinib-resistant CML (MYL-R) identified approximately 30 miRNAs whose expression differed >2-fold in comparison to drug-sensitive MYL cells. In particular, the expression of the miR181 family (a-d) was significantly reduced (~11-25 fold) in MYL-R cells. Incubation of MYL-R cells with a Lyn inhibitor (dasatinib) or nucleofection with Lyn-targeting siRNA increased miR181b and miR181d expression. A similar Lyn-dependent regulation of miR181b and miR181d was observed in imatinib-resistant K562 CML cells. Sequence analysis of potential targets for miR181 regulation predicted Mcl-1, a Bcl-2 family member whose expression is increased in MYL-R cells and drug-resistant leukemias. Inhibition of Lyn or rescue of miR181b expression reduced Mcl-1 expression in the MYL-R cells. To further investigate the mechanism of Mcl-1 repression by miR181, a luciferase reporter construct incorporating the Mcl-1 3' UTR was tested. Over-expression of miR181b reduced luciferase activity, whereas these effects were ablated by mutation of the seed region of the miR181 target site. Lastly, stimulation of Lyn expression by 1,25-dihydroxyvitamin D_3 treatment in HL-60 cells, a cell model of acute myelogenous leukemia, decreased miR181b expression and increased Mcl-1 expression. In summary, our results suggest that Lyn-dependent regulation of miR181 is a novel mechanism of regulating Mcl-1 expression and cell survival.

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Introduction:

The discovery and application of the BCR-Abl inhibitor imatinib has been a major hallmark in the development of kinase inhibitors for cancer chemotherapy. However, despite the success of imatinib for the treatment of chronic myelogenous leukemia (CML) and other cancers, multiple mechanisms of imatinib resistance have been identified. These include BCR-Abl mutations that prevent imatinib binding (i.e. T315I) (Hochhaus et al., 2002; Yamamoto et al., 2004), BCR-Abl over-expression (Hochhaus et al., 2002), and increased expression and activity of Src family kinases (SFKs) or other pro-survival proteins (Illmer et al., 2004; Li, 2008). Recently, Lyn kinase (Lyn) has been implicated in imatinib resistance in both CML cells and patient samples. Over-expression of Lyn, the most abundant SFK in hematopoietic cells, may contribute to drug resistance through increased STAT5 phosphorylation, Bcl-2 expression, and other pro-survival responses (Dai et al., 2004; Donato et al., 2003; Nam et al., 2007). Inhibition of Lyn with Src family kinase (SFK) inhibitors reduced pro-survival signaling and reversed imatinib resistance in CML cells (Ito et al., 2007; Nam et al., 2007). Moreover, the dual-specificity BCR-Abl/SFK inhibitors (i.e. dasatinib, sorafenib, nilotinib) effectively treat patients that are non-responsive to imatinib therapy (Li, 2008; Wu et al., 2008).

MicroRNAs (miRNAs) are small (22-24 nt) non-coding RNA molecules that are key regulators of protein expression through their targeted binding to specific mRNAs. By forming a double-stranded RNA duplex with target mRNAs in the RNA-induced silencing complex (RISC), miRNAs trigger the degradation of the mRNA transcript or directly inhibit protein translation (Ambros, 2001). Over 700 miRNAs have been described in humans, and patterns of deletion, down-regulation, or up-regulation of

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specific miRNAs have been characterized in B cell chronic lymphocytic leukemias (CLL), acute myelogenous leukemia (AML), and CML (Calin et al., 2005; Dixon-McIver et al., 2008; Venturini et al., 2007). Recent studies have demonstrated the importance of the miR181 family (a-d) expression in AML and CLL. MiR181a is involved in hematopoietic differentiation (Chen et al., 2004), and loss of miR181 strongly correlates with a common AML morphological subtype (Debernardi et al., 2007). Moreover, high miR181 (a-d) expression is prognostic for the achievement of complete remission and event free survival in AML patients (Marcucci et al., 2008; Schwind, 2009).

Bcl-2 family members are important pro-survival regulators of apoptosis that have been implicated in the promotion of drug resistance in cell models of leukemia (Bagrintseva et al., 2005; Dai et al., 2004; Shangary and Johnson, 2003). Myeloid Cell Leukemia-1 (Mcl-1) is a Bcl-2 family protein shown to correlate with leukemic relapse in AML and has been directly linked to resistance to chemotherapy (Kaufmann et al., 1998). In addition, Mcl-1 was recently implicated in AML survival in response to FLT-3 internal tandem duplications (ITDs), a common mechanism of resistance in AML that involves the activation of Lyn (Breitenbuecher et al., 2009; Okamoto et al., 2007). Mcl-1 functions at the mitochondria by sequestering the pro-apoptotic BH3-only proteins Bim and Noxa, thereby preventing cytochrome c release and cell death (Warr and Shore, 2008). Mechanisms to inhibit Mcl-1 function include ubiquitination and degradation directed by the MULE/LASU1 E3-ligase (Zhong et al., 2005), and targeting of Mcl-1 mRNA for degradation by miRNA has been described (Chen et al., 2009; Crawford et al., 2009; Mott et al., 2007).

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In this study, we describe a novel mechanism by which Lyn may confer multi-drug resistance in a cell model of CML by repressing the expression of miR181. As our data show, miR181b directly represses Mcl-1 expression and the Lyn-dependent loss of miR181 results in enhanced Mcl-1 levels and increased drug resistance. Thus, this is the first demonstration, to our knowledge, of Mcl-1 as a *bona fide* target of miR181 and suggests that the regulation of miR181 by Lyn in drug-resistant cells may contribute to this important anti-apoptotic mechanism.

Materials and Methods:

Cell culture and reagents

MYL and MYL-R human CML cells were a generous gift from Dr. Hideo Tanaka (Dept. of Haematology and Oncology, Hiroshima University, Hiroshima, Japan). K562-R cells were obtained from Dr. Steven Grant (Massey Cancer Center, Virginia Commonwealth University; K562-R(1)). A separate isolate of imatinib-resistant K562 cells were kindly provided by Dr. Nicholas Donato (Dept. of Internal Medicine, University of Michigan; K562-R(2)). HL-60 cells were obtained from the UNC Tissue Culture Facility. Cells were cultured in RPMI 1640 medium (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals; Norcross, GA) and 1% antibiotic/antimycotic (Invitrogen). The imatinib-resistant cells (MYL-R, K562R) were not continuously cultured in the presence of imatinib; however, imatinib resistance and Lyn over-expression was routinely checked and found to be stably maintained. HEK293T cells were a generous gift from Dr. Channing Der (Dept. of Pharmacology, UNC) and were maintained in DMEM medium (Invitrogen) supplemented with 10% fetal

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bovine serum and 1% antibiotic/antimycotic. Cell cultures were maintained and incubated with drug at 37°C in a 5% CO_2 humidified atmosphere. Imatinib and dasatinib were purchased from LC Laboratories (Woburn, MA). SMARTpool siRNA was purchased from Thermo Scientific (Lafayette, CO). 1,25-dihydroxyvitamin D_3 (1,25-D_3) was purchased from Enzo Life Sciences International (Plymouth Meeting, PA).

Nucleofection

Plasmids and siRNA were incorporated into cells using the Amaxa nucleofection system (Walkersville, MD; >90% transfection efficiency with >90% cell viability, data not shown). Briefly, 1.0-1.5x10^6 cells were washed with PBS and re-suspended in nucleofection solution (Mirus Bio LLC; Madison, WI). Cells were nucleofected using the T-16 nucleofector program and added to 4.5 ml of nutrient-rich media. Cells were cultured in a 5% CO_2 humidified atmosphere at 37°C for 24 hr before experimentation.

