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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 CSCs^{2–5}, and dysregulation of miRNAs has been implicated in tumorigenesis⁶. CSCs in many tumors—including cancers of the breast⁷, pancreas⁸, head and neck⁹, colon^{10,11}, 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 metastatic^{18,19} 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 target^{20–24}, 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 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 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

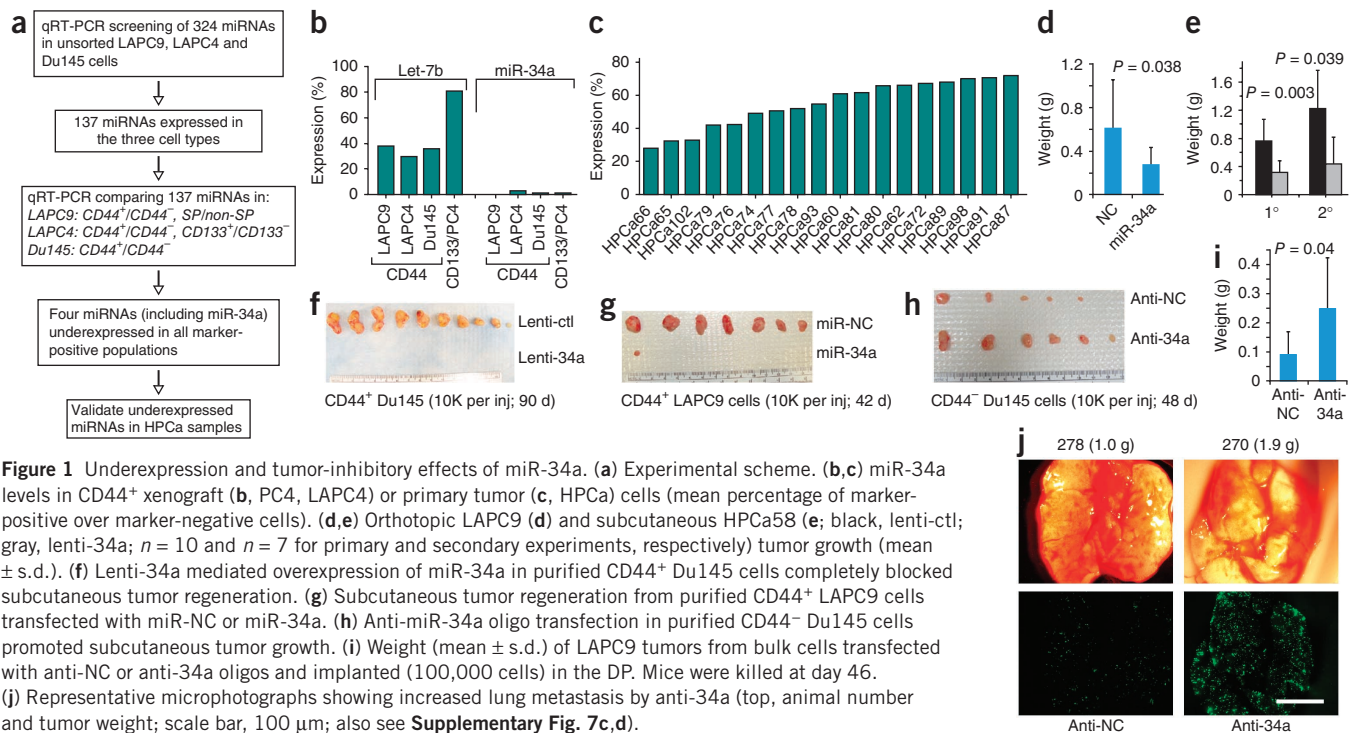
CSCs in order to design CSC-specific therapies. To this end, we used quantitative RT-PCR (qRT-PCR) to compare the miRNA expression^{25,26} of CD44⁺ and CD44[−] prostate cancer cells. The CD44⁺ prostate cancer cell population harbors tumor-initiating and metastatic cells^{18,19} and is enriched in the self-renewal gene *NANOG* (ref. 27). We purified CD44⁺ prostate cancer cells from three xenograft models^{18,19,27,28}—LAPC9, LAPC4 and Du145. For comparison, we also purified LAPC4 CD133⁺ and LAPC9 side-population cells. CD133⁺ prostate cancer cells are clonogenic *in vitro*¹⁷, 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 miRNA⁶ and an important regulator of both normal and cancer stem cells^{3,4}. 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) samples^{27,29,30} 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 cells^{20–24,31}. 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

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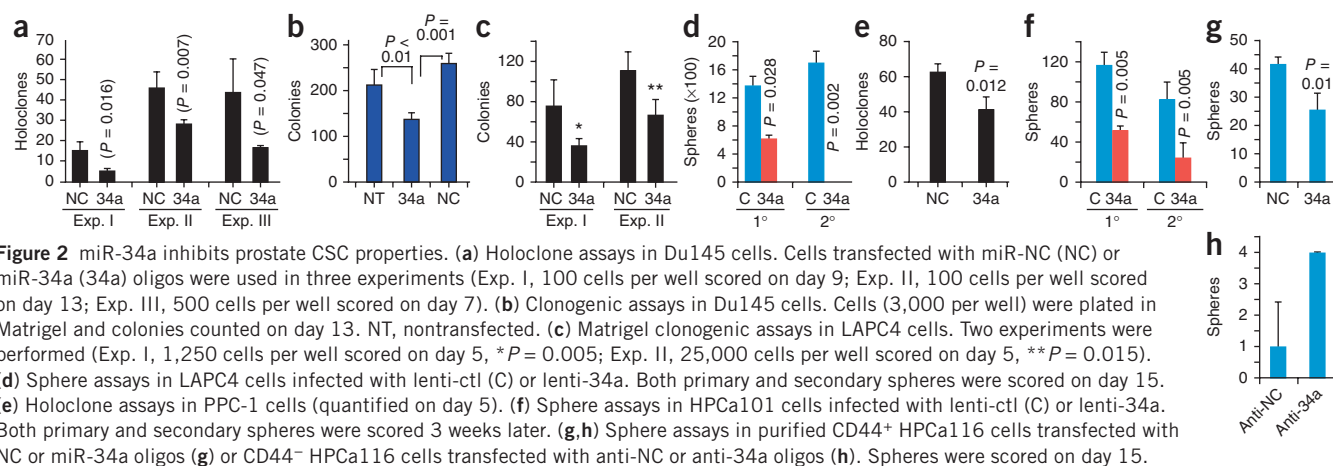
their p53 status (**Supplementary Fig. 1** and **Supplementary Results**). Transfection of synthetic miR-34a oligonucleotides (oligos), but not the negative control (NC) miRNA oligos, induced cell-cycle arrest, apoptosis or senescence in p53-mutant and p53-null prostate cancer cells (**Supplementary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



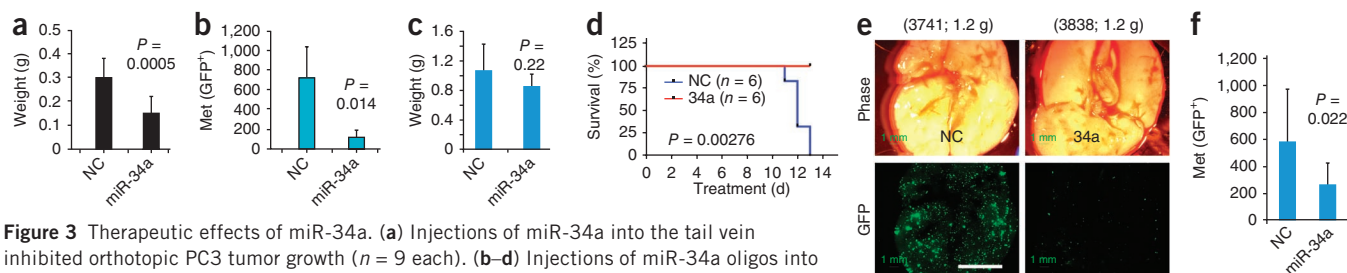
bulk LAPC9 cells transfected with anti-34a oligos generated larger orthotopic tumors than those with anti-NC oligos (Fig. 1i and Supplementary Fig. 7a). Anti-34a also promoted subcutaneous tumor growth in purified CD44⁺ LAPC9 cells (Supplementary Fig. 7b). Notably, in the two orthotopic LAPC9 tumor experiments (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 ($\geq 1 \text{ mm}^3$) 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.

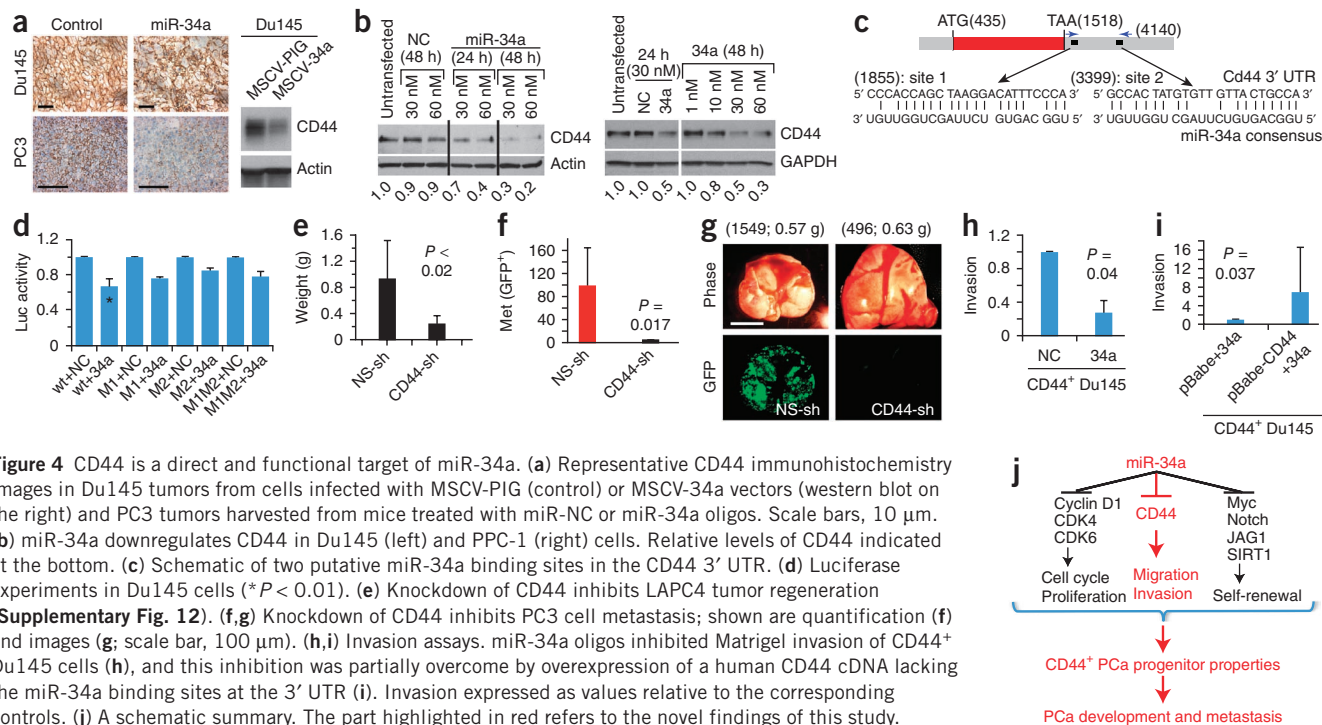
To further investigate the effects of miR-34a on prostate CSC properties, we performed holoclone, clonogenic and sphere formation assays^{18,19,27,32,33}. Prostate cancer cell holoclones contain self-renewing cancer cells³², and sphere-formation assays have been widely used to measure the activity of stem or progenitor cells^{1,33}. 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. 2d,f) 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,e,f 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-34a^{20–24,26,31,34,35}. 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





(Supplementary Fig. 11c) were increased in tumors transfected with anti-34a. The target-prediction program rna22 (ref. 36) revealed two putative miR-34a binding sites in the 3' UTR of CD44 mRNA (Fig. 4c). When we cloned the 3' UTR fragment containing both putative miR-34a binding sites downstream of a luciferase coding sequence (Supplementary Fig. 11d,e), co-transfection of the luciferase reporter and miR-34a oligos into three prostate cancer cell types produced lower luciferase activity than in cells co-transfected with the NC oligos. However, mutation of the seed sequence in either site, especially the distal site, partially abrogated the suppressive effect of miR-34a (Fig. 4d and Supplementary Fig. 11f,g). These results suggest that miR-34a regulates CD44 expression through two binding sites in the 3' UTR of the gene that encodes CD44.

