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

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Cancer stem cells (CSCs), or tumor-initiating cells, are involved in tumor progression and metastasis1. MicroRNAs (miRNAs) regulate both normal stem cells and CSCs2-5, and dysregulation of miRNAs has been implicated in tumorigenesis6. CSCs in many tumors—including cancers of the breast7, pancreas8, head and neck9, colon10,11, small intestine12, liver13, stomach14, bladder15 and ovary16—have been identified using the adhesion molecule CD44, either individually or in combination with other marker(s). Prostate CSCs with enhanced clonogenic17 and tumor-initiating and metastatic18,19 capacities are enriched in the CD44+ cell population, 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 target20-24, 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 antagonirs 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 recreate 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 metastasis1. 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 expression25,26 of CD44+ and CD44− prostate cancer cells. The CD44+ prostate cancer cell population harbors tumor-initiating and metastatic cells18,19 and is enriched in the self-renewal gene NANG (ref. 27). We purified CD44+ prostate cancer cells from three xenograft models18,19,27,28—LAPC9, LAPC4 and Du145. For comparison, we also purified LAPC4 CD133+ and LAPC9 side-population cells. CD133+ prostate cancer cells are clonogenic in vitro27, and the LAPC9 side population is also enriched in tumor-initiating cells28. We first used unsorted cells to measure the levels of 324 sequence-validated human miRNAs and found that 137 miRNAs were expressed at reliably detectable levels (Fig. 1a). We then compared the expression of these 137 miRNAs in marker-positive versus marker-negative prostate cancer cell populations and found that miR-34a (1p36.22) was prominently underexpressed in all CD44+ populations (Fig. 1a), being expressed at < 3% of the level in the corresponding CD44− cells (Fig. 1b). The other two miR-34 family members, miR-34b and miR-34c (11q23.1), did not show consistent differences between CD44+ and CD44− prostate cancer cells (not shown). Underexpression of miR-34a in CD44+ prostate cancer cells was more pronounced than that of let-7b (Fig. 1b), a tumor-suppressive miRNA4 and an important regulator of both normal and cancer stem cells3,4. We also found that miR-34a was underexpressed in LAPC4 CD133+ (Fig. 1b) and LAPC9 side-population (not shown) cells. To validate the underexpression of miR-34a in CD44+ prostate cancer cells and to determine its clinical relevance, we purified CD44+ and CD44− prostate cancer cells from 18 human prostate cancer (HPCa; Supplementary Table 1) samples27,29,30 and compared the expression of miR-34a. CD44+ HPCa cells expressed miR-34a at levels ~25−70% of those in CD44− cells from the same tumors (Fig. 1c). These results suggest that miR-34a is underexpressed in the CD44+ prostate cancer cells in both xenograft and primary tumors.

The expression of miR-34a is regulated by p53, and miR-34a induces apoptosis, cell-cycle arrest or senescence when introduced into cancer cells20-24,31. We found that the expression of miR-34a in ten normal human prostate (NHP) epithelial strains, immortalized (but non-tumorigenic) NHP cells and prostate cancer cell lines correlated with...
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. 1.d and Supplementary Fig. 5). LAPC9 (Fig. 1.d and Supplementary Fig. 5a) and HPCA58 (Fig. 1e) cells transfected with miR-34a produced significantly smaller tumors than the same cells transfected with miR-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. 1e). 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 LPC4 cells transfected with miR-34a oligos (Supplementary Fig. 5a,b) and Du145 cells infected with the MCSV-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-1γ (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 lentiviral miR-34a, tumor regeneration was completely blocked in that tumor incidence was 10/10 for the lentiv-ctl group, whereas the incidence for the miR-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, lentiv-34a infection of CD44+ LAPC9 cells also inhibited tumor regeneration (tumor incidences for the lentiv-ctl and lentiv-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 antagomir) into purified CD44+ Du145 or LAPC9 cells, which are less tumorigenic than the corresponding CD44− cells.18,19 The antagomir-transfected LAPC9 cells showed reduced endogenous miR-34a (Supplementary Fig. 4c) and increased mRNA levels of CDK4 (Supplementary Fig. 4d), a known miR-34a target26, 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,
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 used 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) 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).
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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., F.E.W. 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.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturemedicine/.