Plasmid constructs and site-directed mutagenesis

The mature human miR181b sequence was cloned into the SDSA 3.0 expression plasmid using the BglII and Xho1 restriction sites. After incorporation into cells, this plasmid expresses miR181b concomitantly with GFP. The Mcl-1 3' UTR luciferase expression construct was a generous gift from Dr. Serge Nana-Sinkam (Div. of Pulmonary, Allergy, Critical Care and Sleep Medicine, Ohio State University, Columbus, OH). This is a psicheck-2 plasmid vector that incorporates the 3' UTR of Mcl-1 down-stream of the luciferase gene (Crawford et al., 2009). Site-directed mutagenesis of the miR181b binding site was performed using the following primers: fw: 5'-

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CCATTTAAAAATAGGTATGAATAAGATGACTAAGA*ATAC*TAATGGGGAAGAAC TGCCCTG-3' and reverse: 5'-CAGGGCAGTTCTTCCCCATTA*GTA*TCTTAGTCATCTTATTCATACCTATTTTTA AATGG-3'). The reaction mix included 1-ng template DNA, dNTPs, and forward and reverse primers. Touchdown PCR was performed on a thermocycler (Eppendorf; Hauppauge, NY); after an initial incubation at 95°C for 2 min, the following steps were cycled 18 times: 95°C for 50 sec, 70°C->55°C (each cycle was performed with a temperature 1 degree lower) for 50 sec, and 72°C for 14 min. The PCR product was treated with Dpn1, and DH5α *E. Coli* (Invitrogen) were transformed according to the manufacturer's instructions. Mutation was confirmed by sequencing (UNC Genome Analysis Facility).

Caspase 3 activity assay

To determine caspase 3 activity, cells ($6x10^4$/well) were incubated with drug or vehicle in a 96-well plate. Plates were then centrifuged for 5 min to pellet the cells. Cells were lysed on ice with buffer containing 250 mM HEPES (pH 7.4), 25 mM CHAPS, and 25 mM dithiothreitol. The non-ionic detergent disrupts the plasma membrane and releases the cytosolic proteins without denaturing the caspase protein. The activity of caspase 3 in these samples was determined using the Caspase 3 Fluorimetric Assay Kit (CASP3F-1KT; Sigma Aldrich; St. Louis, MO) according to the manufacturer's instructions. Fluorescence was measured using a FLUOstar Galaxy plate reader (BMG Labtech; Durham, NC) with a 360 nm excitation filter and a 460 nm emission filter.

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Quantitative RT-PCR-based miRNA expression profiling

Total RNA was extracted from $40.0x10^6$ MYL and MYL-R cells using Trizol reagent (Invitrogen) according to the manufacturer's instructions. MiRNA enrichment was then performed using the Small RNA Isolation Kit (SABiosciences; Frederick, MD), and the miRNA purity was assessed using an Agilent Bioanalyzer Chip (UNC Genomics and Bioinformatics Core). Finally, 100-ng miRNA was 3' polyadenylated and converted to cDNA using the First Stand Synthesis Kit (SABiosciences) according to the manufacturer's instructions.

Approximately 100-ng cDNA was added to the Human Cancer RT^2 miRNA qPCR Array (SABiosciences, cat. #: MAH102C; ~1.0 ng/well), and qRT-PCR was performed on an Applied Biosystems (ABI) 7500 Fast Real-Time Thermocycler (Foster City, CA) according to the manufacturer's instructions. Melting curve analysis of the resulting transcript was determined. Analysis of the qRT-PCR data was performed using the RT^2 miRNA PCR Array Data Analysis program (http://www.sabiosciences.com/pcr/arrayanalysis.php).

Quantitative RT-PCR analysis of miR181 expression

MiRNA 181 expression was measured using the Taqman MicroRNA Assay (ABI; miR181b ID#: 001098; miR181d ID#: 001099) according to the manufacturer's instructions with slight modification. Briefly, total RNA was extracted from cells using Trizol reagent. One microgram of RNA was added to the following reaction mix: RT buffer, 1 mM dNTPs, U6 snRNA RT primers (ABI; ID#: 001173), and RT primers

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against miR181b. Twenty-five units of the Multiscribe RT enzyme (ABI) were added, and the reverse transcription reaction was performed on a PCR thermocycler. Twenty-five nanograms of the resulting cDNA were added to a qPCR mix containing the Taqman Fast Universal PCR Master Mix (ABI) and the PCR primer/probe reaction mix against either miR181b or U6 snRNA. Quantitative PCR was performed on an ABI 7500 Real-Time System, and data were analyzed using the 7500 Fast software (ABI).

Western blot analysis

Cells were collected, washed twice with PBS, and lysed using RIPA buffer (without SDS; 150 mM NaCl, 9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 1% NP-40, and 0.5% deoxycholic acid; pH 7.4) supplemented with protease and phosphatase inhibitors (150 μM Na_3VO_4, 0.25 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 10 nM microcystin). The lysate was centrifuged and the protein concentration was determined using Bradford reagent (Thermo Scientific). Sample buffer (2X: 0.5 M Tris, 20% glycerol, 10% β-mercaptoethanol, 0.002 μg/ml Bromphenol blue; pH 6.8) was added to an equal volume of total protein (30-50 μg) and the samples were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a polyvinylidene difluoride membrane (Millipore; Billerica, MA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline supplemented with Tween-20 (TBST; 9.9 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 hr at room temperature. Primary antibodies against Lyn (sc-7274; Santa Cruz Biotechnology; Santa Cruz, CA), Mcl-1 (sc-819; Santa Cruz), GFP (G1544; Sigma), SFK pY416 (2101; Cell Signaling Technology; Beverly, MA), and SFK pY527 (2105, Cell Signaling) were diluted in 1% bovine serum

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albumin/TBST and applied to a membrane overnight at 4°C or for 1 hr at room temperature. Following the primary antibody incubation, membranes were washed three times for 5 min in TBST. Secondary antibodies (horseradish peroxidise-conjugated anti-mouse (sc-2008) or anti-rabbit (sc-2004) immunoglobulin; Santa Cruz) diluted in 1% gelatin/TBST were then applied to the membrane for 1 hr at room temperature. β-actin (sc-47778; Santa Cruz) or α-tubulin (T9026; Sigma) expression was measured as a loading control. Membranes were developed using enhanced chemiluminescence (ECL; GE Healthcare, Piscataway, NJ) with exposure to autoradiography film. Densitometry was performed using a FluorChem FC2 imager (Cell Biosciences, Santa Clara, CA).

Transfection and luciferase activity assay

HEK293T cells ($3x10^5$/well) were plated in poly-L-lysine-treated 6-well plates. The next day, cells were transfected using lipofectamine (Invitrogen) according to the manufacturer's instructions.

Twenty-four hours after transfection, cells were lysed at room temperature with RIPA buffer (without SDS). The lysate was centrifuged for 10 min at 10000 RPM. Ten-microliter aliquots of each sample were applied to a 96-well plate, and 150 µl of the luciferase assay reaction mix (25 mM glycyl glycine, 15 mM $MgSO_4$, 15 mM KPO_4, 4 mM EGTA, pH 7.8) containing 0.1 mg/ml luciferin (Sigma) was added to each well. Luciferase activity was measured on a Pherastar luminometer (BMG Labtech). Western blot analysis of GFP was performed to ensure equivalent transfection, and β-actin expression was determined as a loading control.

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Statistics

Data were analyzed using ANOVA and t-tests where appropriate using Prism 4 software (Graphpad Software; La Jolla, CA).