To determine whether CD44 is a functionally important target of miR-34a in the context of prostate cancer development, we reduced CD44 expression using a lentiviral vector carrying a short hairpin RNA (shRNA) against CD44 (Supplementary Fig. 1d) in LAPC4, PC3 and Du145 cells. Knockdown of CD44 in LAPC4 cells inhibited both orthotopic tumor formation (Fig. 4e) and lung metastasis (Supplementary Fig. 12). In PC3 cells, it markedly inhibited metastasis (Fig. 4f,g and Supplementary Fig. 13) without affecting tumor growth (data not shown). Knockdown of CD44 in Du145 cells inhibited tumor development in both subcutaneous and orthotopic sites (Supplementary Fig. 14a,b) as well as metastasis (not shown). These results not only show that CD44 has a key role in determining the tumorigenic and metastatic capacity of prostate cancer cells but also indicate that knockdown of CD44 phenocopies the anti-prostate cancer effects of miR-34a. Mechanistically, the CD44⁺ prostate cancer cells showed higher migratory (Supplementary Fig. 14c,d) and invasive (Supplementary Fig. 14e) capacities than CD44⁻ cells, and these capacities were partially inhibited by miR-34a (Fig. 4h and Supplementary Fig. 14f,g). 'Rescue' experiments wherein CD44 was overexpressed using a cDNA that lacked the 3' UTR containing the miR-34a binding sites abrogated miR-34a-mediated inhibition

of invasion of CD44⁺ Du145 cells (Fig. 4i), reinforcing the idea that 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 promoter³⁷. Considering the widespread expression of CD44 in CSCs⁷⁻¹⁶ and the functional involvement of CD44 in mediating CSC migration and homing³⁸ 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^{35,39} properties, coupled with the therapeutic effects of miR-34a on lung²⁶ 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., B.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.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)^{25,26}. Briefly, we first isolated total RNA from unsorted 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^{25,26}, 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} . 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 intratumorally²⁶ 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 RNALancerII *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 weight²⁶. 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 mRNAs and found two potential miR-34a binding sites at 3'-UTR (Gi48255940). To characterize the identified sites, we first amplified the 3' UTR of human CD44 from LNCaP genomic DNA using primers 5'-AGAGCTCCACCTACACCA TTATCTTG-3' and 5'-TAAGCTTGGAGTCTTCAGGAGACAC-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, CATTTCCTCA to GCAATCGGT; M2, GTTACTGCCA to CCGCGACAGT) using the QuikChange 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 (3×10^4 cells per well) and co-transfected them with 1 μ g 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- μ m 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 cells (5×10^4) in the upper chamber of the insert and used medium containing 20% FBS in the lower chamber as a chemoattractant. After 22 h, we removed non-invaded (or non-migrated) cells with a cotton swab, stained invaded or migrated cells with HEMA3 (Fisher Scientific), 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 μ g 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**.

SUPPLEMENTARY INFORMATION

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

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This PDF contains:

SUPPLEMENTARY DATA

SUPPLEMENTARY METHODS

SUPPLEMENTARY REFERENCES

SUPPLEMENTARY FIGURES 1-15

SUPPLEMENTARY TABLES 1 and 2

SUPPLEMENTARY RESULTS

The expression levels of miR-34a, but not miR-34b and miR-34c, in normal prostate and prostate cancer cells correlate with the p53 status.

To determine whether miR-34a expression in normal prostate and prostate cancer cells might be regulated by p53, we employed qRT-PCR analysis to correlate the expression levels of endogenous miR-34a (localized on chromosome 1p36.22) with the p53 status in ten prostate cells (**Supplementary Fig. 1a,b**). These cells included primary prostate epithelial cell strains NHP8 (normal human prostate epithelial strain 8) and NHP9 (normal human prostate epithelial strain 9) (4); immortalized NHP9 cells (NHP9-IM) (4); cultured prostate cancer cell lines LNCaP, LNCaP C4-2 (a LNCaP subline), PC3, PPC-1, and Du145; and prostate cancer cells LAPC4 and LAPC9 freshly purified from xenograft tumors. The NHP8, NHP9, NHP9-IM, and two LNCaP lines express wild-type (wt) p53 (1,4), as evidenced by low levels of p53 protein in these cells (**Supplementary Fig. 1b**). LAPC9 cells also expressed wt p53 as revealed by our genomic DNA sequencing of exons 5-8 (data not shown). PC3 and PPC-1 cells were p53 null whereas Du145 and LAPC4 cells harbor mutant p53 (1), as supported by Western blotting analysis (**Supplementary Fig. 1b**). qRT-PCR analysis revealed that the four prostate cancer cells harboring mutant or null p53 displayed much lower levels of miR-34a than the six cell types with wt p53 (**Supplementary Fig. 1a**; note that we utilized PPC-1 cells, which expressed the lowest level of miR-34a mRNA, as the normalization control), suggesting that p53 regulates the baseline miR-34a expression in prostate and prostate cancer cells. Interestingly, among the six p53-wt cells, LNCaP and C4-2 cells expressed higher levels of miR-34a than NHP8, NHP9, NHP9-IM, and LAPC9 cells (**Supplementary Fig. 1a**).

To determine whether p53 may also regulate the baseline expression levels of the other two miR-34 family members, miR-34b and miR-34c, which are localized on chromosome 11 q23.1 and were not differentially expressed between the CD44⁺ and CD44⁻ prostate cancer cells, we also employed qRT-PCR to measure their levels in the same ten prostate cells. In contrast to miR-34a, miR-34b and miR-34c showed similar expression patterns and were not strictly correlated with the p53 status (**Supplementary Fig. 1a**). For

example, in p53 null or mutant cells, although PPC-1, PC3, and LAPC4 cells exhibited undetectable miR-34b and miR-34c, Du145 cells showed extremely high levels of both miRNAs (**Supplementary Fig. 1a**), suggesting p53-independent regulation of miR-34b and miR-34c in certain prostate cancer cells. Similarly, miR-34b and miR-34c levels showed wide variations in the six p53-wt cells.

Finally, we measured the baseline levels of let-7b in the ten prostate cells as this miRNA was also downregulated in the CD44⁺ prostate cancer cells (**Fig. 1b**). The let-7b expression pattern was somewhat like that of miR-34a in that it was much higher in LNCaP and C4-2 cells (**Supplementary Fig. 1a**). However, unlike miR-34a, its expression was readily detectable in p53 mutant or null prostate cancer cells (**Supplementary Fig. 1a**).

Transfection of miR-34a oligos induced cell-cycle arrest, apoptosis or senescence in p53-mutant and p53-null prostate cancer cells.

Since p53 is frequently mutated in advanced prostate cancer, we transfected p53-mutant or null Du145, PC3, and PPC-1 cells with synthetic mature miR-34a oligonucleotides (oligos) or the negative control miRNA (miR-NC or NC) that contains a scrambled sequence and does not specifically target any human gene products (**Supplementary Fig. 1c**). The miR-34a mimics the dicer cleavage product that is loaded into the RISC in the cytoplasm and therefore, no processing of the pre-miRNA is required for it to be activated (thus it represents a mature miRNA). Transfected miR-34a oligos caused inhibitory effects in all three prostate cancer cells (**Supplementary Figs. 2 and 3**). In Du145 cells, miR-34a oligo transfection reduced cell numbers and population doublings (**Supplementary Fig. 2a,b**) as a result of inhibition of cell proliferation (**Supplementary Fig. 2c**). In PC3 cells, miR-34a oligos inhibited population doublings by causing apoptosis (**Supplementary Fig. 3a–c**). In PPC-1 cells, transfected miR-34a oligos inhibited proliferation, increased senescence, and induced apoptosis resulting in reduced total cell numbers and cumulative population doublings (**Supplementary Fig. 3d–g**).

SUPPLEMENTARY METHODS

Cells and animals. We obtained prostate cancer cell lines, LNCaP, LNCaP C4-2, PC3, PPC-1, and Du145, from ATCC and maintained them as described¹⁻³. Primary and immortalized normal human prostate (NHP) epithelial cells were detailed elsewhere⁴. We purified LAPC4 and LAPC9 (and, sometimes, Du145) cells from xenograft tumors (see below)^{3,5-8}. Immune-deficient mice, NOD-SCID (non-obese diabetic severe combined immune deficient) and NOD-SCID γ , were produced mostly from our own breeding colonies and purchased occasionally from the Jackson Laboratories (Bar Harbor) and maintained in standard conditions according to the Institutional guidelines. All animal experiments were approved by our institutional IACCUC.

Prostate cancer cell purification. We routinely maintained human xenograft prostate tumors, *i.e.*, LAPC9 (bone metastasis; AR⁺ and PSA⁺), LAPC4 (lymph node metastasis; AR⁺ and PSA⁺), and Du145 (brain metastasis; AR⁻ and PSA⁻), in NOD-SCID mice. We first purified human prostate cancer cells out of xenografts by depleting murine cells. CD44⁺ and CD44⁻ cells were further purified using fluorescence-activated cell sorting (FACS) with the purities of both populations being >98% (5,6). CD133⁺ and CD133⁻ LAPC4 cells were purified using biotinylated monoclonal antibody to CD133 (AC133) and the magnetic beads (MACS) by following the manufacturer's instructions (Miltenyi Biotech). Post-sort analysis revealed purities of both populations being >95%. We purified the side population (SP) of LAPC9 cells by FACS as previously described³. We obtained primary human prostate tumors (HPCa; Supplementary Table 1) with the patients' consent from Da Vinci robotic surgery. All work with HPCa samples was approved by the M.D. Anderson Cancer Center Institutional Review Board (IRB LAB04-0498). We purified epithelial HPCa cells through a multi-step process and by depleting lineage-positive hematopoietic, stromal, and endothelial cells^{4,7,8}. We then purified Lin⁻CD44⁺ HPCa cells using MACS or FACS (Supplementary Table 1).

