The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44

Can Liu, Kevin Kelnar, Bigang Liu, Xin Chen, Tammy Calhoun-Davis, Hangwen Li, Lubna Patrawala, Hong Yan, Collene Jeter, Sofia Honorio, Jason F Wiggins, Andreas G Bader, Randy Fagin, David Brown & Dean G Tang

Cancer stem cells (CSCs), or tumor-initiating cells, are involved in tumor progression and metastasis. MicroRNAs (miRNAs) regulate both normal stem cells and CSCs, and dysregulation of miRNAs has been implicated in tumorigenesis. CSCs in many tumors—including cancers of the breast, pancreas, head and neck, colon, small intestine, liver, stomach, bladder and ovary—have elucidated the signaling and regulatory mechanisms that are unique to CSCs in order to design CSC-specific therapies. To this end, we used quantitative RT-PCR (qRT-PCR) to compare the miRNA expression of CD44+ and CD44− prostate cancer cells. The CD44+ prostate cancer cell population harbors tumor-initiating and metastatic cancer cells and is enriched in the self-renewal gene NANOG (ref. 27). We purified CD44+ prostate cancer cells from three xenograft models and CD44+ and CD44− prostate cancer cell populations, but whether miRNAs regulate CD44+ prostate cancer cells and prostate cancer metastasis remains unclear. Here we show, through expression analysis, that miR-34a, a p53 target, was underexpressed in CD44+ prostate cancer cells purified from xenograft and primary tumors. Enforced expression of miR-34a in bulk or purified CD44+ prostate cancer cells inhibited clonogenic expansion, tumor regeneration, and metastasis. In contrast, expression of miR-34a antagonists in CD44− prostate cancer cells promoted tumor development and metastasis. Systemically delivered miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice. We identified and validated CD44 as a direct and functional target of miR-34a and found that CD44 knockdown phenocopied miR-34a overexpression in inhibiting prostate cancer regeneration and metastasis. Our study shows that miR-34a is a key negative regulator of CD44+ prostate cancer cells and establishes a strong rationale for developing miR-34a as a novel therapeutic agent against prostate CSCs.

Many human cancers contain CSCs, which possess an enhanced tumor-initiating capacity, can self-renew, partially reconstitute the cellular heterogeneity of the parental tumor, and seem to be generally more resistant than other cancer cells to conventional anticancer therapeutics. Because of these properties, CSCs have been linked to tumor recurrence and distant metastasis. Consequently, it is essential to elucidate the signaling and regulatory mechanisms that are unique to CSCs in order to design CSC-specific therapies. To this end, we used quantitative RT-PCR (qRT-PCR) to compare the miRNA expression of CD44+ and CD44− prostate cancer cells. The CD44+ prostate cancer cell population harbors tumor-initiating and metastatic cancer cells and is enriched in the self-renewal gene NANOG (ref. 27). We purified CD44+ prostate cancer cells from three xenograft models and CD44+ and CD44− prostate cancer cell populations, but whether miRNAs regulate CD44+ prostate cancer cells and prostate cancer metastasis remains unclear. Here we show, through expression analysis, that miR-34a, a p53 target, was underexpressed in CD44+ prostate cancer cells purified from xenograft and primary tumors. Enforced expression of miR-34a in bulk or purified CD44+ prostate cancer cells inhibited clonogenic expansion, tumor regeneration, and metastasis. In contrast, expression of miR-34a antagonists in CD44− prostate cancer cells promoted tumor development and metastasis. Systemically delivered miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice. We identified and validated CD44 as a direct and functional target of miR-34a and found that CD44 knockdown phenocopied miR-34a overexpression in inhibiting prostate cancer regeneration and metastasis. Our study shows that miR-34a is a key negative regulator of CD44+ prostate cancer cells and establishes a strong rationale for developing miR-34a as a novel therapeutic agent against prostate CSCs.

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their p53 status (Supplementary Fig. 1 and Supplementary Results). Transfection of synthetic miR-34a oligonucleotides (oligos), but not the negative control (NC) miRNA oligos, induced cell-cycle arrest, apoptosis or senescence in p53-mutant and p53-null prostate cancer cells (Supplementary Figs. 2 and 3 and Supplementary Results).

