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

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Abstract

MicroRNAs (miRNAs) are ~22-nucleotide RNAs that can pair to sites within messenger RNAs to specify posttranscriptional repression of these messages. Aberrant miRNA expression can contribute to tumorigenesis, but which of the many miRNA-target relationships are relevant to this process has been unclear. Here, we report that chromosomal translocations previously associated with human tumors disrupt repression of *High Mobility Group A2* (*Hmga2*) by *let-7* miRNA. This disrupted repression promotes anchorage-independent growth, a characteristic of oncogenic transformation. Thus, losing miRNA-directed repression of an oncogene provides a mechanism for tumorigenesis, and disrupting a single miRNA-target interaction can produce an observable phenotype in mammalian cells.

Hmga2 codes for a small, nonhistone, chromatin-associated protein that has no intrinsic transcriptional activity but can modulate transcription by altering the chromatin architecture (1,2). *Hmga2* is primarily expressed in undifferentiated proliferating cells during embryogenesis and in a wide variety of benign and malignant tumors (3–6). In many of these tumors, a chromosomal translocation at 12q15 truncates the human *HMGA2* open reading frame (ORF), typically retaining the three DNA-binding domains of HMGA2 while replacing the spacer and the acidic domain at the C terminus by any of a wide variety of ectopic sequences (3–5,7–10) (SOM text) (Fig. 1A). The loss of the C-terminal region is nearly always presumed to be the cause of oncogenic transformation. However, the translocations also replace the 3′ untranslated region (3′ UTR), and large fragments of the *Hmga2* 3′ UTR confer repression to luciferase reporters, which has led to the idea that transformation might be caused by the loss of repressive elements in the UTRs (11). Indeed, chromosomal rearrangements in some tumors leave the ORF intact but disrupt the 3′ UTR, and this is associated with over-expression of the wild-type Hmga2 protein (3,4,10). Moreover, transgenic mice overexpressing wild-type Hmga2 have similar phenotypes to those expressing the truncated protein; both develop abdominal lipomatosis, then lymphomas, pituitary adenomas, and lung adenomas (2,12,13).

The *Hmga2* 3′ UTR has seven conserved sites complementary to the *let-7* RNA (14), a miRNA expressed in later stages of animal development (15), leading us to suspect that disrupting *let-7* regulation of *Hmga2* might lead to oncogenic transformation. Consistent with this idea, introducing *let-7* RNA repressed Hmga2 in F9 cells (Fig. 1C), an undifferentiated embryonic carcinoma cell line that does not express detectable *let-7* RNA (Fig. 1B). Moreover, introducing a 2′-*O*-methyl oligonucleotide (16,17) complementary to *let-7* RNA enhanced Hmga2 in NIH3T3 cells (Fig. 1D), a cell line that naturally expresses *let-7* (Fig. 1B). These effects were

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specific in that they did not occur with non-cognate miRNAs (m*let-7* and miR-101, Fig. 1C) or a noncognate inhibitor (miR-124 2′-*O*-Me, Fig. 1D).

To test whether *let-7* directly targets the *Hmga2* 3′ UTR, we constructed reporters with the wild-type 3′ UTR (Luc-wt) and the UTR with point mutations disrupting all seven sites (Luc-m7), the four distal sites (Luc-m4), or the two most distal sites (Luc-m2) (Fig. 2A). In F9 cells, the degree of repression corresponded to the number of intact sites and depended on cotransfection of the *let-7* miRNA, whereas cotransfection of an unrelated miRNA had little effect (miR-124, Fig. 2B). The repression profile inverted when the *let-7* miRNA was replaced with a mutant miRNA, m*let-7* (Fig. 2B), which was designed to recognize the mutant sites instead of the wild-type sites (Fig. 2A). This rescue of repression with compensatory changes in the miRNA confirmed targeting specificity and the importance of direct pairing between the sites and the miRNA.

To examine repression directed by endogenous *let-7*, we repeated the reporter assays using NIH3T3 cells and HeLa cells, which naturally express *let-7* (Fig. 1B). In both cell types, reporter repression depended on the wild-type sites, as would be expected if the endogenous *let-7* miRNA directed repression (Fig. 2C). Adding exogenous *let-7* RNA enhanced repression, suggesting that *let-7* RNA was subsaturating in these cells. Adding mutant *let-7* also caused reporters with mutant sites to be repressed. Adding m*let-7* or miR-124 decreased repression of the reporter with wild-type sites (particularly in HeLa cells), as if the transfected miRNA was competing with endogenous *let-7* RNA for a limiting factor.

