miR-10b expression in breast cancer stem cells supports self-renewal through negative PTEN regulation and sustained AKT activation

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Abstract

Cancer stem cells (CSCs) are linked to metastasis. Moreover, a discrete group of miRNAs (metastamiRs) has been shown to promote metastasis. Accordingly, we propose that miRNAs that function as metastatic promoters may influence the CSC phenotype. To study this issue, we compared the expression of 353 miRNAs in CSCs enriched from breast cancer cell lines using qRT-PCR analysis. One of the most altered miRNAs was miR-10b, which is a reported promoter of metastasis and migration. Stable overexpression of miR-10b in MCF-7 cells (miR-10b-OE cells) promoted higher self-renewal and expression of stemness and epithelial-mesenchymal transition (EMT) markers. In agreement with these results, inhibiting miR-10b expression using synthetic antisense RNAs resulted in a decrease in CSCs self-renewal. Bioinformatics analyses identified several potential miR-10b mRNA targets, including phosphatase and tensin homolog (PTEN), a key regulator of the PI3K/AKT pathway involved in metastasis, cell survival, and self-renewal. The targeting of PTEN by miR-10b was confirmed using a luciferase reporter, qRT-PCR, and Western blot analyses. Lower PTEN levels were observed in CSCs, and miR-10b depletion not only increased PTEN mRNA and protein expression but also decreased the activity of AKT, a downstream PTEN target kinase. Correspondingly, PTEN knockdown increased stem cell markers, whereas AKT inhibitors compromised the self-renewal ability of CSCs and breast cancer cell lines overexpressing miR-10b. In conclusion, miR-10b regulates the self-renewal of the breast CSC phenotype by inhibiting PTEN and maintaining AKT pathway activation.

Keywords hsa-miR-10b; microRNAs; non-coding RNAs; tumor-initiating cancer cells

Subject Categories RNA Biology; Signal Transduction; Stem Cells

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Introduction

Studies in cancer biology support the existence of a small proportion of cancer cells with high self-renewal and differentiation potential. These cells, called cancer stem cells (CSCs), are responsible for tumor initiation and maintenance [1]. Whether these CSCs arise by mutations in normal tissue stem cells or progenitor cells or from differentiated cells from different cancer subtypes is currently unknown [2]. These cells have been proposed to promote tumor relapse and metastasis because of their intrinsic resistance to anti-oncogenic treatments, their self-renewal, and their potential for multi-lineage differentiation [3].

Breast adenocarcinomas and cell lines derived from them have been shown to contain functional CSCs [4]. In these cells, diverse metastatic features have been uncovered, such as epithelial-mesenchymal transition (EMT) signatures and overexpression of master stem cell transcription factors [5]. Similarly, metastasis drivers can enhance CSC properties [6]. For example, forced expression of a metastasis effector, such as Ras homolog family member C (RhoC), increased the proportion of stem/progenitor cancer cells identified by the aldehyde dehydrogenase (ALDH) marker [7]. In this sense, other master regulators of metastasis could sustain the CSC phenotype.

MicroRNAs (miRNAs) are a group of small RNAs that can negatively control gene expression through the RNA-induced silencing complex (RISC). A single miRNA can control several different mRNAs and promote or suppress specific cellular processes [8,9]. Aberrant miRNA expression is associated with diverse pathophysiological conditions, including cancer. In particular, miRNAs that control central metastasis regulators have recently been shown to regulate cell invasion. These metastamiRs have been reviewed elsewhere [10,11]. We propose that miRNAs acting as metastasis promoters may influence CSC capabilities such as self-renewal and differentiation.

To test this hypothesis, we analyzed the expression profiles of 353 miRNAs in CSCs of the luminal and basal breast cancer cell lines MCF-7 and MDA-MB-231, respectively, and compared them to the expression profiles of non-stem cells. We detected numerous

deregulated miRNAs; prominent among these differentially expressed miRNAs, we found the previously described metastamiR miR-10b. *In vitro* and *in vivo* assays demonstrated that miR-10b promotes CSC features such as self-renewal and stemness. With the aid of target predictors and a luciferase reporter assay, we found that phosphatase and tensin homolog (PTEN) is a bona fide miR-10b target. Lower PTEN levels were observed in CSCs, and depletion of miR-10b in several cell lines not only increased PTEN mRNA and protein expression but also decreased AKT activity, a downstream PTEN target kinase. Finally, both activation of PTEN and AKT inhibition decreased the self-renewal ability of CSCs and breast cancer cells overexpressing miR-10b (miR-10b-OE cells).