To determine whether miR-34a can inhibit tumor development, we manipulated miR-34a levels (Supplementary Fig. 4) in a variety of prostate cancer cell types and then implanted the cells subcutaneously or orthotopically in the dorsal prostate in NOD-SCID mice (Fig. 1a and Supplementary Fig. 5). LAPC9 (Fig. 1d and Supplementary Fig. 5a) and HPCa58 (Fig. 1e) cells transfected with miR-34a produced significantly smaller tumors than the same cells transfected with NC oligos. LAPC9 cells are androgen dependent, whereas HPCa58 cells were from an early-generation xenograft tumor (Supplementary Methods). miR-34a also inhibited the secondary transplantation of HPCa58 cells (Fig. 1c). miR-34a showed similar tumor-inhibitory effects on androgen-dependent LAPC4 (Supplementary Fig. 5b) and androgen-independent DU145 (Supplementary Fig. 5d) and PPC-1 (Supplementary Fig. 5g) cells. As expected, miR-34a–transfected prostate cancer cells showed miR-34a levels at several orders of magnitude higher than cells with miR-NC (Supplementary Fig. 4a). In contrast to freshly transfected cells, the residual tumors showed only a marginal or no increase in miR-34a levels (Supplementary Fig. 4b), suggesting that transfected mature miR-34a oligo were gradually lost in vivo and explaining why miR-34a–overexpressing prostate cancer cells still regenerated some tumors. To complement the oligo transfection studies, we also infected prostate cancer cells with lentiviral or retroviral vectors encoding pre-miR-34a (Supplementary Fig. 1d) before implantation. The viral vector–mediated overexpression of miR-34a also inhibited tumor regeneration of LAPC4 (Supplementary Fig. 5c), DU145 (Supplementary Fig. 5e,f), and LAPC9 (not shown) cells. Notably, LAPC9 and LAPC4 cells transfected with miR-34a oligos (Supplementary Fig. 5a.b) and DU145 cells infected with the MSCV-34a retroviral vectors (Supplementary Fig. 5e) all developed fewer tumors compared to the corresponding controls ($P < 0.01$ for tumor incidence). Histological and immunohistochemical examination of tumor sections (Supplementary Fig. 6) showed increased necrotic areas and reduced Ki-67$^+$ cells in miR-34a transfected tumors, which also showed increased expression of HP-1$^+$(a protein that is associated with cell-cycle arrest and senescence). These overexpression experiments in unfractionated prostate cancer cells show that miR-34a possesses strong tumor-inhibitory effects.

To evaluate whether miR-34a–mediated inhibition of tumor development might be due to an effect on the CSC populations, we performed tumor growth experiments using purified CD44$^+$ or CD44$^-$ prostate cancer cells that had been subjected to manipulation of miR-34a levels. When we infected purified CD44$^+$ DU145 cells with lenti-34a, tumor regeneration was completely blocked in that tumor incidence was 10/10 for the lenti-ctl group, whereas the incidence for the lenti-34a group was 0/10 (Fig. 1f). When we transfected CD44$^+$ LAPC9 cells with miR-NC or miR-34a oligos, tumor incidence was 7/7 and 1/8, respectively ($P = 0.016$), and the only tumor observed in the miR-34a group was much smaller (0.03 g versus the mean tumor weight of 0.5 g for the miR-NC group) (Fig. 1g). Similarly, lenti-34a infection of CD44$^+$ LAPC9 cells also inhibited tumor regeneration (tumor incidences for the lenti-ctl and lenti-34a groups were 7/7 and 2/7, respectively; $P = 0.01$) (Supplementary Fig. 5h).