Having established that the sites within the *Hmga2* 3′ UTR could mediate *let-7*–directed repression, we tested whether disrupting this repression could promote oncogenic transformation. Assays for anchorage-independent growth were performed with NIH3T3 cells, which form colonies in soft agar when stably transfected with a potent oncogene. Stably transfecting a vector expressing wild-type *Hmga2* did not significantly increase the number of colonies compared with transfecting an empty vector, whereas transfecting a vector expressing a truncated *Hmga2* (Hmga2-tr) (Fig. 3A), shown previously to promote anchorage-independent growth (18), produced significantly more colonies (Fig. 3B). As in human tumors, Hmga2-tr lacked the spacer, the acidic domain, and the entire 3′ UTR with its miRNA complementary sites (Fig. 3A). The increase in colonies was attributed to the loss of *let-7* repression rather than the truncation of the protein because stably expressing Hmga2-m7, which had the full ORF but disrupted *let-7* complementary sites (Fig. 3A), produced at least as many colonies as Hmga2-tr, whereas stably expressing Hmga2-ORFtr, which had the truncated ORF but intact miRNA complementary sites (Fig. 3A), produced a number comparable to that of Hmga2-wt (Fig. 3B). Stably expressing Hmga2-m4, which retained the first three *let-7* sites, led to an intermediate number of colonies.

We next tested whether the stably transfected cells used to assay anchorage-dependent growth were also able to form subcutaneous tumors in nude mice. Consistent with our soft-agar results, tumors were observed when injecting cells expressing constructs with mutated *let-7* sites: After 5 weeks, three of four mice injected with Hmga2-m7 cells and two of four mice injected with Hmga2-m4 cells had tumors at the sites of injection. One of four injected with Hmga2-tr cells and one of four injected with Hmga2-ORFtr also had tumors, whereas no tumors were observed 5 weeks after injecting cells stably transfected with either wild-type Hmga2 or empty vector.

Taken together, our results support the proposal that the *let-7* miRNA acts as a tumor-suppressor gene (19,20) and indicate that a major mechanism of oncogenic *Hmga2* translocations associated with various human tumors is the loss of *let-7* repression. Thus, loss of miRNA-directed repression of an oncogene is another type of oncogene-activating event that should be considered when investigating the effects of mutations associated with cancer.

Likewise, mutations that create miRNA-directed repression of tumor-suppressor genes might also impart a selective advantage to the tumor cells. In this regard, we note that *Hmga2* translocations frequently append the *Hmga2* 3′ UTR to the 3′ end of known tumor-suppressor genes, including *FHIT*, *RAD51L1*, and *HEI10* (5,8,9), suggesting that *let-7*–directed repression of these translocation partners might cooperate with disrupting *Hmga2* repression to promote tumorigenesis.

Vertebrate miRNAs can each have hundreds of conserved targets and many additional nonconserved targets (15,21–25), all of which have confounded exploration of the biological impact of particular miRNA-target relationships. In worms, flies, and plants, repression of particular targets is known to be relevant because genetic studies have investigated what happens when that target alone is not repressed by the miRNA (26–28). Because of the possibility that multiple interactions might need to be perturbed to observe a phenotypic consequence in mammals, it has been unclear whether the importance of a particular mammalian miRNA-target interaction can be demonstrated experimentally. Our results, combined with previous cytogenetic studies that speak to the effect of misregulating the endogenous human *HMGA2* gene, show that disrupting miRNA regulation of *Hmga2* enhances oncogenic transformation, thereby demonstrating that disrupting miRNA regulation of a single mammalian gene can have a cellular phenotype in vitro and a clinical phenotype in vivo.

Supplementary Material

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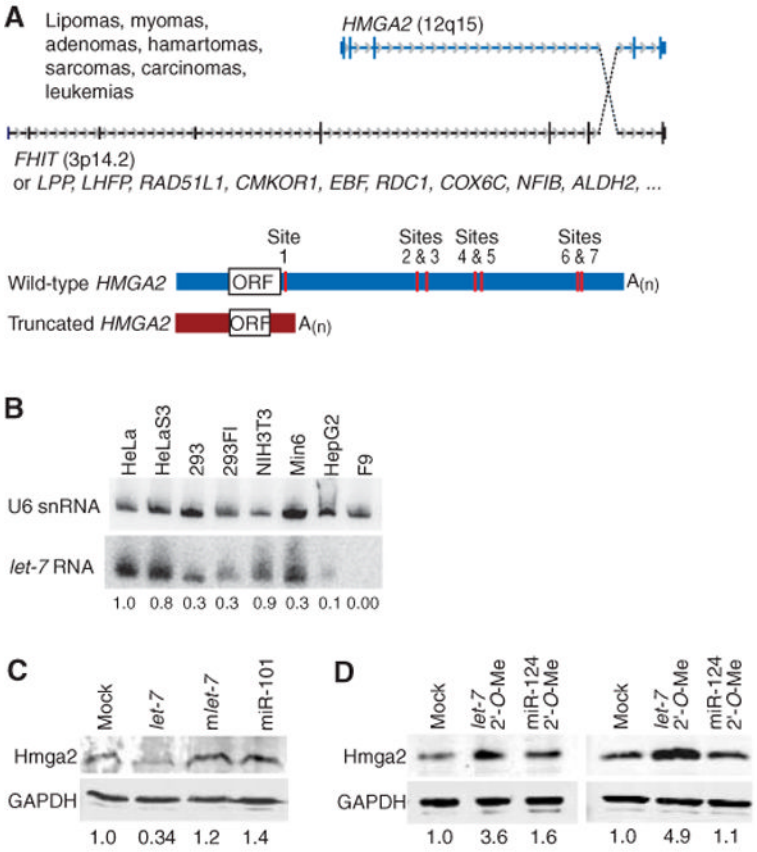