Results:

Lyn kinase over-expression in MYL-R cells confers drug resistance

Previous studies have demonstrated that Lyn is important for MYL-R survival and imatinib resistance (Ito et al., 2007). To compare Lyn expression and activity, lysates of MYL and MYL-R cells were Western blotted with antibodies that recognize total Lyn, active Lyn (P-Y397 (P-Y416 Src)), or inactive Lyn (P-Y507 (P-Y527 Src)). As shown in figure 1A, the Lyn B splice form (53 kDa) was significantly over-expressed and phosphorylated on the activation loop (Y397), indicating increased activity in MYL-R cells. Similarly, the Lyn A splice form (56 kDa), though reduced in expression, was also more highly phosphorylated on this residue in the MYL-R cells (Fig. 1A). Loss of the inactivating Y507 phosphorylation was observed only for Lyn B, suggesting the elevated activity state of this kinase in MYL-R cells. Importantly, the increase of Lyn activity was confirmed in these cells using a novel peptide-based fluorescent Lyn biosensor (Wang et al., 2010a).

Lyn hyper-activation has been attributed to imatinib resistance in MYL-R cells (Ito et al., 2007). In addition, incubation of MYL-R cells with gemcitabine, Ara-C, adaphostin, or Immucillin H failed to activate caspase 3 in these cells, demonstrating that MYL-R are highly anti-apoptotic (Zimmerman et al., unpublished results). To investigate the importance of Lyn in cell survival, MYL-R cells were transfected with

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Lyn-directed siRNA or non-targeting control siRNA and then exposed to 1 μM imatinib. This treatment resulted in a partial knockdown of Lyn and a significant increase in caspase 3 activation (Fig. 1B and 1C). However, the magnitude of caspase activation was less than in MYL cells exposed to the same dose of imatinib (data not shown); this may be due to incomplete silencing of Lyn expression (Fig. 1B). In addition, pre-treatment with PP2, a Lyn and SFK inhibitor, significantly elevated caspase activity in MYL-R cells treated with imatinib, an effect not observed after pre-treatment with the inactive inhibitor analog, PP3 (data not shown).

MiR181 expression is reduced in MYL-R cells

MiRNAs are small (20-22 nt) non-coding RNA regulators of protein expression that have been implicated in the progression and survival of numerous cancers (Hammond, 2006). To identify miRNAs involved in anti-apoptosis, MYL and MYL-R cells were profiled for miRNA expression using the Human Cancer RT^2 miRNA qPCR Array (SA Biosciences; MAH102C). This qRT-PCR-based assay analyzed the expression of 88 known human miRNAs previously associated with cancer. Duplicate experiments demonstrated that 15 miRNAs had a >2-fold increase in expression in MYL-R cells relative to MYL cells (Fig. 2A) and that 15 miRNAs showed a >2-fold decrease in relative expression (Fig. 2B). Importantly, we observed a strong down-regulation of the miR181 family (a-d) of miRNAs in MYL-R cells (Fig. 2B and 2C). This family of miRNA is highly conserved (Fig. 2D), and loss of miR181 expression is prognostic for aggressive AML (Marcucci et al., 2008; Schwind, 2009). To confirm the array results, individual qRT-PCR of miR181b and miR181d was performed; the results of these

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analyses showed that the expression of both miRNAs was reduced approximately 10-fold in MYL-R cells (Supplemental Fig. 1).

Lyn inhibition increases miR181 expression in MYL-R cells

To determine whether hyper-activation of Lyn regulated miR181 expression in MYL-R cells, cells were treated with dasatinib and miRNA expression was determined by qRT-PCR. As shown in figure 3, dasatinib treatment inhibited Lyn and increased miR181b expression in MYL-R cells in a dose-dependent manner (Fig. 3A and 3B). Dasatinib (1 nM) treatment produced minimal effects on cell viability (~15-20% cell loss), as determined by MTS assay and cell proliferation (data not shown). In addition, nucleofection of MYL-R cells with Lyn-targeted siRNA significantly elevated miR181b expression in comparison to control siRNA-treated cells (Fig. 3C). K562-R cells are a model of imatinib-resistant CML, and over-expression of Lyn has been attributed to the mechanism of resistance in these cells (Dai et al., 2004; Donato et al., 2003). To further determine whether Lyn regulates miR181 expression, we tested two independently subcloned imatinib-resistant K562-R cell lines, termed K562-R(1) and K562-R(2). Similar to MYL-R cells, dasatinib treatment of K562-R cells inhibited Lyn activity and increased miR181b expression (Fig. 3E-G). In addition, dasatinib treatment increased miR181d expression in each imatinib-resistant cell line (Fig. 3D-F). These data suggest that hyper-activation of Lyn represses miR181 expression in imatinib-resistant CML.

Mcl-1 is a target of miR181b

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To investigate the potential significance of miR181 to cell survival, we sought to identify protein targets for miR181. Public database bioinformatic algorithms (www.targetscan.org) predict a binding site for miR181 on the 3' UTR of Mcl-1, a Bcl-2 family member and anti-apoptotic protein (Fig. 4A) (Warr and Shore, 2008). Mcl-1 expression inversely correlates with leukemia chemosensitivity (Kaufmann et al., 1998), and Western blot analysis determined that Mcl-1 expression was higher in MYL-R cells (Fig. 4B). Inhibition of Lyn with dasatinib decreased Mcl-1 expression in a dose-dependent manner, demonstrating the importance of Lyn activity in regulating Mcl-1 expression (Fig. 4C).

To determine whether miR181b regulated Mcl-1 expression, MYL-R cells were nucleofected with a miR181b-expressing plasmid vector (SDSA-miR181b) or empty vector (SDSA 3.0) control. Expression of GFP confirmed nucleofection and plasmid processing (Fig. 5A). In addition, qRT-PCR confirmed miR181b expression in these experiments (data not shown). As shown in figure 5, over-expression of miR181b in MYL-R cells significantly decreased Mcl-1 expression in comparison to the empty vector control (Fig. 5A).

MiRNAs regulate protein expression by binding to the 3' UTR of target mRNA and initiate mRNA degradation or inhibit translational processing (Ambros, 2001). To determine whether miR181-dependent inhibition of Mcl-1 expression was due to a direct interaction, HEK293T cells were co-transfected with the miR181b construct or empty vector and a luciferase-reporter construct containing the 3' UTR of Mcl-1 mRNA (Fig. 5B). Using this approach, we observed that over-expression of miR181b significantly inhibited luciferase activity in comparison to the vector control cells (Fig. 5C). Because

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the miRNA "seed region" is critical for the recognition of miRNA targets, complementary nucleotides within this region were mutated in the 3' UTR of the Mcl-1 luciferase reporter (Fig. 5B). These mutations ablated the miR181b-dependent repression of luciferase activity (Fig. 5C), whereas mutation of nucleotides outside the seed region failed to affect this process (data not shown). These data suggest that miR181b directly interacts with the 3' UTR of Mcl-1 mRNA to inhibit Mcl-1 protein expression, and nucleotides within the seed region of this binding site are important to mediate this effect.

Lyn regulates miR181b and Mcl-1 expression in AML

The HL-60 cell line is a commonly used cell model for the study of acute myelogenous leukemia (AML). Previously, it was shown that 1,25-D_3 treatment increased the expression of Lyn in these cells (Wang et al., 2000). To determine whether manipulation of Lyn expression in HL-60 cells affected miR181b and Mcl-1 expression, we treated these cells with 1,25-D_3 for up to 96 hr. A strong increase in active Lyn expression was observed by Western blotting these samples. In agreement with our data obtained with the MYL-R and K562R cells, the increase in Lyn expression correlated with repression of miR181b and increased Mcl-1 expression in a time-dependent manner (Fig. 6A and 6B). Thus, these results indicate that Lyn can affect Mcl-1 expression through modulation of miR181b expression in an AML cell line.

