MicroRNA-7, a Homeobox D10 Target, Inhibits p21-Activated Kinase 1 and Regulates Its Functions

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Abstract

MicroRNAs are noncoding RNAs that inhibit the expression of their targets in a sequence-specific manner and play crucial roles during oncogenesis. Here we show that microRNA-7 (miR-7) inhibits p21-activated kinase 1 (Pak1) expression, a widely up-regulated signaling kinase in multiple human cancers, by targeting the 3′-untranslated region (UTR) of Pak1 mRNA. We noticed an inverse correlation between the levels of endogenous miR-7 and Pak1 expression in human cancer cells. We discovered that endogenous miR-7 expression is positively regulated by a homeodomain transcription factor, HoxD10, the loss of which leads to an increased invasiveness. HoxD10 directly interacts with the *miR-7* chromatin. Accordingly, the levels of Pak1 protein are progressively up-regulated whereas those of miR-7 and its upstream activator HoxD10 are progressively down-regulated in a cellular model of breast cancer progression from low to highly invasive phenotypes. Furthermore, HoxD10 expression in highly invasive breast cancer cells resulted in an increased miR-7 expression but reduced Pak1 3′-UTR-luciferase activity and reduced Pak1 protein. Finally, we show that miR-7 introduction inhibits the motility, invasiveness, anchorage-independent growth, and tumorigenic potential of highly invasive breast cancer cells. Collectively, these findings establish for the first time that Pak1 is a target of miR-7 and that HoxD10 plays a regulatory role in modifying the expression of miR-7 and, consequently, the functions of the miR-7-Pak1 pathway in human cancer cells. [Cancer Res 2008;68(20):8195–200]

Introduction

MicroRNAs (miR) are endogenous, noncoding RNAs that regulate gene expression in a sequence-specific manner in plants and animals. MicroRNAs are derived from long transcripts, which undergo trimming by Dicer and Drosha complexes into mature microRNAs before feeding into the RNA interference pathway (1). In addition to cleaving mRNA molecules, most of the animal microRNAs pair with their target sequence in the 3′untranslated region (UTR) of mRNA with imperfect complementarity and leads to interference with mRNA translation (2). In the recent years, it is becoming apparent that many microRNAs play crucial roles during development and oncogenesis (1–6). MicroRNA genes are present in the cancer-associated genomic regions or in fragile sites (3). In general, microRNAs are believed to be part of a network wherein a modest change in the level of one will set up a chain reaction and feedback pathways involving multiple microRNAs and affecting multiples targets of the same or different pathways (7). Many microRNAs are implicated as tumor suppressors or as oncogenes and found to be down-regulated or up-regulated in human tumors, including breast cancer (2–6). In fact, there is considerable excitement about the prospect of using microRNAs for anticancer therapy (8).

The progression of a number of human cancers, including breast cancer, to more invasive phenotypes and the invasiveness of experimental model systems have been linked with the up-regulation and activation of p21-activated kinase 1 (Pak1; refs. 9, 10). The Pak family of serine/threonine kinases play a pivotal role in physiologic processes including motility, survival, invasion, and mitosis (10). Paks are widely expressed in a variety of tissues and are often up-regulated or hyperactivated in a variety of human cancers such as breast cancer (9, 10) and gliomas (11). Due to the signal-dependent or constitutive hyperactivation of Pak1, there is an increased emphasis in designing specific inhibitors to interfere with its activation process (12–14). Given the diversity and overlapping nature of Pak regulators and effectors, it is likely that some of these inhibitors will have to overcome the expected problem of specificity and redundancy before clinical development.

