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A methylation-based regulatory network for microRNA 320a in chemoresistant breast cancer

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Running title: miR-320a mediates chemoresistance

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Nonstandard abbreviations used: TRPC5: transient receptor potential channel C5; NFATC3: nuclear factor of activated T cells isoform c3; miR-320a: microRNA 320a; ETS-1, v-ets erythroblastosis virus E26 oncogene homolog 1; ADM: adriamycin; PTX: paclitaxel.

Abstract

We previously demonstrated that the over-expression of transient receptor potential channel C5 (TRPC5) and nuclear factor of activated T-cells isoform c3 (NFATC3) are essential for cancer chemoresistance, but how TRPC5 and NFATC3 are regulated was still unclear. In this study, microRNA 320a (miR-320a) was found to be down-regulated in chemoresistant cancer cells. MiR-320a directly targeted TRPC5 and NFATC3, and down-regulation of miR-320a triggered TRPC5 and NFATC3 over-expression. In chemoresistant cells, down-regulation of miR-320a was associated with regulation by methylation, which implicated promoter methylation of the miR-320a coding sequence. Furthermore, the transcription factor v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS-1), which inhibited miR-320a expression, was activated in chemoresistant cancer cells; such activation was associated with hypomethylation of the ETS-1 promoter. Lastly, the down-regulation of miR-320a, and high expression of TRPC5, NFATC3, and ETS-1 were verified in clinically chemoresistant samples. Low expression of MiR-320a was also found to be a significant unfavorable predictor for clinic outcome. In conclusion, miR-320a is a mediator of chemoresistance by targeting TRPC5 and NFATC3. Expression of miR-320a is regulated by methylation of its promoter and that of ETS-1.

Key words: miR-320a; chemoresistance, methylation

Introduction

Chemoresistance is one of the main causes of failure in chemotherapy. One well-demonstrated mechanism for the acquisition of chemoresistance is the over-expression of drug transporters in cancer cells (Li et al., 2007;Liu et al., 2005). The p-glycoprotein (p-gp) encoded by the *ABCB1* gene is typical of these. P-gp is frequently associated with drug-resistant phenotypes, so understanding its mechanism of over-expression may provide promising strategies for overcoming chemoresistance. In our previous study, we found that over-expression of the *ABCB1* gene in chemoresistant cancer cells is controlled by transient receptor potential channel C5 (TRPC5) (Ma et al., 2012), which can be used as an chemoresistance marker in breast cancers (Ma et al., 2014). TRPC5 is highly expressed in chemoresistant human breast cancer cells, induces the activation of nuclear factor of activated T-cells isoform c3 (NFATC3), and NFATC3 directly stimulates *ABCB1* gene expression. However, it is not clear how the expression of TRPC5 and NFATC3 are regulated in chemoresistant cancer cells.

Alterations of tumor suppressors and proto-oncogenes can be mediated by abnormalities in the quality or quantity of microRNAs (miRNAs), single-stranded 19–25 nucleotide short RNAs that modulate gene expression at the post-transcriptional stage by targeting mRNAs, and the binding sites commonly occur within the 3'-untranslated region (UTR) of mRNAs. In chemoresistant cancer cells, miRNAs regulate features closely associated with chemoresistance, including apoptosis, chemotherapeutic agent response, DNA damage repair, cell cycle, stemness

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transition, and mesenchymal transition in cancer cells (Kutanzi et al., 2011;Sarkar et al., 2010),

Quantitative defects in miRNAs are mediated by several mechanisms and recent studies have shown the involvement of methylation events (Saito and Jones, 2006). Human DNA methylation usually occurs at the CpG dinucleotide, and CpG islands (CpGis) are regions with a high density of these dinucleotides. Methylation at CpGis in promoter regions usually leads to gene suppression. Because the expression of miRNA is controlled by either the promoters of their host genes or separate promoters of their own, silencing or activating miRNAs by promoter hyper- or hypo-methylation is emerging as a mechanism of producing quantitative miRNA defects. However, relatively little is known about the transcriptional regulation of miRNA genes. To date, among the >100 miRNAs associated with cancer, only a few studies have characterized their regulation by methylation (Li et al., 2010;Datta et al., 2008;Furuta et al., 2010;He et al., 2011;Alpini et al., 2011;Wong et al., 2011), and to the best of our knowledge, few of these miRNAs have been directly demonstrated to be associated with chemoresistance.

Therefore, in this study, we set out to explore the dysregulation of miRNA by methylation in chemoresistance. Based on our previous study (Ma et al., 2012), we found that decreased expression of miR-320a regulated both TRPC5 and NFATC3 up-regulation, as well as the chemoresistance in adriamycin (ADM)-resistant breast cancer cells (MCF-7/ADM). The decrease of miR-320a was associated with hypermethylation of its promoter and that of its suppressor v-ets erythroblastosis virus

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E26 oncogene homolog 1 (ETS-1). Dysregulation of miR-320a was found in clinically chemoresistant samples and was associated with an unfavorable outcome of chemotherapy.

Materials and methods

Cell cultures

MCF-7/WT cells were purchased from the ATCC (USA), and HCT-8 and fluorouracil (5-FU)-resistant HCT-8 cells were purchased from Keygen Biotech (China). Adriamycin (ADM)- and paclitaxel (PTX)-resistant human breast cancer cells (MCF-7/ADM and MCF-7 PTX) were derived as previously described (Ma et al., 2012) by treating MCF-7 cells with stepwise increasing concentrations of ADM and PTX over 8 months. All cells were cultured in RPMI supplemented with 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin.

Identification of miRNAs targeting TRPC5 and NFATC3

To search for the miRNAs targeting TRPC5 and NFATC3, Targetscan and miRDB software (www.targetscan.org; www.mirdb.org/) were applied together.

To verify the binding of miRNA-320a to TRPC5 and NFATC3, the 3'-UTR regions of TRPC5 and NFATC3 mRNAs were cloned into the dual-luciferase reporter vector pmirGLO (Promega, USA) with the following primers. Sequences in capitals are complementary to the TRPC5 or NFATC3 3'-UTR on mRNA; lowercase letters are the restriction enzyme recognition sequence: TRPC5 forward: 5' cacaactcgagTGCTCTCAGAGGTGACACAG3'; reverse: 5'aaggatccTGAGTTAGGAATGTAGCCCA-GG3'; NFATC3 forward:

5'taattctaggcgatcgctcgagGGTGACTCTGAATGGTGGGG3'; reverse: 5' gcggccagcggccgcggatccAACTGAGGAGCCTCAACTGC 3'.

HEK293 cells were then transfected with luciferase reporter vector by Lipofectamine™ 2000 (Invitrogen, USA) together with a mimic of endogenous miR-320a (miR-320a mimic, RiboBio Co., Ltd, PRC), or with scrambled miRNA mimic (control). Luciferase activity was assessed with a Dual-Luciferase Reporter Assay System kit (Promega, USA).

qRT-PCR and real-time PCR

miRNA expression was analyzed by qRT-PCR with an All-in-One™ miRNA qRT-PCR detection kit (GeneCopoeia, USA). Briefly, total RNA was extracted with TRIzol (Invitrogen, USA) and reverse-transcribed in the presence of a poly-A polymerase with an oligo-dT adaptor. qRT-PCR was then performed with miR-320a primers (MIMAT0000510, GeneCopoeia, USA) at 58°C for 20 s, 72°C for 10 s, 40 cycles with an iQ5 Real Time PCR Detection System (Bio-Rad, USA). The expression of miR-320a was normalized to that of U6 small RNA.

mRNA expression was analyzed by real-time PCR. RNAs were reverse-transcribed by Superscript II reverse transcriptase (Invitrogen, USA). Real-time PCRs were performed using IQTM SYBR Green Supermix (Bio-Rad, USA) for 40 cycles of 95°C for 15 s and 60°C for 40 s. The primer pairs for TRPC5 were: forward: 5'CCACCAGCTATCAGATAAGG3', reverse 5'CGA AACAAGCCACTTATACCC3' (Liu et al., 2007); for NFATC3 were: forward 5'GTCAGGCCTTGGCCCTGCAG3', reverse 5'CGCTGGGAGCACTCAACG

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GG3' 3'; and for ETS-1 were: forward 5'CTGCGCCCTGGGTAAAGA3', reverse: 5'CCCATAAGATGTCCCCAACAA3'(Chan et al., 2011).

Western blot

Whole-cell lysates of treated cells were prepared and separated by 10% SDS-PAGE. The antibody binding was evaluated with an Odyssey imaging system (LI-COR Biosciences, USA). Anti-TRPC5, NFATC3, and ETS-1 mAbs were from Abcam (USA).

IC_{50} of ADM

MCF-7/ADM, PTX, and HCT-8/5-FU cells were transfected with miR-320a mimic, seeded into in 96-well plates, and treated with different concentrations of ADM, PTX, or 5-FU for 48 h. To measure the number of viable cells, the supernatant was discarded and 100 μL of MTT solution (0.5 mg/ml) was added to form formazan crystals, which were later dissolved in solubilization solution. Absorbance was read at 570 nm to calculate the IC_{50} by nonlinear regression with GraphPad prism software.

Construction of the TRPC5 over-expression vector

The 3'UTR region of the TRPC5 gene was cloned into pcDNA3 plasmid and mutated with the Hieff Mut^{TM} Site-Directed Mutagenesis Kit (Yeasen, Shanghai, China) at the site of miR-320a targeting. The coding sequence of the human TRPC5 gene was then cloned and fused with the mutated 3'UTR region.

5'RACE assay analysis of the transcriptional start site (TSS) of miR-320a

Total RNA was extracted from MCF-7/WT cells with TRIzol (Invitrogen, USA). Alkaline phosphatase and tobacco acid pyrophosphatase were used to remove the free

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5'-phosphates and cap structures of RNA. The decapped RNA was ligated to RNA adapter oligonucleotide and reverse-transcribed. Nested PCR was applied at 55°C for 30 s and 72°C for 2 min for 25 cycles; the primers were: 5'TCATCCTTTTTCGCCCTCTC3' for the outer gene-specific primer; and 5'ACTCCGGGAAGAACCGGGAAG 3' for nested gene-specific primer.

The PCR products were separated on 3% agarose gel, inserted into pUC19-T plasmid, and transfected into the DH5α *E. coli* system on LB plates (100 μg/ml ampicillin with IPTG and X-Gal). Three *E. coli* colonies were selected randomly. Then plasmids were extracted, digested by EcoRI/HindIII, and separated on 3% agarose gel to select the correctly-cloned 5'RACE sequence. The selected 5'RACE sequence was sequenced and blasted against the human genome sequence.