Discussion:

These studies describe three important findings: one, that the hyper-activation of Lyn suppresses the expression of miR181; two, that miR181b represses the expression of

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a key anti-apoptotic protein, Mcl-1; and three, loss of miR181 upon Lyn activation may represent a novel mechanism of drug resistance in leukemia. This mechanism was observed in cell models of CML and AML, and the specific role of Lyn was confirmed by both dasatinib treatment and siRNA against Lyn. Considerable evidence now supports the role of Lyn as a "compensatory oncogene" in imatinib-resistant CML and specific subtypes of AML. Lyn is over-expressed in drug-resistant cell lines and patient samples (Dai et al., 2004; Donato et al., 2003; Wu et al., 2008) and activated in response to Flt3 activation or Flt3-ITD mutation (Okamoto et al., 2007). Using a model of Lyn-dependent CML, we observed strong repression of the miR181 family of miRNAs. Previously, low miR181 expression has been associated with poor prognosis in AML, whereas high miR181 (a-d) expression is prognostic for event free survival in AML patients (Marcucci et al., 2008; Schwind, 2009).

Although our studies primarily focused on miR181b, this family of miRNAs is highly conserved. Previous studies suggest Bcl-2 as a target for repression by miR181 microRNAs (Chen et al., 2010; Neilson et al., 2007). Comparison of the potential 3' UTR targeting sequence of Bcl-2 with that of Mcl-1 demonstrates a remarkable homology between these sequences, which includes conservation within the seed sequence. The seed sequence is a key region of complementary necessary for the targeting of miRNAs (Wang et al., 2010b). Our results demonstrated that mutation of the nucleotides complementary to the miR181 seed sequence in the 3' UTR of Mcl-1 ablated the effects of miR181b. Whether miR181 directly targets Bcl-2 has yet to be confirmed; however, this is would establish the importance of the miR181 family in the regulation of mitochondria-mediated apoptosis.

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Previous studies have identified multiple miRNAs that are altered in drug-resistant cancer, suggesting that some miRNAs have tumor suppressive effects, whereas others regulate cell survival (Hummel et al., 2010). A recent study profiled miRNAs from imatinib-resistant CML patients who did not have BCR/Abl mutations (San Jose-Eneriz et al., 2009). These authors identified 19 miRNAs that were differentially expressed between drug-resistant patients and drug-responders. In agreement with their results, we observed down-regulation of the expression of miR183 and two members of the Let7 family (Let 7a, Let7b). However, in contrast to studies reporting decreased miR10a expression in drug-resistant CML, we observed increased miR10a expression in the MYL-R cells. Many of the other miRNAs reported by San Jose-Eneriz et al. were either unchanged or not present on our arrays.

Previous studies have identified miRNAs that target Mcl-1 (Chen et al., 2009; Crawford et al., 2009; Mott et al., 2007), and we measured the expression of these miRNAs in our cell lines; however, the expression of these miRNAs were not implicated in the mediation of Mcl-1 regulation in MYL and MYL-R cells, suggesting that they may contribute to Mcl-1 expression in a cell-type specific manner. In addition to the miR181 family, we observed many unique differences in miRNA expression between MYL and MYL-R cells (miR10a, miR128a, miR132, miR150, miR155, miR183, miR196a, miR212). It is possible that some of these miRNAs may play a role in the mediation of drug resistance, and this will be a focus of future research.

Recently, Chen et al. demonstrated that miR181a over-expression sensitized cells to radiation (Chen et al., 2010). Moreover, Studzinski and colleagues have demonstrated that 1,25-D_3 treatment of HL-60 cells not only increases Lyn expression, but also

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decreases miR181 expression and increases Mcl-1 expression (Wang et al., 2000; Wang et al., 2009; Wang and Studzinski, 1997). This work was published independently over a course of a decade. Our study is in agreement with their data, and, importantly, we believe our study provides further mechanistic insight to these observations. Finally, Studzinski and colleagues determined that 1,25-D_3 treatment decreases sensitivity to drugs that induce apoptosis (Xu et al., 1993); this observation is in agreement with our data that suggests that Lyn-dependent loss of miR181 may contribute to the development of imatinib resistance.

While the mechanism of Lyn repression of miR181 is not known, miRNA expression can be regulated by both genetic and epigenetic mechanisms. For instance, the role of BCR-Abl in the silencing of miR328 in CML through a MAPK-dependent manner was recently shown (Eiring et al., 2010). Furthermore, activating mutations of the c-Kit kinase induce a MYC-dependent repression of miR29b in AML (Liu et al., 2010). In addition to transcription factor (CEBPα, Myc, etc.)-dependent mechanisms, kinases may regulate miRNA expression through post-transcriptional events, such as that observed with LIN28 (Khusial et al., 2009; Newman et al., 2008). Future studies will aim to determine the mechanism of Lyn-dependent effects on miR181 expression.

Molecular Pharmacology Fast Forward. Published on August 6, 2010 as DOI: 10.1124/mol.110.066258
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Footnote:

This work was supported by grants from the American Heart Association [09PRE2200044] and the National Institutes of Health [T32-GMOO8581-10].

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Legends for Figures:

Figure 1: Lyn kinase confers imatinib resistance in MYL-R cells. A) $2x10^6$ cells were lysed and Western blotted using the indicated antibodies (pY507, inhibitory phosphorylation site; pY397, autophosphorylation site). β-actin expression was similarly measured as a loading control and densitometry was performed. Representative data is shown. B and C) $1x10^6$ MYL-R cells were nucleofected with siRNA against Lyn or non-targeting control siRNA. B) Forty-eight hr later, cells were lysed and Western blot analysis was performed using antibodies against Lyn and β-actin and densitometry was performed. Representative data is shown. C) Cells were plated ($6x10^5$/well) in a 96-well plate and treated with imatinib (1.0 μM) or DMSO for the indicated time. Cells were then lysed and caspase 3 activity was measured. Data represent the mean +/- SEM of triplicate samples.

Figure 2: Loss of miR181 expression in MYL-R cells. A and B) MiRNA expression was profiled in MYL and MYL-R cells by qRT-PCR using the SuperArray™ cancer array. The heat map depicts fifteen miRNAs that had a >2-fold A) increase or B) decrease in expression in MYL-R cells in comparison to MYL cells. Arrows denote miR181 family members. Data represent duplicate experiments performed with triplicate samples. C) The fold change in miR181 expression in MYL-R cells in comparison to MYL cells. D) Alignment of human miR181 miRNAs (5'->3') using ClustalW software. An asterisk denotes a conserved nucleotide.

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Figure 3: Lyn kinase inhibits miR181 expression. A and B) $2x10^6$ MYL-R cells were treated for 24 hr with the indicated dose of dasatinib or DMSO. A) Total RNA was extracted from $1x10^6$ cells, and miR181b expression was measured. Data represent the mean +/- SEM of triplicate samples. B) $1x10^6$ cells were lysed, and Western blot analysis was performed using the indicated antibodies. Representative data is shown. C) $1x10^6$ MYL-R cells were nucleofected with siRNA against Lyn or non-targeting control siRNA. Forty-eight hr later, RNA was extracted and miR181b expression was determined. Data represent the mean +/- SEM of duplicate experiments performed with triplicate samples (**, p=0.0036). D-G) $2x10^6$ cells were treated for 24 hr with 1 nM dasatinib or DMSO. D-F) Total RNA was extracted from $1x10^6$ D) MYL-R, E) K562-R(1), and F) K562-R(2) cells, and miR181b and miR181d expression was measured. Data represent the mean +/- SEM of duplicate experiments performed with triplicate samples. G) $1x10^6$ cells were lysed, and Western blot analysis was performed using the indicated antibodies. Representative data is shown.

Figure 4: Mcl-1, a predicted target of miR181, is over-expressed in MYL-R cells. A) Diagram of the predicted miR181 binding site in the Mcl-1 3' UTR (www.targetscan.org). B) $2x10^6$ cells were lysed, and Western blot analysis was performed with the indicated antibodies. Samples were loaded in duplicate. Representative data is shown. C) $2x10^6$ MYL-R cells were treated for 24 hr with the indicated dose of dasatinib or DMSO. Cells were lysed and Western blotted with the indicated antibodies. Representative data is shown.