In spite of an established role of Pak1 in normal physiologic or cancerous states, it remains unclear whether Pak1 is targeted by microRNAs in cancer cells. With the computational microRNA prediction tool mirBASE (15), we have identified hsa-mir-7-1 (termed miR-7) as a potential miRNA for targeting Pak1 mRNA. MiR-7 is an intronic miRNA that resides in the first intron of heterogenous ribonuclear protein K gene on chromosome 9 and is conserved across all species. In *Drosophila* photoreceptor cells, miR-7 controls epidermal growth factor receptor (EGFR) signaling and promotes photoreceptor differentiation (16). More recently, miR-7 has been shown to inhibit EGFR expression in glioblastoma (17). We have now discovered an inverse correlation between the levels of endogenous miR-7 and Pak1 expression and that miR-7 down-regulates Pak1 expression by targeting the 3′-UTR of *Pak1* mRNA and also inhibits the invasiveness of cancer cells. Here we have also defined the *cis* acting elements in the miR-7 promoter region involving positive regulation by a homeobox transcription factor, HoxD10, a gene product responsible for the inhibition of invasiveness of cancer cells (6, 18).

Materials and Methods

Cell lines and transfections. Cell lines were cultured in DMEM/F-12 with 10% fetal bovine serum. Transfections for miRNA mimics and plasmids were done with Oligofectamine and Fugene, respectively.

Plasmids, luciferase assay, and Western blotting. Pak1 3′-UTR and microRNA-7 promoter region were cloned into pGL3 control vector and pGL3 basic vector, respectively. HoxD10 plasmid was kindly provided by

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Dr. Nancy Boudreau (University of California, San Francisco, San Francisco, CA). Luciferase assays and Western blotting were done as described in ref. 11.

Quantitative PCR of microRNAs. RNA was isolated with mirVana miRNA kit. For quantitative analysis of miRNAs, two-step TaqMan real-time PCR was done with reagents from Applied Biosystems.

Migration, soft-agar, xenograft, and confocal studies. Migration, invasion, soft-agar, and confocal studies were done as described in ref. 19.

Chromatin immunoprecipitation was done with a kit from Upstate Biotechnology.

Results and Discussion

miR-7 targets the 3′-UTR of Pak1 and inhibits its expression. To search for microRNAs that might regulate the Pak1 expression, we screened the 3′-UTR region of human Pak1 mRNA against the public database for possible complementation of a minimum of 8 bp to the seed region of miRNAs (15). This exercise resulted in identification of miR-7 and miR-465 as candidate micro-RNAs for Pak1. MiR-7 is human miRNA (Fig. 1*A*) whereas miR-465 is of murine origin, and both were predicted to target Pak1 in multiple species (Supplementary Fig. S1*A*). To directly evaluate the effects of miR-7 and miR-465 on Pak1 expression, a number of human cancer cell lines were transfected with miR-7, miR-465, or negative control (designated miR-con) miRNA mimic, and Pak1 expression levels were determined by Western blotting. MiR-7 and miR-465 inhibited the levels of Pak1 protein but not Pak2, actin, or vinculin (Fig. 1*B*; Supplementary Fig. S1*B*). To show the direct targeting of the 3′-UTR region by the microRNAs, we next cloned the 3′-UTR region of Pak1, complementary to miR-7 or miR-465, into the pGL3-luciferase reporter (Pak1 3′-UTR-luc). We found that transfection of miR-7 or miR-465 along with the respective Pak1 3′-UTR-luc into