Cloning and activity analysis of miR-320a promoter

Genomic DNA was extracted from MCF-7/WT cells with a Wizard Genomic DNA purification kit (Promega, USA), from which the putative promoter at chr8: 22,103,522-5,556 was amplified at 95°C for 30 s, 58°C for 30 s, and 68°C for 2 min 40 s for 30 cycles with the primers: forward: 5'cggggtaccGTCTGAACTGAAATTCACTGATTCCTTC3'; reverse: 5'ccgctcgagCCTAGGTATGGCATTCGAGGCTC3' (lowercase letters: restriction enzyme-recognition sequences of KpnIF and XhoIR). The PCR products were separated on 3% agarose gel and gel-purified (DongSheng Biotech, PRC). The purified PCR product was then cut by the KpnIF/XhoIR system, inserted into pGL3 basic plasmid (Promega, USA), transfected into the DH5α *E. coli* system, and

amplified on LB plates (100 μg/ml ampicillin). Seven positively-transfected *E. coli* colonies were selected randomly and the insertion of PCR product was verified by KpnIF/XhoIR cutting followed by 1% agarose gel separation. The separated PCR product was then sequenced and blasted against the human genome sequence. pGL3 plasmid with correct insertion of the promoter region was later transfected into HEK293 cells with Lipofectamine™ 2000 (Invitrogen, USA) and luciferase activity was assessed with a Dual-Luciferase Reporter Assay System kit (Promega, USA).

Hypomethylation of miR-320a

MCF-7/ADM cells were treated with 5 μM 5-aza-2'-deoxycytidine (DAC, Sigma, USA) for 48 h, then the RNA or protein was extracted for qRT-PCR or western blot.

ETS-1 targeting of the miR-320a promoter

The promoter and 5'-UTR region were cloned together into pGL3 basic plasmid as described above with the primers forward: 5' cggggtaccGTCTGAACTGAAATTCACTGATTCCTTC3', reverse: 5' ccgctcgagGCCGCCTGATAAATACTGTGG3'. The cloned region was verified by sequencing and blasting, pGL3 plasmid with correct insertion of the promoter region was later transfected together with pcDNA3 plasmid over-expressing ETS-1 into HEK293 cells with Lipofectamine™ 2000 (Invitrogen, USA), and the luciferase activity was assessed by the Dual-Luciferase Reporter Assay System kit (Promega, USA).

Bisulfite sequencing PCR (BSP) of miR-320a and ETS-1 promoters

CpG plot online software (https://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/)

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was used to analyze CpGis on promoters.

Total DNA was extracted (Wizard genomic DNA purification kit, Promega, USA) and bisulfite-converted by an EpiTect Bisulfite Kit (Qiagen, USA). The DNA was then amplified by PCR at 95°C for 30 s, 60°C for 30 s, and 72°C for 40 s for 30 cycles with the miR-320a primers: forward: 5'TGGATTAYGAGGTTAGGAGTT3'; reverse: 5'ACCYCTCCTAACCYAATA3'; ETS-1 outer: forward: 5'ATGTTGTTATTGGGAGGGGGY3'; reverse: 5'ATTCCCRCTACCTTTCTTTCC3'; ETS-1 nested: forward: 5'GGGGTTTTAYGTATTGT3', 5'TTCRCTCTCRATCTCC (because CpG sites are unavoidable in a primer, degenerate bases 'Y' represent 'C' and 'T' in forward primers, 'R' represent 'G' and 'A' in reverse primers). The PCR products were cloned into pDM19-T plasmid (Takara, USA) and sequenced.

Chromatin Immunoprecipitation (ChIP) Assay

The binding sites of ETS-1 on miR-320a were predicted by Jaspar online software (jaspar.genereg.net/); three sites (S1-S3, supplemental file 4) showed the highest probability of binding, which was later confirmed using a ChIP Assay kit (Beyotime, China). Briefly, MCF-7/ADM cells in T75 disks were fixed with 1% formaldehyde for 10 min at 37°C followed by neutralization with 125 mM glycine. The cells were then lysed on ice in SDS lysis buffer supplemented with 1 mM PMSF (Beyotime, ST506). Then the lysate was sonicated on ice (10-s pulse, 60-s break, seven times, 10% amplitude; Sonics) to break the genome into 200-1000 bp fragments. Two micrograms of primary antibody (ETS-1, Santa Cruz, USA, or pre-immune IgG

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as negative control) was added to 2 ml of Protein G-pretreated lysate to allow precipitation of ETS-1-associated genomic DNA fragments by overnight rotation at 4°C, then washed with different buffers. The DNA-protein cross-links were reversed and chromatin DNA was purified and subjected to PCR analysis. The primer pairs for S1 were forward 5'-GGCGTTTCCTTCCGACATGTTGCCT-3', reverse 5'-AAAGGCTCTCACCTGGGTGGTCC-3'; S2 forward 5'-TCATCCTTTTTCGCCCTCTCAACC-3', reverse 5'-ATCTTGGCGCGGGGCGGAAGTGACGTTA-3'; S3 forward 5'-CTGATAAATACTGTGGCCCAGTCC-3', reverse 5'-CACAACCTCACCTGCAACGCGACCA-3'.

Clinical sample collection and fluorescence *in situ* hybridization (FISH) of miRNA

Each patient involved in this study provided written informed consent. All procedures for enrolling patients or sample analysis were conducted with the approval of the Ethics Committees of the University and Hospital. Tissue samples from biopsy and surgical resection were collected from female breast cancer patients receiving anthracycline-taxane-based neoadjuvant chemotherapy in Wuxi Cancer Hospital (The Affiliated Hospital of Jiangnan University, Wuxi, Jiangsu, China). Patients were stratified according to chemotherapeutic response. Tumor responders were defined as having a reduction in total tumor size (breast primary + axillary nodes) by ≥90% at the time of surgery; reduction by <10% were defined as non-responders.

Samples were embedded in TissueTek OCT (Sakura, Japan), frozen, and cut into

8 μm sections. The sections were washed with PBS and permeated with 0.4% TritonX-100. TE buffer (100 mM Tris-HCl, 50 mM EDTA, 1 μg/ml Proteinase K) was used to expose the miRNAs in sections and stopped by 4% PFA. To reduce non-specific signals, cells were washed with 0.25% acetic anhydride. 5'TAMRA-labeled miRNA detection probe against has-mir-320a (20 nM) was hybridized with the sections at 54°C for 16 h and treated with NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, 20 μg/ml RNase A) to stop the reaction. The sections were then stained with DAPI (4', 6-diamidino-2-phenylindole). Fluorescence was then detected under a confocal system (Nikon, Japan). The miRNA expression was determined by the intensity of staining (no staining, 0; weak staining, 1; moderate staining, 2; strong staining, 3) multiplied by the extent of staining (0%, 0; 1-24%, 1; 25-49%, 2; 50-74%, 3; 75-100%, 4) (Pan et al., 2011).

Immunohistochemistry

Immunohistochemistry was performed on TissueTek OCT-frozen sections using either TRPC5 or NFATC3 mAb (Abcam, USA); nuclei were identified by DAPI. Antibody reactivity was detected by the HRP/DAB system or FITC fluorescence to determine the expression of TRPC5 and NFATC3 respectively; the staining score was calculated as described above.

Statistical analysis

All data are expressed as mean ± SEM (n = 3). Student's t test was used for analysis ($p < 0.05$ was considered statistically significant). Non-linear regression was used to analyze the dose-response to ADM. IC_{50}s were calculated by GraphPad Prism.

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Kaplan-Meier estimators of the survival, area under the receiver operating characteristic curve (AUC), and Spearman correlation were analyzed using SPSS.

Results

Targeting of TRPC5 and NFATC3 by miR-320a

Based on our previous findings that TRPC5 is critical for the induction of chemoresistance by activating NFATC3 and thus triggering p-gp expression (Ma et al., 2012), we predicted the potential miRNAs targeting TRPC5 and TRPC5-related pathways by applying online Targetscan and miRDB software together. miR-320a was selected because it was predicted to target both TRPC5 and NFATC3 (Figure 1 A). The luciferase reporter assay was then used to verify this prediction. We found that miR-320a suppressed the expression of luciferase by targeting the 3'-UTRs of the TRPC5 and NFATC3 mRNAs, and mutation of the 3'-UTRs destroyed the homology with miR-320a at its target site (Figure 1 B). The theoretical binding sites predicted by the online software were not totally supported in a real biological system. Indeed, the interaction between miR-320a and NFATC3 was not affected by mutation in the 3rd binding site, so this predicted site of miR-320a binding does not exist. Therefore, these data suggest that TRPC5 and NFATC3 are targeted by miR-320a.

The interactions between miR-320a and its targets were then confirmed in chemoresistant MCF-7/ADM cells, which express high levels of TRPC5 and NFATC3 compared with MCF-7/WT cells (Ma et al., 2012); Figure 1 C). MCF-7/ADM cells were transfected with an miR-320a mimic designed to

over-express the endogenous miRNA (miR-320a mimic), and the expression of TRPC5 and NFATC3 were analyzed. When the mimic was introduced into MCF-7/ADM cells, this forced expression of miR-320a induced down-regulation of TRPC5 and NFATC3 at both the mRNA and protein levels (Figure 1 C and D).

MiR-320a modulates chemoresistance

To define the role of miR-320a in chemoresistance, qRT-PCR was performed and showed that miR-320a expression was significantly decreased in MCF-7/ADM cells compared with their parental control MCF-7/WT cells (Figure 2 A), suggesting that reduction of miR-320a contributes to the high expression of TRPC5 and NFATC3 in MCF-7/ADM cells.

Previously, we had demonstrated that suppression of the high TRPC5 and NFATC3 activity reduces the chemoresistance of MCF-7/ADM cells (Ma et al., 2012). Therefore, we hypothesized that over-expression of miR-320a, which down-regulated TRPC5 and NFATC3, may also play a role in reducing chemoresistance. To test this hypothesis, MCF-7/ADM cells were transfected with the miR-320a mimic. We found that over-expression of miR-320a sensitized the cells to ADM; the half-maximal inhibitory concentration (IC_{50}) of ADM decreased in MCF-7/ADM cells when miR-320a was over-expressed (Figure 2 B). Furthermore, a TRPC5 over-expression vector was constructed by fusing the coding sequence of TRPC5 with its 3'UTR in pcDNA3 vector, in which the 3'UTR was mutated so that the miR-320a could not target the mRNA of TRPC5. When the MCF-7/ADM cells were transfected with the empty vector, the miR-320a mimic decreased their chemoresistance (Figure 2 C, 1st

and 2nd bars), suggesting that the empty vector does not influence the chemoresistant feature. In addition, over-expression of TRPC5 increased the chemoresistance slightly compared with the empty vector ($p = 0.36$) (Figure 2 C, 1st and 3rd bars), suggesting the chemoresistance cannot increase indefinately with the over-expression of TRPC5, probably because distribution of TRPC5 in the plasma membrane is already overloaded in MCF-7/ADM cells. When the miR-320a mimic was transfected into the cells with mutated TRPC5, the chemoresistance could no longer be decreased (Figure 2 C, 3rd and 4th bars). On one hand, this result suggested that the mutated TRPC5 retains its original activity in mediating chemoresistance; when the endogenous TRPC5 is targeted by the miR-320a mimic, the TRPC5 with a mutated 3'UTR from the over-expression vector could act as a substitute. On the other hand, it suggested that miR-320a modulates chemoresistance *via* the TRPC5 signaling pathway.