Results

miRNA expression in breast CSCs

To analyze the expression of miRNAs in CSCs derived from breast cancer cell lines, we used magnetic-activated cell sorting (MACS) and a panel of cell surface proteins. For luminal breast cancer cells, we used CD44, a cell membrane glycoprotein that facilitates cancer cell invasion and metastasis and that has been widely used as a CSC marker in breast cancer [12,13]. The left panel of Figs 1A and EV1A shows that CSC enrichment was successful, as assessed by serial mammosphere-forming assays. Similar results were observed using extreme limiting dilution assays (ELDAs) [14] (Figs 1B and EV1C). In addition, higher expression of two stemness markers, SRY-box2 (SOX2) and POU class 5 homeobox 1 (OCT4), three EMT markers, snail family zinc finger 1 (SNAIL), twist family bHLH transcription factor 1 (TWIST) and vimentin, and the metastasis marker RhoC was observed in these cells (Fig 1C and D). Because basal breast cancer has a stem-like phenotype, CD44 is expressed in almost 100% of MDA-MB-231 cells. Nevertheless, a subpopulation defined by the cell surface marker epithelial cell adhesion molecule (EpCAM) has an enhanced stemness phenotype [15,16], including higher expression of stemness and EMT markers (Fig 1E), an increased number of stem-like cells, as assessed by serial mammosphere-forming assays (Figs 1A, right panel and EV1B), and a higher number of colony-forming cells, as assessed by ELDA (Figs 1F and EV1C). In both CSC-enriched populations, we analyzed 353 miRNAs by qRT-PCR using TaqMan Low Density Arrays (TLDA) (Dataset EV1). In these assays, we found 142 deregulated miRNAs (119 up-regulated and 33 down-regulated) in MCF-7 luminal CSC and nine deregulated miRNAs (five up-regulated and four down-regulated) in MDA-MB-231 basal CSCs. Nine miRNAs were regulated in both cell lines (Fig 1G), including miR-10b. It has been previously reported that this miRNA is regulated by TWIST [17], a central stemness transcription factor in breast cancer [18]. In addition, miR-10b has been proposed to target Homeobox D10 (HOXD10), which promotes the activation of metastasis drivers such as RhoC. We corroborated this finding with a similar gene signature observed in MCF-7/CD44⁺ cells (Fig 1D). Metastasis is a key cancer hallmark that is intimately associated with the stem cell phenotype [6]. For these reasons and because no direct relationship between miR-10b and stemness functions has been proposed, we analyzed this potential relationship.

miR-10b expression controls self-renewal capacity and an EMT/CSC signature

To determine whether miR-10b regulates the CSC stemness phenotype, we performed loss- and gain-of-function experiments. CD44⁺ MCF-7 or EpCAM⁺ MDA-MB-231 breast cancer cells were transiently transfected with a synthetic anti-miR-10b. This anti-miR induced a significant decrease in miR-10b expression and a concomitant loss of the mammosphere-forming ability of enriched CSCs (Fig 2A, upper panel). Similarly, miR-10b inhibition decreased the number of mammospheres in sorted EpCAM⁺ MDA-MB-231 cells (Fig 2B). Additionally, as previously reported, miR-10b inhibition in MDA-MB-231 cells reduced migration (Fig EV2A) [19,20]. Finally, we found that depleting miR-10b in CD44⁺ MCF-7 cells decreased the number of colonies in soft agar assays (Fig EV2B), another surrogate marker for stem cell phenotypes [21]. These data indicate that miR-10b represents an important component of the clonal potential and migration capabilities of breast CSCs.

For gain-of-function studies, the pre-miR-10b sequence was cloned and stably transfected into MCF-7 breast cancer cells (creating MCF-7-miR-10b-OE cells) and HER2⁺ SKBR-3 cells (creating SKBR-3-miR-10b-OE cells). miR-10b overexpression increased the percentage of CD44⁺ MCF-7 cells from 6.8 to 14.5% (Fig EV2C). Similarly, SKBR-3-miR-10b-OE cells presented an increase in the number of ALDH-positive cells, from 12.0 to 16.6% (Fig EV2E). As expected, serial mammosphere assays showed a reproducible increase in mammosphere-forming ability in these cells (Fig 2A, lower panel). miR-10b also regulated the anchorage-independent growth of MCF-7 cells and CSCs derived from them, a reported cancer stem cell phenotypic trait (Fig EV2B, lower panel, and D) [21]. In addition, miR-10b overexpression increased the levels of E-cadherin, a well-known marker for mammosphere formation [22] (Fig EV2G). Finally, we found a significant ($P = 0.04$) increase in the number of CSCs in the MCF-7-miR-10b-OE cell line, as assessed with an *in vivo* limiting dilution assay using nu/nu mice (Figs 2C, left panel and EV2F upper panel). This effect was not restricted to a single cell line, as overexpression of miR-10b in SKBR-3 cells also increased the number of CSCs in *in vivo* assays ($P = 0.007$) (Figs 2C, right panel and EV2F, lower panel). Tumor morphology between cell lines was similar (Fig EV3).

We then hypothesized that stemness and EMT factors could be modified by miR-10b overexpression. We performed experiments with either miR-10b overexpression or inhibition in four breast cancer cell lines and in the CD44⁺ MCF-7 subpopulation. Twenty-four hours after transient transfection of miR-10b in MCF-7 and SKBR-3 cells, OCT4/3, SOX2, vimentin, and SNAIL expression increased compared with the miR-Scr-OE control, which resembled the expression pattern of isolated CSCs (Fig 2D). We then selected two cell lines with high miR-10b expression (T47-D and MDA-MB-231 cells, Fig EV3C), as well as CD44⁺ MCF-7 cells, and transiently transfected specific anti-miRs. After 24 h, we observed decreased (< 0.75) expression of stemness and EMT genes after anti-miR-10b transfection compared with the anti-miR-control. These results support the participation of miR-10b in the stemness phenotype. Interestingly, the regulation of miR-10b by TWIST [17], a central EMT and stemness transcription factor in breast CSCs [18], further supports this hypothesis.

