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Supporting Online Material for

Disrupting the Pairing Between *let-7* and *Hmga2* Enhances Oncogenic Transformation

Christine Mayr, Michael T. Hemann, David P. Bartel*

*To whom correspondence should be addressed. E-mail: dbartel@wi.mit.edu

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Supplemental Online Material

Supplemental Text

Because of restrictions in the number of permitted references, we were unable to cite some translocation partners and associated tumors reported in the literature. These included refs (*1-6*).

Materials and Methods

Northern blot

Total RNA was isolated from HeLa cells, 293 cells, NIH3T3, Min6, HepG2 and F9 cells (all purchased from ATCC) using Trizol (Ambion), and 20 µg was loaded per lane. RNA blotting was performed as described (*7*) (http://web.wi.mit.edu/bartel/pub/protocols/), with the following DNA oligo probes: *let-7*, 5'-CAACCTACTACCTCA; U6 snRNA, 5'-TTGCGTGTCATCCTTGCGCAGG. The *let-7* probe was designed to detect *let-7a*, *let-7b*, *let-7c* and *let-7d*; it hybridizes to *let-7* nucleotides 2-15, which are identical for these *let-7* family members.

Western blot

F9 cells were transfected using Lipofectamine 2000 (Invitrogen) in 6-well plates (4.0×10^5 cells/well) with 5.0 µg pUC19 (used as carrier) and 50 nM miRNA duplex (Dharmacon; *let-7a*, 5'-UGAGGUAGUAGGUUGUAUAGUUU and 5'-ACUAUACAAUCUACUGUCUUUCC; m*let7a*, 5'-UGCGUUAGUAGGUUGUAUAGUUU and 5'-ACUAUACAAUCUACUGGCGUUCC; miR-101, 5'-UACAGUACUGUGAUAACUGAAG and 5'-UCAGUUAUCACAGUACUGUAUA). Six hours after transfection media was changed, and cells were harvested after 48 hours. NIH3T3 cells were transfected in 6-well plates (2.3×10^5 cells/well) with 5.0 µg pUC19 and 50 nM 2'-*O*-methyl oligonucleotides (Dharmacon) complementary to *let-7a* RNA (5'-GAGAUCUUCACUAUACAACCUACUACCUCAACCUUAGAG) or to miR-124a (5'-GAGAUCUUCGGCAUUCACCGCGUGCCUUAACCUUAGAG) as a control (*8, 9*). After 24 and 48 hours cells were lysed in Laemmli buffer (Biorad). Samples were run in 15% Tris-HCL gels, transferred to a PVDF membrane (Biorad), blocked with Odyssey blocking buffer (Licor) and probed with anti-HMGA2 antibody (1:4000; a generous gift from M. Narita and S. Lowe) and anti-GAPDH antibody (1:20000; Sigma). Blots were scanned and bands were quantitated using the Odyssey Infrared Imager (Licor).

Constructs

The 3' UTR of the mouse *Hmga2* cDNA (BC052158) was subcloned into pCR2.1-TOPO (Invitrogen) for site-directed mutagenesis. To disrupt each *let-7* complementary site, the nucleotides that paired to nucleotides 3 and 5 of the miRNA were substituted (Fig. 2A), using the Quikchange site-directed mutagenesis kit (Stratagene). The UTR fragment was then cloned back into the vector containing the *Hmga2* cDNA (pCMV•SPORT6.1). To construct the *Renilla* luciferase reporters, wild-type and mutant *Hmga2* 3' UTRs were amplified (PCR primers, 5'-GCGTCTCGAGGGGCGCCGACATTC and 5'-GGCGCGGCCGCAGTCAGAGGGCACAC) and cloned into the XbaI and NotI sites of pIS1 (*10*). To construct the mammalian expression vectors, *Hmga2* inserts were excised from the pCMV•SPORT6.1 constructs using EcoRI restriction sites and cloned into pcDNA3.1 (Invitrogen).

Luciferase Assays
F9, NIH3T3, and HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) in 24-well plates (F9: 1.0 x 10^5 cells/well; NIH3T3 and HeLa: 0.5 x 10^5 cells/well) with 25 ng firefly luciferase control reporter plasmid pISO (*10*) and 100 ng *Renilla* luciferase reporter plasmid, and 1.25 µg pUC19 (used as carrier), with or without 25 nM miRNA duplex. Firefly and *Renilla* luciferase activities were measured 24 hours after transfection with the Dual-luciferase assay (Promega). *Renilla* activity was normalized to firefly activity to control for transfection efficiency. To account for differences in plasmid preparations, values were then normalized to those of the reporter in F9 cells without added miRNA.