Transient transfection with oligos. We plated bulk or purified CD44⁺ prostate cancer cells 24 h before transfection with 33 nM of miR-34a or non-targeting negative control miRNA (miR-NC) oligos (Ambion) by using Lipofectamine 2000 (Invitrogen). Alternatively, we transfected bulk or purified CD44⁻ prostate cancer cells with 33 nM of anti-miR-34a (anti-34a) or anti-miR-NC (anti-NC) oligos (Ambion). In some experiments (see below), oligos were electroporated into prostate cancer cells. We generally harvested the transfected cells for *in vitro* or *in vivo* studies after culturing for overnight to 24 h.

Retroviral and lentiviral mediated overexpression of miR-34a. Basic retroviral and lentiviral procedures were previously described^{4,7} and the key vectors used in the present study were presented in Supplementary Fig. 1d. An MSCV retroviral vector directing the expression of pre-miR-34a (MSCV-34a) and the empty control vector, MSCV-PIG (Puromycin-IRES-GFP), were used in previous studies⁹. prostate cancer cells were infected with the retroviral supernatant for 48 h in the presence of 8 μ g ml⁻¹ polybrene. Two days after infection, puromycin was added to the media at 3 μ g ml⁻¹, and cell populations were selected for 2 weeks. A lentiviral vector encoding pre-miR-34a (lenti-34a) and the control vector

(lenti-ctl) (Supplementary Fig. 1d) were obtained from Systems Biosciences (SBI). Lentivirus was produced in 293FT packaging cells and titers determined for GFP using HT1080 cells. prostate cancer cells were infected at an MOI of 10 - 20 and harvested 48–72 h post-infection.

Experiments correlating miR-34a levels in normal prostate and prostate cancer cells with the p53 status. We employed qRT-PCR to quantify the levels of miR-34a, and, for comparisons, of miR-34b, miR-34c, and let-7b in NHP8, NHP9, and NHP9-IM, LNCaP, LNCaP C4-2, PC3, PPC-1, Du145, LAPC4, and LAPC9 cells. For qRT-PCR analysis¹⁰, we prepared total RNA from these cells and assayed the levels of miR-34a (assay ID 000426, TaqMan miRNA Assay, ABI), miR-34b (ABI assay ID 000427), miR-34c (ABI assay ID 000428), and hsa-let-7b (let-7b; ABI assay ID 000378).

BrdU incorporation assays, senescence β -gal (SA- β gal) staining, Western blotting, immunofluorescence, flow cytometry analysis (FACS), and immunohistochemistry (IHC). These procedures were previously described²⁻⁸. For Western blotting of p53 in ten prostate cells, protein lysate (50 μ g) was separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with a monoclonal Ab to p53 (clone MO-1). For characterization of the knockdown effect of pGIPz-CD44shRNA, PC3 or Du145 cells were infected with this vector or the pGIPz control vector (MOI 20; 72 h) and cells were harvested for Western blotting of CD44 or β -actin (loading control) (see Supplementary Fig. 1d).

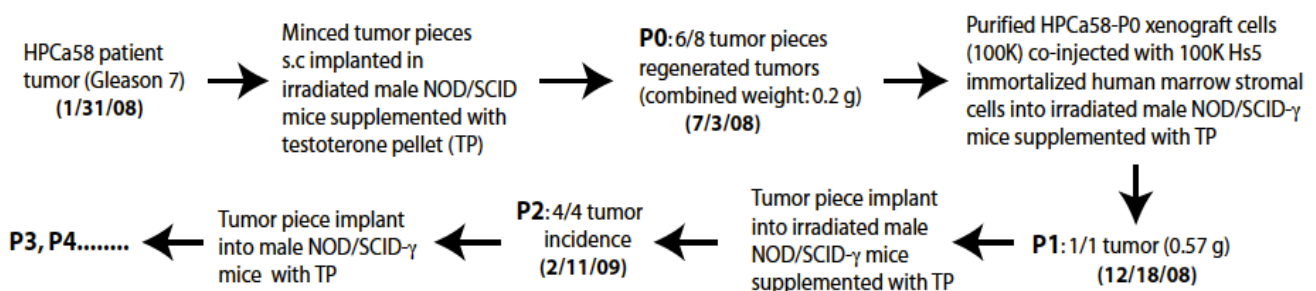
***In vitro* effects of miR-34a overexpression on bulk prostate cancer cells.** For studies in Du145 cells, we first electroporated cells (Bio-Rad GenePulserXcell, 150 mV, 25 mS) in 200 μ l of serum-free OPTIMEM in triplicate with 1.6 μ mol L⁻¹ miR-34a or miR-NC oligos. Immediately after electroporation, we added 800 μ l of serum-containing medium to each cuvette, and plated one million live cells in triplicate on d 0. At the end of 1 week, cells were dissociated, counted, re-electroporated (600,000 cells/well in triplicate), and plated. We repeated this process one week later at d 14 and terminated the whole experiment at the end of the third week (d 21). We then determined the cumulative cell numbers and population doublings (PDs). For BrdU incorporation assays, we transfected Du145 cells with miR-NC or miR-34a oligos (33 nM) using Lipofectamine and plated cells at two different densities (*i.e.*, 10,000 or 5,000 cells/well) on glass coverslips. Cells were terminated either 2 or 5 d after plating and used in BrdU staining.

For studies in PC3 cells, we also electroporated cells with miR-NC or miR-34a oligos, plated one million cells of each type in triplicate, and cultured them in RPMI-1640 plus 7% FBS. At the end of one week, cells were photographed and then dissociated, counted, re-electroporated (600,000 cells each in triplicate), and replated. The procedure was repeated at the end of the second week and at the end of the third week (*i.e.*, 21 d), cells were harvested and experiments terminated. We electroporated PPC-1 cells with miR-NC or miR-34a oligos on d 0 and carried out subsequent experiments as for PC3 cells except that we enumerated cells every 2–3 d. For BrdU assays, we transfected PPC-1 cells with miR-NC or miR-34a oligos (33 nM) for 24 h and then plated 15,000 cells each on glass coverslips. 24 h later, we terminated cells and performed BrdU staining.

Clonal, clonogenic, and sphere-formation assays. For *holoclone assays*^{8,11}, we plated prostate cancer cells at a clonal density (*i.e.*, 100 cells/well) in a 6-well dish, counted the number of holoclones several days later, and presented the percentage of cells that established a holoclone as cloning efficiency. For *clonogenic assays*^{5,7,8}, we plated cells generally at 1,000 cells/well in Matrigel (MG) or methylcellulose (MC) at 1:1 ratio in 100–200 μ l and enumerated colonies 1–2 weeks after plating. For *sphere-formation assays*^{5,7,8}, we generally plated cells at 5,000–10,000 cells/well in serum-free prostate epithelial basal medium (PrEBM) supplemented with 4 μ g ml⁻¹ insulin, B27 (Invitrogen), and 20 ng ml⁻¹ EGF and bFGF in ultra-low attachment (ULA) plates. Floating spheres that arose in 1–2 weeks were counted. For sphere-formation assays in HPCa cells, we purified HPCa cells from human primary tumors, *i.e.*, HPCa101 (Gleason 9), HPCa107 (Gleason 7), HPCa109 (Gleason 7), and HPCa112 (Gleason 6), and infected with lenti-ctl or lenti-34a vectors (MOI 20) overnight. Next day, equal numbers of live cells (20,000/well) were plated in triplicate in ULA plates in serum-free medium containing B27, EGF, and bFGF and spheres enumerated at 11 d (for HPCa107), 33 d (for HPCa109), or 9 d (for HPCa112) after plating. For all above experiments, we run a minimum of triplicate wells for each condition and repeated experiments whenever feasible.

Tumor transplantation experiments. Basic procedures for subcutaneous (s.c) and orthotopic (DP) tumor transplantations can be found in our earlier publications³⁻⁸. For *tumor experiments in LAPC9 cells*, we acutely purified LAPC9 cells from the maintenance tumors and transfected with miR-34a or miR-NC oligos (33 nM). 24 h later, 100,000 cells each were implanted, in 50% Matrigel, into the DP of intact male NOD-SCID mice. For *tumor experiments in LAPC4 cells*, we freshly purified LAPC4 cells from xenograft tumors and transfected with miR-NC or miR-34a oligos (33 nM). 100,000 cells each were injected s.c in male NOD-SCID mice. Alternatively, purified LAPC4 cells were infected with either the control (lenti-ctl) or lenti-miR-34a (lenti-34a) lentiviral vectors (both at an MOI of 10). 24 h after infection, 10,000 cells each were injected s.c in male NOD-SCID mice. For *tumor experiments in Du145 cells*, in addition to oligo transfection, we also infected cultured Du145 cells with either the control retroviral vector (MSCV-PIG) or a retroviral vector encoding miR-34a (MSCV-34a)(9), followed by puromycin selection and s.c injection in Matrigel. Alternatively, Du145 cells were infected with lenti-ctl or lenti-34a vectors (MOI 10) and, 24 h after infection, 10,000 cells of each type ($n = 10$) were injected s.c in NOD-SCID mice. For *tumor experiments in PPC1 cells*, we electroporated cultured PPC-1 cells with miR-34a or NC oligos (1.6 μ M or 5 μ g) on d 0. We injected 500,000 live cells s.c in NOD-SCID mice and measured tumor volumes, using a digital caliper, starting from d 3. On d 7, 13, 20, and 25, we injected miR-NC or miR-34a oligos mixed with siPORT amine (Ambion) intra-tumorally¹⁰.

Experiments with HPCa58 early-generation xenograft tumors. HPCa58 xenograft tumor



was established using the *Scheme* below. Briefly, the primary tumor pieces were first implanted into γ -irradiated (4 Gy; X-ray) male NOD-SCID mice. The P0 xenograft tumors were pooled and used to purify out human prostate cancer cells as described above, which were then co-injected with the Hs5 immortalized human marrow stromal cells in male NOD-SCID γ mice. Subsequent passaging of the first-generation (P1) xenografts was performed by implanting tumor pieces or by injecting purified HPCa58 cells alone without Hs5 cells. We have utilized similar strategies to establish about 8 early-generation human prostate cancer xenografts (including HPCa87 and HPCa91 xenografts; see Supplementary Table 1). These xenograft tumors were of the human origin and morphologically epithelial with detectable cytokeratin 8 and 18. RT-PCR analysis detected *AR* whereas Western blotting detected racemase expression in most xenografts (Chen et al., manuscript in preparation). For the present study, HPCa58 cells were purified from a P3 xenograft tumor (see *Scheme*) and infected with lenti-ctl or lenti-34a vectors (MOI 20). 24 h later, 100,000 cells of each were s.c injected into the NOD-SCID γ mice. The 1° tumors were harvested 21 d later and 10,000 purified GFP⁺ (*i.e.*, infected) tumor cells from respective 1° tumors were injected and the 2° tumors were harvested 26 d later.