Figure 1. Breast CSC enrichment and miRNA expression.

- A** Breast cancer cell lines were sorted using CD44 for luminal MCF-7 cells (left panel) or EpCAM for MDA-MB-231 cells (right panel). Serial mammosphere assays were performed using 4,000 cells for each cell fraction, counting spheres after 3 weeks. Plots show the mean $\pm$ SEM of mammospheres formed with 4,000 cells from three independent experiments performed in triplicate. An ANOVA and Dunnett's multiple comparison test was used to compare the groups ($*P < 0.05$).
- B** Extreme limiting dilution assay (ELDA) of MCF-7 cells. Sorted cells were serially diluted (1,250, 625, 312, 156, and 78 cells) and then seeded in 96-well plates. After 1 week, the cells were fixed, stained, and counted. The lower limit for well repopulation in CD44⁺ cells was 312 cells, compared with 615 cells for both unsorted and CD44⁻ populations. The data obtained were evaluated using the ELDA software. *P*-values that test the single-hit hypothesis using the likelihood ratio test are shown. The curves are depicted in Fig EV1.
- C** Relative expression of the stemness factors SOX2 and OCT4/3 and the EMT markers SNAIL, vimentin, and TWIST in MCF-7/CD44⁺ cells. The fold change was calculated using GAPDH as an internal control using the $2^{-\Delta\Delta CT}$ method comparing stem or non-stem cells versus the total cell line. The box and whiskers graph shows the range, median, and quartile interval values from three independent experiments.
- D** miR-10b overexpression affecting stemness and EMT factors. Relative quantification of miR-10b (relative to RNU6B), HOXD10 and RHOC (relative to GAPDH) was performed in MCF-7/CD44⁺ fractions compared to non-enriched cells. Box plots show the range, median, and quartile interval values from three independent experiments.
- E** Relative expression of stemness factors SOX2 and OCT4/3 and EMT markers SNAIL and vimentin in MDA-MB-231/EpCAM⁺ cells. The fold change was calculated using GAPDH as an internal control using the $2^{-\Delta\Delta CT}$ method comparing stem or non-stem cells versus the total cells. The box and whiskers graph shows the range, median, and quartile interval values from three independent experiments.
- F** Extreme limiting dilution assay (ELDA) of MDA-MB-231 cells. Sorted cells were serially diluted (1,250, 625, 312, 156, and 78 cells) and then seeded in 96-well plates. After 1 week, the cells were fixed, stained, and counted. The lower limit for well repopulation in EpCAM⁺ cells was 156 cells compared with 625 cells for both unsorted and EpCAM⁻ populations. The data obtained were evaluated using the ELDA software. *P*-values that test the single-hit hypothesis using the likelihood ratio test are shown. The curves are depicted in Fig EV1.
- G** Nine miRNAs are commonly regulated in both CD44⁺ and EpCAM⁺ breast cancer cells. Quantitative RT-PCR of miRNAs was performed on sorted MCF-7 and MDA-MB-231 cell populations using TaqMan Low Density Arrays (TLDA). Results were plotted as a heat map comparing CD44⁺ to CD44⁻ cells (MCF-7) and EpCAM⁺ to EpCAM⁻ cells. The scale is shown below the map.

Figure 2.

Figure 2. Self-renewal ability is controlled by miR-10b expression in MCF-7 cells.

- A Upper panel, left: MCF-7/CD44⁺ cells were transfected with anti-miR-10b or a scrambled control, and qRT-PCR was performed. Upper panel, right: An aliquot of these cells was subjected to mammosphere-forming assays. The graph shows the mean $\pm$ SEM of the number of formed mammospheres per 4,000 seeded cells in three independent experiments. Lower panel, left: Expression of miR-10b relative to RNU6B in MCF-7 cells stably transfected with a plasmid containing the miR-10b gene versus an empty vector. Results are the mean $\pm$ SEM values from three independent experiments. Lower panel, right: miR-10b-overexpressing MCF-7 cells analyzed by serial mammosphere assays. The mean $\pm$ SEM of the number of formed mammospheres per 4,000 seeded cells in three independent experiments was plotted on each bar.
- B Mammosphere formation was performed by seeding 4,000 MDA-MB-231/EpCAM⁺ cells, and after 3 weeks, the cells were disaggregated, counted, and reseeded. Stable miR-10b-overexpressing cells from three independent clones were tested in three passages, and cell number was plotted as the mean $\pm$ SEM.
- C *In vivo* limiting dilution assays. miR-10b-OE or control cells were subcutaneously injected in the backs of mice at three doses (1,000, 10,000, and 50,000 cells for MCF-7 and 5,000, 10,000, and 20,000 for SKBR-3). Mice were sacrificed after 8 weeks, and tumor burden was analyzed using the ELDA software. Plots are depicted in Fig EV2. The tables show the number of mice with tumors versus the total number of mice injected. Below the tables, the estimated frequency (freq) of stem cells for each group and *P*-values are shown. Stem cell frequency for MCF-7 cells was 1 in 34,252 (range 94,888–12,364) in miR-10b-OE cells versus 1 in 238,965 (range 1,817,801–31,414) in miR-scr cells. Stem cell frequency for SKBR-3 cells was 1 in 5,013 (range 11,135–2,257) in miR-10b-OE cells versus 1 in 18,778 (range 45,397–7,767) in miR-scr cells.
- D Stemness and EMT marker mRNA levels following miR-10b modulation. miR-10b, OCT4/3, SNAIL, and vimentin expression, was quantified in breast cancer cell lines using transient transfection of miR-10b versus a miR-Scr vector (left) or anti-miR-10b versus anti-miR-ctl (right). Log-scale plots show the mean and SEM for the relative expression of each gene from triplicate independent transient transfections, each performed three times. A Mann-Whitney *U*-test was used to compare expression between groups. SOX2, OCT4/3 and vimentin were significantly modulated by up- or down-regulation of miR-10b (*P* = 0.0043, *P* = 0.0054 and *P* = 0.000908, respectively).

