YMTHE, Volume *27*

Supplemental Information

miR-221/222-Mediated Inhibition of Autophagy Promotes Dexamethasone Resistance in Multiple Myeloma

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Supplemental Information

Figure S1

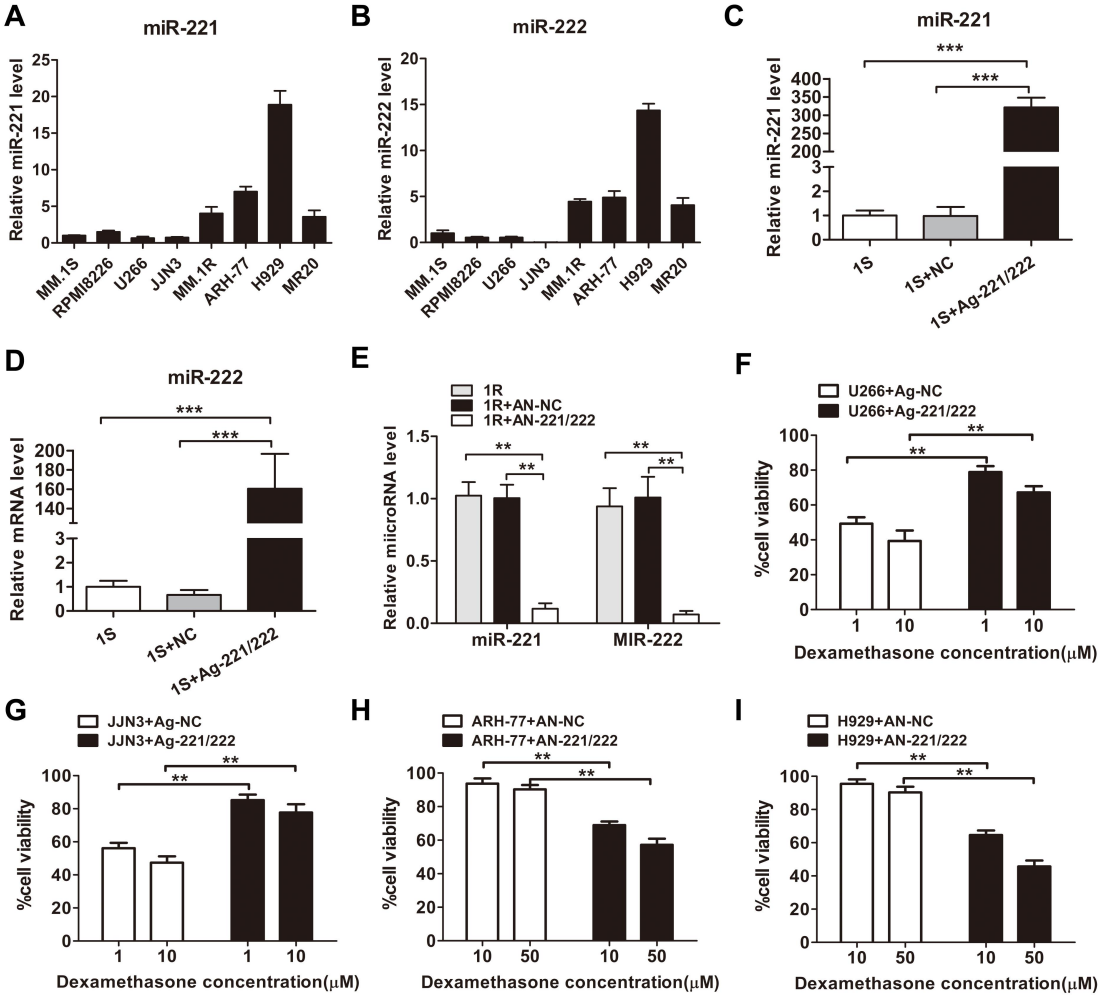

Figure S1. Expression levels of miR-221 and miR-222 in MM cell lines.