Therefore, these data suggested that down-regulation of miR-320a is involved in the development of chemoresistance.

Down-regulation of miR-320a is associated with methylation in its promoter region

We next studied the mechanism of miR-320a down-regulation in chemoresistant MCF-7/ADM cells. Because miRNAs are transcribed from the genome by RNA polymerase II (Lee et al., 2004) and controlled by promoter elements that show great similarity to protein-coding genes (Rodriguez et al., 2004;Saito and Jones, 2006), the expression of miRNAs can be controlled by methylation of their promoter regions (Saito and Jones, 2006). The down-regulation of miR-320a suggested that it might be

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suppressed by methylation. To further clarify the mechanism, we investigated the methylation status of miR-320a.

miRNAs are either located within the introns of protein-coding genes or in intergenic regions, and are transcribed from promoters of the host gene or their own promoters. We found that no protein-coding genes overlapped with the coding sequence of miR-320a (miRBASE database), so this miRNA might have its own promoter. Therefore, the promoter region of miR-320a was identified by locating the transcriptional start site (TSS) with 5'-rapid amplification of cDNA ends (5'-RACE, Supplemental figure 1 A). The 5'RACE product was cloned into pUC19-T plasmid and transfected into the DH5α *E. coli* system for amplification and DNA sequencing. Three positively-transfected *E. coli* colonies were selected randomly and the amplified pUC19-T was cut with restriction enzymes. The second colony (Lane 3 in Supplemental figure 1B) was chosen because two clear bands were generated after enzyme cutting of pUC19-T within the colony (lane 4 in Supplemental figure 1B)); one band was the empty pUC19-T plasmid, while the other was the 5'RACE product. The enzyme-cutting results of the second colony not only suggested a successful 5'RACE assay that generated a product of ~1000 bp, but also indicated that the pUC19-T plasmid was correctly cloned with the 5'RACE product, which was necessary for reliable DNA sequencing in the subsequent processes. The 5'RACE products that were cloned within the pUC19-T plasmid were then sequenced and blasted with the human genome (Supplemental file 1, Tables S1.1 and S1.2). The TSS was then identified as the nucleobase guanine at 965 bp upstream of the miR-320a

coding sequence. According to the USCS database (GRCh37/hg19), the TSS was at chr8:22,103,522 (Supplemental figure 1 A and supplemental file 1).

We then analyzed the promoter activity around the TSS and identified a region -2035 bp from the TSS to the TSS itself (chr8: 22,103,522-5,556) as a putative promoter region (Supplemental file 2). This region was later cloned into the upstream of the luciferase gene of the pGL3 basic plasmid (Supplemental figure 2), a plasmid lacking promoter and enhancer sequences, thus allowing the study of putative regulatory sequences. Analysis of the luciferase activity induced by the promoter showed that insertion of the region from -2035 bp to the TSS induced strong luciferase expression (Figure 3 A), indicating that this region displays promoter activity.

The methylation status of miR-320a was then determined. According to the UCSC database, several CpGi regions cover the whole promoter region and coding sequence of miR-320a. As methylation at both the promoter and 5'-UTR region (Brenet et al., 2011) inhibits gene expression, the methylation status of both the promoter and the 5'-UTR region (TSS to coding sequence) was analyzed. CpG plot software identified three CpGi regions (Supplemental file 2). Methylation of these regions was then analyzed by bisulfite sequencing PCR (BSP). The results showed that the promoter methylation was significantly increased in ADM cells (Figure 3 B a and b), however, there was no significant methylation in the 5' -UTR (Figure 3 B c), suggesting that down-regulation of miR-320a is regulated by methylation of its promoter.

5-aza-2'-deoxycytidine (DAC) was then used to hypomethylate DNA, and this abolished most of the promoter methylation of miR-320a (Supplemental figure 3). The expression of miR-320a, TRPC5, and NFATC3 were then examined and showed that when miR-320a was hypomethylated, its expression increased significantly (Figure 3 C) while the expression of TRPC5 and NFATC3 decreased (Figure 3 D).

Down-regulation of miR-320a is associated with ETS-1 hypermethylation in chemoresistant cells

A recent report showed that miR-320a expression is suppressed by the proto-oncogenic transcription factor ETS-1 (v-ets erythroblastosis virus E26 oncogene homolog 1) (Tang et al., 2012a). Based on that work, we analyzed the activity of ETS-1 in chemoresistant cells. First, consistent with the previous work, we confirmed that the promoter and 5'-UTR region of miR-320a were able to induce luciferase activity in pGL3 plasmid. However, over-expression of ETS-1 significantly suppressed the luciferase activity (Figure 4 A). Then we analyzed the ETS-1 expression in MCF-7 cells and found significantly increased expression in chemoresistant MCF-7/ADM cells compared with MCF-7/WT cells (Figure 4 B - D). The increased ETS-1 was found to aggregate within the nucleus (Figure 4 D), where most of the transcription factors reside. Then, we analyzed the binding activity of ETS-1 on the promoter and 5'UTR of miR-320a by ChIP assay in MCF-7/ADM cells. Three theoretical binding sites were found by Jaspar online software (Supplemental file 4), and we confirmed that site 2 (S2) was the real binding site in MCF-7/ADM cells because the ETS-1 antibody was able to pull down the DNA sequence in S2,

while pre-immune IgG antibody (negative control) failed to pull down this part of the DNA (Figure 4 E). On the other hand, sites 1 and 3 (S1 and S3) did not bind to ETS-1 in MCF-7/ADM cells, because although the PCR product showed the correct sizes of S1 and S3 when the binding sites were pulled down by ETS-1 antibody, the negative control also showed the same bands at the same sizes, indicating that these results are false-positives. Taken together, these results suggest that ETS-1 is elevated and activated to regulate miR-320a in MCF-7/ADM cells.

We then studied the methylation of ETS-1 using BSP in chemoresistant cells. As shown by the CpG plot, a CpGi (230 bp) was found immediately upstream of the ETS-1 coding sequence (Supplemental file 3). Since this CpGi contained relatively more bases, nested BSP was applied to enhance the quality of the PCR. We found that the promoter of ETS-1 was highly methylated in MCF-7/WT cells, but strong demethylation occurred in MCF-7/ADM cells (Figure 4 F). Therefore, these data suggest that the over-expression of ETS-1 is regulated by promoter hypomethylation in MCF-7/ADM cells.

Effect of MiR-320a in chemoresistance of other cancer cells

We then tested our findings in different chemoresistant cell lines. Paclitaxel (PTX)-resistant MCF-7 cells (MCF-7/PTX) and fluorouracil (5-FU)-resistant HCT-8 cells (colorectal adenocarcinoma, HCT-8/5-FU) were tested for miR-320a, TRPC5, and NFATC3 expression. The miR-320a expression was significantly decreased, while TRPC5 and NFATC3 were increased in both MCF-7/PTX and HCT-8/5-FU cells compared with their parental controls (MCF-7/WT and HCT-8/WT) (Figure 5 A

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a-c). At the same time, restoring the activity of miR-320a markedly decreased the chemoresistance in MCF-7/PTX cells (Figure 5 B), but only slightly in HCT-8/5-FU cells.

Low miR-320a expression is associated with clinical chemoresistance and predicts outcomes of breast cancers

The activity of miR-320a was then tested in clinical samples of breast tumors from patients receiving anthracycline-taxane-based neoadjuvant chemotherapy. Tumors with a reduction in size <10% in response to this therapy were considered to be chemoresistant (termed non-responders). Post-chemotherapy samples from non-responders and responders were compared to identify the miR-320a expression changes in chemoresistance and chemosensitivity. We found that miR-320a expression was significantly decreased in non-responders, i.e. chemoresistant samples. At the same time, the expression of TRPC5, NFATC3, and ETS-1 was significantly increased in non-responders (Figure 6 and table 1), and the expression of miR-320a was significantly and negatively associated with the expression of TRPC5, NFATC3, and ETS-1 when analyzed by the Spearman correlation coefficient. Therefore, our data suggested the implication of miR-320a, TRPC5, NFATC3, and ETS-1 in clinical chemoresistance.

We later analyzed the predictive role of miR-320a in clinical samples by analyzing the distant relapse-free survival (DRFS) from the miRNA profiling data of Buffa *et al.* (GSE22216) (Buffa et al., 2011) involving 210 patients with 10-years of follow-up of clinical and demographic information. The samples were obtained at

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surgery followed by adjuvant chemotherapy and/or adjuvant hormone therapy. The patients were divided into ER-negative (ER-) and ER-positive (ER+) groups (patients aged >50 years were excluded as they were considered as a single group and treated with different regimes as indicated by Buffa *et al*). Association of miR-320a with DRFS was analyzed by Kaplan-Meier. In the ER- groups (Figure 7 A), the miR-320a failed to predict the DRFS. By contrast, in the ER+ groups, miR-320a displayed clinical relevance for patient survival (Figure 7 B). We also tested the how well miR-320a predicted DRFS by measuring the area under the receiver operating characteristic curve (AUC) in the same data set of Buffa *et al*. A significant AUC for prediction of response to DFRS was found for miR-320a in the ER+ group (Figure 7 C).

Later, the predictive power of miR-320a was verified in the studies of Lyng *et al.* which included three miRNA profiling data sets for ER+ breast cancer patients (GSE37405) (Lyng et al., 2012); one of these data sets was abandoned because it lacked the detection of miR-320a. In the other two data sets (n = 40 and n = 51 (the author indicated that the latter data set contained 52 individuals, but only 51 were found in the uploaded files)), all patients received adjuvant tamoxifen as the only therapy and the DFRS was recorded. In both groups, miR-320a was still able to predict the distant recurrence ratio (Figure 7 D and E).

Discussion

MiRNAs have attracted great interest from oncologists due to their versatile

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ability to regulate nearly every critical aspect of carcinogenesis: proliferation/apoptosis (He et al., 2007), motility (Tavazoie et al., 2008), angiogenesis (Dews et al., 2006), and the microenvironment (Soon and Kiaris, 2013) of cancer cells. Compared with targeting one molecule, interrupting one or more of the above signaling networks could be more effective in overcoming cancer (Hanahan and Weinberg, 2011). Because a single miRNA can target different genes that may intensively participate the whole process of developing a certain cancer property (Rajewsky, 2006;Krek et al., 2005), miRNA provides a promising therapeutic strategy in cancer treatment.