Data information: A two-tailed Student's *t*-test was used to compare the two groups (**P* < 0.05).

Targeting PTEN with miR-10b sustains AKT activation and promotes self-renewal in MCF-7 CSCs

To further explore miR-10b's mechanism of action, we performed an *in silico* search for putative targets using the ComiR and TargetScan tools. Both programs reported higher scores for the mRNAs of the cancer-related genes ASLX1, BAP1, GATA3, NCOR1, PI3KCA, and PTEN. Because PTEN has previously been shown to be required to sustain breast CSCs [11,23] is co-regulated with miR-10b after TGF β exposure [24] and its levels decreased in stem cells derived from MDA-MB-231 and MCF-7 cell lines (to 81.4% in MDA-MB-231/EpCAM⁺ cells and less than 1% in MCF-7/CD44⁺ cells by quantitative RT-PCR), we chose to focus on this gene and the pathway it regulates. Figure 3A shows that a conserved miRNA regulatory element (MRE) for miR-10b is found 145 bp downstream of the PTEN coding sequence (ComiR and TargetScan scores of 0.875 and -0.21 , respectively). Similarly, a miR-10b MRE is located 170 bp downstream of the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) coding sequence. Therefore, these two putative targets of miR-10b, which are related to the AKT pathway, were assessed using 3'-UTR luciferase reporter assays. Briefly, firefly luciferase was engineered to examine the 3'-UTR MRE of PTEN, PIK3CA, or HOXD10 (positive control). These constructs were transiently cotransfected with anti-miR-10b or anti-miR-ctl in MCF-7 and T47-D cells. In T47-D cells, which have higher miR-10b expression levels (Fig EV3C), miR-10b inhibition increased PTEN and HOXD10 reporter activities, whereas miRNA overexpression in SKBR-3 cells inhibited reporter activity (Fig 3B). The luciferase activity of a UTR with a mutated miR-10b MRE seed sequence was not modulated by either miR-10b inhibition or overexpression. Although not statistically significant, we also found that using anti-miR-10b or miRNA overexpression, miR-10b regulation induced similar changes in MCF-7 cells, which express lower levels of this miRNA (Fig 3B). No changes were observed in PIK3CA reporter activity, demonstrating that miR-10b's effects are restricted to PTEN in this signaling pathway.

To further support the specific regulation of PTEN by miR-10b, we performed a series of gain- or loss-of-function analyses using a panel of breast cancer cell lines. In these experiments, we inhibited or overexpressed miR-10b and measured PTEN or PIK3CA

expression by qRT-PCR. As shown in Fig 3C, miR-10b overexpression decreased PTEN expression, but not PIK3CA expression, in MCF-7 cells, whereas inhibition of miR-10b in MDA-MB-231 increased PTEN expression. We next expanded the analysis to a larger panel of cell lines (SKBR-3, T47-D, CD44⁺ MCF-7, MCF-7, and MDA-MB-231 cells), performing both gain- and loss-of-function experiments in each cell line or subpopulation and plotted the relative levels of miR-10b and PTEN. Figure 3D shows that miR-10b expression levels were inversely correlated with PTEN levels ($r = -0.80$), whereas PIK3CA expression was not ($r = 0.43$). This result was also corroborated by Western blot analyses, where gain- or loss-of-expression assays showed that miR-10b regulated PTEN in four cellular backgrounds (Fig 3E). To support these data, PTEN immunohistochemistry was performed on tumor xenografts in mice with MCF-7 cells stably overexpressing miRNAs. Lower PTEN protein expression was observed in tumors from MCF-7/miR-10b-OE cells (Fig 3F).