(A-B) qRT-PCR analysis of miR-221 and miR-222 expression levels in MM cell lines. (C-D) qRT-PCR analysis of miR-221 and miR-222 expression levels in MM.1S cells 48 h after transfection with agomir-NC (1S+NC) or agomir-221/222 (1S+Ag-221/222). Nontransfected (1S) MM cells were used as controls.*** $p<0.001$. (E) qRT-PCR analysis of miR-221 and miR-222 expression levels in MM.1R cells transfected with antagomir-NC (AN-NC) or antagomir-221/222 (AN-221/222) for 48 h. Nontransfected MM.1R cells (1R) were used as controls. ** $p<0.01$. (F-I) U266 and JJN3 cells were transfected with 100 nM of agomir-221/222 (Ag-221/222) or agomir-NC (Ag-NC). ARH-77 and H929 cells were transfected with 200 nM of antagomir-221/222 (AN-221/222) or antagomir-NC (AN-NC). Then the cells were treated with Dex at the indicated concentrations. After 48 h, cell viability was measured using CCK-8 assay. Data were shown as mean ± SD from three independent experiments. ** $p<0.01$.

Figure S2

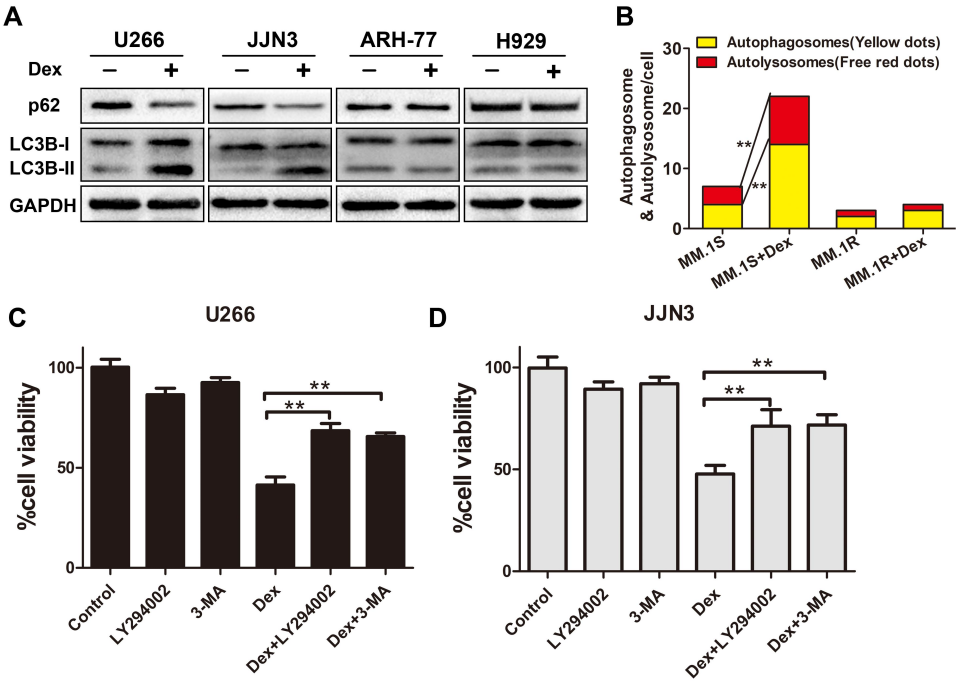

Figure S2. Dex induces pro-death autophagy in MM cells.

(A) Western blot analysis of p62 and LC3B expression in U266, JJN3, ARH-77 and H929 cells after treatment with Dex for 24 h. (B) MM.1S and MM.1R cells expressing GFP-mCherry-LC3 fusion protein were treated with Dex for 24 h. The mean number of autophagosomes and autolysosomes per cell is represented by yellow and red, respectively. ** $p<0.01$. (C-D) Cell viability of U266 and JJN3 cells pretreated with autophagy inhibitor 3-MA or Ly294002 for 2h followed by Dex (10 μM) for 48 h was assessed using CCK-8 assay. ** $p<0.01$.