MiR-320a showed such potential to treat cancer chemoresistance. To date, only a few studies have implicated miR-320a in cancers (Wee et al., 2012;Tang et al., 2012b), but the mechanism of action was unclear. Here, we not only showed that dysregulation of miR-320a was involved in the chemoresistance of cancer cells, but also demonstrated that it regulated the critical TRPC5-NFATC3 network during the developing of chemoresistance. Previously, we showed that the effect of high TRPC5 expression on NFATC3 is to activate a calcium signaling pathway, which triggers the translocation of NFATC3 into the nucleus. The translocated NFATC3 binds to the promoter of the *ABCB1* gene and induces *ABCB1* expression. However, TRPC5 does not directly contribute to the high expression of NFATC3. Until this study, we were able to explain the reason for the high expression of TRPC5 and NFATC3; that is because they are both regulated by miR-320a. This is not the first miRNA that has been found to regulate several essential factors in one signaling network. The

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miR-200 family has been found to regulate the epithelial mesenchymal transition (EMT) of cancer cells by targeting modulators of the EMT, such as ZEB1, ZEB2, and SIP1 (Peter, 2009), and restoring the activity of miR-200 reverses the EMT and EMT-related chemoresistance (Li et al., 2009). Therefore, our studies and those of others suggest that focusing on one signaling pathway is a good way to explore and understand the synergic regulation of miRNA.

MiR-320a is generally down-regulated in breast cancers and reverse-regulated by activation of ETS-1 (Wee et al., 2012;Tang et al., 2012b); our study further explained how methylation is involved in miR-320a down-regulation and ETS-1 up-regulation, especially in chemoresistant breast cancer cells. Previously, Wee *et al.* (Wee et al., 2012) analyzed the methylation status of miR-320a in nine breast cancer cell lines, and argued that its down-regulation in these cells was independent of methylation. However, we not only found, for the first time, the effect of miR-320a in chemoresistant cancer cells, but also that miR-320a can be regulated by methylation. The difference between our results and those of others may be due to the differences in the regulatory systems of chemoresistant and normal cancer cells, suggesting that methylation of the miR-320a coding sequence is an effective signature to distinguish chemoresistant from chemosensitive cancer cells. In addition, we noted that Wee *et al.* (Wee et al., 2012) only characterized the promoter of miR-320a by luciferase reporter assay, while by combining 5'RACE with luciferase assays, we were able to locate the promoter and 5'-UTR more precisely. These regions covered more than 2000 bp upstream of miR-320a and included three main CpGis, one of which was newly

identified by us and shown to be hypermethylated in chemoresistant cancer cells.

After DAC globally hypomethylated the MCF-7/ADM cells, the chemoresistant characters of the cells, including down-regulation of miR-320a as well as up-regulation of TRPC5 and NFATC3, were reversed, confirming that methylation is involved in miR-320a regulation, as well as the relationship between miR-320a and TRPC5/NFATC3. It should be noted that because there is no effective way to specifically demethylate the promoter of miR-320a, we cannot exclude the influence of other methylation-regulated factors that could regulate miR-320a at the same time. Therefore, based on the current results we still cannot draw an explicit conclusion that promoter methylation is the cause of miR-320a down-regulation, and can only conclude that the methylation is involved in regulating miR-320a.

The changes of TRPC5 and NFATC3 tended to be greater than miR-320a, suggesting that other factors controlled by methylation also regulate TRPC5 and NFATC3, and this needs further study. It should be noted that DAC treatment did not dramatically (but significantly) restore the expression of miR-320a; this is probably due to the continuing inhibitory effect of ETS-1 in MCF-7/ADM cells. However, neither of these factors completely inhibited miR-320a because a small amount was still detectable in MCF-7/ADM cells (Figure 2 A). Therefore, both methylation and ETS-1 only partially and independently regulate miR-320a expression, and deletion of methylation partially diminishes the inhibition of miR-320a.

These data also demonstrate how the opposite status of DNA methylation contributed to the development of chemoresistance in the same population of cells.

The complicated mechanism of regulation by methylation is triggered in tumor cells when they confront a disadvantageous environment and is determined by the pressure of selection. Genes that inhibit tumor progression are suppressed by methylation, while those that promote proliferation are highly activated. Finally, the most favorable changes are selected and MCF-7/ADM cells develop from MCF-7/WT cells, making them no longer sensitive to ADM.

Methylation-based therapies with DAC have already been approved by the US Food and Drug Administration to treat myelodysplastic syndromes and chronic myelomonocytic leukemia (Kaminskas et al., 2005;Steensma, 2009). However, DAC is a nonspecific agent so that global hypomethylation by DAC may trigger tumor progression (Gius et al., 2004). Furthermore, appropriate techniques to detect DNA methylation-based biomarkers clinically are lacking. On the other hand, specific interfering miRNAs are relatively easily realized by miRNA mimics or inhibitors, and FISH can effectively detect miRNA markers on small tumor sections. Here, we also demonstrated an easy and effective way of using miRNA tools to detect and inhibit chemoresistance, suggesting good potential for the development of miRNA-based therapies.

When the miR-320a-mediated chemoresistance was examined in PTX-resistant MCF-7 cells and 5-FU-resistant HCT-8 cells, we found that although miR-320a was down-regulated in both cell lines, it only significantly modulated chemoresistance in breast cancer cells, suggesting that a role of miR-320a in chemoresistance may be found more frequently in breast cancers. The hypothesis was then supported by the

clinical studies, in which chemoresistant breast cancers were accompanied by low miR-320a expression.

Neoadjuvant chemotherapy samples are good sources of chemoresistant/sensitive cancer cells; tumors that do not respond to chemotherapy (non-responders) are defined as chemoresistant and *vice versa*. In this study, we confirmed the high expression of TRPC5 and NFATC3 and low expression of miR-320a in non-responders to two-agent neoadjuvant chemotherapy, suggesting that miR-320a is a common factor hijacked by cancer cells to induce chemoresistance regardless of the type and number of chemotherapeutic drugs.

Chemoresistant cancer cells have greater opportunities for relapse because they may gain features of stem/mesenchymal cells that easily metastasize (Polyak and Weinberg, 2009). Indeed, by mediating chemoresistance, miR-320a also showed its clinical relevance and predictive power on DRFS in ER+ breast cancer patients, but not in ER- patients who by nature have a poor prognosis (Putti et al., 2005); higher miR-320a expression in ER- patients seems not to reverse their malignancy. However, ER+ breast cancer patients have lower risk of recurrence and a better 10-year survival ratio, but 30-50% develop resistance to adjuvant tamoxifen or other chemotherapies through mechanisms that remain largely unclear (Early Breast Cancer Trialists Collaborative Group (EBCTCG) studies). Based on our study, we suggest that low expression of miR-320a in ER+ patients contributes to their drug resistance, and acts as an unfavorable breast cancer prognostic marker for DRFS or recurrence-free ratio.

In figure 8, we provide a schematic of the regulatory network of miR-320a.

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Hyper- and hypomethylation of the promoters of miR-320a and ETS1 respectively suppress miR-320a expression in chemoresistant cancer cells, thus increasing the expression of TRPC5 and NFATC3, which are targeted by miR-320a and essential for p-gp-induced chemoresistance. Our study provides potential therapeutic targets for the suppression of chemoresistance in breast cancers.

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Authorship contribution

Participated in research design: DX He, X Ma, J Jin

Conducted experiments: XT Gu, L Jiang

Performed data analysis: DX He, XT Gu

Wrote or contributed to the writing of the manuscript: DX He, X Ma, J Jin

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Footnotes:

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Figure Legends

Figure 1. miR-320a targets both TRPC5 and NFATC3. (A) Pairing of the miR-320a seed sequence and the respective mutant (Mut) sequences with 3'-UTRs of TRPC5 and NFATC3 mRNA. There were three binding site of miR-320a on the 3'-UTR of NFATC3 mRNA. (B) Binding of miR-320a onto TRPC5 and NFATC3 mRNA was verified by dual-luciferase reporter assay with an miR-320a mimic (320a); a scrambled mimic was set as control. The binding was abolished by sequence mutation (Mut) of mRNAs. *p <0.05, **p <0.001 *vs* control; *(blue) p <0.05, **(blue) p <0.01 *vs* wild-type 3'-UTR. (C and D) The interaction between miR-320a and TRPC5/NFATC3 was analyzed in chemoresistant MCF-7/ADM cells. TRPC5 and NFATC3 were over-expressed in MCF-7/ADM *versus* MCF-7/WT cells, and the over-expression was diminished by an miR-320a mimic (320a) in MCF-7/ADM cells as assessed by RT-PCR (C) and western blot (D; TRPC5: ~111 kDa; ~116 kDa). The scrambled mimic was used as a control. *p <0.05 *vs* mimic control, ***p <0.0001 *vs* MCF-7/WT.

Figure 2. MiR-320a mediates chemoresistance in MCF-7/ADM cells. (A) Expression of miR-320a was decreased in chemoresistant MCF-7/ADM (ADM) cells *versus* its parental control MCF-7/WT (WT) cells when analyzed by qRT-PCR. (B) An miR-320a mimic (320a) diminished chemoresistance in terms of the IC_{50} in MCF-7/ADM cells compared with MCF-7/ADM cells transfected with a scrambled mimic (Control). *p <0.05 *vs* control. (C) A TRPC5 over-expression vector with a mutated 3'UTR region was constructed. The empty vector was first transfected into

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MCF-7/ADM cells together with the miR-320a mimic or a scrambled mimic, and the influence of the vector on the chemoresistance was analyzed by MTT. Then the mutated TRPC5 was over-expressed together with the miR-320a mimic or a scrambled mimic in MCF-7/ADM cells; when the miR-320a failed to target TRPC5, its anti-chemoresistant ability was abolished as determined by IC_{50}.

Figure 3. Hypermethylation of miR-320a promoter in MCF-7/ADM cells. (A) To analyze the promoter activity of miR-320a, the 2035-bp promoter region was inserted into pGL3 plasmid to analyze luciferase activity, and the empty pGL3 plasmid was set as control. ***p <0.0001 *vs* control. (B) Increase of DNA methylation of the miR-320a gene promoter. DNA methylation profile of 13 CpG dinucleotides in the promoter region (a) and two 5'-UTR region (c) was analyzed by 5 independent BSP tests (one line of 13 rings represents one individual test), the methylative ration was calculated as number of methylated CpG dinucleotides / total number of CpG dinucleotides. Black rings represent methylated CpG dinucleotides, hollow rings are unmethylated. ***p <0.0001 *vs* MCF-7/WT. (B b) Representative BSP chromatograms analyzing the promoter of miR-320a in MCF-7/WT and ADM cells. The sequencing chromatograms analyzing the 3rd to the 5th CpG dinucleotides (+81 to 92 of the CpGi) are shown. Blue , cytosine; red, thymine; green, adenine; black, guanine; squares, sites containing 5-methylcytosine in MCF-7/ADM *vs* WT cells. The first line of nucleotide sequence is the DNA sequence before bisulfate transition; CpG dinucleotides are underlined. (C) miR-320a expression was rescued by 5 μM DAC. As a result, the expression of TRPC5 and NFATC3 decreased (D). *p <0.05 *vs*

MCF-7/ADM cells not treated with DAC.