Next, to assess the importance of PTEN on the stemness phenotype, we inhibited this protein using RNA interference with Dicer-substrate RNAs and measured the expression of stem cell markers. As shown in Fig 3G left panel, inhibiting PTEN increased the expression of the stem markers OCT4 and SNAIL, supporting the role of PTEN in the breast CSC phenotype. Overexpressing miR-10b in PTEN-depleted cells only increased the levels of SOX2 and vimentin, without further affecting OCT4/3 and SNAIL levels (Fig 3G, right panel). This could be due to a larger decrease in PTEN levels due to the additive effects of miR-10b and the PTEN shRNA, which in turn could regulate additional genes. These results, and the fact that miR-10b overexpression and PTEN depletion did not induce an additive increase in mammosphere formation, support the notion that miR-10b acts through PTEN (Fig 3G, right panel). Furthermore, PTEN knockdown in an additional cell line (T47-D) induced a recovery in the mammosphere depletion produced by miR-10b inhibition (Fig 3H). We next cloned the PTEN open reading frame, including the miR-10b MRE in its 3'-UTR or an alternative version containing a mutated miR-10b MRE. As expected (Fig 3I), we found that cells expressing the mutated version of PTEN presented higher PTEN levels. In line with this, expressing the mutated version of PTEN decreased the stem cell phenotype as assessed by the stem cell marker SNAIL.

Figure 3.

Figure 3. miR-10b targets PTEN but not PIK3CA mRNA.

- A miR-10b miRNA recognition elements (MREs) in the 3'-UTRs of PTEN and HOXD10 genes. The coding DNA sequence (CDS) and MRE are shown in red and blue, respectively. The new PTEN MRE is located 145 bp downstream of the CDS, with its pairing sequence located between nucleotides 2,388 and 2,410 of the main PTEN transcript isoform. Below each depiction, an alignment of each mRNA and miR-10b is shown.
- B MCF-7, SKBR-3, and T47-D breast cancer cells were transfected with gain- or loss-of-function constructs/shRNAs and their respective controls (a miR-10b-OE plasmid or anti-miR-10b). MREs of the validated target HOXD10 and of the putative targets PTEN and PIK3CA were cloned into pMIR-Report, and dual luciferase reporter assays were performed. A mutated control miR-10b (MRE MUT PTEN) was also included. Bars show the mean $\pm$ SEM from six independent transfections. A two-tailed Student's *t*-test was used to compare the differences between groups ($*P < 0.05$).
- C PTEN and PIK3CA expression after miR-10b modulation. Left panel shows miR-10b, PTEN, and PIK3CA expression by qRT-PCR in MCF-7 cells with ectopic miR-10b expression. Right panel shows miR-10b, PTEN, and PIK3CA expression by qRT-PCR in MDA-MB-231 cells after antagomiR-10b transfection. Data (mean $\pm$ SEM) are from three independent transient transfections performed by triplicate.
- D To further confirm PTEN targeting, we performed gain- or loss-of-function experiments in breast cancer cell lines (MCF-7, MCF-7/CD44⁺, SKBR-3, and T47-D) by overexpressing miR-10b or inhibiting it with a miR-10b-OE plasmid or anti-miR-10b shRNAs. PTEN and miR-10b levels were measured by qRT-PCR. The log₂-fold change of PTEN (left panel) and PIK3CA (right panel) mRNA levels obtained through the $2^{-\Delta\Delta CT}$ method were plotted against their respective miR-10b expression levels. Correlation analysis was performed [PTEN ($r = -0.80$), PIK3CA ($r = 0.43$)].
- E PTEN protein modulation by miR-10b. Upper panel: PTEN protein levels in SKBR-3 and MCF-7 cells were analyzed by Western blot. GAPDH was used as a loading control with cells cultured as in (B). Lower panel: Densitometric analysis of Western blot assays performed with three biological replicates. Bars represent mean $\pm$ SEM.
- F PTEN immunohistochemical analysis of MCF-7-miR-10b-OE or control MCF-7-miR-Scr-OE tumor xenografts.
- G miR-10b overexpression does not increase mammosphere formation in PTEN-depleted cells. MCF-7-miR-Scr-OE cells (left panels) and MCF-7-miR-10b-OE (right panels) were transfected with a Dicer-substrate RNA directed toward PTEN, and after 24 h, quantitative RT-PCR assays for PTEN, SOX2, OCT4/3, SNAIL, and vimentin were performed. Additionally, the same cells were analyzed for mammosphere formation. The graph shows the mean and standard error of the relative increase of each marker or number of mammospheres in PTEN-depleted cells (black bars) and in cells transfected with a scrambled control (white bars) in three independent biological experiments performed in triplicate.
- H T47-D cells were transfected with antagomiR-10b, a dsRNA for PTEN or both and mammospheres produced as described in the Material and Methods. Bars show mean and SEM from three independent experiments. $*P = 0.003$ by Student's *t*-test, after ANOVA showing statistical differences in groups.
- I PTEN overexpression compensates for miR-10b overexpression. Left panel: MDA-468 cells were transfected with a plasmid overexpressing wild-type PTEN or a version containing a mutated MRE in its 3'-UTR, with or without a plasmid containing miR-10b and PTEN mRNA expression was measured by end-point RT-PCR. Right panel: Relative expression of stemness factor SNAIL. The plots show the relative expression from biological triplicate analyses (mean and SE shown).