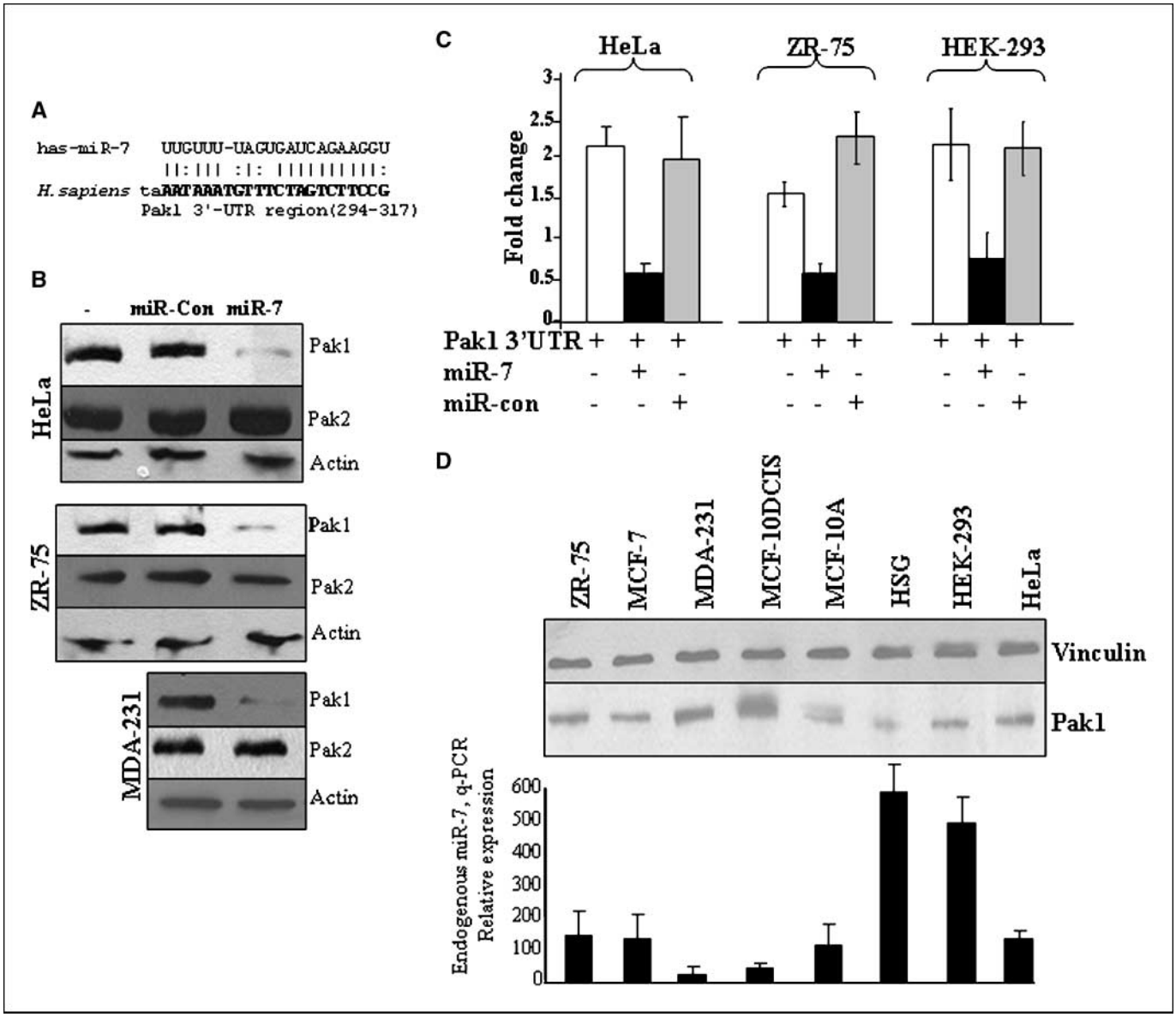

Figure 1. miRNA-7 inhibits Pak1 expression. *A,* sites of miRNA-7 seed matches in the Pak1 3′-UTR. *B,* Western blot of Pak1, Pak2, and actin in HeLa, ZR-75, and MDA-231 cells after 48 h of transfection with miR-7 or miR-con. *C,* normalized Pak1 3′-UTR-luc reporter activity in HeLa, ZR-75, and HEK-293 cells after 48 h of transfection of miR-7 or miR-con. *Columns,* mean of three experiments; *bars,* SD. *D,* Western blot for Pak1 expression and relative expression of miR-7 by quantitative PCR (*q-PCR*) in the indicated cell lines. *Columns,* three experiments; *bars,* SD.