Monitoring metastasis. For metastasis analysis^{5,8}, we first observed tumor-bearing animals for symptoms such as hunched posture, irregular breathing and gait, and paraplegia. When systemic symptoms or primary tumor burden became obvious or when the animals became moribund, we sacrificed them by CO₂ euthanization and cervical dislocation. We then performed comprehensive necropsy to isolate individual organs, which were examined for gross metastases. Finally, GFP⁺ metastatic foci in each organ (primarily, the lung) were examined and quantified under a Nikon SMZ1500 whole-mount epifluorescence dissecting microscope.

Measuring cell migration by time-lapse videomicroscopy

We seeded bulk Du145 or purified CD44⁺ and CD44⁻ cells onto the glass-bottom dish (CELLviewtm, 4 compartments, Greiner Bio-One GmbH) and cultured them overnight to create a monolayer. We introduced a homogeneous ‘wound’ track using the tip of a fine forceps. Cells were washed with PBS to remove the debris and smoothen the wound edges. We then placed cells in the culture chamber connected to the time-lapse microscope (Nikon, BioStation IM). We acquired phase-contrast images of at least 20 selected fields of each group at the interval of 30 min for a total of 24 h. We analyzed images using the NIS Elements software (Nikon, NIS elements- 2.35) and quantified cell migration by measuring the time required to close the induced wounds.

Statistical analyses. In general, we used unpaired two-tailed Student’s *t*-test to compare differences in cell numbers, cumulative PDs, percentages of CD44⁺, % BrdU⁺ or SA- β gal⁺ cells, cloning efficiency, tumor weights, migration, invasion, and other related parameters. We employed Fisher’s Exact Test and χ^2 test to compare incidence and latency. We used the Log-Rank test to analyze the survival curves and ANOVA (F-test) to compare differences in multiple groups. In all these analyses, a *P* < 0.05 was considered statistically significant.

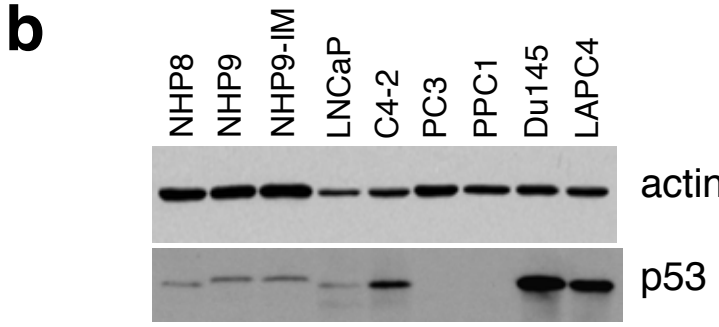
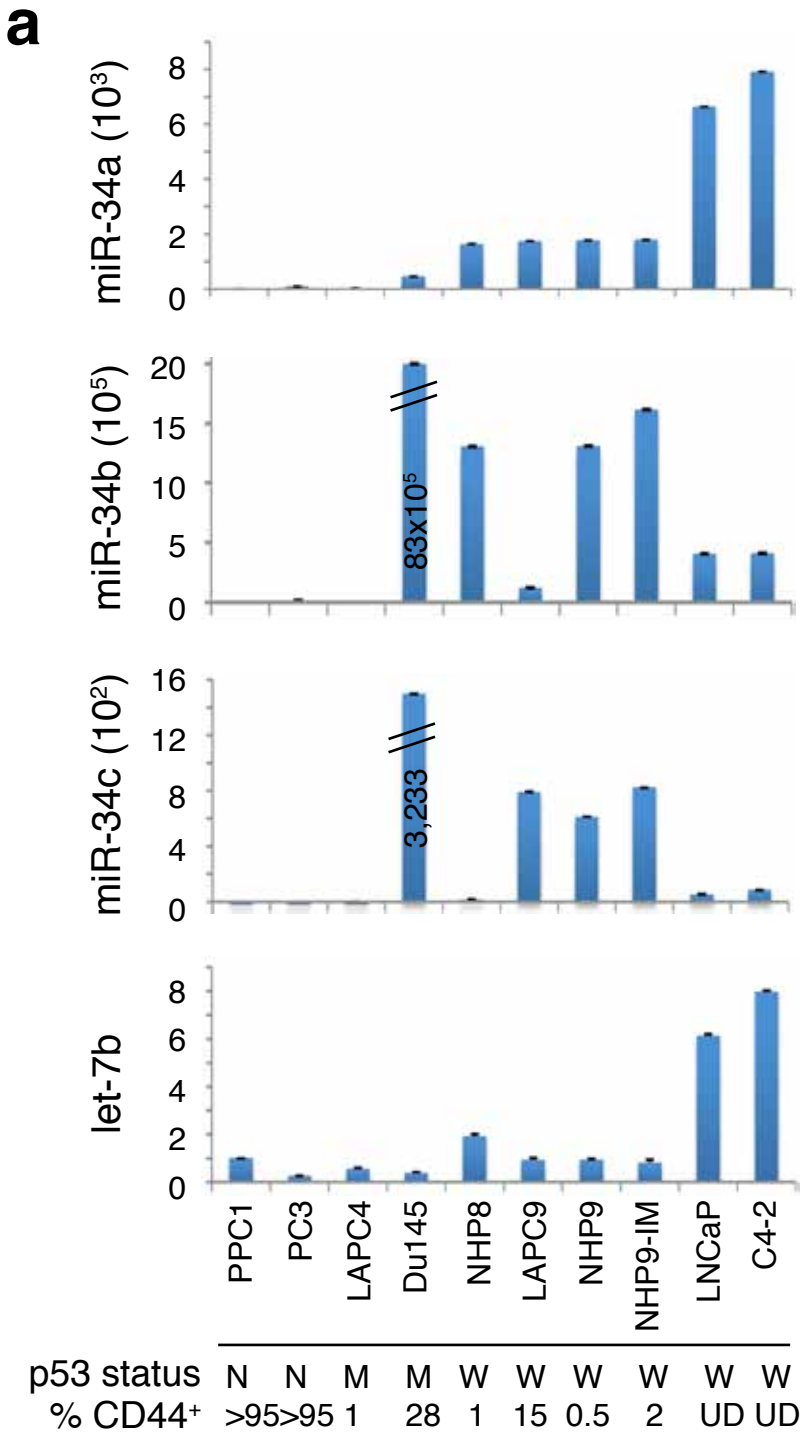
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Supplementary Figure 1. miR-34a levels in prostate cells correlate with p53 status.

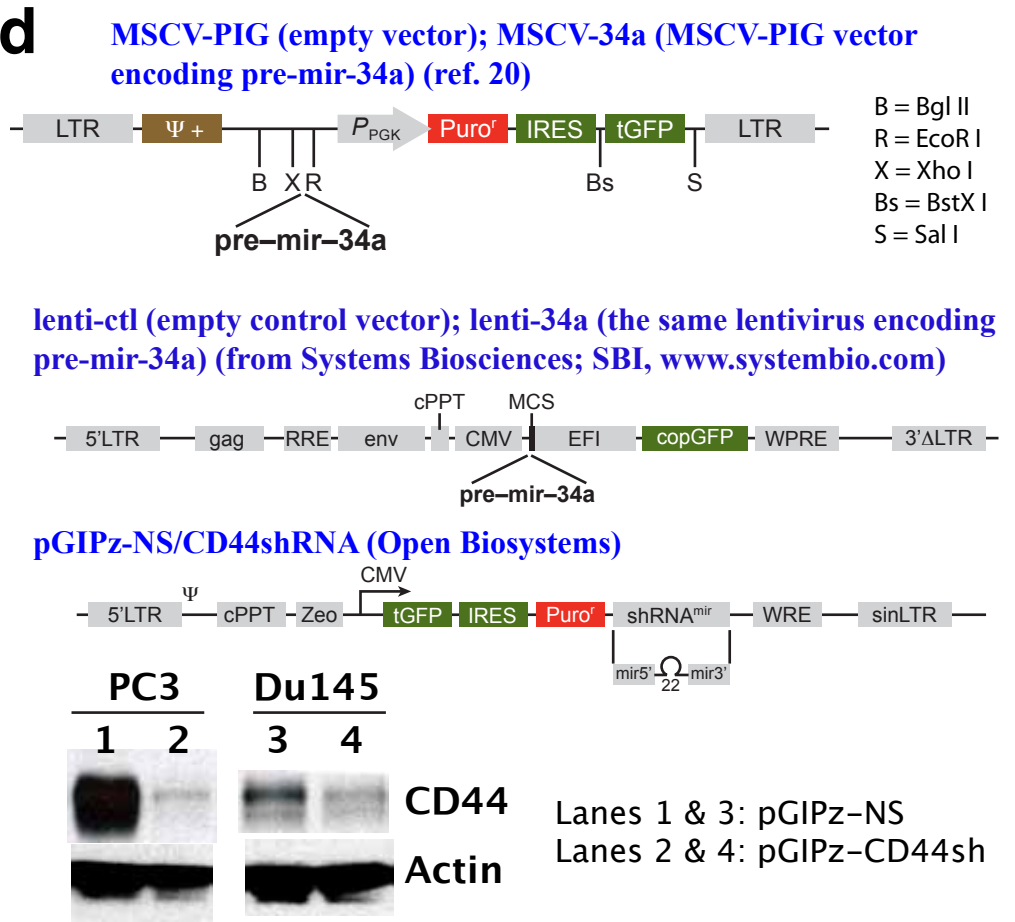
- (a)** qRT-PCR quantification of miR-34a, miR-34b, miR-34c, and let-7b in 10 prostate cell lines. The relative expression levels (mean $\pm$ S.D) are presented by setting the miRNA levels in PPC-1 cells as one. Shown below the bar graphs are the p53 statuses (N, null; M, mutant; W, wild-type) and the % of CD44⁺ cells in each cell type as determined by flow cytometry or immunofluorescence staining (U.D, undetectable).
- (b)** Western blotting of p53 in prostate (cancer) cells.
- (c)** The four oligonucleotides (oligos) used in the current study. All oligos were obtained from Ambion and at least 3 studies (references indicated) have utilized and characterized miR-34a and miR-NC oligos. Use of anti-miR-NC oligos has been published in at least one study (i.e., reference 25) and anti-miR-34a was characterized in the present study.
- (d)** Retroviral and lentiviral vectors utilized in the present study. Shown at the bottom was the characterization of the knockdown effect of pGIPz-CD44shRNA by Western blotting.