Fig. 1.

Chromosomal translocations involving *HMGA2*, and the influence of *let-7* on protein expression. (**A**) Translocations involving *HMGA2* and numerous translocation partners (3–10) (SOM text). These translocations generate a truncated *HMGA2* mRNA lacking the *let-7* complementary sites of the wild-type mRNA and are associated with the indicated tumors. In its 3′ UTR, human *HMGA2* has seven *let-7* complementary sites, all of which are conserved in the mouse, rat, dog, and chicken (14). $A_{(n)}$, polyadenylate tail. (**B**) RNA blot detecting *let-7* RNA in different cell lines. The blot was reprobed for U6 small nuclear RNA (snRNA), and *let-7* signal normalized to that of U6 is indicated. (**C**) Western blot monitoring endogenous Hmga2 48 hours after transfection of F9 cells with the indicated miRNA duplex. The blot was also probed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the normalized Hmga2 signal is indicated. (**D**) Western blot monitoring endogenous Hmga2 at 24 (left) and 48 (right) hours after transfection of NIH3T3 cells with the indicated 2′-*O*-methyl oligonucleotide (2′-*O*-Me). The blot was probed also for GAPDH, and the normalized Hmga2 signal is indicated.

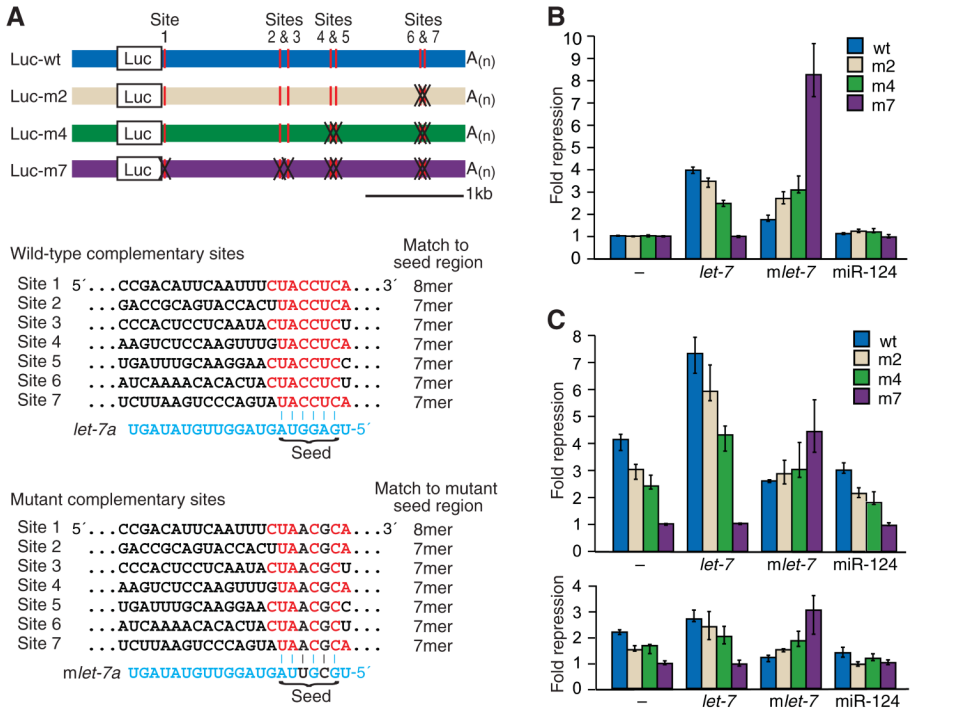

Fig. 2.