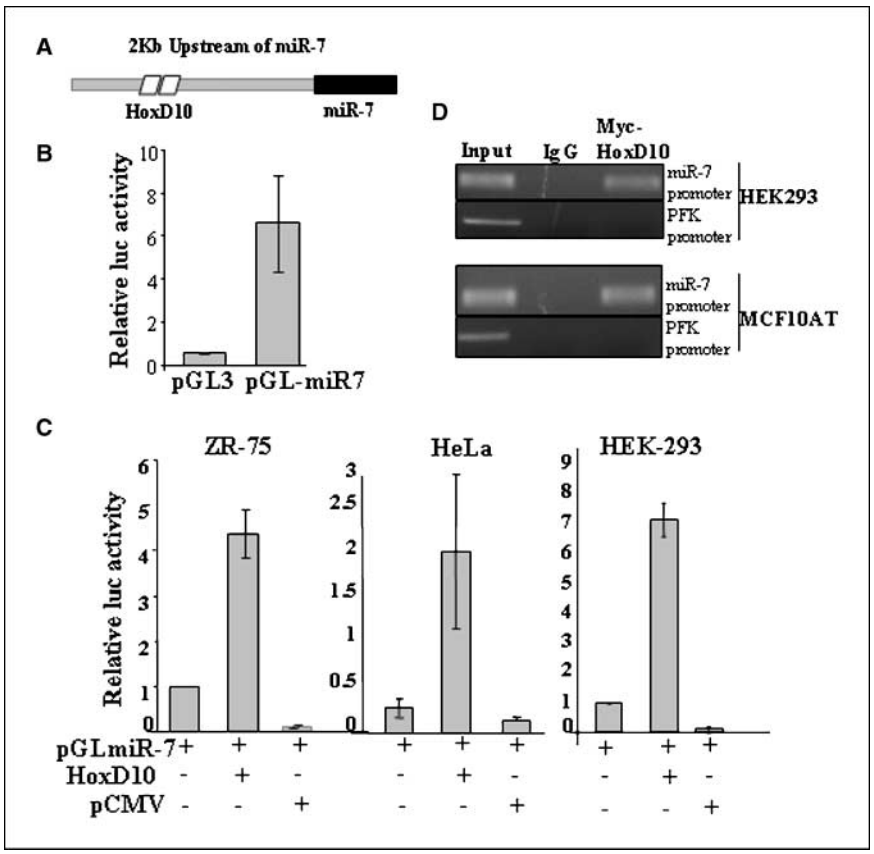

Figure 2. HoxD10 regulation of the miR-7 promoter. *A,* schematic representation of miR-7 putative promoter with the binding sites for HoxD10. *B,* normalized pGL-miR-7-luc activity in HeLa cells. *Columns,* mean of three experiments; *bars,* SD. *C,* normalized pGL-miR-7-luc activity in the indicated cells transfected with HoxD10 or pCMV control vector. *Columns,* mean of three experiments; *bars,* SD. *D,* recruitment of Myc-HoxD10 into the *miR-7* gene chromatin (240 bp; −1,139 to −899) by chromatin immunoprecipitation in HEK-293 and MCF10-AT cells.

various human cancer cell lines resulted in a significant inhibition of luc activity from the reporter, whereas there was no such inhibitory effect of the control miR-con (Fig. 1*C*; Supplementary Fig. S1*C*). These findings suggest that miR-7 or miR-465 directly targets the 3′-UTR of Pak1 and selectively inhibits Pak1 expression. To assess the relationship between the endogenous levels of Pak1 and its microRNAs, we next determined the levels of miR-7, as well as Pak1 protein, by quantitative PCR in a variety of exponentially growing cancer cell lines. In general, cells with increased levels of miR-7 contain reduced levels of Pak1 protein as compared with cells with low levels of miR-7 and high levels of Pak1 (Fig. 1*D*). Inspite of repeated attempts, we were unable to detect miR-465 in human cells (data not shown), presumably due to its restricted expression in murine systems. Therefore, we have studied the regulation of Pak1 expression and functions by miR-7 in the subsequent studies.