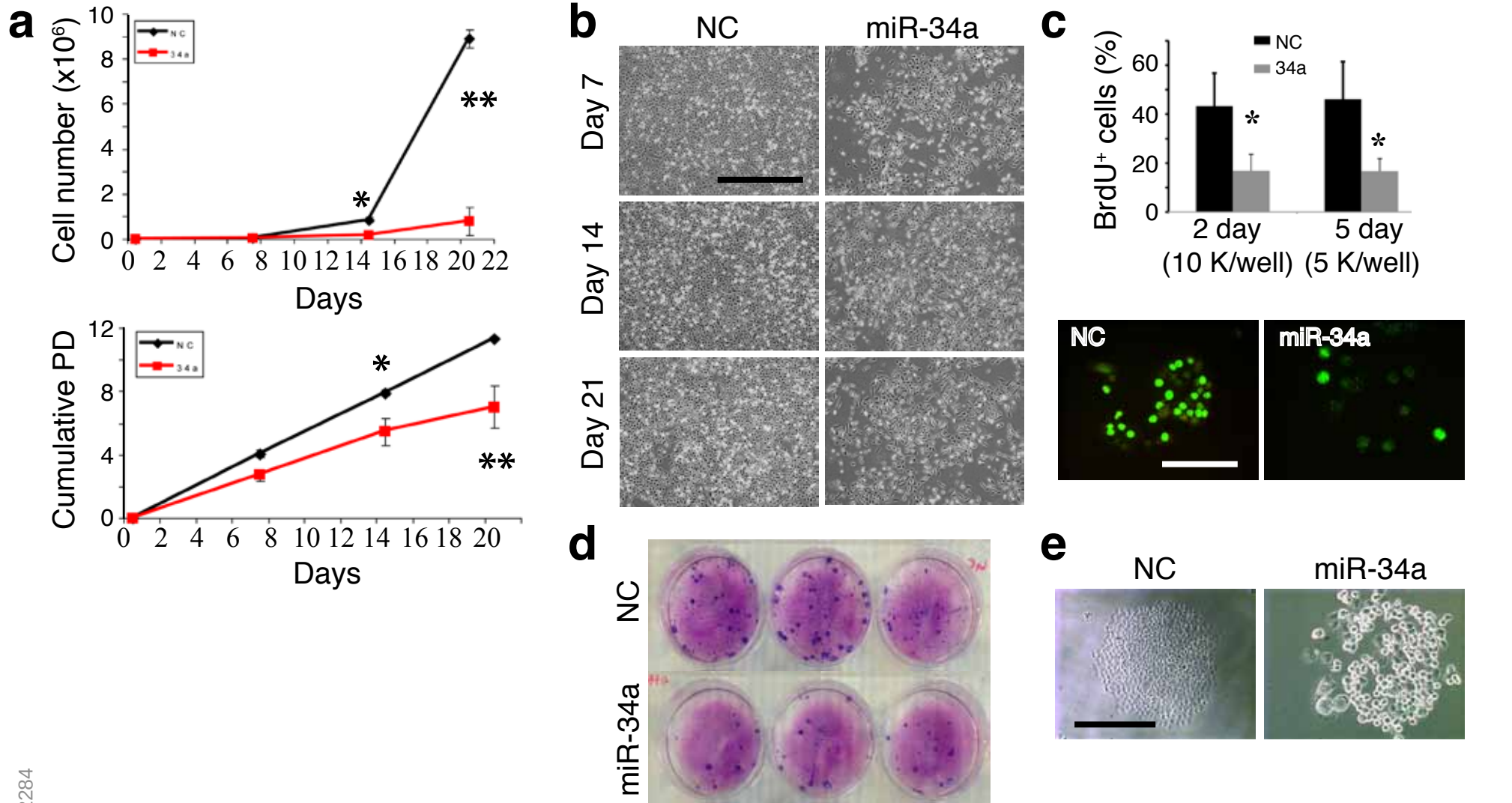
Supplementary Figure 1



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Name	Source	Ref.
miR-34a (mature) oligos	Ambion	26,31,32
miR-NC (neg. control) oligos	Ambion	26,31,32
anti-miR-34a oligos	Ambion	this study
anti-miR-NC oligos	Ambion	25





Supplementary Figure 2. miR-34a inhibits Du145 cell proliferation and clonal expansion.

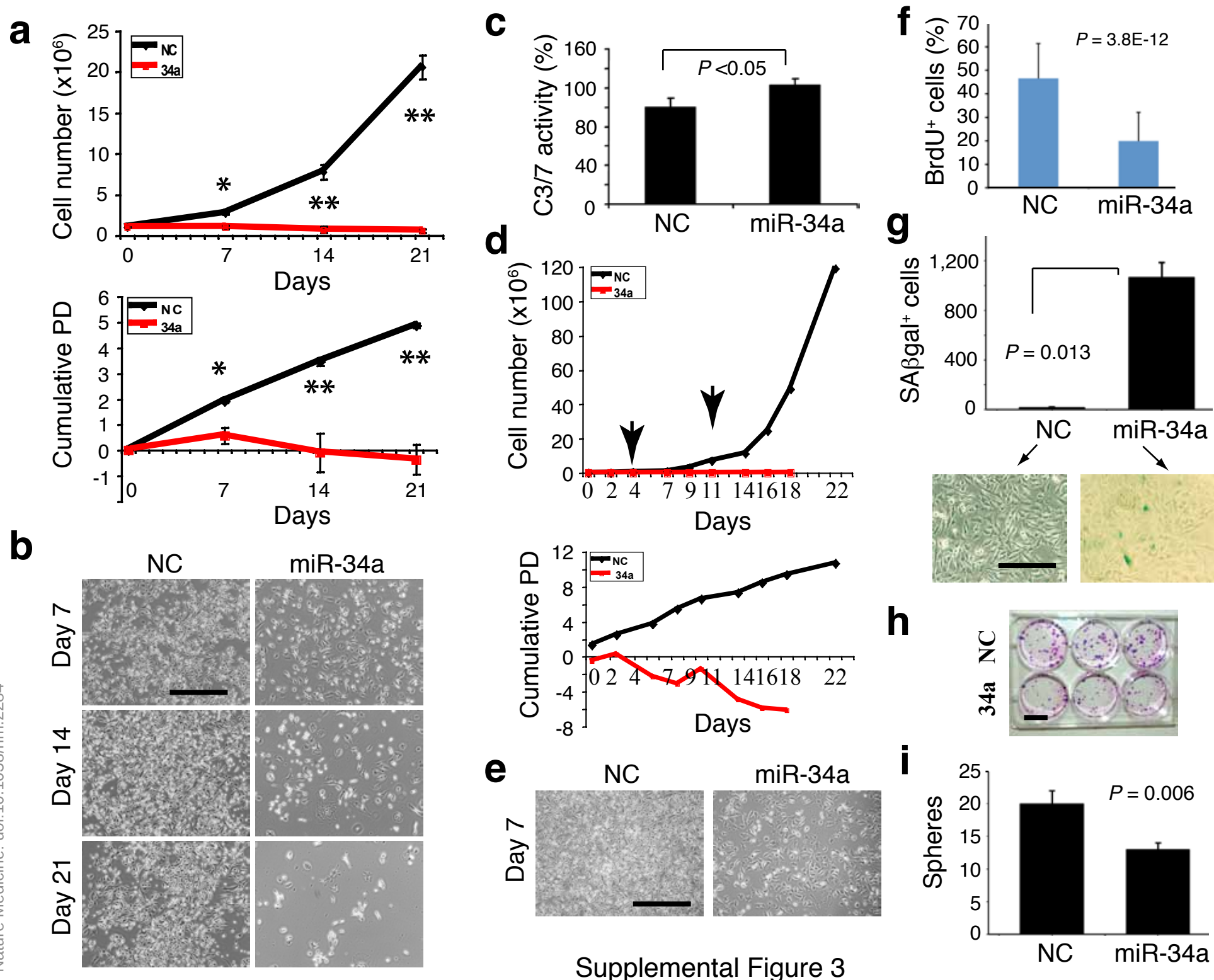
(a,b) Exogenous miR-34a reduces Du145 cell number. Plotted is the cumulative cell numbers or population doublings (PDs) as a function of time and bars represent the mean $\pm$ S.D (* P < 0.05; ** P < 0.001). Shown in b are representative microphotographs (scale bar, 10 μ m).

(c) miR-34a inhibits Du145 cell proliferation. Presented is the mean % of BrdU-positive cells counted from a total of 800–1,000 cells performed under two conditions (* P < 0.001). Below are representative images (scale bar, 10 μ m) of BrdU staining in the 2-d samples.

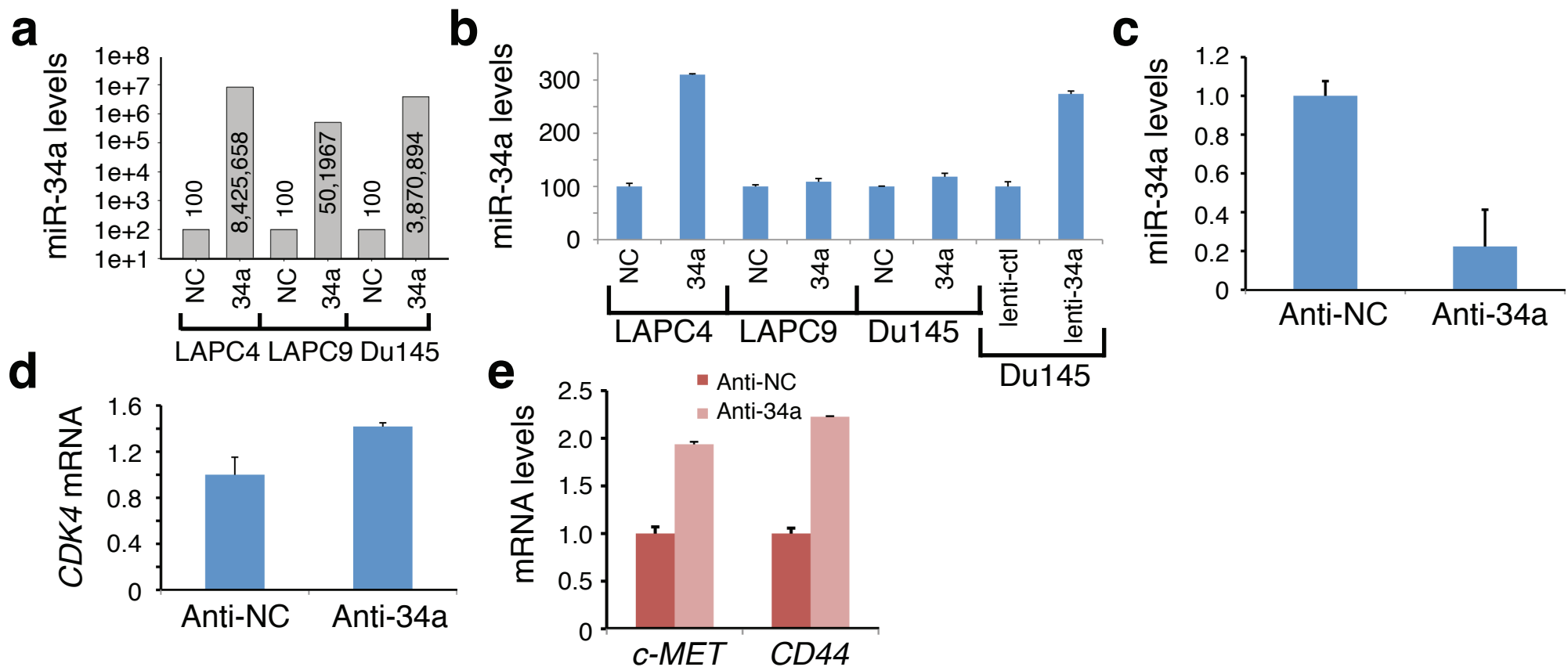
(d,e) miR-34a inhibits Du145 clonal expansion. Cells transfected with miR-NC or miR-34a oligos (33 nM) were plated in triplicate at 100 cells/well. The experiment was terminated at 9 d and wells were Giemsa-stained (d). Shown in e are clonal images (scale bar, 10 μ m). Results shown in d and e were representative of two independent experiments.