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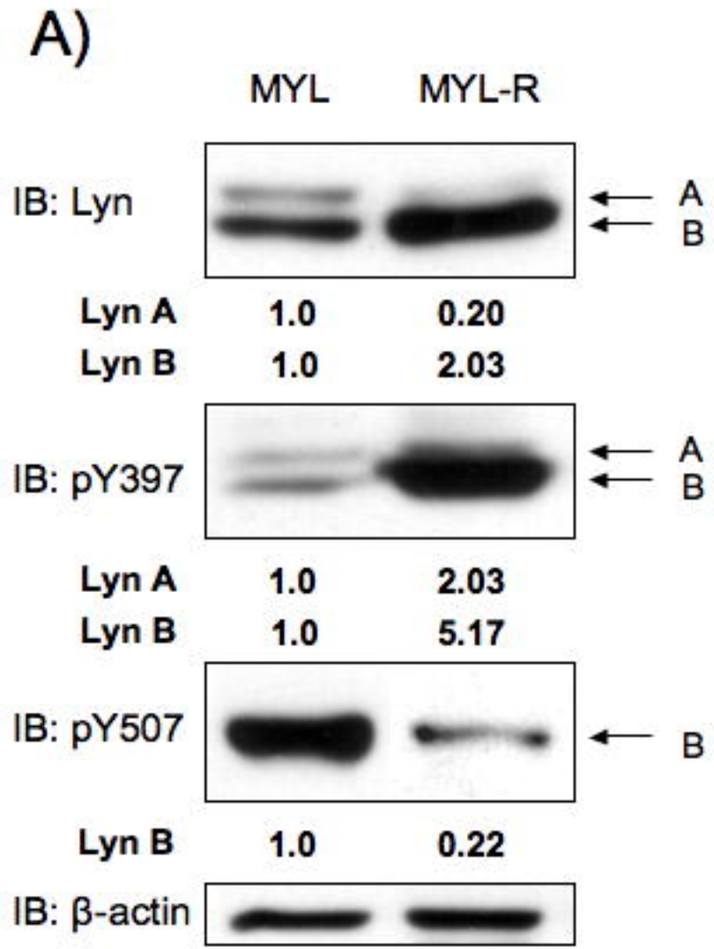
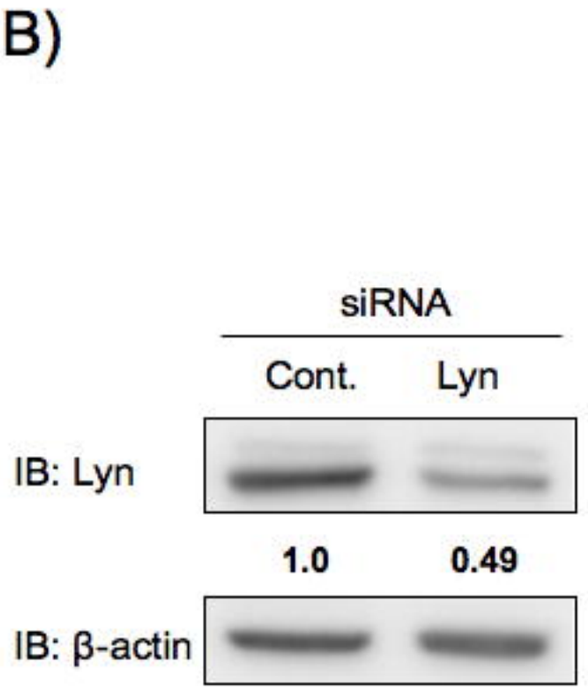
Figure 5: MiR181b inhibits Mcl-1 expression. A) $1x10^6$ MYL-R cells were nucleofected with the SDSA 3.0 miR181b vector or empty vector (SDSA 3.0). Forty-eight hr later, cells were lysed, and Western blot analysis was performed with the indicated antibodies. Representative data is shown. B) Diagram of luciferase constructs containing the Mcl-1 3' UTR. Alignment of miR181b with the WT and mutant Mcl-1 3' UTR sequences. An asterisk denotes a nucleotide mutation in the predicted miR181b binding site. C) HEK293T cells were co-transfected with the indicated SDSA 3.0 and psicheck Mcl-1 3' UTR vectors. Twenty-fours hr later, cells were lysed, and luciferase activity was measured. Data represent the mean +/- SEM of duplicate experiments performed with triplicate samples (***, $p<0.001$). E) Western blot analysis was performed with the indicated antibodies. Representative data is shown.

Figure 6: Enhancement of Lyn expression in HL-60 cells decreases miR181b expression and increases Mcl-1 expression. A and B) $1x10^6$ HL-60 cells were treated with 10 ng/ul 1,25-D_3 for the indicated time. A) Total RNA was extracted from cells, and miR181b expression was measured. Data represent the mean +/- SEM of triplicate samples. B) Cells were lysed, and Western blot analysis was performed using the indicated antibodies. Representative data is shown.