MiR-7 is directly regulated by the transcription factor HoxD10. To gain insights into the mechanisms by which miR-7 might be regulated in a physiologic setting, we analyzed the upstream 2-kb region of the putative *miR-7* promoter for the presence of binding motifs for various transcription factors using the PROMO[3] search program. This analysis suggested that miR-7 resides within the first intron of heterogenous ribonuclear protein K gene on chromosome 9. We found the presence of two perfect consensus motifs for HoxD10 within the −958 to −968 and −1,019 to −1,028 regions from the transcriptional initiation site of miR-7 (Fig. 2*A*). Because HoxD10 has been intimately linked with the invasion and metastatic potential of human breast cancer cells (6, 18) and because HoxD10 expression is lost as a function of invasion (18) and reintroduction of HoxD10 restores nontumorigenic phenotypes in invasive breast cancer cells (18), we decided to focus on HoxD10 as a potential upstream modulator of miR-7 expression.

To study the effect of HoxD10 on the expression of miR-7, we next cloned the putative *miR-7* promoter into a TATA-less basic pGL3-luc reporter (pGLmiR-7) and established the functionality of pGLmiR-7 in HeLa cells (Fig. 2*B*). We next showed that transient expression of HoxD10, but not control vector, efficiently stimulates the transcription of miR-7 from the pGLmiR-7 reporter in multiple human cancer cell lines (Fig. 2*C*). To show the potential recruitment of HoxD10 to the *miR-7* gene, we next performed chromatin immunoprecipitation analysis of the *miR-7* chromatin. Due to the lack of a good chromatin-immunoprecipitation–grade HoxD10 antibody, we used a Myc-tagged HoxD10 expression vector in these studies. We found that Myc-HoxD10 is indeed recruited to the *miR-7* promoter region encompassing −1,139 to −899 (240 bp), which included both HoxD10 binding sites. PFK promoter was used as a negative control, which lacks HoxD10 binding sites (Fig. 2*D*). Together, these observations suggest that HoxD10 directly interacts with the putative *miR-7* promoter and that miR-7 expression is positively regulated by the transcription factor HoxD10.

HoxD10 regulation of Pak1 expression via miR-7. Because miR-7 targets Pak1 (this study) and because HoxD10 positively regulates the expression of miR-7 (this study) and the fact that the loss of HoxD10 (6, 18) is a major contributor of cancer cell invasiveness which is also driven by Pak1 (10, 19), the above findings suggest an interesting possibility of a dynamic relationship

[3] http://alggen.lsi.upc.es/

between Pak1, miR-7, and HoxD10, and the potential role of HoxD10 in the cellular action of the miR-7-Pak1 axis in cancer cells. To examine these possibilities in the context of breast cancer progression to more aggressive phenotypes, we next analyzed the levels of Pak1, miR-7, and HoxD10 in exponentially growing cell lines derived from the MCF-10A model system (20). This model consists of four isogenic, human breast cancer cell lines with distinct invasive and metastatic potential (19, 20). Interestingly, we found that the level of Pak1 protein is progressively up-regulated whereas that of miR-7 as well as HoxD10 is progressively down-regulated from low invasive MCF10A to highly invasive MCF-10DCIS cells (Fig. 3*A*). To validate these results, we next showed that transient overexpression of HoxD10 leads to up-regulation of miR-7 (Fig. 3*B*) and down-regulation of Pak1 3′-UTR-luc activity in the highly invasive MCF-10DCIS cells as well as in HEK-293 cells (Fig. 3*C*). The noticed regulation of miR-7 and Pak1-UTR activity by HoxD10 was also accompanied by a corresponding down-regulation of Pak1 protein in HEK-293 and MCF10DCIS cells (Fig. 3*D*). In brief, these results suggested a regulatory role of HoxD10 in modifying the expression of miR-7 and, consequently, the functions of the miR-7-Pak1 pathway in human cancer cells.