Figure 4. Over-expression of ETS-1 and hypomethylation of the ETS-1 promoter in MCF-7/ADM cells. (A) ETS-1 inhibited the promoter activity of miR-320a. The promoter (including the 5'-UTR) of miR-320a was cloned into pGL3 basic plasmid and co-transfected with pcDNA3 plasmid over-expressing ETS-1 in HEK293 cells (ETS-1); empty pcDNA3 plasmid was used as control. The luciferase activity was then measured. *p <0.05 *vs* empty pcDNA3 plasmid. (B–D) Expression and location of ETS-1 in MCF-7/ADM cells was increased and it aggregated in the nucleus when analyzed by RT-PCR, western blot (~55 kDa), and confocal microscopy. **p <0.001 *vs* MCF-7/WT. (E) ETS-1 was activated in MCF-7/ADM cells by binding of the miR-320a promoter and 5'UTR. Three binding sites (S1, 192 bp; S2, 146 bp; S3, 144 bp) were analyzed by ChIP assay. ETS-1 antibody (or pre-immune IgG as negative control) was added to the cell lysate to allow precipitation of genomic DNA fragments associated with ETS-1. The DNA-protein cross-links were then reversed and DNA was analyzed by PCR with primers specific to S1–S3. (F a) Decrease of DNA methylation of the ETS-1 gene promoter. DNA methylation profile of 36 CpG dinucleotides in the promoter region was analyzed by BSP. Black circles represented methylated CpG dinucleotides, hollow rings are unmethylated. ***p <0.0001 *vs* MCF-7/WT. (F b) Representative BSP chromatograms analyzing the promoter of ETS-1 in MCF-7/WT and ADM cells. The sequencing chromatograms analyzing the 18^{th} to 20^{th} CpG dinucleotides (+112 to 128 of the CpGi) are shown. Blue, cytosine; red, thymine; green, adenine; black, guanine; squares, sites containing

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5-methylcytosine in MCF-7/ADM *vs* WT cells. The first line of nucleotide sequence is the DNA sequence before bisulfate transition; CpG dinucleotides are underlined.

Figure 5. Expression of miR-320a in other chemoresistant cancer cells. (A) miR-320a (a) was down-regulated, while TRPC5 (b), and NFATC3 (c) were up-regulated in paclitaxel-resistant MCF-7/PTX cells and fluorouracil-resistant HCT-8/5-FU cells as measured by RT-PCR. (B) miR-320a-mediated chemoresistance in MCF-7/PTX and HCT-8/5-FU cells was analyzed by MTT. **p <0.01 *vs* chemosensitive MCF-7/WT and MCF-7/WT cells. *p <0.05 *vs* untransfected with miR-320a mimic.

Figure 6. Dysregulation of miR-320 (63× oil-immersion), TRPC5 (100×), NFATC3 (63× oil-immersion), and ETS-1 (63× oil-immersion) was analyzed in post-chemotherapeutic samples (n = 8) from patients receiving anthracycline-taxane-based neoadjuvant chemotherapy. The sections were stained with DAPI (blue) to identify the nucleus. The miR-320a was then detected by FISH with red fluorescence; TRPC5, NFATC3, and ETS-1 were detected by immunohistochemical staining with DAB and green fluorescence, respectively.

Figure 7. Predictive role of miR-320a on distant relapse-free survival (DRFS). Kaplan-Meier plots of DRFS based on miR-320a expression in ER- (A) and ER+ (B) patients in the GSE22216 breast cancer data set (n = 210). To define low-(red) and high-(black) expressers, samples were ranked based on miR-320a expression and the top 50% were defined as high-expressers. (C) The predictive power of miR-320a on DRFS in ER+ individuals was tested by area under the receiver operating

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characteristic curve (AUC). (D and E) Kaplan-Meier plots of recurrence-free ratio based on the miR-320a expression in patients in the GSE37405 breast cancer data set (n = 40 and 51, respectively).

Figure 8. Network of miR-320a-regulated chemoresistance of cancer. Downregulation of miR-320a originates from hypermethylation of the promoter of miR-320a and hypomethylation of that of ETS1. The mRNAs of TRPC5 and NFATC3 are no longer targeted by miR-320a and highly expressed, which in turn triggers p-gp activation and chemoresistance.

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Table 1. Statistical summary of the expression of miR-320a, TRPC5, NFATC3, and ETS-1 in clinical samples

		miR-320a	**TRPC5**	**NFATC3**	**ETS-1**
Expression Score	Responder (n = 4)	4.00 ± 0.70	4.25 ± 0.62	4.75 ± 0.71	3.16 ± 0.44
	Non-responder (n = 4)	1.98 ± 0.28	8.22± 0.90	7.23 ± 0.70	5.48 ± 0.49
	P value	0.0371	0.0109	0.0481	0.0168
Correlation *vs* miR-320a	Correlation coefficient	N/A	-0.77	-0.78	-0.72
	P value		0.023	0.023	0.045

Figure 1

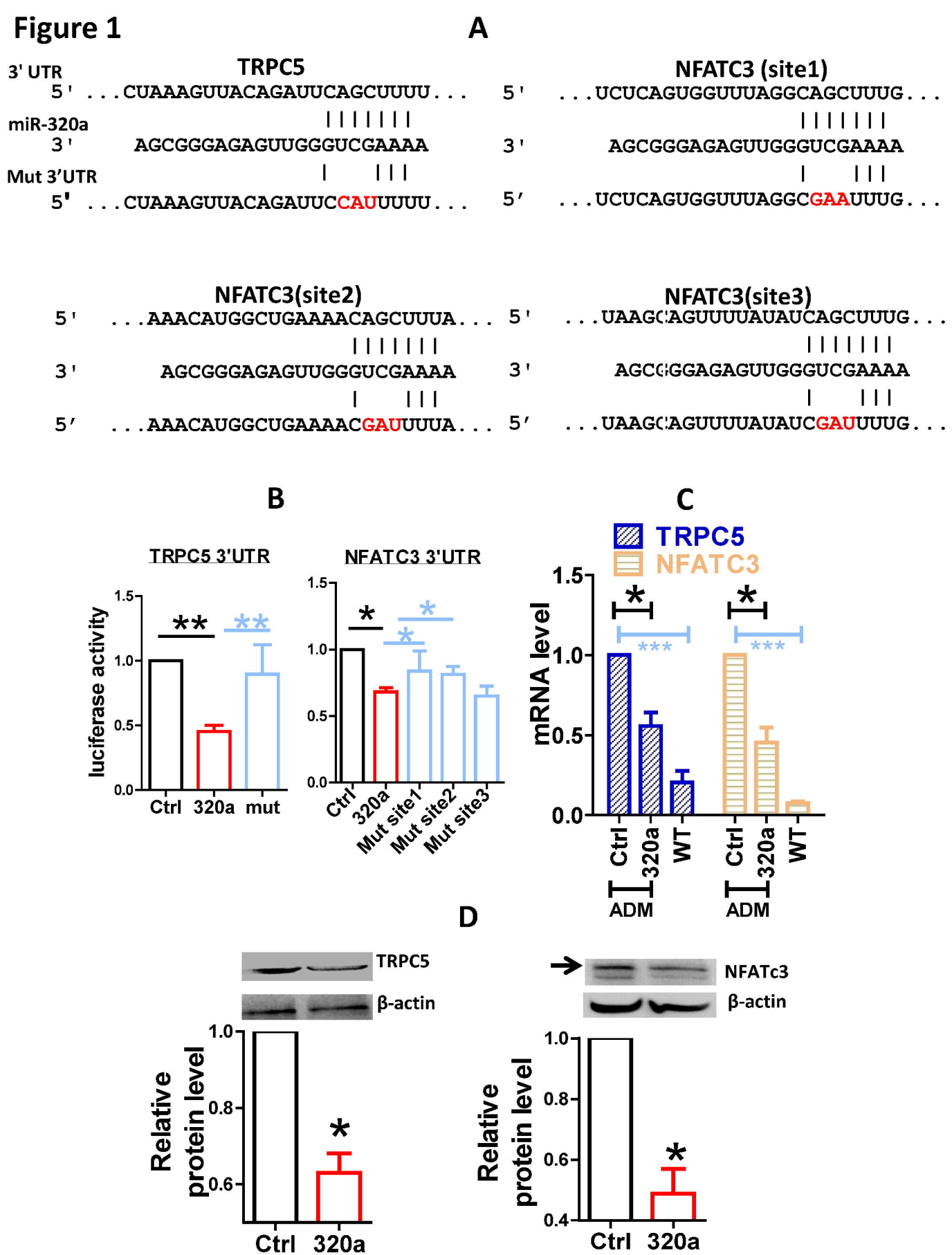

A
TRPC5
3' UTR
5' ...CUAAAGUUACAGAUUCAGCUUUU...
miR-320a
3' AGCGGGAGAGUUGGGUCGAAAA
Mut 3'UTR
5' ...CUAAAGUUACAGAUUCCAUUUUU...
NFATC3 (site1)
5' ...UCUCAGUGGUUUAGGCAGCUUUG...
3' AGCGGGAGAGUUGGGUCGAAAA
5' ...UCUCAGUGGUUUAGGCGAAUUUG...
NFATC3(site2)
5' ...AAACAUGGCUGAAAACAGCUUUA...
3' AGCGGGAGAGUUGGGUCGAAAA
5' ...AAACAUGGCUGAAAACGAUUUUA...
NFATC3(site3)
5' ...UAAGCAGUUUUAUAUCAGCUUUG...
3' AGCGGGAGAGUUGGGUCGAAAA
5' ...UAAGCAGUUUUAUAUCGAUUUUG...
B
TRPC5 3'UTR
NFATC3 3'UTR
luciferase activity
1.5
1.0
0.5
0.0
**
**
*
*
*
Ctrl
320a
mut
Ctrl
320a
Mut site1
Mut site2
Mut site3
C
TRPC5
NFATC3
mRNA level
1.5
1.0
0.5
0.0
*
*
***
***
Ctrl
320a
WT
Ctrl
320a
WT
ADM
ADM
D
TRPC5
β-actin
NFATc3
β-actin
Relative protein level
1.0
0.8
0.6
*
Ctrl
320a
Relative protein level
1.0
0.8
0.6
0.4
*
Ctrl
320a