Supplementary Figure 3. Effects of miR-34a overexpression on PC3 and PPC-1 cells.

- (a,b) Exogenous miR-34a reduces PC3 cell number. Cumulative cell numbers and PDs were presented and bars represent the mean $\pm$ S.D (* $P < 0.05$; ** $P < 0.001$). Shown in b are representative microphotographs of treated PC3 cells at 7, 14, and 21 d post treatment (scale bar, 10 μ m).
- (c) miR-34a induces apoptosis in PC3 cells. Samples harvested at the end of 21 d (above) were used in DEVDase assays, which measure caspase-3 or 7 (C3/7) activities.
- (d,e) miR-34a transfection reduces PPC-1 cell number. PPC-1 cells were electroporated with miR-NC or miR-34a oligos on d 0 and subsequent experiments were carried out as for PC3 cells except that cells were enumerated every 2-3 d using triplicate samples and re-electroporation was done on d 4 and 11, respectively (arrows). Cumulative cell numbers and PDs were presented (d). Shown in e are representative microphotographs (scale bar, 10 μ m) of treated PPC-1 cells at d 7.
- (f-i) miR-34a inhibits PPC-1 cell proliferation and induces senescence. Presented in f is the % of BrdU⁺ cells (mean $\pm$ S.D; $n = 3$). g, 100,000 PPC-1 cells transfected with NC or miR-34a oligos were plated for SA- β gal staining. Shown are total number of SA- β gal⁺ cells in each well ($n = 3$) and representative microphotographs (below; scale bar, 10 μ m). h, Holoclone assays in PPC-1 cells. 500 cells/well were plated in triplicate and holoclones imaged on d 5 (scale bar, 25 μ m). i, Sphere-formation assays. 1,000 PPC-1 cells transfected with miR-NC or miR-34a oligos were plated in triplicate in 6-well ULA plates. Spheres were counted on d 10. Bars are mean $\pm$ SD ($n = 3$).



Supplemental Figure 3



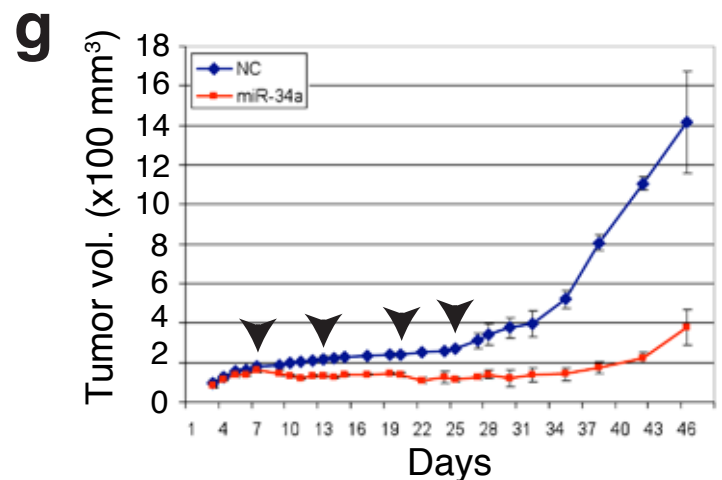
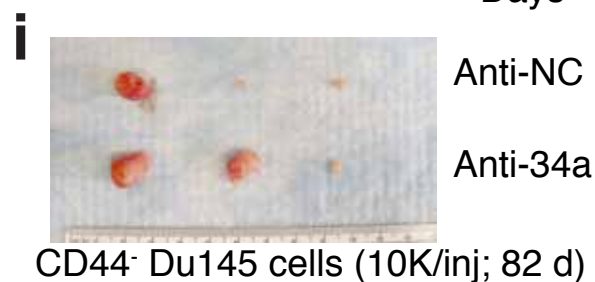
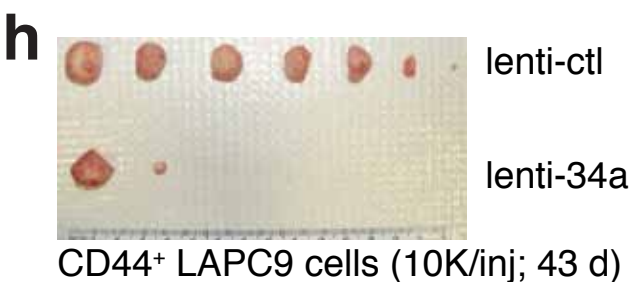
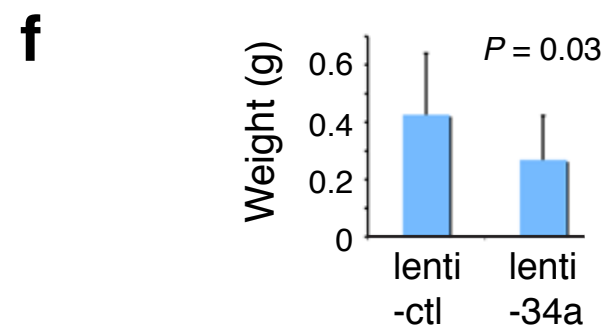
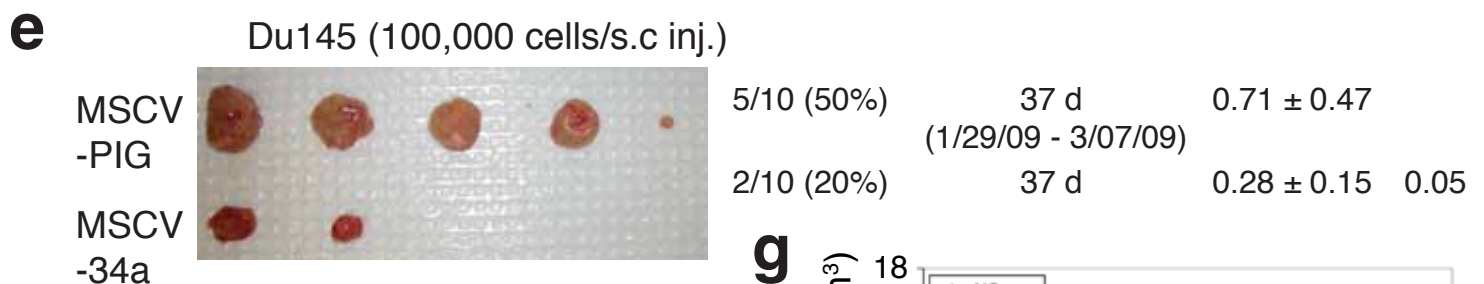
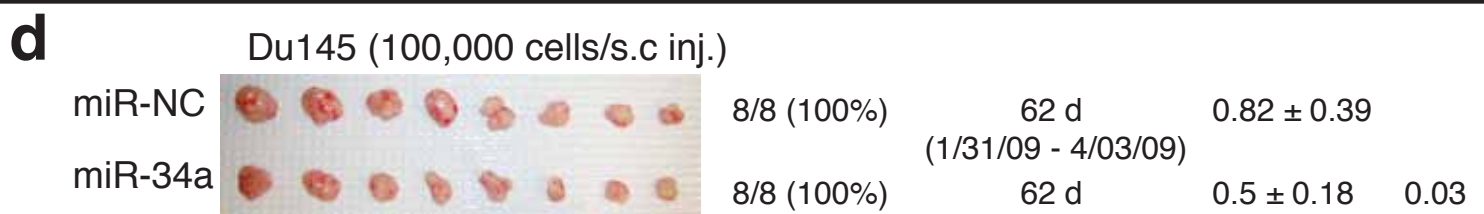
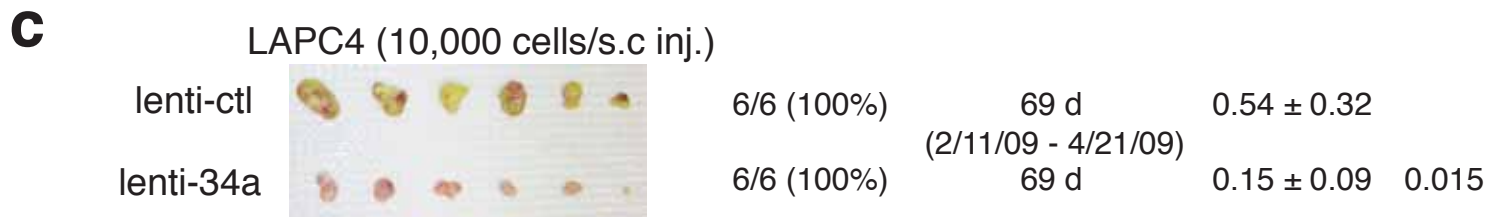
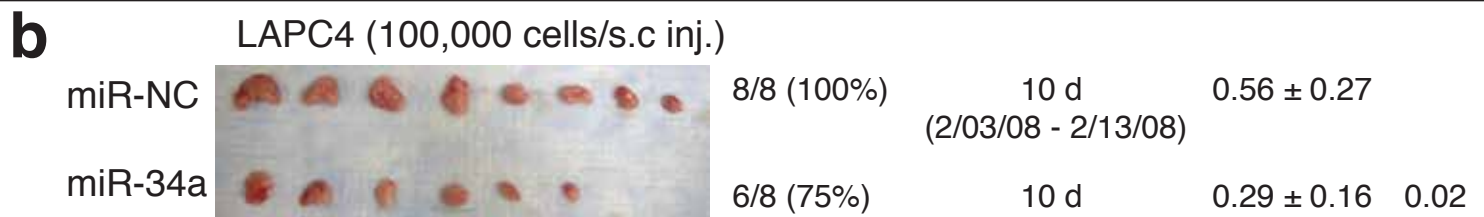
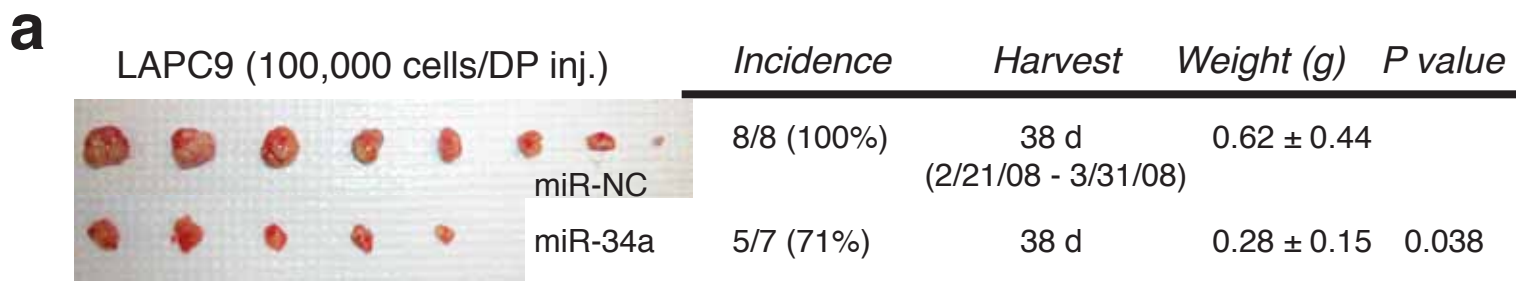
Supplementary Figure 4. Validations of miR-34a and anti-miR-34a.