We also performed the opposite experiments by introducing an anti-sense inhibitor of miR-34a (that is, anti-34a or miR-34a antagonist) into purified CD44$^+$ DU145 or LAPC9 cells, which are less tumorigenic than the corresponding CD44$^-$ cells. The antagonist–transfected LAPC9 cells showed reduced endogenous miR-34a (Supplementary Fig. 4c) and increased mRNA levels of CDKN1B (Supplementary Fig. 4d), a known miR-34a target, validating the specificity of anti-34a. We observed that CD44$^+$ DU145 cells transfected with anti-34a developed larger tumors than those with anti-NC oligos (0.2 g versus 0.05 g; $P = 0.038$) (Fig. 1h), which we verified in a repeat experiment (Supplementary Fig. 5i). Likewise, in two independent experiments,
bulk LAPC9 cells transplanted with anti-34a oligos generated larger orthotopic tumors than those with anti-NC oligos (Fig. 1i and Supplementary Fig. 7a). Anti-34a also promoted subcutaneous tumor growth in purified CD44+ LAPC9 cells (Supplementary Fig. 7b). Notably, in the two orthotopic LAPC9 tumor experiments (Fig. 1i and Supplementary Fig. 7a), we observed lung metastasis in 5/9 (56%; for anti-NC) and 8/11 (73%; for anti-34a) tumor-bearing mice, respectively. When we quantified the GFP-bright foci (≥100 µm) in the five anti-NC- and eight anti-34a mouse lungs, the latter showed higher levels of metastasis (Fig. 1j and Supplementary Fig. 7c,d). Taken together, these in vivo experiments in purified prostate cancer cells suggest that miR-34a negatively regulates the tumor-initiating capacity of prostate CSCs.

To further investigate the effects of miR-34a on prostate CSC properties, we performed holoclone, clonogenic and sphere formation assays18,19,27,32,33. Prostate cancer cell holoclones contain self-renewing cancer cells32, and sphere-formation assays have been widely used to measure the activity of stem or progenitor cells1,33. We first established stringent competition assays in which clones (holoclones formed in culture dishes), colonies (formed in Matrigel or methylcellulose) and (floating) spheres were all of clonal origin (Supplementary Fig. 8). Under these conditions, miR-34a overexpression inhibited holoclone formation, clonogenic capacity, or sphere establishment in DU145 (Fig. 2a,b and Supplementary Fig. 2d,e), LAPC4 (Fig. 2c,d) and PPC-1 (Fig. 2e and Supplementary Fig. 3h,i) cells. In addition, miR-34a inhibited sphere formation by primary HPCA cells (Fig. 2f and Supplementary Fig. 9a). HPCA cells overexpressing miR-34a formed tiny or differentiated spheres (Supplementary Fig. 9b). Notably, miR-34a overexpression abrogated secondary sphere establishment (Fig. 2f,d) and inhibited sphere formation in purified CD44+ HPCA116 cells (Fig. 2g). By contrast, anti-34a increased the inherently low sphere-forming capacity of CD44+ HPCA116 cells several-fold (Fig. 2h). These observations collectively indicate that miR-34a negatively regulates prostate CSC properties.

Subsequently, we performed four sets of therapeutic experiments (Fig. 3 and Online Methods) in NOD-SCID mice with established prostate tumors. We first observed that repeated intratumoral injections of miR-34a into subcutaneous PPC-1 tumors halted tumor growth (Supplementary Fig. 5g). We then established orthotopic PC3 tumors and, 3 weeks later, injected miR-34a or miR-NC oligos complexed with a lipid-based delivery agent26 into the tail veins of mice every 2 d. Systemically delivered miR-34a reduced PC3 tumor burden by 50% (Fig. 3a). In two therapeutic experiments with orthotopic LAPC9 tumors, miR-34a reduced lung metastasis (Fig. 3b,c,e and Supplementary Fig. 10) without affecting tumor growth (Fig. 3c). miR-34a also extended the survival of tumor-bearing mice (Fig. 3d). These results indicate that miR-34a has therapeutic efficacy against established prostate tumors.

Cyclin D1, CDK4 and 6, E2F3, N-Myc, c-MET and BCL-2 have been reported to be direct targets of miR-34a20,21,23,31,34,35. A survey of some of these molecules revealed that miR-34a affected the levels of cyclin D1, CDK4, CDK6 and c-MET in our prostate cancer models (Supplementary Figs. 4d,e and 6d,e). There was a consistent and strong inverse correlation between miR-34a levels and CD44 (Fig. 4a,b, Supplementary Figs. 1a, 4e and 11a–c and Supplementary Table 2). For example, CD44 protein and CD44* prostate cancer cells were reduced in miR-34a–treated tumors (Fig. 4a). Transfected miR-34a downregulated CD44 in prostate cancer cells (Fig. 4b and Supplementary Fig. 11a,b). By contrast, CD44 mRNA (Supplementary Fig. 4e) and protein