These results, together with previous results showing that PTEN depletion induces the expression of stem markers and the expansion of prostate and leukemia CSCs [11,25], further demonstrate the importance of this miRNA in CSC maintenance.

miR-10b expression increases AKT activation

Constitutive activation of the PI3K pathway has been previously reported as a key component of breast and prostate CSC maintenance [23,25,26]. To assess whether miR-10b inhibition of PTEN could regulate this cascade, we analyzed AKT activation, a key downstream kinase in this pathway. Thus, p-AKT (Ser473) levels were analyzed in CD44⁺/sorted cell fractions using Western blot analysis in MCF-7 cell line. As shown in Fig 4A and B, AKT phosphorylation was detected in CD44⁺ cells but barely or not at all in CD44⁻ or non-sorted cells. We then exposed cells overexpressing miR-10b to insulin and measured several PI3K signaling molecules. As expected, p-AKT was barely detectable in MCF-7/miR-Scr-OE cells exposed to serum compared with cells overexpressing miR-10b, where serum increased AKT phosphorylation in MCF-7/miR-10b cells. Insulin induced phosphorylation in both control and miR-10b-overexpressing cells (Fig 4B, upper panel). To support these results, AKT kinase assays for a downstream PI3K effector, GSK-3, were performed. As shown in Fig 4B upper panel, AKT kinase activity was detected only in MCF-7/miR-10b-OE cells exposed to serum or insulin. We then assessed the effect of PTEN expression in cells overexpressing miR-10b. As shown in Fig 4B lower panel, PTEN enforced expression decreased the levels of p-AKT in SKBR-3 cells overexpressing miR-10b. In line with this, cells overexpressing both miR-10b and PTEN presented higher p-AKT levels than cells overexpressing only PTEN.

To assess the functional impact of this pathway on the stemness effects of miR-10b, CD44⁺ cells were transiently transfected with

anti-miRs, and clonogenic assays were performed in the presence of PI3K activators or inhibitors. Although insulin did not significantly affect the colony-forming ability of anti-miR-ctrl-transfected cells, PI3KCA inhibitors significantly reduced the number of colony-forming cells. These results demonstrate the importance of the PI3K/AKT pathway in CD44⁺ CSCs. Moreover, miR-10b inhibition did not produce any additive effects on the clonal inhibition mediated by PI3KCA inhibitors (Fig 4C). Finally, we measured the impact of PI3K modulation on the miR-10b overexpression effects on clonogenicity. As expected, even though we detected no differences in the number of colonies after an insulin stimulus, PI3K inhibitors had a significant negative effect on the self-renewal capacity of MCF-7 miR-10b-OE cells (Fig 4D).

Interestingly, although insulin induced a similar degree of AKT phosphorylation, only cells overexpressing miR-10b effectively phosphorylated the downstream GSK-3 kinase. At least three different phosphorylation sites can activate the AKT kinase. In particular, phosphorylation at Thr308 can stabilize kinase functions and induce cell transformation, suggesting that additional phosphorylation events are needed for miR-10b to complete AKT signaling [27]. The lack of differential changes in clonogenic assays in cells with overexpressed or repressed miR-10b after blocking the PI3K cascade supports the role of this pathway in miR-10b's stem cell effects.

A recent report revealed that PTEN-deficient prostate and breast tumors depend on AKT2 for maintenance [28]. Because the phenotype elicited by miR-10b could be reminiscent of PTEN disruption, miR-10b overexpression may render cancer cells sensitive to AKT2 inhibition. Conceivably, even with functional PTEN tumors, AKT2 inhibition could be used to target CSCs if this alteration proves to be synthetically lethal. Further studies are required to test this idea. With the advent of new PI3K inhibitors [29] now in clinical trials, elucidating potentially informative therapeutic response markers, including miR-10b, should be pursued.

Figure 4. The effects of miR-10b on stemness are mediated by the PI3K pathway.

- A** AKT phosphorylation in CSCs from MCF-7 cells sorted by CD44. GAPDH protein levels were used as a loading control.
- B** Upper panel: Cultures were serum-starved for 18 h and then stimulated with insulin. PTEN, PI3KCA, GSK-3, and phospho-AKT Ser473 protein levels were analyzed by Western blot. GAPDH was used as a loading control. AKT kinase activity was analyzed in these cells by immunoprecipitating phosphorylated AKT proteins with a p-AKT-Ser473 antibody and using chimeric GSK-3 as a substrate. Lower panel: SKBR-3 cells were transiently transfected with a plasmid containing an open reading frame of PTEN, miR-10b or both and phospho-AKT, AKT or tubulin levels analyzed by Western blot assays. Control is empty vector-transfected cells.
- C** Clonogenic ability of miR-10b-depleted CD44⁺ MCF-7 cells after PI3K pathway inhibition. Cultures were serum-starved for 18 h and then stimulated with insulin or exposed to the PI3K pathway inhibitors wortmannin or LY294002. Cells transfected with anti-miR-10b or a scrambled control (anti-miR-ctl) were seeded in soft agar at clonal density and exposed to insulin or PI3KCA inhibitors. After 3 weeks, colonies were quantified. Bars show the mean $\pm$ SEM for three independent experiments.
- D** miR-10b overexpression does not induce additional changes in cell colony numbers in response to insulin or PI3K inactivation. MCF-7 stable miR-Scr-OE or miR-10b-OE cells were seeded in soft agar at clonal density and exposed to insulin or PI3KCA inhibitors. After 3 weeks, colonies were quantified. Bars show the mean $\pm$ SEM for three independent experiments.