Figure S3

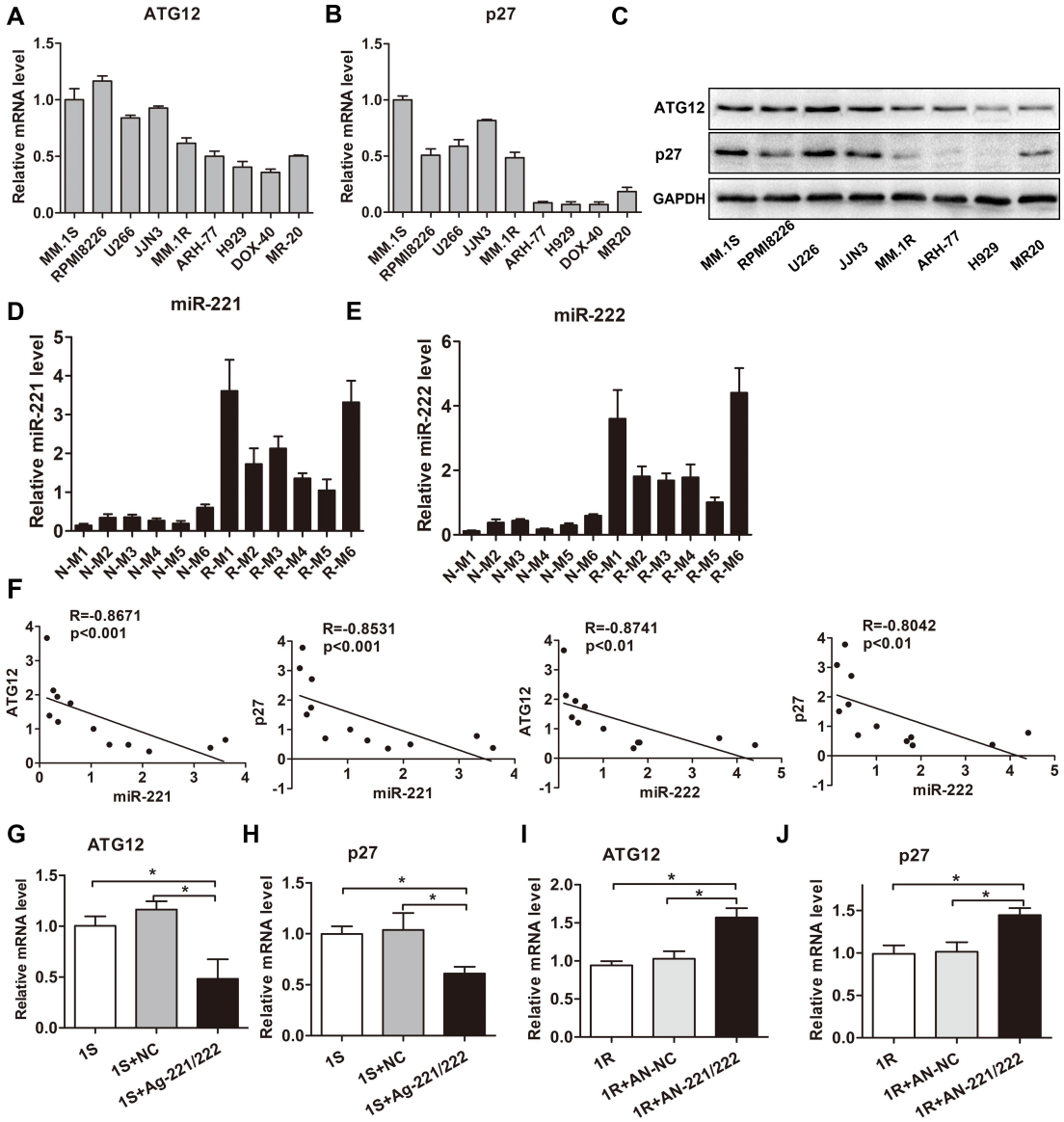

Figure S3. miR-221/222 regulate the expression levels of ATG12 and p27 in MM cells.