**Figure 2** miR-34a inhibits prostate CSC properties. (a) Holoclone assays in DU145 cells. Cells transfected with miR-NC (NC) or miR-34a (34a) oligos were in three experiments (Exp. I, 100 cells per well scored on day 9; Exp. II, 100 cells per well scored on day 13; Exp. III, 500 cells per well scored on day 7). (b) Clonogenic assays in DU145 cells. Cells (3,000 per well) were plated in Matrigel and colonies counted on day 13. NT, nontransfected. (c) Matrigel clonogenic assays in LAPC4 cells. Two experiments were performed (Exp. I, 1,250 cells per well scored on day 5, *P = 0.005; Exp. II, 25,000 cells per well scored on day 5, **P = 0.015). (d) Sphere assays in LAPC4 cells infected with lenti-ctl (C) or lenti-34a. Both primary and secondary spheres were scored on day 15. (e) Holoclone assays in PPC-1 cells (quantified on day 5). (f) Sphere assays in HPCA101 cells infected with lenti-ctl (C) or lenti-34a. Both primary and secondary spheres were scored 3 weeks later. (g,h) Sphere assays in purified CD44+ HPCA116 cells transfected with NC or miR-34a oligos (g) or CD44+ HPCA116 cells transfected with anti-NC or anti-34a oligos (h). Spheres were scored on day 15.

**Figure 3** Therapeutic effects of miR-34a. (a) Injections of miR-34a into the tail vein inhibited orthotopic PC3 tumor growth (n = 9 each). (b–d) Injections of miR-34a oligos into the tail vein inhibited metastasis (GFP+ foci in the endpoint lungs; mean ± s.d., n = 6 per group) of orthotopic LAPC9-GFP tumors (b) without significantly affecting tumor growth (c) and extended mouse survival (d; Kaplan–Meier analysis and log-rank test). (e,f) The fourth set of therapeutic experiments in LAPC9 cells. Representative lung images (e, animal number and tumor weight indicated on top; scale bar, 100 µm) and quantification of lung metastases (f; mean ± s.d., n = 10 per group).
Figure 4 CD44 is a direct and functional target of miR-34a. (a) Representative CD44 immunohistochemistry images in Du145 tumors from cells infected with MSCV-PIG (control) or MSCV-34a vectors (western blot on the right) and PC3 tumors harvested from mice treated with miR-NC or miR-34a oligos. Scale bars, 10 µm. (b) miR-34a downregulates CD44 in Du145 (left) and PPC-1 (right) cells. Relative levels of CD44 indicated at the bottom. (c) Schematic of two putative miR-34a binding sites in the 3′ UTR. (d) Luciferase experiments in Du145 cells (*P < 0.01). (e) Knockdown of CD44 inhibits LAPC4 tumor regeneration (Supplementary Fig. 12). (f,g) Knockdown of CD44 inhibits PC3 cell metastasis; shown are quantification (f) and images (g; scale bar, 100 µm). (h,j) Invasion assays. miR-34a oligos inhibited Matrigel invasion of CD44+ Du145 cells (h), and this inhibition was partially overcome by overexpression of a human CD44 cDNA lacking the miR-34a binding sites at the 3′ UTR (i). Invasion expressed as values relative to the corresponding controls. (j) A schematic summary. The part highlighted in red refers to the novel findings of this study.

CD44 is a direct and functional target of miR-34a. By contrast, overexpression of CD44 did not significantly relieve the inhibition of prostate cancer cell proliferation by miR-34a (Supplementary Fig. 15). We have shown that miR-34a is underexpressed in tumorigenic CD44+ prostate cancer cells and that it has potent antitumor and antimetastasis effects. Our results establish miR-34a as a key negative regulator of CD44+ prostate cancer cells and CD44 as an important target of miR-34a. Our findings suggest that reduced expression of miR-34a in prostate CSCs contributes to prostate cancer development and metastasis by regulating expression of CD44 and the migratory, invasive and metastatic properties of CSCs (Fig. 4j). It is noteworthy that p53, which directly activates miR-34a, also negatively regulates CD44 through a noncanonical p53-binding site in the promoter. Considering the widespread expression of CD44 in CSCs and the functional involvement of CD44 in mediating CSC migration and homing and in metastasis of many cancers, the suppression of CD44 by miR-34a reveals a previously unknown signaling pathway that regulates prostate CSCs (Fig. 4j). The emerging role of miR-34a in regulating other CSC properties, coupled with the therapeutic effects of miR-34a on lung and prostate tumors (this study), establishes a strong rationale for developing miR-34a as a therapeutic agent that targets prostate CSCs.