Figure 2

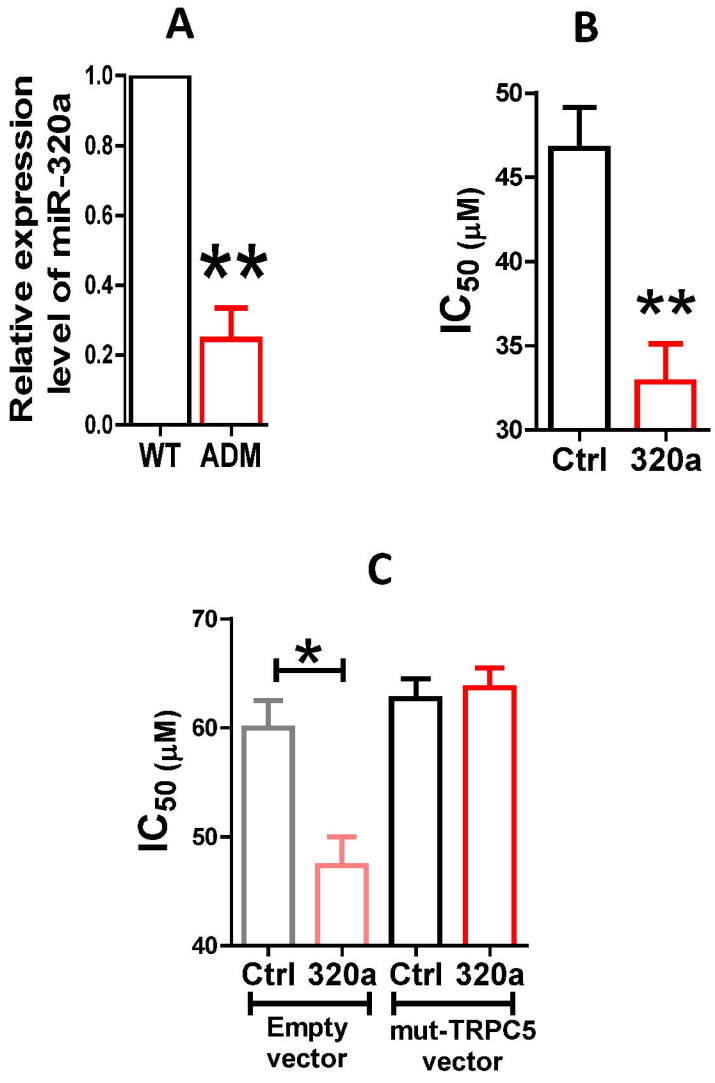
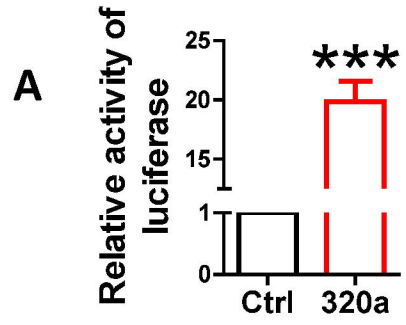
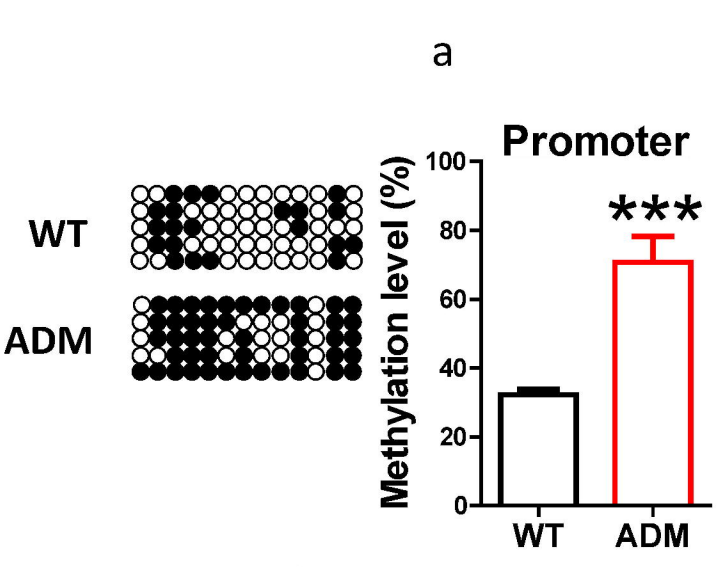
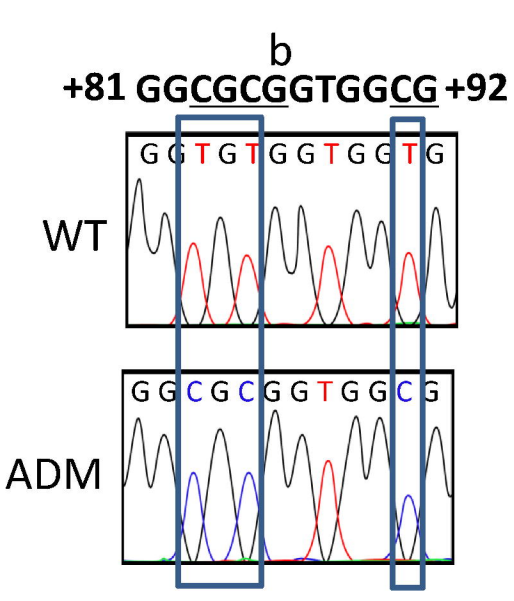
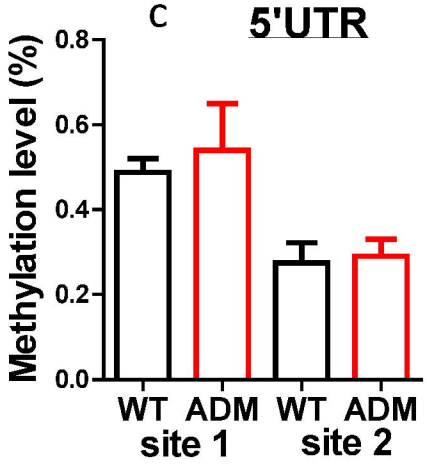

A
Relative expression level of miR-320a
1.0
0.8
0.6
0.4
0.2
0.0
**
WT
ADM
B
IC50 (μM)
50
45
40
35
30
**
Ctrl
320a
C
IC50 (μM)
70
60
50
40
*
Ctrl
320a
Ctrl
320a
Empty vector
mut-TRPC5 vector

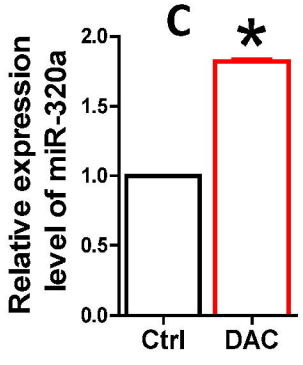
Figure 3

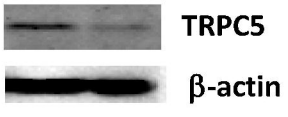

A
Relative activity of luciferase
25
20
15
1
0
***
Ctrl
320a

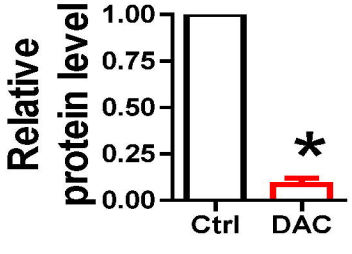

B
a
WT
ADM
Promoter
Methylation level (%)
100
80
60
40
20
0
***
WT
ADM

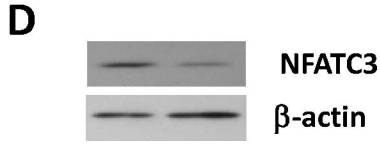

b
+81 GGCGCGGTGGCG +92
WT
ADM
G G T G T G G T G G T G
G G C G C G G T G G C G


C
5'UTR
Methylation level (%)
0.8
0.6
0.4
0.2
0.0
WT ADM WT ADM
site 1
site 2


C
Relative expression level of miR-320a
2.0
1.5
1.0
0.5
0.0
*
Ctrl
DAC


TRPC5
β-actin


Relative protein level
1.00
0.75
0.50
0.25
0.00
*
Ctrl
DAC


D
NFATC3
β-actin

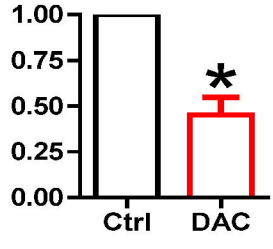

1.00
0.75
0.50
0.25
0.00
*
Ctrl
DAC

Figure 4

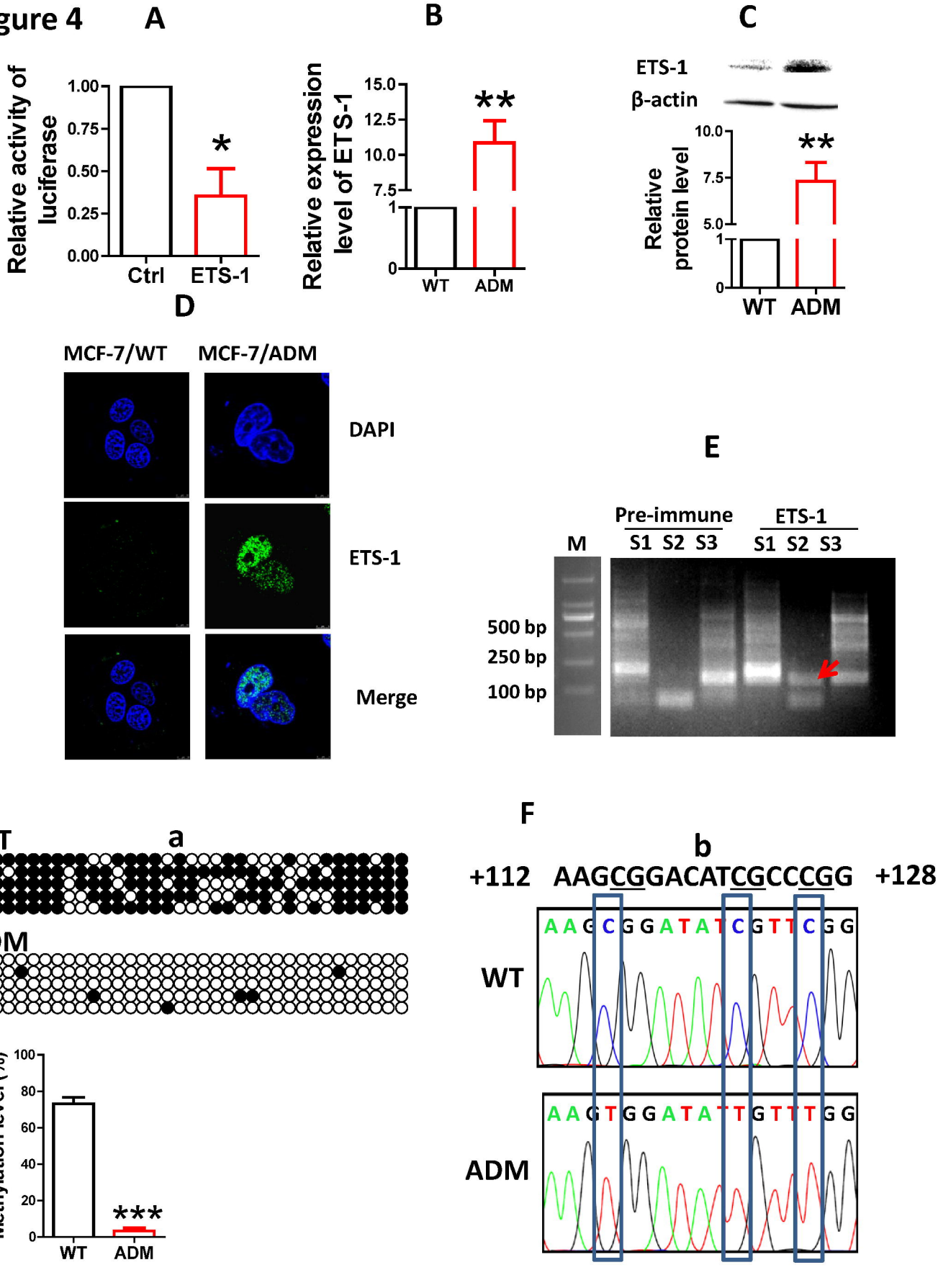

A
Relative activity of luciferase
1.00
0.75
0.50
0.25
0.00
*
Ctrl
ETS-1
B
Relative expression level of ETS-1
15.0
12.5
10.0
7.5
1
0
**
WT
ADM
C
ETS-1
β-actin
Relative protein level
10.0
7.5
5.0
1
0
**
WT
ADM
D
MCF-7/WT
MCF-7/ADM
DAPI
ETS-1
Merge
E
M
Pre-immune
ETS-1
S1 S2 S3
S1 S2 S3
500 bp
250 bp
100 bp
WT
a
ADM
Methylation level (%)
100
80
60
40
20
0
***
WT
ADM
F
b
+112 AAGCGGACATCGCCGG +128
WT
ADM