Data information: A two-tailed Student's t-test was used to compare the two groups (* $P < 0.05$).

Further supporting the importance of PTEN regulation by miR-10b, we analyzed the possible correlation between PTEN and miR-10b expression and prognosis in two previously published breast cancer cohorts [30,31]. Figure EV4 shows that patients with tumors with low PTEN mRNA levels had a higher relapse probability than those whose tumors had higher levels. Thus, patients whose tumors presented higher miR-10b expression had a higher distant relapse probability.

In conclusion, our results show for the first time that by modulating the PTEN/PI3K/AKT pathway, miR-10b is a *bona fide* regulator of stemness in breast cancer cells. More research is required to investigate the possible use of this miRNA as a biomarker.

Materials and Methods

Cell culture, cell sorting, flow cytometry, and pharmacological treatments

MCF-7, MDA-MB-231, T47-D and SKBR-3 human cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA.) and cultured in the recommended medium. Cell lines were dissociated using Accutase (Millipore, Darmstadt, Germany), and magnetic cell sorting was performed using the MiniMACS separator, LS columns and CD44-MicroBeads (human) or EpCAM-MicroBeads (human) (Miltenyi Biotec, Bergisch Gladbach, Germany). The proportion of CD44⁺ and EpCAM-positive cells was estimated using flow cytometry (FACS ARIA and FACS DIVA software, BD Biosciences, Franklin Lakes, USA). For ALDH^{br} cell flow cytometry, one million SKBR3-control and SKBR3-miR-10b cells were grown in 12.5 cm² plates under gentle agitation for 3 days. Upon cell

disaggregation, ALDH enzymatic activity was measured according to ALDEFLUOR assay manufacturer's guidelines (StemCell Technologies, BC, Canada). DEAB (diethylaminobenzaldehyde), an ALDH inhibitor, was used at a 1.5 mM concentration as a control. Insulin (100 µg/ml), wortmannin (100 nM), and LY294002 (50 nM) (Sigma-Aldrich, St. Louis, MO, USA) were used as described.

Mammosphere assays

Single cells were plated in 6-well plates (Corning) covered with 1% agarose at a density of 2,000 cells/ml at each passage. Cells were grown in DMEM/F12 medium supplemented with B27 (Invitrogen), 20 ng/ml EGF, 5 mg/ml insulin (BD Biosciences), and 0.5 mg/ml hydrocortisone. Mammospheres were collected by gentle centrifugation (130 g) after 7–10 days and dissociated enzymatically (10 min in 0.05% trypsin, 0.53 mM EDTA-4Na; Invitrogen). For stable cell lines and MACS fractions, we used three serial passages. The number of mammospheres after 10 days was counted manually using an inverted microscope. For immunohistochemistry, mammospheres were fixed in 4% formaldehyde in PBS and embedded in paraffin. Tissue sections were deparaffinized, hydrated, and, after blocking, incubated with an anti-E-cadherin antibody (Cell Signaling, #31955, MA, USA). Antibody binding was visualized with an HRP-conjugated secondary antibody (SignalStain Boost IHC Detection Reagent, Cell Signaling #8114, MA, USA).

Soft agar for colony formation assays

Cells (1,250) suspended in DMEM with 0.33% UltraPure™ Low Melting Point Agarose and 5% fetal bovine serum (FBS) were overlaid on 24-well plates containing a 0.5% agar base with DMEM and 5% FBS (Life Technologies, CA, USA). All samples were plated in triplicate. The plates were incubated at 37°C in a humidified incubator for 21 days. For visualization, 0.01% crystal violet was added, and the cells were incubated overnight at 4°C. Colonies were counted using ImageJ. For AKT modulation, recombinant human insulin (100 µg/ml), wortmannin (100 nM), and LY294002 (50 nM) (Sigma-Aldrich, St. Louis, MO USA) were used in suspension with the agar base and soft agar.

Extreme limiting dilution assays

In vivo and *in vitro* limiting dilution assays were analyzed using the Extreme limiting dilution assay (ELDA) software [14], which uses a generalized linear model to compare active cell frequencies (stem cells) in cell populations.

RNA isolation and qRT-PCR

RNA was purified from the cell lines using the TRIzol (Invitrogen, CA, USA) and GlycoBlue co-precipitant reagents. Reverse transcription of miRNA was performed using multiplex pools for miRNA expression analysis (miRNA Reverse Transcription kit, Life Technologies, CA, USA). miRNA profiling analysis was performed using a TaqMan Low Density Array 1.0 (Life Technologies, CA, USA). qRT-PCR for miR-10b, RNU6B, PTEN, and TWIST was performed using specific TaqMan probes. The remaining mRNAs were quantified using the Maxima SYBR Green/ROX kit (Fermentas Inc., MA, USA).