METHODS
Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS
C.L., K.K., R.L., X.C. and L.P. designed and performed the experiments with help from C.J., T.C.-D., H.L., S.H., H.Y., J.F. and A.G.B. R.F. provided all HPCA samples. C.L. and D.G.T. prepared the manuscript. D.G.T., with help from D.B., designed the experiments and supervised the whole project. All authors discussed the results and commented on the manuscript.

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ONLINE METHODS

Quantification of mature miRNA levels using qRT-PCR. We quantified miRNA levels using TaqMan MicroRNA Assays (Applied Biosystems)\(^{25,26}\). Briefly, we first isolated total RNA from xenograft-derived LAPC9, LAPC4 and Du145 cells and recovered small RNA fractions (<200 nucleotides) using the mirVANA PARIS miRNA Isolation Kit (Ambion). We measured RNA concentrations using absorbance at 260 nm. We used the small RNA fractions from unsorted cells to measure the levels of a library of 324 sequence-validated human miRNAs and then compared the expression of 137 miRNAs in CD44\(^+\) and CD44\(^-\) LAPC9, LAPC4 and Du145 cells, side-population and non–side-population LAPC9 cells, and CD133\(^+\) and CD133\(^-\) LAPC4 cells (Fig. 1a). For qRT-PCR analysis\(^{25,26}\), we defined the threshold cycle (C\(t\)) as the fractional cycle number at which fluorescence exceeds the fixed threshold of 0.2. Quantitative miRNA expression data were analyzed using dC\(t\) (the C\(t\) value normalized to internal ‘housekeeping’ miRNAs such as miR-24 and miR-103) and ddC\(t\) (difference between the dC\(t\) of positive population and that of the negative population) values for each of the miRNAs. When necessary, we converted ddC\(t\) to percentage of expression using the formula \(2^{-\text{ddC}_t}\). We used total RNA (10 ng) for all other measurements of individual miRNA levels, including those in primary tumor–derived cells.

Therapeutic experiments. We performed four sets of therapeutic experiments. (i) We repeatedly injected subcutaneous PPC-1 tumors intratumorally\(^{26}\) with miR-NC or miR-34a oligos mixed with siPORT amine (Ambion). (ii) We implanted 500,000 PC3-GFP cells in the dorsal prostate of male NOD-SCID mice and allowed tumors to develop for 3 weeks. Starting from day 22, we implanted 500,000 PC3-GFP cells each in the dorsal prostate of NOD-SCID mice. On day 22, animals were randomly assigned to (i) We implanted 500,000 PC3-GFP cells in the dorsal prostate of male NOD-SCID mice and allowed tumors to develop for 3 weeks. Starting from day 22, we implanted 500,000 PC3-GFP cells each in the dorsal prostate of NOD-SCID mice. On day 22, animals were randomly assigned to

Migration and invasion assays, CD44 knockdown and ‘rescue’ experiments. We performed knockdown experiments using pGIPz-CD44shRNA (CD44-sh) or pGIPz-NS (non-silencing) lentiviruses (Open Biosystems) at a multiplicity of infection (MOI) of 20 (see Supplementary Fig. 1d for vectors and knockdown effects). We performed invasion assays in CD44\(^+\) and CD44\(^-\) Du145 cells using Matrigel Invasion Chamber (8-µm pore size, BD). We carried out migration assays in a similar way but without the Matrigel. In some experiments, purified CD44\(^+\) Du145 cells were first transfected with NC or miR-34a oligos. We seeded prostate cancer cells in 24-well plates (3 × 10\(^4\) cells per well) and co-transfected them with 1 µg reporters with 24 pmol miR-34a or miR-NC together with Renilla luciferase internal normalization plasmid (pRL-CMV). We determined the ratio of firefly to Renilla luciferase activity with a dual luciferase assay (Promega) 48 h later.

Additional methods. Detailed methodology is described in Supplementary Methods.