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ONLINE METHODS

Quantification of mature miRNA levels using qRT-PCR. We quantified miRNA levels using TaqMan MicroRNA Assays (Applied Biosystems). Briefly, we first isolated total RNA from xenograft-derived cells and then 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 RNAs 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 xenograft-derived cells and then 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 RNAs 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, we defined the threshold cycle (Ct) as the fractional cycle number at which fluorescence exceeds the fixed threshold of 0.2. Quantitative miRNA expression data were analyzed using dCt (the Ct value normalized to internal ‘housekeeping’ miRNAs such as miR-24 and miR-103) and ddCt (difference between the dCt of positive population and that of the negative population) values for each of the miRNAs. When necessary, we converted ddCt to percentage of expression using the formula 2−ddCt.

Therapeutic experiments. We performed four sets of therapeutic experiments. (i) We repeatedly injected subcutaneous PPC-1 tumors intratumorally 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 injected miR-34a or NC oligos complexed with RNAI-LaceNerII in vivo delivery reagent (BIOO Scientific) into tail veins of randomly selected mice (n = 9 for each group) every 2 d at a rate of 1 mg of oligos per kg of body weight. All animals were killed after the fifth injection, and DP tumors were isolated and analyzed. (iii) We implanted 500,000 LAPC9-GFP cells each in the dorsal prostate of NOD-SCID mice. On day 22, animals were randomly assigned to miR-34a and NC groups (n = 6 for each), injected in the same way, and killed when they became moribund. The experiment was ended 13 d after initiation of injections. We removed tumors and lungs as well as several other organs including the pancreas, lymph nodes, liver and kidney to assess metastasis. Representative lung images were captured and quantified for metastases (GFP+ foci). (iv) We carried out the same procedure as in (iii) but with more animals (n = 10 for each group) and more injections (15).

miR-34a binding sites, site-specific mutagenesis and luciferase experiments. We used rna22 program (ref. 36; http://cbcsrv.watson.ibm.com/rna22.html) to compute putative target sites for miR-34a in the human CD44 miRNAs and found two potential miR-34a binding sites at 3′-UTR (G48259940). To characterize the identified sites, we first amplified the 3′ UTR of human CD44 from LNCap genomic DNA using primers 5′-AGAGCTCCACCTACACCA TTAATTCG-3′ and 5′-TAAGCTTGAAAGTCTTGACACAC-3′. The 2.55-kb PCR fragment was cloned into pGEM-T vector (Promega) and its sequence confirmed. For site-specific mutagenesis, we mutated the regions in the CD44 3′ UTR complementary to the seed sequence of miR-34a (M1, CATTTCCTCAAGATCGCTGTT; M2, GGTTAATCGCC to CCGCGACAGT) using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene). For luciferase assays, we cloned wild-type or mutant CD44 3′ UTRs into the HindIII and SacI sites of the 3′-UTR/pMir vector (Ambion). We seeded prostate cancer cells in 24-well plates (3 × 104 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.

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 CD44− and CD44+ Du145 cells with pBabe-puro (vector) or pBabe-CD44 (Addgene) retroviruses in the presence of 8 µg ml−1 polybrene. After 24 h, we transfected cells with miR-34a oligos (24 h) before invasion assays. After 24 h, we removed non-invaded (or non-migrated) cells with a cotton swab, stained invaded or migrated cells in 24-well plates (3 × 104 cells per well) and counted them under a microscope. For the rescue experiments, we infected CD44+ Du145 cells with pBabe-puro (vector) or pBabe-CD44 (Addgene) retroviruses in the presence of 8 µg ml−1 polybrene. After 24 h, we transfected cells with miR-34a oligos (24 h) before invasion assays. In these experiments (n = 3–4), the percentage of invaded cells was converted into an invasion index, which was considered as one in all control groups.

Additional methods. Detailed methodology is described in Supplementary Methods.