(A-C) qRT-PCR and western blot analysis of ATG12 and p27 expression levels in MM cell lines. (D, E) qRT-PCR analysis of miR-221 and miR-222 expression levels in plasma cells (PCs) from patients with newly diagnosed MM (N-M) and patients with relapsed/refractory MM (R-M). (F) Correlations of miR-221 and miR-222 expression levels with ATG12 and p27 mRNA levels in MM patients were analyzed by way of Spearman correlation test. (G, H) qRT-PCR analysis of ATG12 and p27 mRNA levels in MM.1S cells transfected with agomir-NC (NC) or agomir-221/222 (Ag-221/222). (I, J) qRT-PCR analysis of relative mRNA levels of ATG12 and p27 in MM.1R cells transfected with antagomir-NC (AN-NC) or antagomir-221/222 (AN-221/222). * $p<0.05$

Figure S4

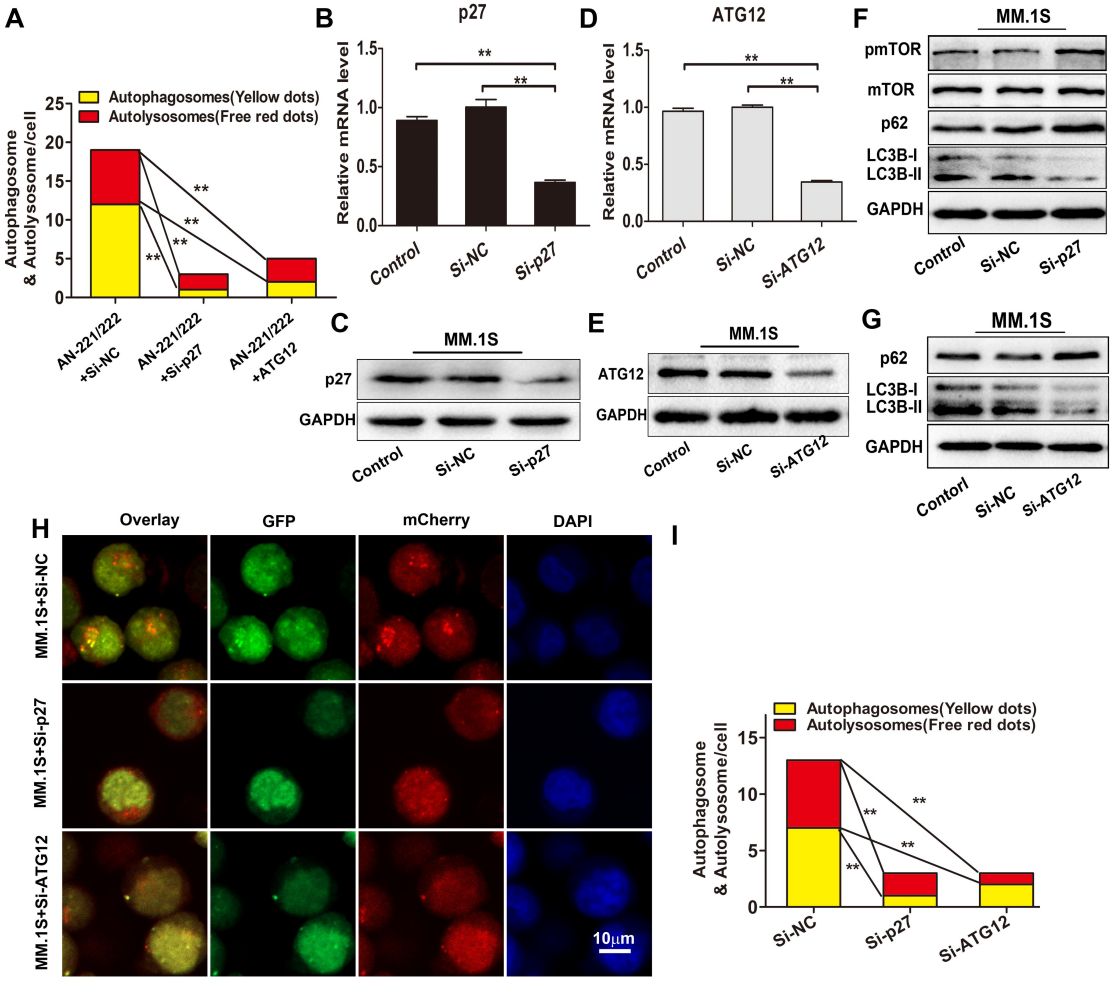

Figure S4. Knockdown of ATG12 or p27 decreases autophagy in MM cells.