MiR-7 suppresses the motility, invasiveness, anchorage independence, and tumorigenesis of breast cancer cells. To further delineate the potential effects of miR-7 down-regulation of Pak1 on the biology of breast cancer cells, we next examined the effects of miR-7 on the biology of MDA-MB231 cells, an established model system for highly invasive and tumorigenic breast cancer cells. We found that miR-7–mediated down-regulation of Pak1 in MDA-MB231 cells was accompanied by a profound inhibition of the cell motility, cell invasiveness, the ability of cells to grow in an anchorage-independent manner, and the tumorigenic potential of cells in nude mice (Fig. 4*A–C*). These findings raise the possibility that miR-7 could be potentially developed as an agent to inhibit the expression and tumor-promoting functions of Pak1. This is important because Pak1 or its effectors have been shown to be up-regulated in a number of human cancers, including breast cancer and gliomas (10–12). Accordingly, a number of academic groups and pharmaceutics have initiated research programs to target Pak1 kinase activity for cancer therapy (13, 14).

While this study was near its completion, we noticed an interesting report describing the ability of miR-7 to also inhibit EGFR and insulin receptor substrate 1 by directly targeting the 3′-UTRs of their mRNAs (17). This is understandable but exciting, given the fact that microRNAs are expected to target multiple mRNAs. Furthermore, microRNAs are expected to work as part of a network and could affect the components of the same pathway at multiple levels (7). Because Pak1 signaling also affected EGFR (10) and Akt (a target of IRS1), we next examined the levels of EGFR and IRS-1 proteins in the cell lysates used above in this study. As expected, transfection of miR-7 down-regulates the levels of