Figure 5

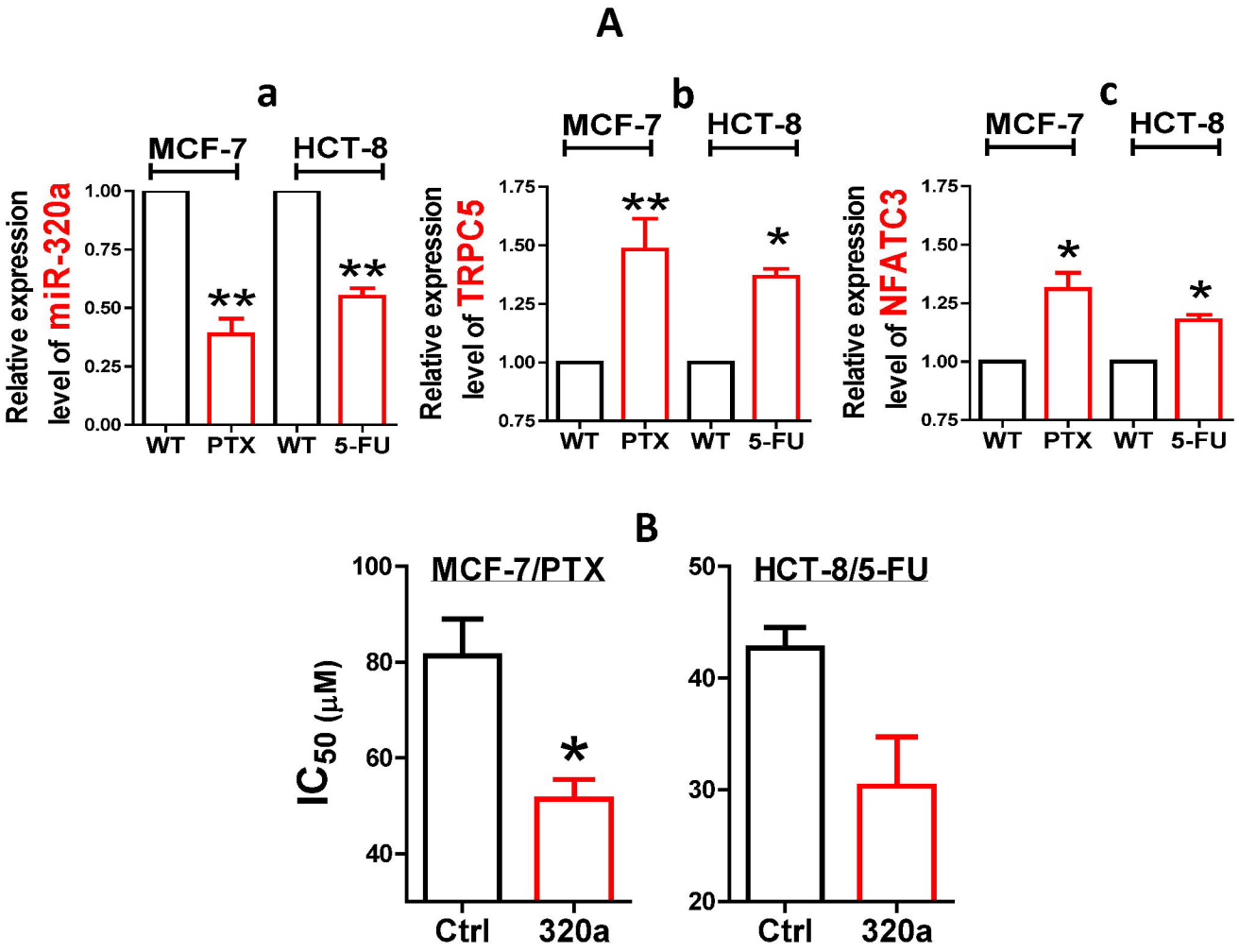
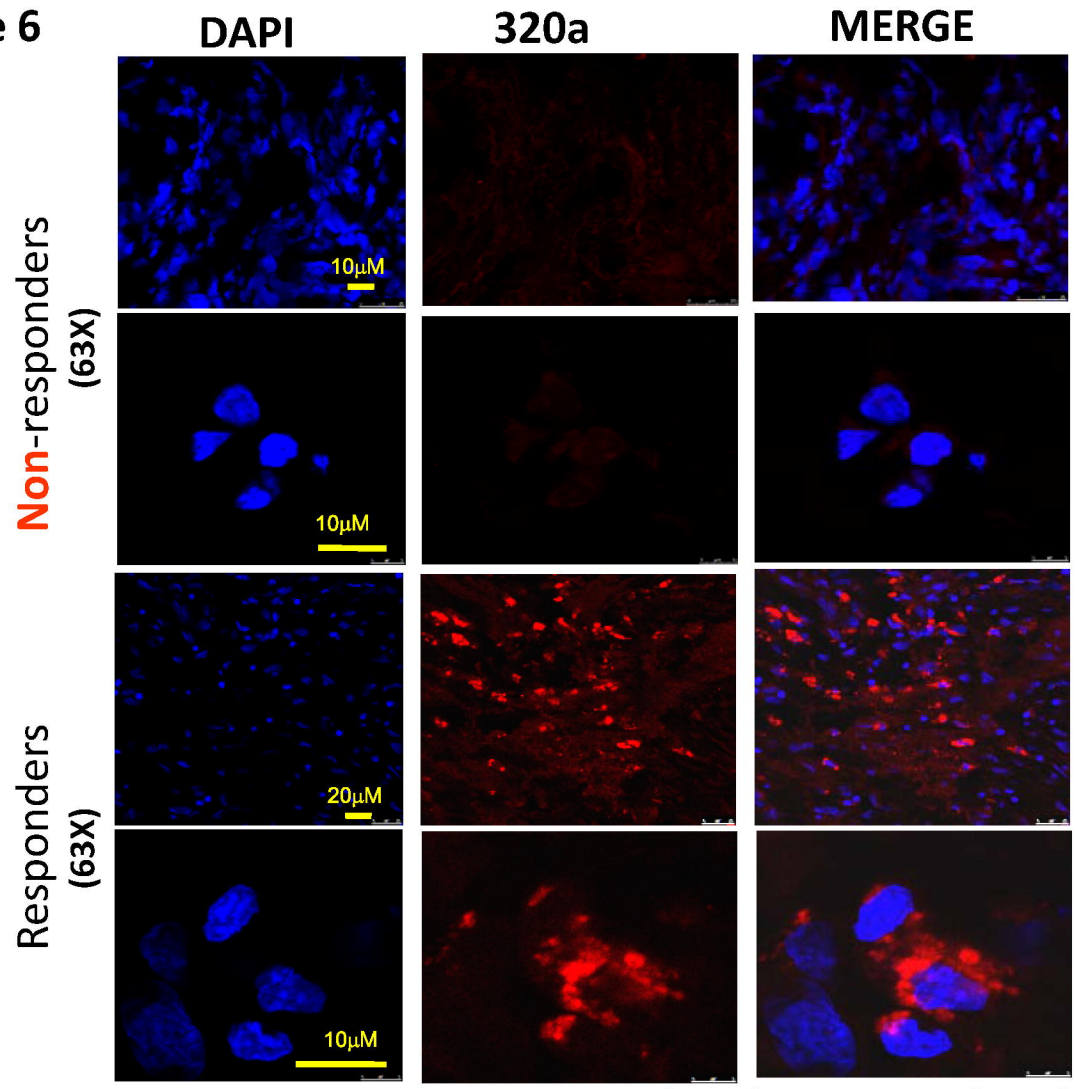

A
a
MCF-7
HCT-8
Relative expression level of miR-320a
1.00
0.75
0.50
0.25
0.00
**
**
WT
PTX
WT
5-FU
b
MCF-7
HCT-8
Relative expression level of TRPC5
1.75
1.50
1.25
1.00
0.75
**
*
WT
PTX
WT
5-FU
c
MCF-7
HCT-8
Relative expression level of NFATC3
1.75
1.50
1.25
1.00
0.75
*
*
WT
PTX
WT
5-FU
B
MCF-7/PTX
HCT-8/5-FU
IC50 (μM)
100
80
60
40
*
Ctrl
320a
50
40
30
20
Ctrl
320a