Luciferase reporter assays showing the influence of miRNA-target pairing. (**A**) Design of Luciferase constructs. The 3′ UTR of murine *Hmga2* was appended to the Luciferase ORF (Luc). *let-7* complementary sites are indicated (vertical red lines), as are mutant sites (black Xs). The Luc-m7, Luc-m4, and Luc-m2 were identical to Luc-wt, except they had seven, four, and two mutant sites, respectively. Each mutant site had two point substitutions that disrupted pairing to *let-7* but created pairing to mutant *let-7* (m*let-7*, bottom, in blue). The seven sites were identified in a search for conserved 7- and 8-nucleotide motifs (7mer and 8mer) matching the seed region of *let-7* (15). (**B**) Reporter repression in F9 cells supplemented with the indicated miRNA. Bars are colored to indicate the number of sites mutated in the reporter. Shown are median repression values, with error bars indicating 25th and 75th percentiles; n = 12, except experiments with no added miRNA (–), in which n = 36. Within each quartet, activity was normalized to that of the Luc-m7 reporter, except for the m*let-7* quartet, for which activity was normalized to that of the Luc-m7 reporter with noncognate miRNA (miR-124). (**C**) Reporter repression in NIH3T3 cells (top) or HeLa cells (bottom) supplemented with the indicated miRNA, performed and displayed as in (B). We also noticed two additional *let-7* complementary sites in the murine *Hmga2* mRNA, but located in the 5′ UTR. When tested in luciferase reporter assays, these sites mediated little or no repression in the different cell lines, and the mutant sites did not respond to m*let-7*, indicating that any effects observed with the 5′ UTR sites were not miRNA specific.

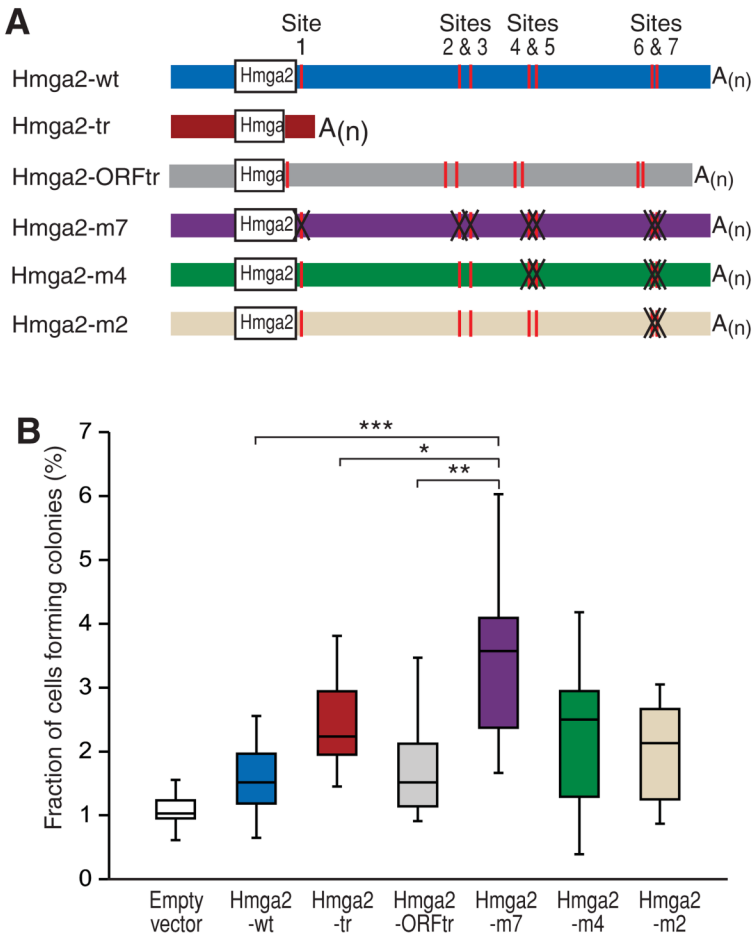

Fig. 3.

Soft-agar assay for anchorage-independent growth. (**A**) Hmga2 constructs used for stable transfection, depicted as in Fig. 2A. (**B**) Colony formation. For cells stably transfected with the indicated vector, the percentage that yielded colonies after 28 days is plotted (horizontal line, median; box, 25th through 75th percentile; error bars, range; $n = 12$ from four independent experiments, each in triplicate). All but Hmga2-wt yielded a significantly higher number of colonies than did the empty vector (Mann-Whitney test for each, $P < 0.05$). When compared with Hmga2-wt, a significantly higher number of colonies was observed for Hmga2-tr ($P = 0.003$). Hmga2-m7 showed significantly more colonies than any of the other constructs tested ($P < 0.05$ for each; *, $P = 0.041$; **, $P = 0.002$; ***, $P = 10^{-6}$). No significant difference was observed between Hmga2-wt and the construct with the truncated ORF ($P = 0.61$).