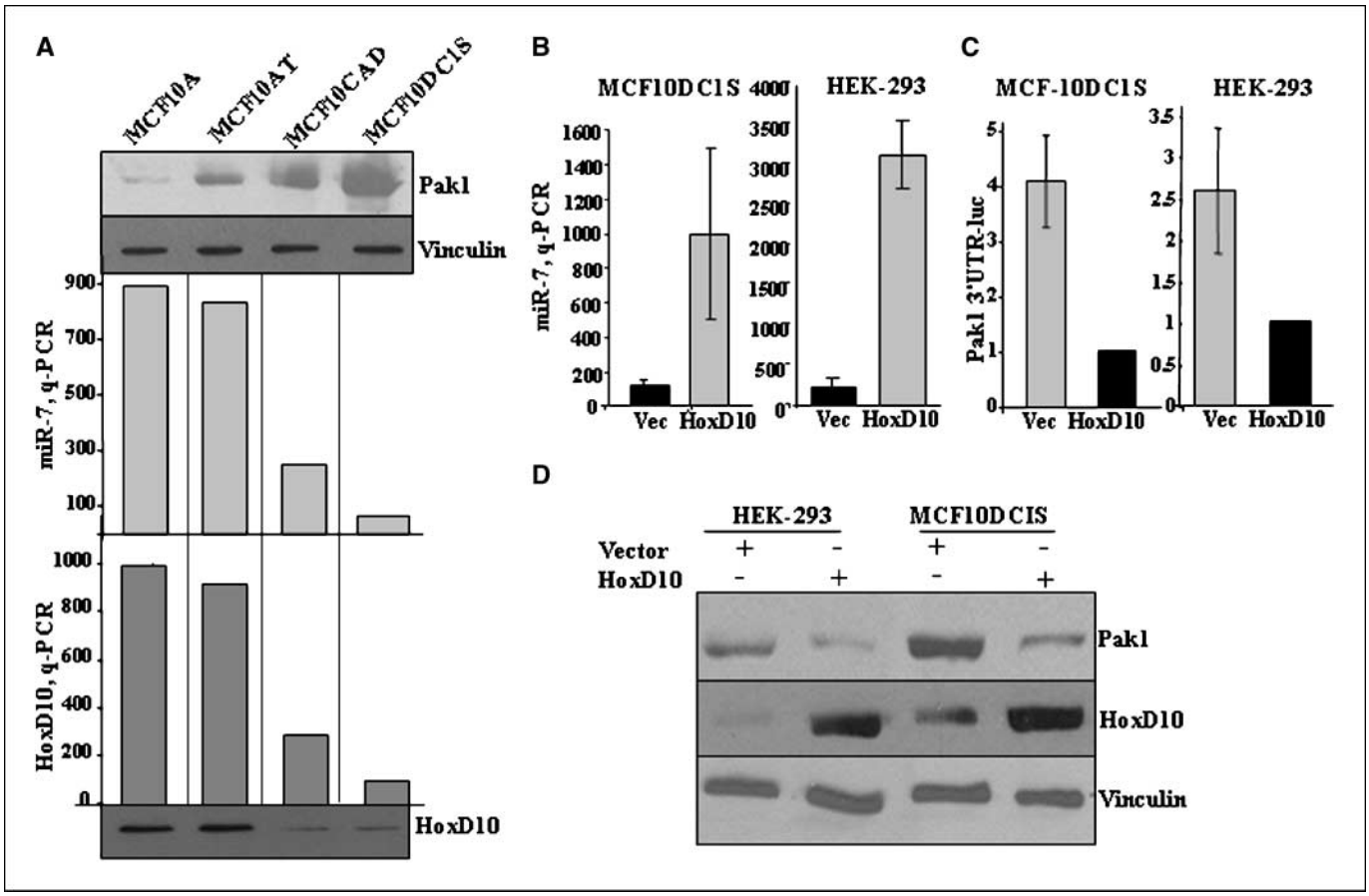

Figure 3. Dynamic correlation of Pak1, miR-7, and HoxD10 in the breast cancer progression model. *A,* Western blot analysis of Pak1 and HoxD10 and quantitative PCR analysis of the endogenous miR-7 and HoxD10 in the MCF10A model system. *B,* effect of HoxD10 expression on the levels of miR-7 in MCF-10-DCIS and HEK-293 cells. *Columns,* mean of three experiments; *bars,* SD. *C,* normalized Pak1 3′-UTR-luc activity in MCF10DCIS and HEK-293 cells transfected with HoxD10 or control vector. *Columns,* mean of three experiments; *bars,* SD. *D,* Western blot analysis of Pak1 and vinculin in MCF10-DCIS and HEK-293 cells transfected with HoxD10 or control vector.