Figure 1





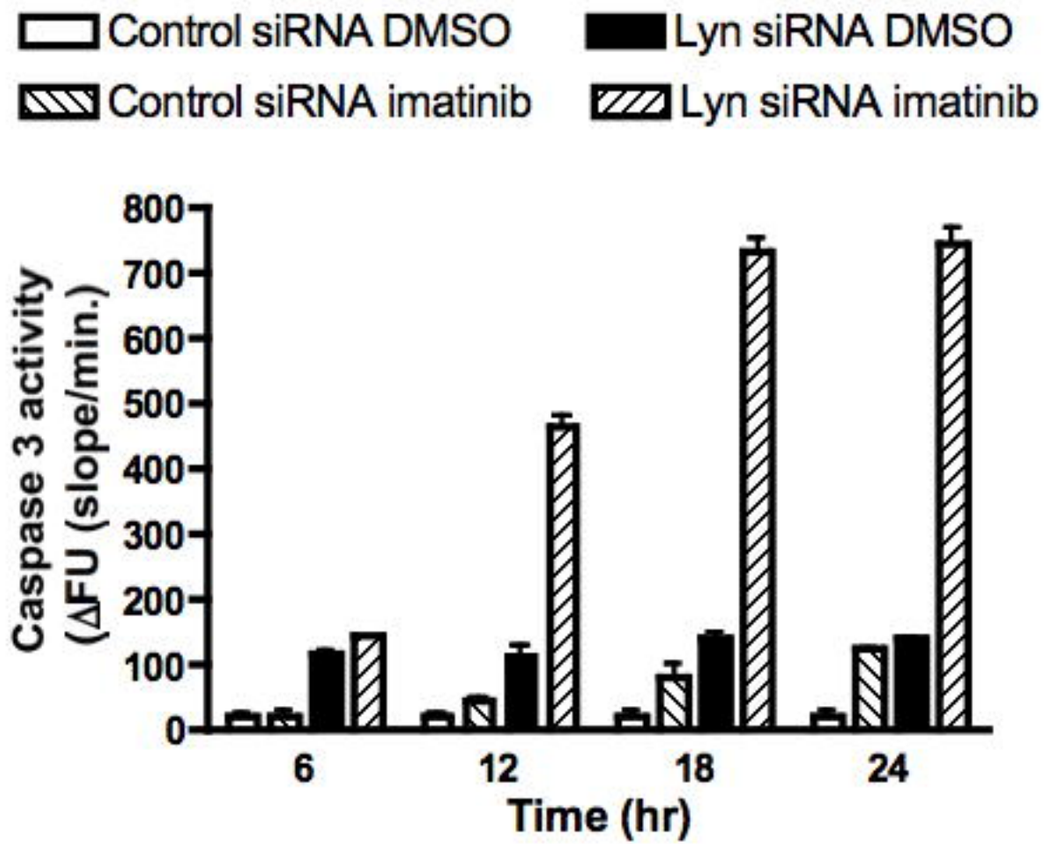
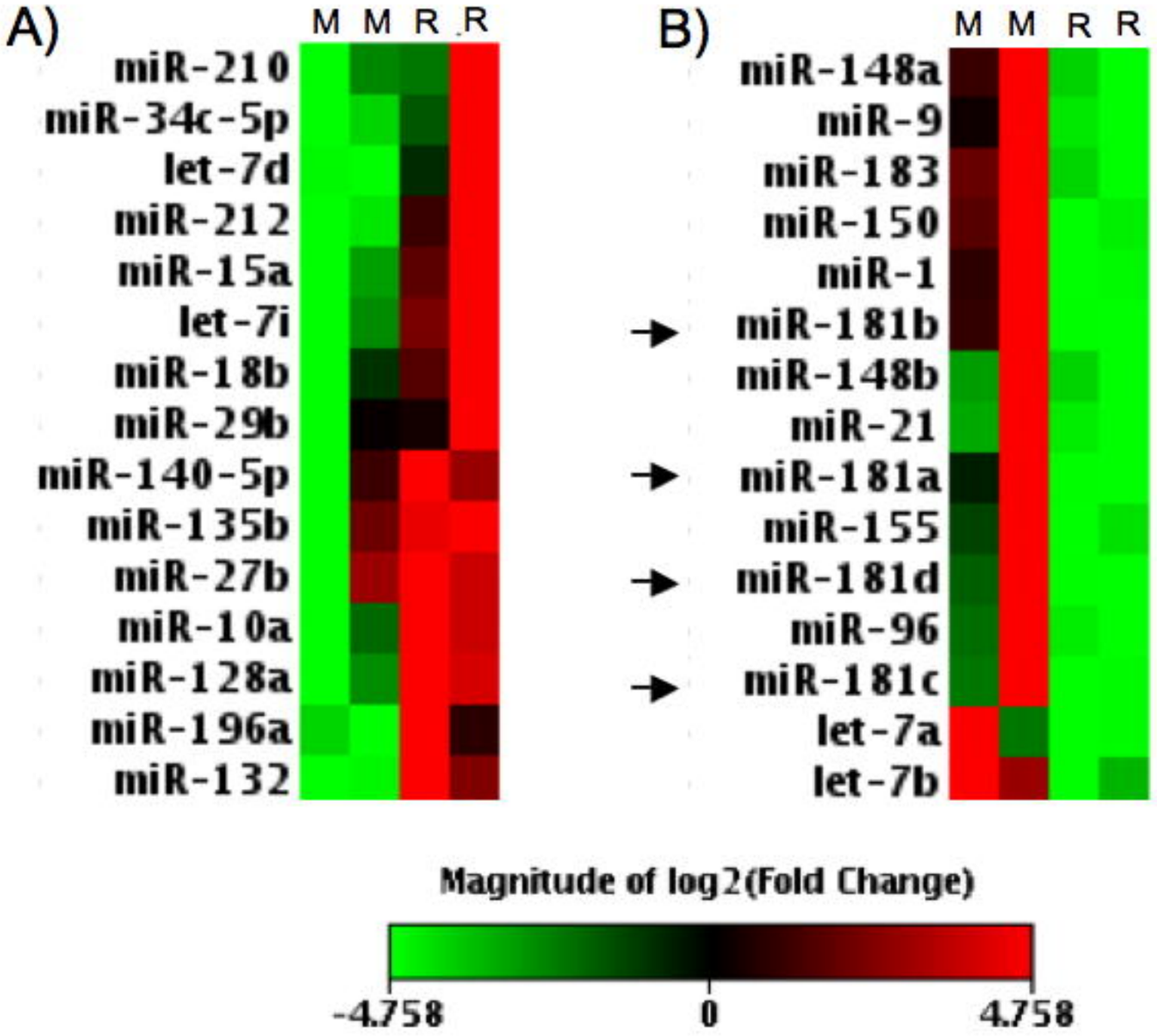


Figure 2



C)

microRNA	MYL-R Fold change
181a	-11.21
181b	-13.59
181c	-12.95
181d	-25.78

D)

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hsa-miR-181b      AACAUUCAUUGCUGUCGGUGGGU
hsa-miR-181d      AACAUUCAUUGUUGUCGGUGGGU
hsa-miR-181a      AACAUUCAACGCUGUCGGUGAGU
hsa-miR-181c      AACAUUCAAC-CUGUCGGUGAGU
                  ********    ******** **
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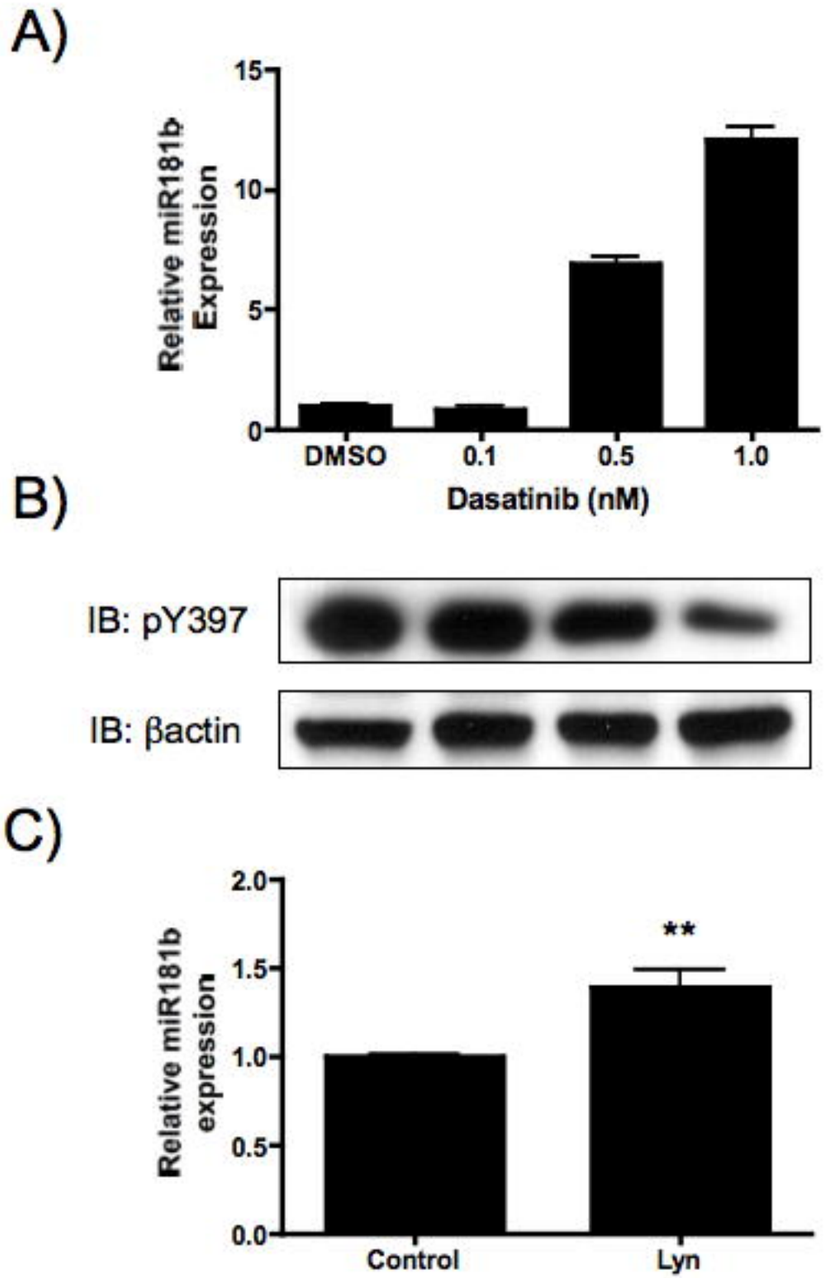
Figure 3