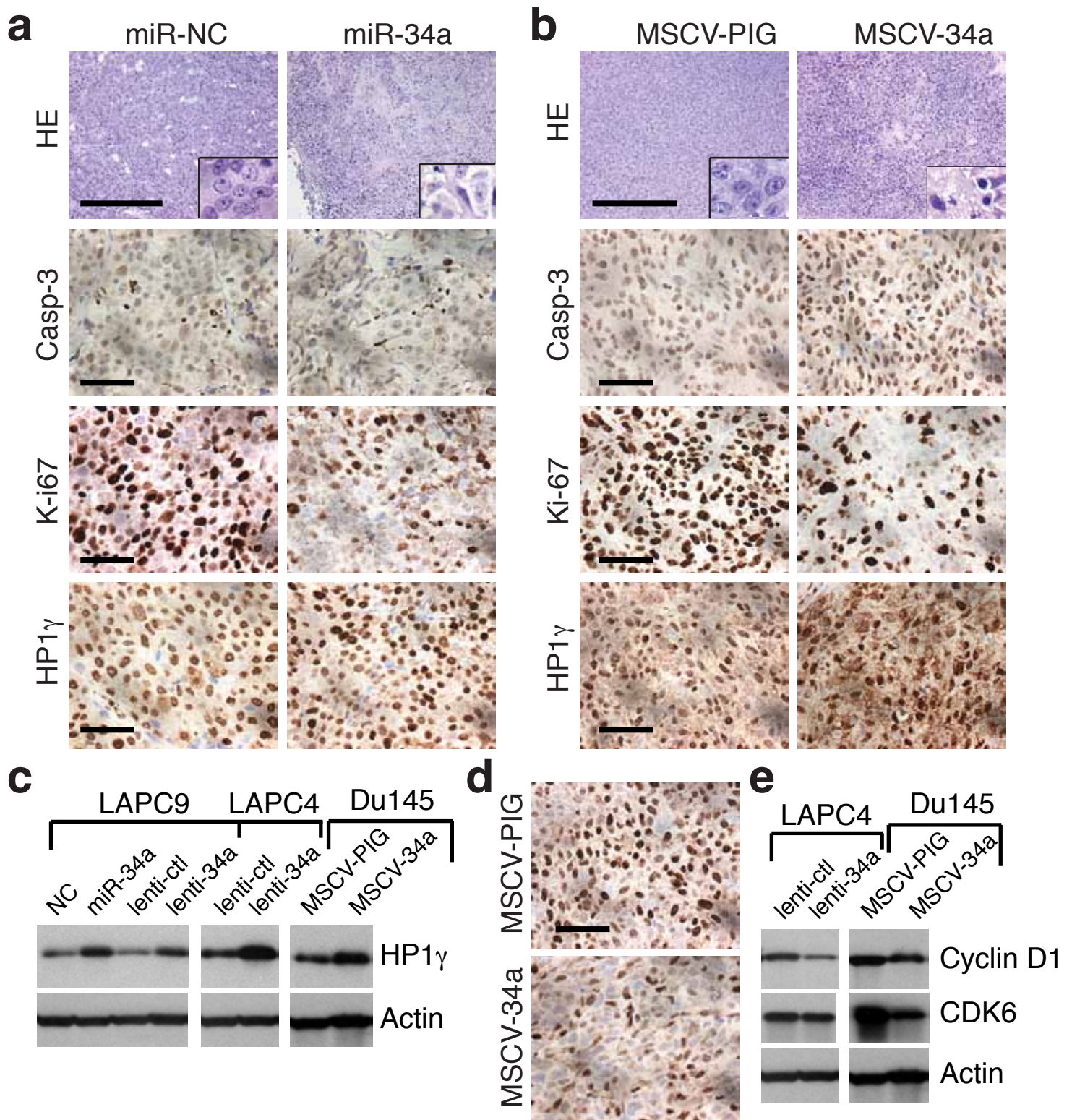
- (a)** PCa cells freshly transfected with miR-34a oligos showed miR-34a levels several orders of magnitude higher than those transfected with miR-NC oligos. The indicated PCa cells purified from xenograft tumors were transfected with miR-34a or miR-NC oligos and, 24 h later, were harvested and used in tumor experiments whereas a small number of cells were set aside and used in qRT-PCR measurement of miR-34a. Shown are the mean miR-34a levels (in log scale; $n = 3$) in miR-34a transfected cells relative to those in the miR-NC transfected cells (actual mean values indicated in the bars).
- (b)** Loss of miR-34a in residual tumors. Residual tumors (see Supplementary Fig. 5) derived from cells transfected with miR-34a oligos or from Du145 cells infected with lenti-34a were used in qRT-PCR analysis of miR-34a. Shown are the miR-34a levels (in linear scale; mean $\pm$ S.D) relative to the respective control tumors.
- (c)** Anti-miR-34a reduces endogenous miR-34a levels. LAPC9 cells were transfected with anti-NC or anti-34a oligos (33 nM) and harvested 24 h later to prepare total RNA for qRT-PCR analysis of miR-34a. Presented are the relative levels normalized to the control miR-191 and miR-24 with the miR-34a in anti-NC-transfected cells set at one.
- (d)** Anti-miR-34a increases the *CDK4* mRNA levels. LAPC9 cells prepared as above were used in qRT-PCR measurement of *CDK4* mRNA (see Supplementary ref. 10). The abundance of *CDK4* mRNA was expressed as the levels relative to the anti-NC transfected cells.
- (e)** Anti-miR-34a increases the mRNA levels of *c-MET* and *CD44*. Du145 tumors from *CD44*⁺ cells transfected with anti-34a or anti-NC were used in qRT-PCR of *c-MET* and *CD44* mRNAs (mean $\pm$ S.D; $n = 3$).

Supplementary Figure 5. Effects of miR-34a on tumor growth

Indicated are tumor incidence (tumors developed/numbers of injections; %), harvest time (including actual injection and termination dates), mean tumor weight (in grams), and the *P* values for tumor weights. Gross tumor images are not to the same scale.

- (a) miR-34a oligo transfection inhibits orthotopic LAPC9 tumor regeneration.
- (b) miR-34a oligo transfection inhibits LAPC4 tumor growth.
- (c) miR-34a overexpression by lentiviral infection inhibits LAPC4 tumor growth. Note that all lenti-ctl tumors were green whereas most lenti-34a tumors were white and had little GFP-positive cells, suggesting that the small lenti-34a tumors were derived from the uninfected cells or from the infected cells that had lost miR-34a expression.
- (d) miR-34a oligo transfection inhibits Du145 tumor growth.
- (e) miR-34a overexpression by retroviral infection inhibits Du145 tumor regeneration. The MSCV-34a tumors were ~3 times smaller than the control tumors but the difference was at the statistical borderline due to small numbers of animals in each group that developed tumors.
- (f) miR-34a overexpression by lentiviral infection inhibits Du145 tumor growth. Tumors were harvested at 49 d.
- (g) miR-34a inhibits PPC-1 tumor development. Animals were terminated on d 46. Arrowheads indicate repeated intra-tumoral oligo injections. Shown are the tumor volumes (mean $\pm$ S.D; $n = 7$ for each group) measured on the indicated time points (d).
- (h) miR-34a re-expression in purified CD44⁺ LAPC9 inhibits tumor regeneration.
- (i) Anti-miR-34a promotes tumor growth of purified CD44⁺ Du145 cells. This represents an independent repeat experiment to Fig. 1h.



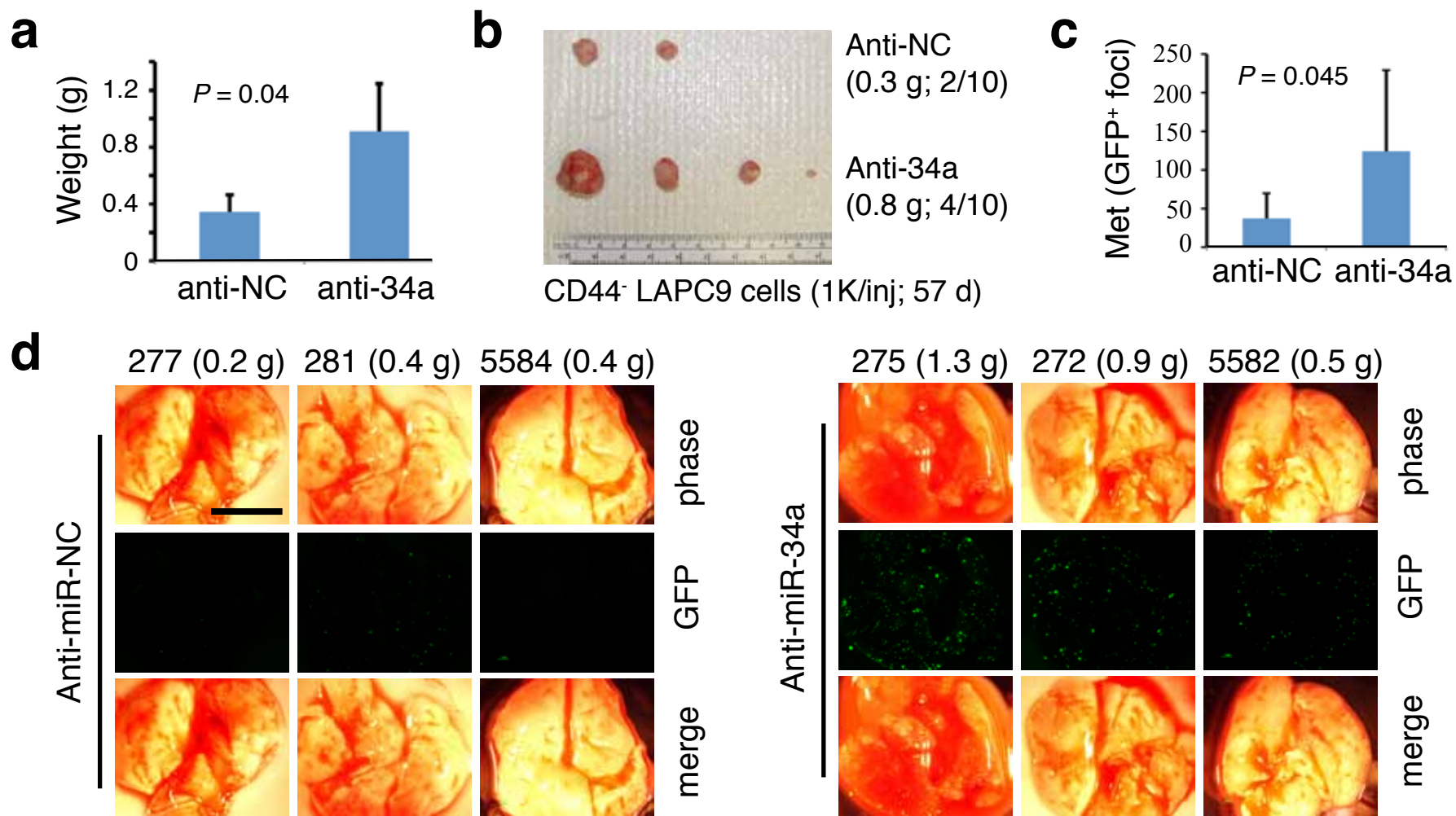


Supplementary Figure 6. Characterizations of miR-34a-overexpressing tumors.