(A) MM.1R cells expressing GFP-mCherry-LC3 fusion protein were co-transfected with antagomir-221/222 and siRNAs targeting ATG12 or p27 for 48 h. The mean number of autophagosomes and autolysosomes per MM.1R cell is represented by yellow and red, respectively. **p<0.01. (B-E) qRT-PCR and western blot analysis of ATG12 and p27 expression levels in MM.1S cells transfected with siRNA targeting ATG12 or p27. **p<0.01. (F, G) Western blot analysis of pmTOR, mTOR, LC3B and p62 protein expression in MM.1S cells transfected with p27 siRNA, ATG12 siRNA or siRNA-NC for 72 h. (H) Representative confocal images of MM.1S cells expressing GFP-mCherry-LC3 fusion protein and transfected with p27 siRNA, ATG12 siRNA or siRNA-NC for 48 h. (I) The mean number of autophagosomes and autolysosomes per MM.1S cell is represented by yellow and red, respectively. ** p<0.01.

Figure S5

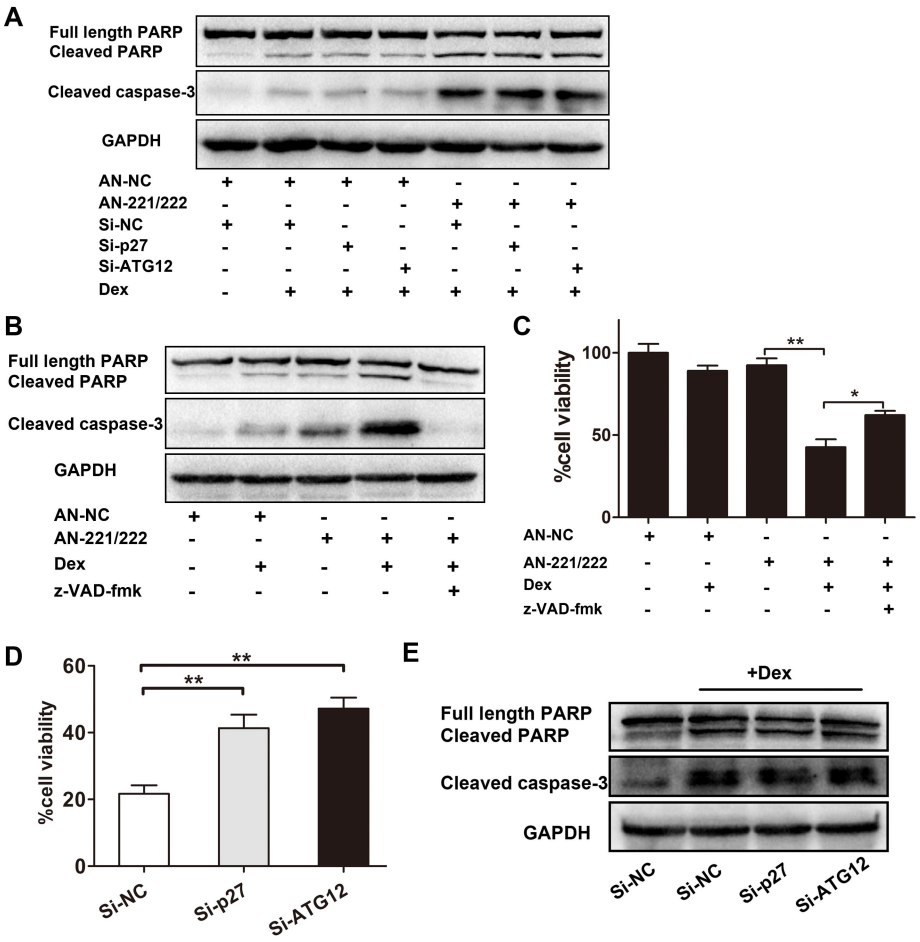

Figure S5. Knockdown of ATG12 or p27 inhibits Dex-induced autophagic cell death in MM cells. (A) MM.1R cells co-transfected with antagomir-221/222 or antagomir-NC and siRNA targeting ATG12 or p27 were treated with Dex (50 μM) for 48 h. Apoptosis was examined by detecting cleavage of caspase-3 and PARP using western blot analysis. (B-C) MM.1R cells transfected with antagomir-NC or antagomir-221/222 were pretreated with pan-caspase inhibitor z-VAD-fmk (50 μM) for 2 h followed by Dex (50 μM) for 48 h. Apoptosis was examined by detecting cleavage of caspase-3 and PARP with western blot analysis (B) and cell viability was assessed using CCK-8 assay (C). (D-E) MM.1S cells transfected with siRNA targeting p27 or ATG12 were treated with Dex (1 μM). After 48 h, cell viability was evaluated using CCK-8 assay (D) and apoptosis was examined by detecting cleavage of caspase-3 and PARP using western blotting (E). * p<0.05, ** p<0.01.