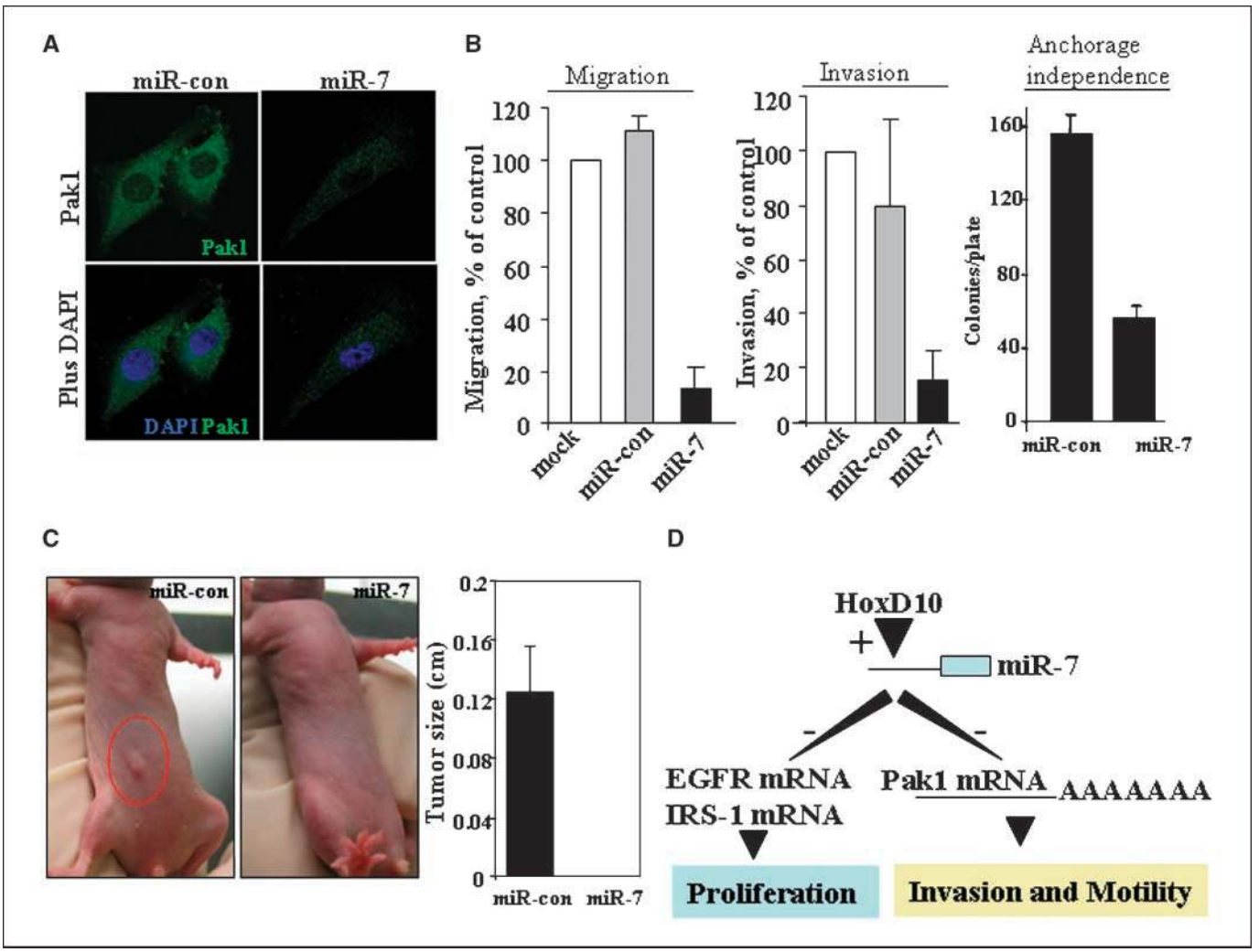

Figure 4. Biological effects of miR-7 in breast cancer cells. *A,* confocal analysis of Pak1 expression in miR-7–transfected MDA-231 cells. *DAPI,* 4′,6-diamidino-2-phenylindole. *B,* effect of miR-7 or miR-con on the motility, invasiveness, and anchorage independence of MDA-231 cells. *C,* effect of miR-7 on the tumorigenic potential of MDAMB-231 cells in nude mice. *D,* an integrated working model of the findings presented here.

EGFR and IRS1 proteins in HeLa, ZR-75, and MDA-231 cells (Supplementary Fig. S2). We further observed that the levels of EGFR and IRS1 correlated well with the levels of Pak1 but were inversely related with the endogenous miR-7 status in human cell lines, with the exception of IRS1, in the MCF-10A model system (Supplementary Fig. S3*A* and *B*). Interestingly, we found that HoxD10 overexpression in HEK-293 or MCF-10DCIS cells also leads to a distinct down-regulation of EGFR and IRS-1 (Supplementary Fig. S4), presumably due to increased expression of miR-7 (this study; Fig. 3*B*).

In summary, the findings presented here in conjunction with recent reports on miR-7 and HoxD10 suggest a model wherein HoxD10, a known regulator of cancer invasiveness, is likely to control the levels of Pak1 protein (and of EGFR and IRS1 and, perhaps, other targets yet to be described) and thus affect the cancerous phenotypes via the miR-7 pathway. Because reintroduction of HoxD10 into MDA-MB231 cells has previously been shown to compromise invasiveness (18), these results now suggest a potential role of the noted reduction in the Pak1 level due to increased expression of miR-7. In addition, our present findings also connect the HoxD10/miR-7/Pak1 pathway with a recently described invasion-promoting role of Twist regulation of miR-10B, which in turn has been shown to inhibit the translation of HoxD10 (6). In light of the observations presented here, it is possible that inhibition of HoxD10 will lead to repression of miR-7 level and up-regulation of Pak1 expression and its functions (Fig. 4*D*). To have a comprehensive understanding of the emerging significance of microRNA network and the engagement of multiple targets in shaping the behavior of the cancer cells, it will be important to keep investigating the above hypotheses and test the lead microRNA in more complex model systems in due course.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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