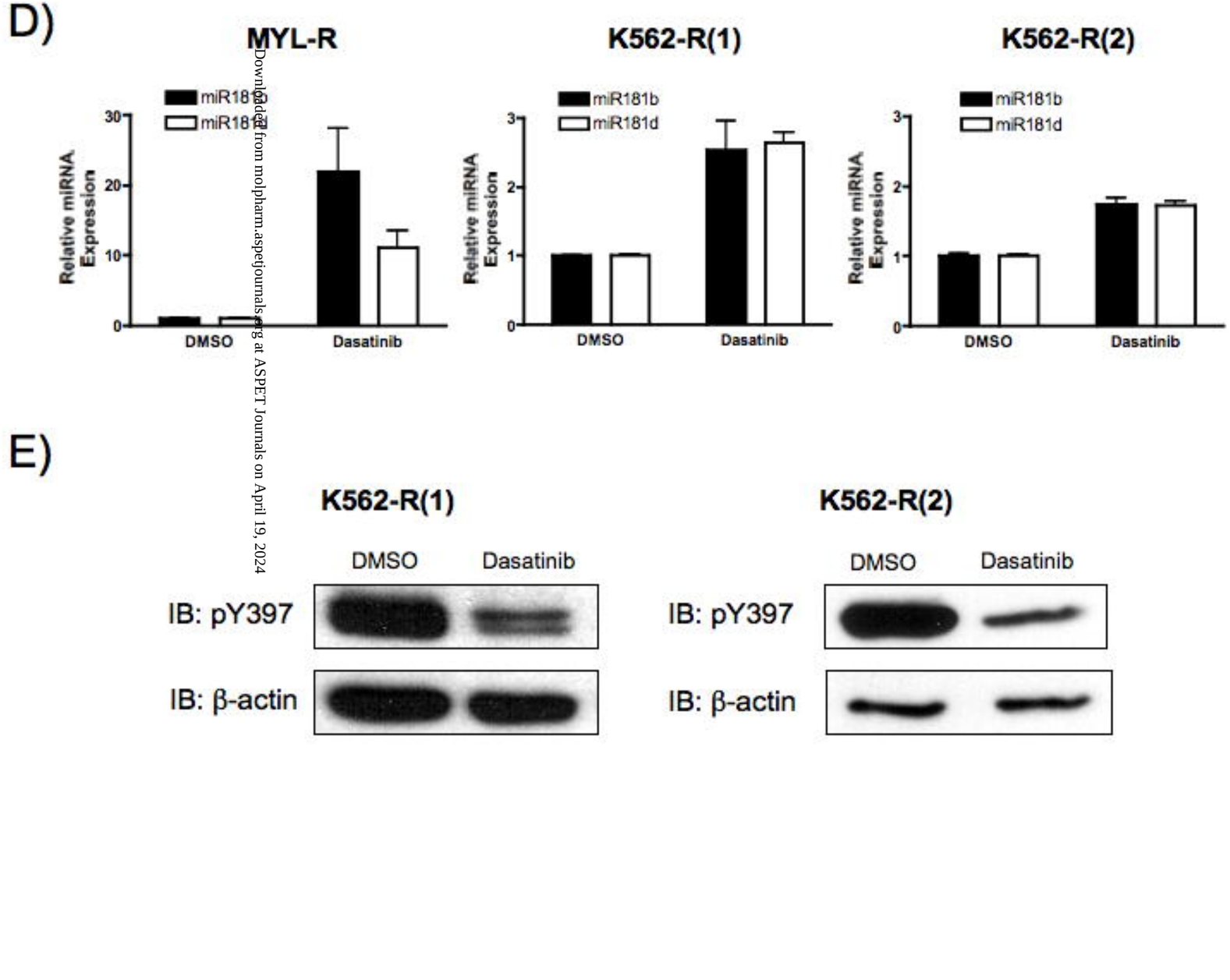
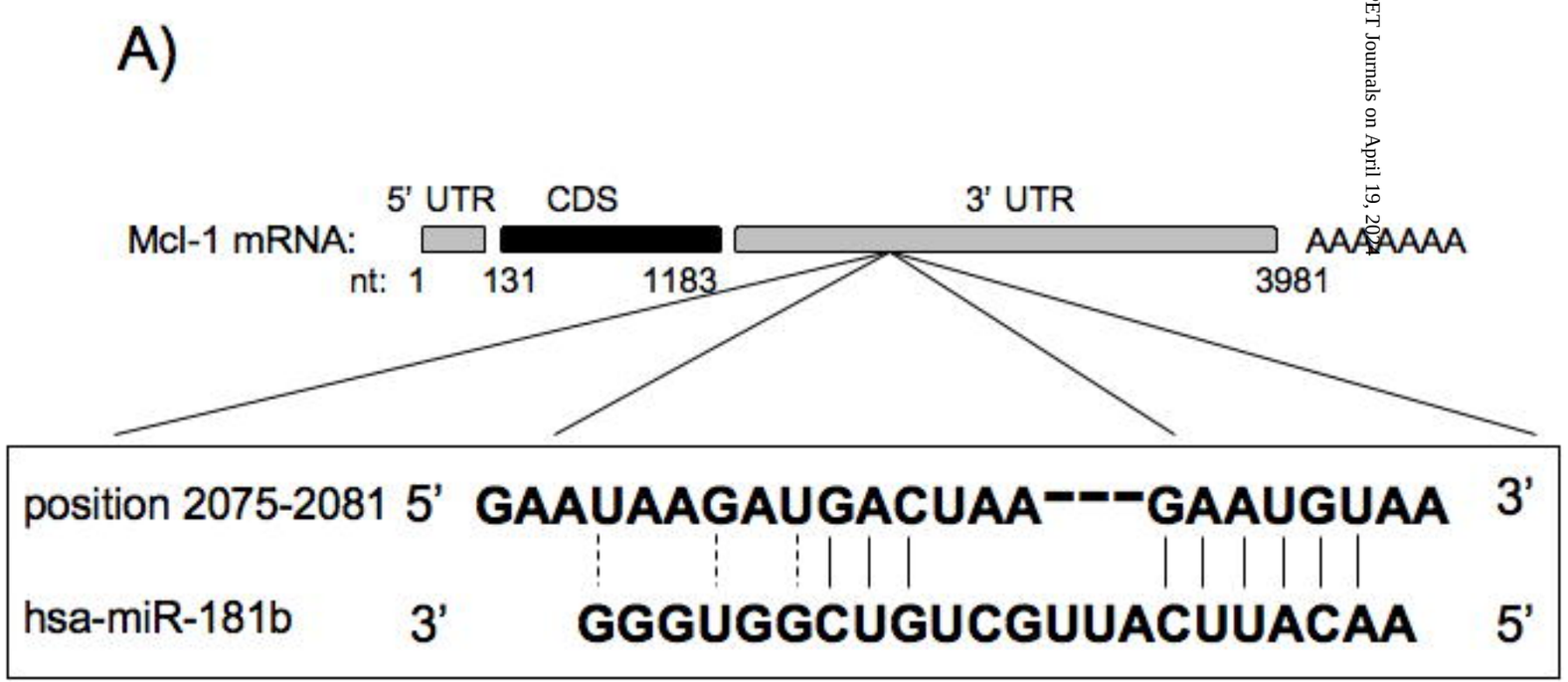
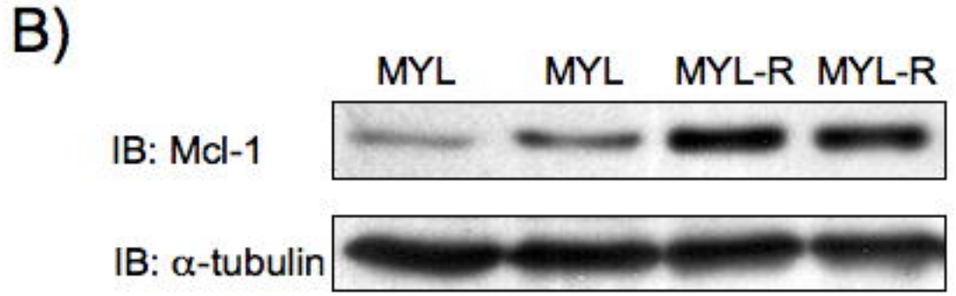