Constructs, transfections, knockdowns, and luciferase assays

A pre-miR-10b sequence was synthesized by IDTX and cloned into a BLOCK-iT Pol II miR RNAi Expression Vector (Invitrogen, CA, USA). The miR-10b construct and a non-targeting (scrambled) control were transfected into MCF-7 cells using Lipofectamine 2000 (Invitrogen, CA, USA), and stable cell lines were obtained after two rounds of selection using 5 µg/ml blasticidin (Invitrogen, CA, USA). The putative miR-10b MREs of the human PTEN, PIK3CA, and HOXD10 mRNAs were synthesized as 50-nt oligonucleotides and inserted between the SpeI and HindIII sites of the pMIR-Report plasmid. A mutated version of PTEN was constructed in the same way, but the core 5 nt of the MRE were deleted. MCF-7 cell lines with stable overexpression of miR-10b or a non-targeting control were seeded onto 96-well plates (1×10^4 cells/well). Cells were transfected with pMIR-Report constructs (50 ng/well) and pRL-SV40 *Renilla* luciferase (10 ng/well). All transfections were performed at least in triplicate using the Escort Reagent (Sigma-Aldrich, MO, USA). Cell lysates were prepared with Passive Lysis Buffer (Promega) 48 h after transfection, and luciferase activities were measured using the Dual Luciferase Reporter Assay kit (Promega, WI, USA). MCF-7 cell fractions were maintained in DMEM, grown to 60% confluency, transfected with anti-miRs using Lipofectamine 2000 (Life Technologies, CA, USA) for 4 h, and then incubated 24 h before each experiment. For PTEN knockdown assays, Dicer-substrate RNA duplexes (TriFecta, IDT Technologies, Coralville, IA) directed against PTEN were transfected into MCF-7-miR-Scr-OE cells using Lipofectamine 2000. We then selected the best performing dsRNA (with a decrease of 69%) to analyze stemness markers by qRT-PCR. For miR-10b function recovery experiments, the complete open reading frame and the 3'-UTR including the miR-10b MRE of PTEN were obtained by RT-PCR using the oligonucleotides 5'-CAGCAGCTTCTGCC ATCTCT and 5'-AGGTATATACATGACACAGCTACAC and cloned into pcDNA3.1 vector to obtain pcDNA-PTEN plasmid. Then, the MRE for miR-10b was mutated using the QuickChange Lightning kit (Agilent Technologies) following the manufacturer's instructions. The oligonucleotides used were as follows: forward: 5'-CCC TAT ACA TCC AGA **ATT CTT** TGA CAC TTG TTG TCC reverse: 5'-GGA CAA CAA GTG TCA AAG AAT TCT GGA TGT ATA GGG (in bold the mutation site).

Western blot and kinase assays

MCF-7 cell fractions and stable cell lines were serum-starved for 16 h and then treated with 10 ng/ml of rh-insulin (R&D Systems, MN, USA) in serum-free medium. Cell lysates were obtained using RIPA buffer with a protease inhibitor cocktail consisting of PMSF (100 µM) and Na_3VO_4 (0.2 mM) (Thermo Scientific Inc., MA, USA). Antibodies directed against p-AKT Ser473 (#9271, Cell Signaling Technologies, MA, USA), PTEN (D4.3, #9188, Cell Signaling Technologies, MA, USA), PI3KCA (C73F8, #4249, Cell Signaling Technologies, MA, USA), and GAPDH (Santa Cruz Biotechnology, CA, USA) were used for Western blot analyses. Cell extracts were analyzed by IP/Kinase assay; kinase reactions were performed in the presence of 200 µM of cold ATP and 1 µg of GSK-substrate. GSK-3 phosphorylation was measured by Western blot using phospho-GSK-3α/β (Ser21/9, 37F11) (Cell Signaling Technologies, #9327, MA, USA).

Animal studies

All mouse experiments were conducted in accordance with standard operating procedures approved by the University Committee on the Use and Care of Animals at the Metropolitan Autonomous University, Mexico. The nu/nu mice were purchased and maintained at UPAEL-UAM or INCAN (Instituto Nacional de Cancerología) (DF, Mexico). Cancer cells were subcutaneously injected in the backs of mice at three doses (50,000, 100,000, and 200,000 cells), and 100 μ l of a mixture of 1:1 PBS and growth factor-reduced (GFR) Matrigel (BD Bioscience, Franklin Lakes, USA) was used as a vehicle. Results were analyzed with the ELDA software (see above).

Statistical analysis

Data are presented as the mean $\pm$ SEM. Statistical significance was set at $P < 0.05$. The specific statistical tests used are mentioned in the Figure legends. Analyses were performed using GraphPad Prism for Windows version 5.03 (GraphPad Software, San Diego, CA, USA).

Expanded View for this article is available online.

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Author contributions

IB-O, ME, GC-C, FL, DC-A, and AS provided experimental data and reagents. IB-O, VM, and JM-Z carried out data analysis. IB-O, VM, and JM-Z designed experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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