Figure 6


DAPI
320a
MERGE
Non-responders (63X)
Responders (63X)
10μM
10μM
20μM
10μM

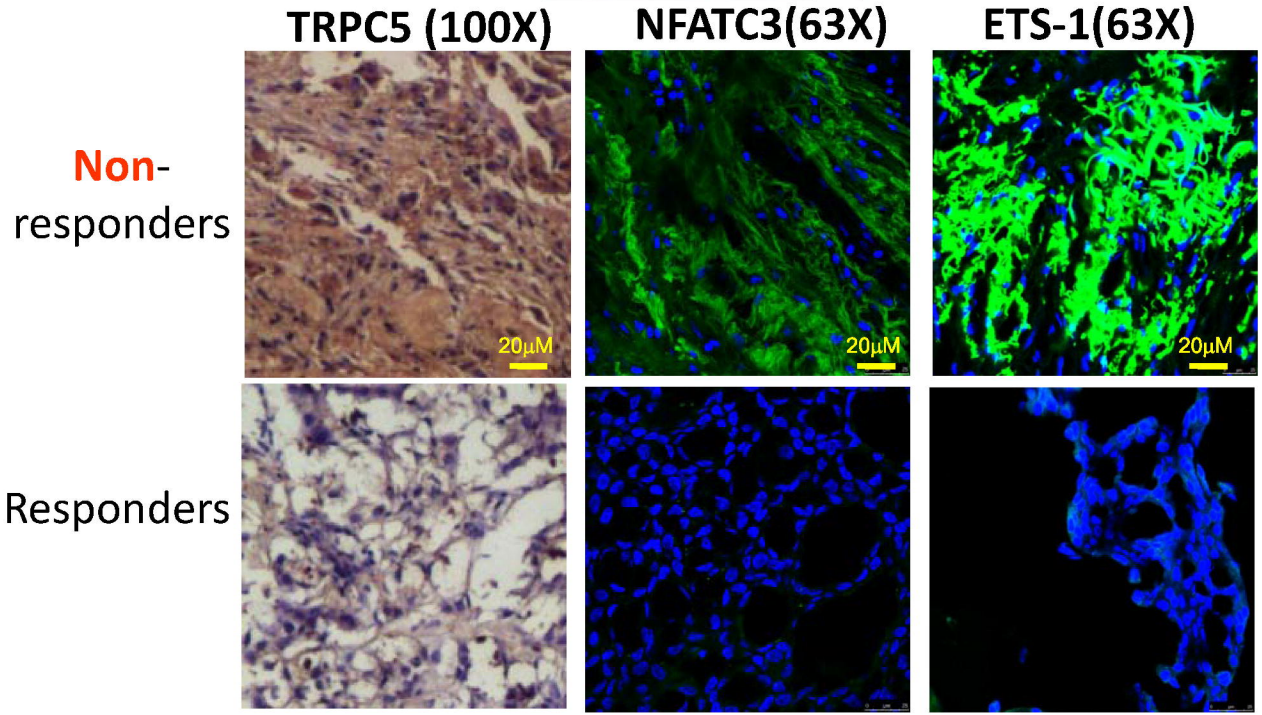
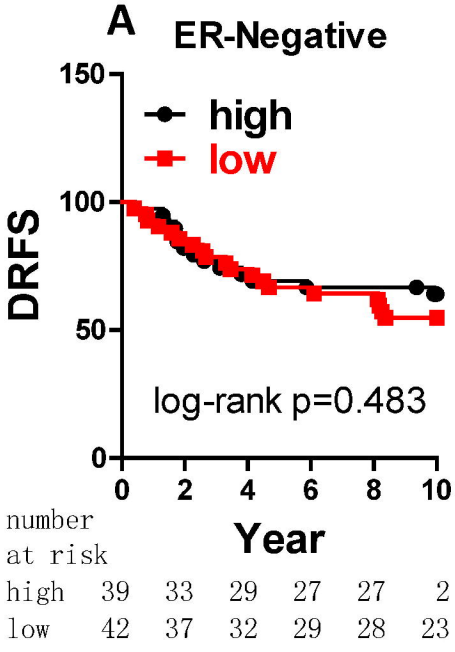
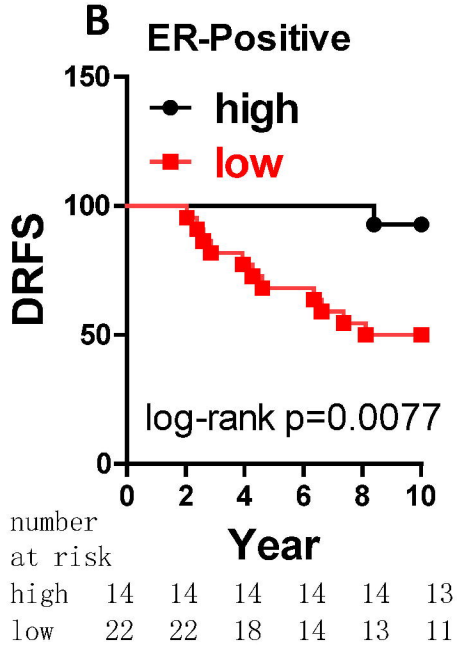
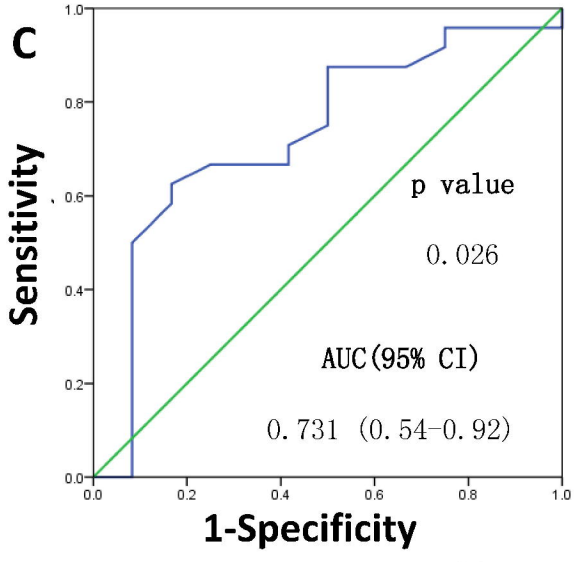
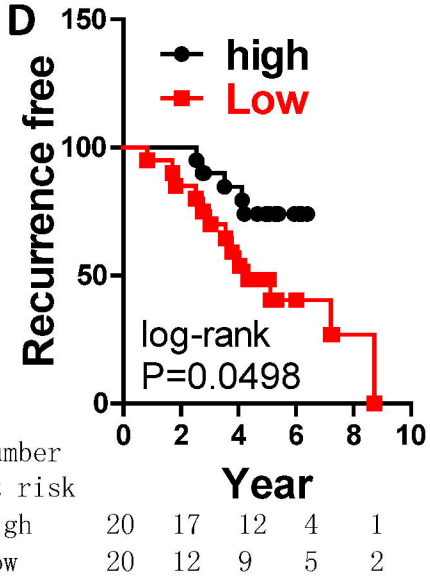

TRPC5 (100X)
NFATC3(63X)
ETS-1(63X)
Non-responders
Responders
20μM
20μM
20μM

Figure 7


A
ER-Negative
high
low
DRFS
log-rank p=0.483
Year
number at risk
high 39 33 29 27 27 2
low 42 37 32 29 28 23


B
ER-Positive
high
low
DRFS
log-rank p=0.0077
Year
number at risk
high 14 14 14 14 14 13
low 22 22 18 14 13 11


C
Sensitivity
1-Specificity
p value
0.026
AUC(95% CI)
0.731 (0.54-0.92)


D
high
Low
Recurrence free
log-rank
P=0.0498
Year
number at risk
high 20 17 12 4 1 1
low 20 12 9 5 2 1

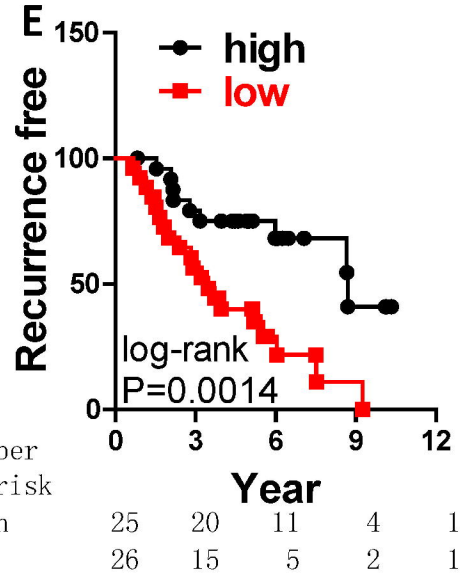
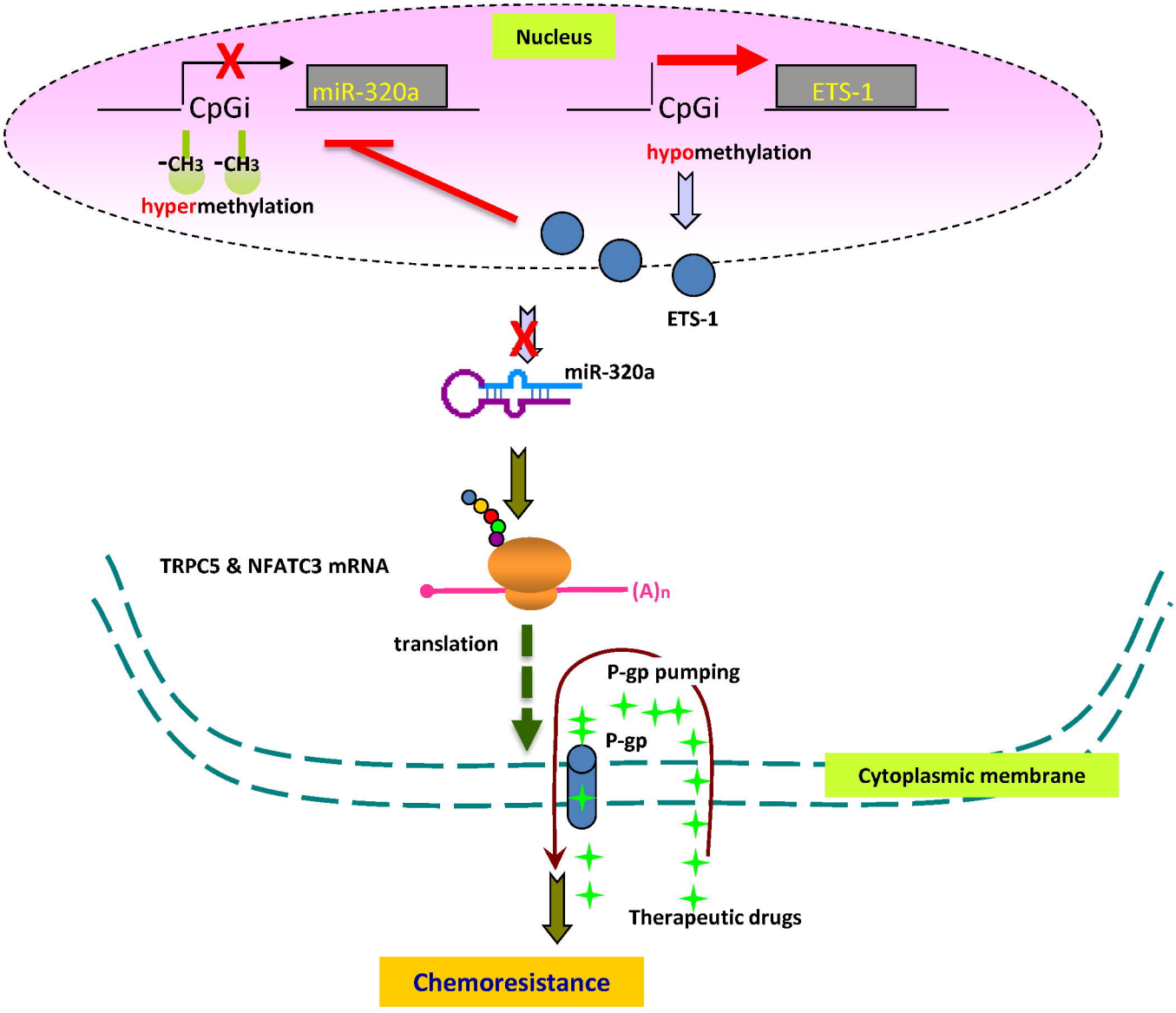

E
high
low
Recurrence free
log-rank
P=0.0014
Year
number at risk
high 25 20 11 4 1
low 26 15 5 2 1

Figure 8


Nucleus
CpGi
miR-320a
CpGi
ETS-1
-CH3 -CH3
hypermethylation
hypomethylation
ETS-1
miR-320a
TRPC5 & NFATC3 mRNA
(A)n
translation
P-gp pumping
P-gp
Cytoplasmic membrane
Therapeutic drugs
Chemoresistance