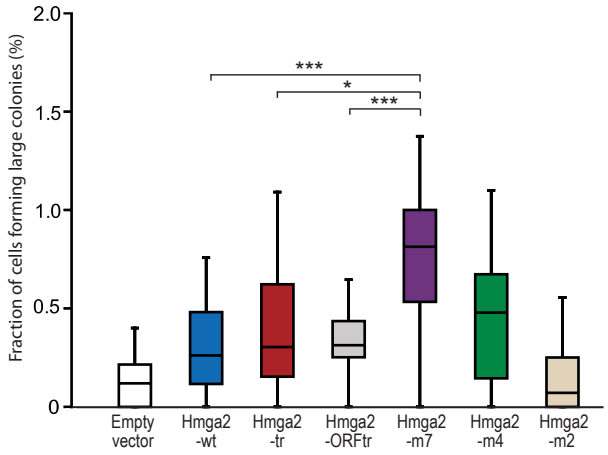
Soft-agar Assay
NIH3T3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS (Invitrogen) and 1% Penicillin/Streptomycin (Invitrogen) and transfected using the Nucleofector kit (Amaxa). Marker-selected populations of NIH3T3 cells were obtained by growing the transfectants with G418 (400 µg/ml) for no more than 14 days. This time was limited to avoid outgrowth of particularly rapidly growing transfectants. Compared to the endogenous Hmga2 level in a population stably transfected with empty vector, the bulk populations stably transfected with *Hmga2* vectors overexpressed Hmga2 protein by no more than 4.5 fold, as determined by Western blot. Soft-agar assays were performed as described (*11*), except the population of stably transfected cells were used rather than clonal lines. 5 x 10^4 stably transfected NIH3T3 cells were suspended in 0.5% Noble Agar (Sigma) in Ham's F12 medium (Cellgro), supplemented with 12% FCS and G418 (400 µg/ml), and plated in triplicates over a first layer of 0.5% Noble Agar in Ham's F12 medium. The cells were grown at 37° C and 5% $C0_2$, and colonies were counted at day 14 and day 28 but did not differ at these two time points. Colonies of eight cells or more were counted (Fig. 3B), as were colonies of at least 32 cells (fig. S1). Four independent transfections were done for each of the constructs. Each transfection was plated in triplicate. For each plate, 30 fields of about 30 cells were counted.

Tumor formation in nude mice
Tumorigenicity was evaluated after injecting 1 x 10^6 stably transfected cells subcutaneously into the rear flanks of athymic nude mice (NCRNU-M-M; Taconic), again using populations of stably transfected cells (selected using G418 for 14 days). For each construct we did four injections, each one into a different mouse. Every mouse was injected with two different constructs; one in the left side and the other in the same region of the right side. All constructs were injected at the same time. Every three days mice were evaluated for tumor development, and tumors were measured to obtain growth curves (fig. S2).

Statistics
The soft agar data were first analyzed by Kruskal-Wallis test to find out if there were significant differences in the percentage of colonies formed per plated cells between the different vectors ($P < 0.0001$). Then for each pair of vectors, we compared the percentages of colonies, performing Mann-Whitney tests. All calculations were done in SPSS version 14.

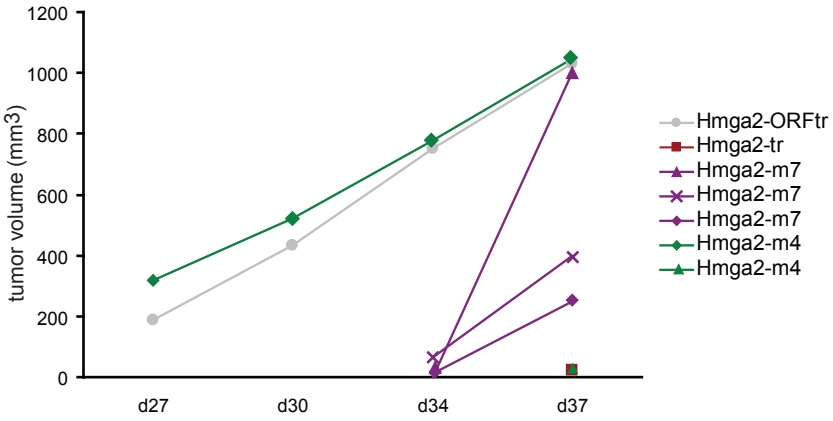
References

1. M. M. Petit *et al.*, *Genomics* **57**, 438 (1999).
2. E. F. Schoenmakers, C. Huysmans, W. J. Van de Ven, *Cancer Res.* **59**, 19 (1999).
3. M. Nilsson, F. Mertens, M. Hoglund, N. Mandahl, I. Panagopoulos, *Cytogenet. Genome Res.* **112**, 60 (2006).
4. M. D. Odero *et al.*, *Leukemia* **19**, 245 (2005).
5. K. Kurose *et al.*, *Genes Chromosomes Cancer* **27**, 303 (2000).
6. T. Takahashi *et al.*, *Genes Chromosomes Cancer* **30**, 196 (2001).
7. N. C. Lau, L. P. Lim, E. G. Weinstein, D. P. Bartel, *Science* **294**, 858 (2001).
8. G. Hutvagner, M. J. Simard, C. C. Mello, P. D. Zamore, *PLoS Biol* **2**, E98 (2004).
9. G. Meister, T. Tuschl, *Nature* **431**, 343 (2004).
10. B. P. Lewis, I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel, C. B. Burge, *Cell* **115**, 787 (2003).
11. M. Fedele *et al.*, *Oncogene* **17**, 413 (1998).



Formation of large colonies. For cells stably transfected with the indicated vector, the percentage that yielded large colonies (more than 32 cells) after 28 days is plotted (median, horizontal line; 25th through 75th percentile, box; range, error bars; n=12 from four independent experiments, each in triplicate). All but Hmga2-m2 yielded a significantly higher number of colonies than did the empty vector (Mann-Whitney test for each, $P < 0.05$). Hmga2-m7 showed significantly more colonies than any of the other constructs tested ($P < 0.02$ for each; *, $P = 0.002$; ***, $P < 0.0004$). No significant difference was observed between Hmga2-wt and the construct with the deleted ORF ($P = 0.6$).

Mayr et al., fig. S2



Growth curves of tumors in nude mice. The constructs used for stable transfectants are depicted in Fig. 3A.