Figure 4





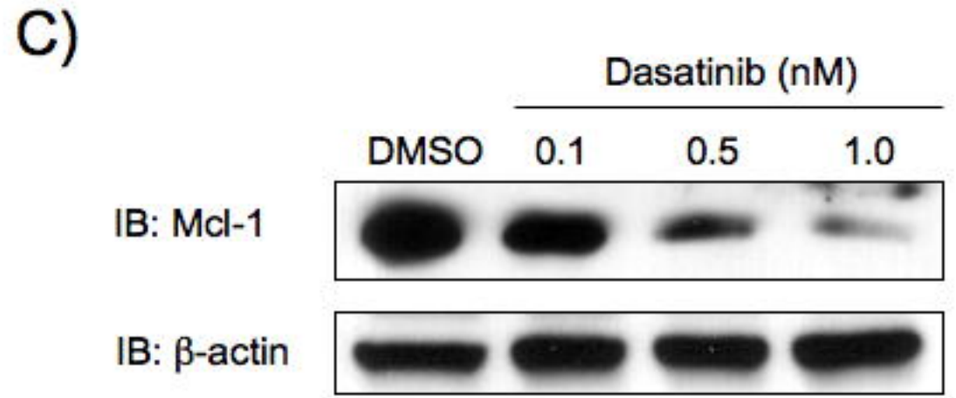


Figure 5

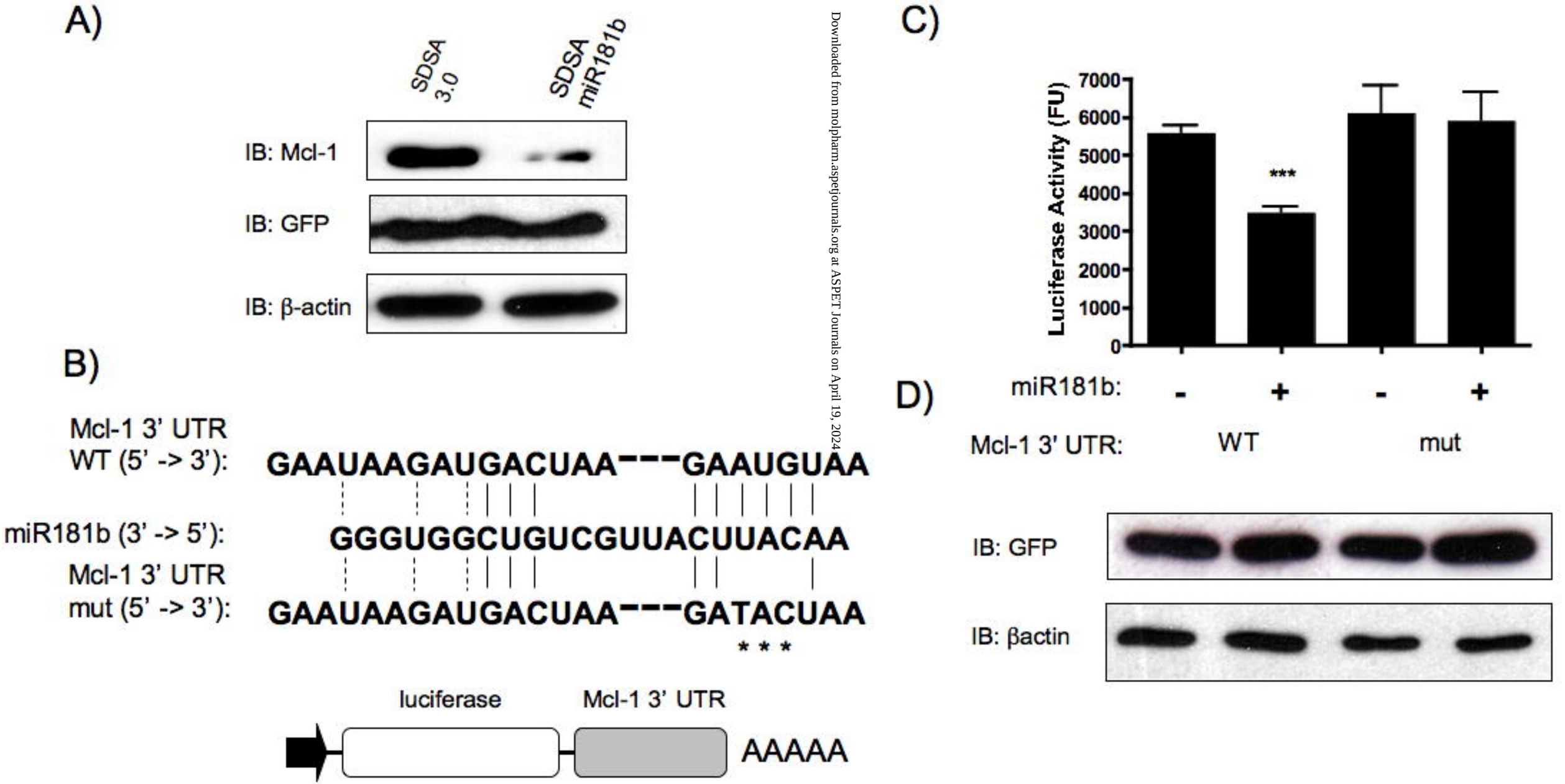
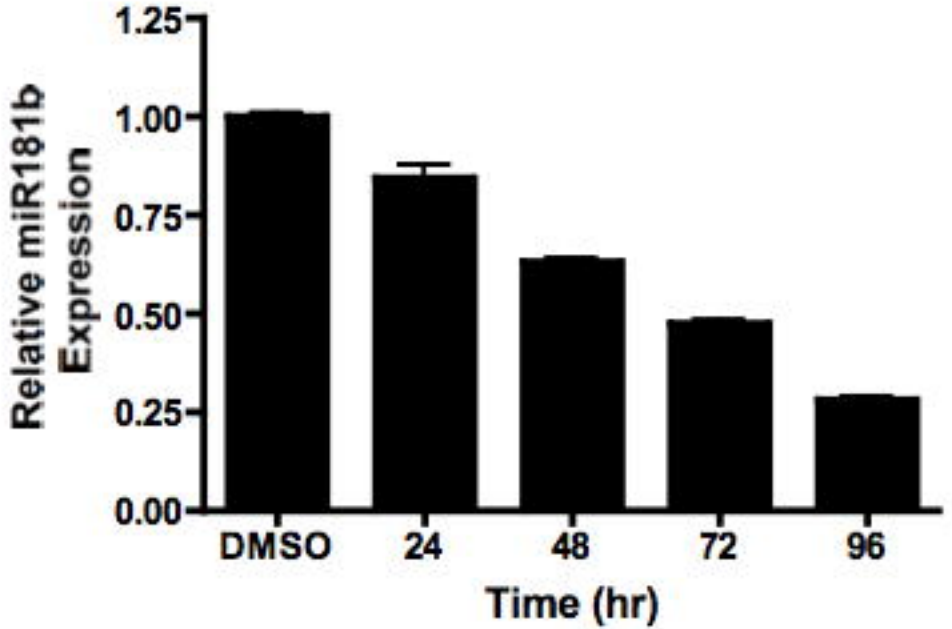


Figure 6

A)



B)