Figure S6

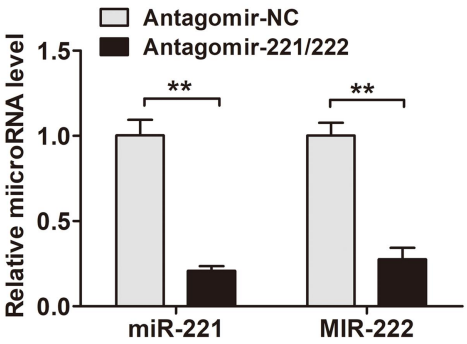

Figure S6. qRT-PCR analysis of miR-221 and miR-222 expression levels in tumor tissues from MM.1R-xenografted mice treated with antagomir-NC and antagomir-221/222. ** $p<0.01$.

Table S1. Clinical information of healthy donors and MM patients .

	Number	Gender, Male/Female	Age, median (range) years	Isotype	International Staging System (ISS) stage
Healthy donor	11	7/4	57 (37-70)	-	-
Newly diagnosed MM	20	14/6	59 (45-76)	11 IgG Kappa 3 IgG Lambda 2 IgA Kappa 2 IgA Lambda 1 IgD Lambda 1 Lambda	ISS I: 2 ISS II: 7 ISS III: 11
Relapsed/ Refractory MM	21	15/6	60 (42-80)	10 IgG Kappa 2 IgG Lambda 4 IgA Kappa 3 IgA Lambda 2 IgD Lambda	ISS I: 2 ISS II: 6 ISS III: 13

Table S2. Dex sensitivity of MM cell lines.

MM cell lines	IC50 (mean±SD)	Dex sensitivity
MM.1S	0.091 ± 0.02 μM	Sensitive
U266	2.02 ± 0.504 μM	Sensitive
JJN3	4.021 ± 0.602 μM	Sensitive
RPMI 8226	14.96 ± 1.873 μM	Sensitive
H929	>500 μM	Resistant
MR20	>500 μM	Resistant
MM.1R	>500 μM	Resistant
ARH-77	>500 μM	Resistant

MM Cells were seeded in 96-well plates (3×10^4 cells/well) in the presence of different concentrations of Dex. After 48 h, CCK-8 assay was performed. The concentration at which Dex produced IC50 was then calculated. Dex sensitivity was defined as follow: sensitive, IC50 < 50 μM.

Table S3. Primers for qRT-PCR used in this study.

Prime name	Sequence
ATG12 forward	5′-CCACTGAGGGTTCAGATGATAGA-3′
ATG12 reverse	5′-TGGGCAACAAGACCGAAAC-3′
p27 forward	5′-GCAGGAGAGCCAGGATGTCAG-3′
p27 reverse	5′-TGCGTGTCCTCAGAGTTAGCC-3′
GAPDH forward	5′-GGTGAAGGTCGGAGTCAACGG-3′
GAPDH reverse	5′-CCTGGAAGATGGTGATGGGATT-3′

Table S4. siRNA sequences of genes used in this study.

Target name	Sequence
Si-ATG12-001	5′-AGAAGTTGGAACTCTCTAT-3′
Si-ATG12-002	5′-GGGATGAACCACAAAGAAA-3′
Si-ATG12-003	5′-AAGTGGGCAGTAGAGCGAACACGAA-3′
Si-P27-001	5′-GGAGCAATGCGCAGGAATA-3′
Si-P27-002	5′-GCAAGTACGAGTGGCAAGA-3′
Si-P27-003	5′-GCACTGCAGAGACATGGAA-3′
Si-NC	5′-TTCTCCGAACGTGTCACGT-3′