(a,b) Paraffin-embedded sections of LAPC9 tumors (a) derived from cells transfected with miR-NC or miR-34a oligos (Supplementary Fig. 5a) or Du145 tumors (b) derived from cells infected with MSCV-PIG or MSCV-34a (Supplementary Fig. 5e) were used in HE or IHC staining for the molecules indicated. Depicted in the insets in the top rows is a more differentiated morphology of the cells in miR-34a overexpressing tumors. Shown below are representative images displaying reduced Ki-67 and increased HP1 γ but no changes in active caspase-3 in the miR-34a overexpressing tumors. Scale bars, 20 μ m.

(c) Increased HP-1 γ in tumors derived from PCa cells overexpressing miR-34a by Western analysis.

(d,e) IHC (d) and Western blotting (e) showing reduced cyclin D1 and CDK6 in Du145 tumors from cells infected with MSCV-34a and LAPC4 tumors from cells infected with lenti-34a vectors.



Supplementary Figure 7. Anti-miR-34a enhances LAPC9 tumor growth and lung metastasis.

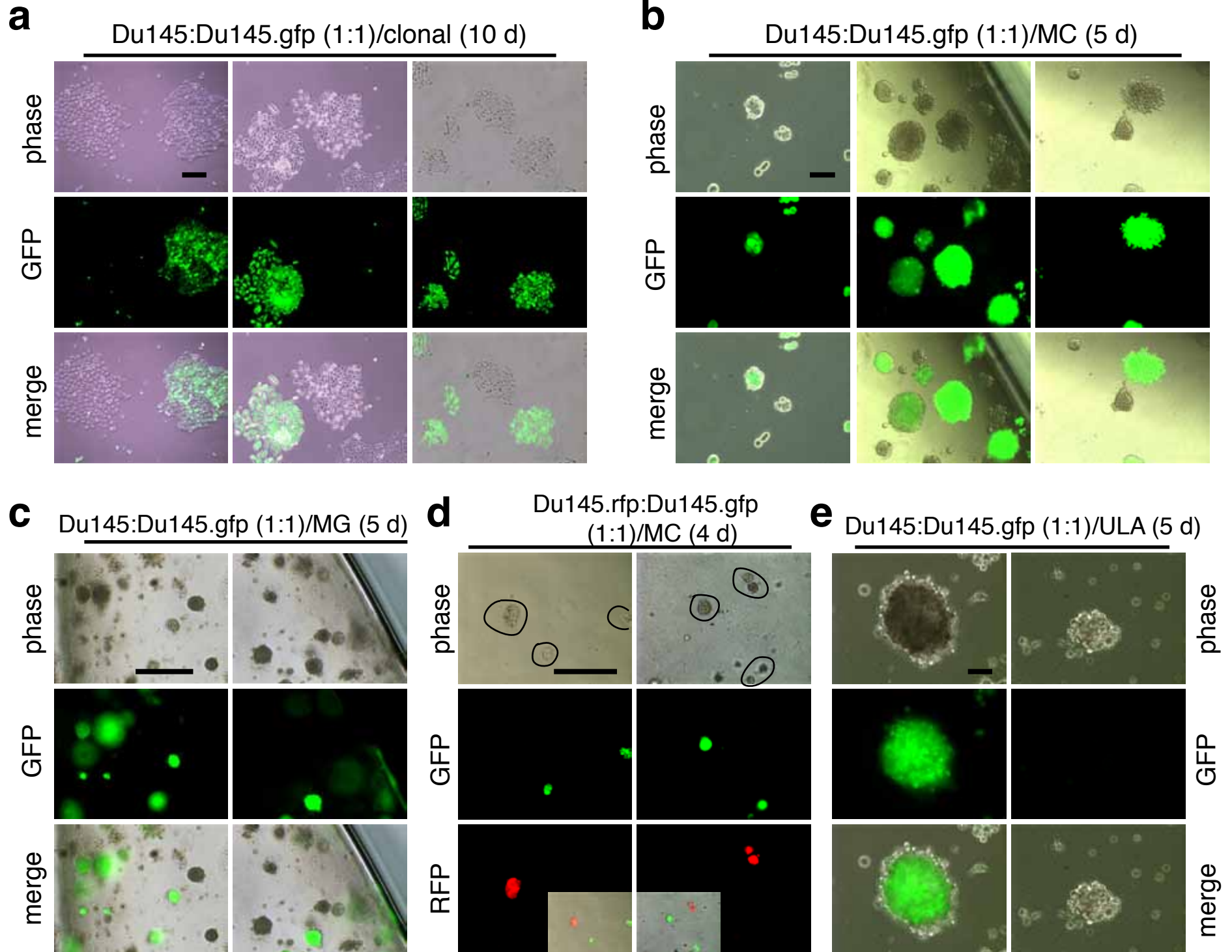
(a) Anti-miR-34a promotes orthotopic LAPC9 tumor growth. This represents a repeat experiment to Fig. 1i. Shown are the mean weights of tumors derived from LAPC9-GFP cells transfected with anti-NC or anti-34a oligos and implanted in the DP of intact male NOD-SCID mice (sacrificed at 50 d). Tumor incidences for both groups were 4/7.

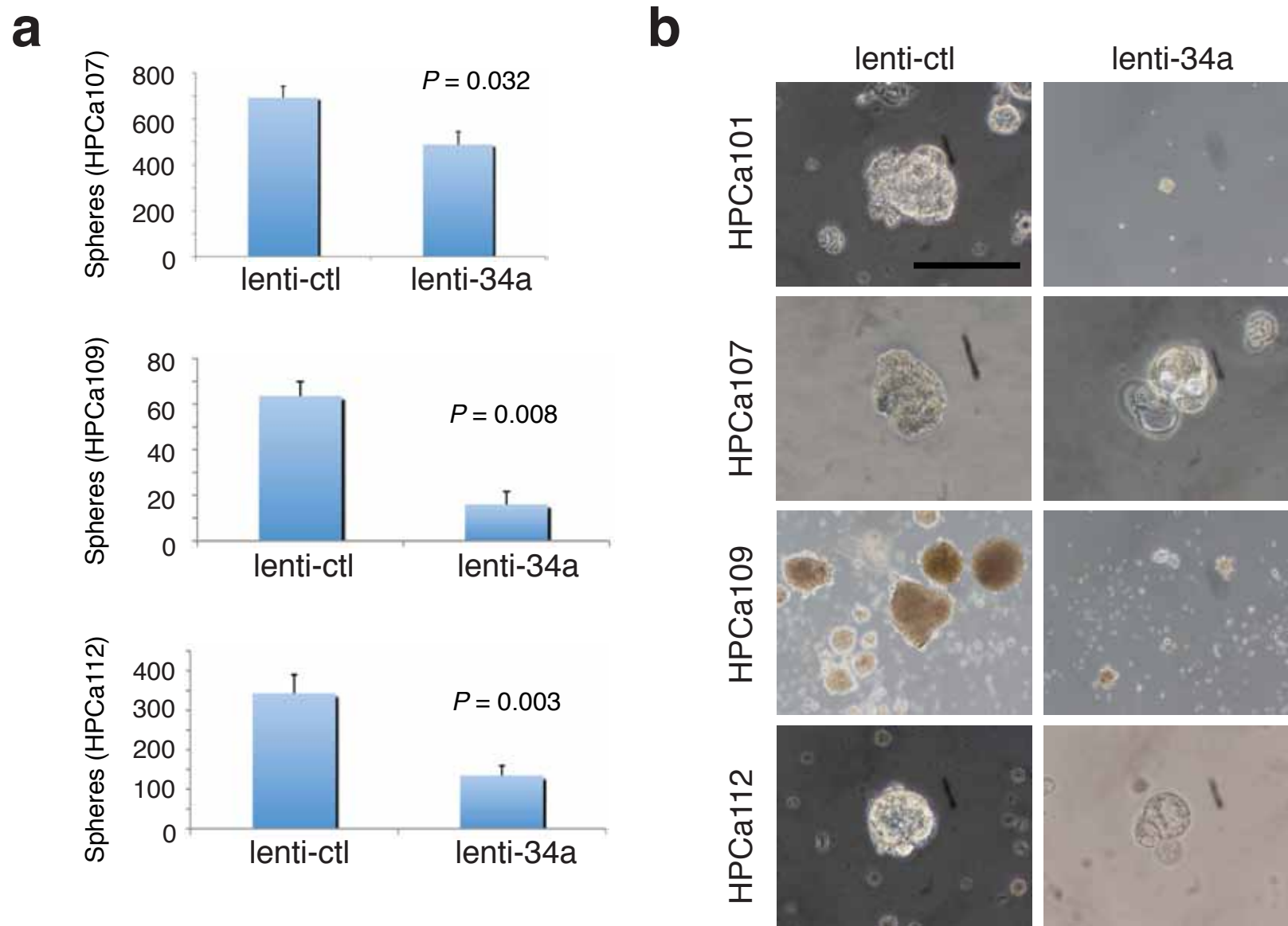
(b) Anti-34a promotes tumor growth of purified CD44⁺ LAPC9 cells. Shown are the end-point tumors derived from CD44⁺ LAPC9 cells transfected with anti-NC or anti-34a and s.c implanted in male NOD-SCID mice (euthanized at 60 d).

(c,d) Anti-miR-34a promotes orthotopic LAPC9 lung metastasis with data pooled from the two orthotopic tumor experiments (Fig. 1i and Supplementary Fig. 7a). Shown in d (and in Fig. 1j) are representative phase and GFP images of 4 lungs from each group (scale bar, 100 μ m) and in c is the quantification of GFP⁺ foci/lung.

Supplementary Figure 8. Competition experiments demonstrating clonality of PCa cell colonies and spheres.

- (a) Clonal assays. Du145 cells were mixed with Du145-GFP cells at 1:1 ratio (50 cells each) and plated in 6-well plate. Images were taken on d 10 and shown are 3 representative fields.
- (b,c) Clonogenic assays in methylcellulose (MC) or in Matrigel (MG). Du145 cells were mixed with DU145-GFP cells at 1:1 ratio and a total of 6,000 cells were plated for clonogenic assays in MC (b) or in MG (c). Photos were taken on d 5 after plating and shown are representative fields.
- (d) Clonogenic assays in MC. Du145-RFP cells were mixed with Du145-GFP cells at 1:1 ratio and a total of 2,000 cells were plated in MC. Images were taken on d 4 and shown are two representative fields.
- (e) Sphere-formation assays. Du145 cells were mixed with Du145-GFP cells at 1:1 ratio and a total of 2,000 cells were plated in ULA plates. Photos were taken on d 5 after plating and shown are a representative GFP⁺ (left) and GFP⁻ (right) sphere.
- Scale bars, 20 μ m.





Supplementary Figure 9. miR-34a inhibits prostasphere formation in HPCa cells.

Presented in **a** are the numbers of spheres formed by HPCa cells freshly purified from the indicated patient primary tumors and infected with either lenti-ctl or lenti-34a vectors. Bars represent the mean $\pm$ s.d ($n = 3-6$). Shown in **b** are representative images of spheres formed by primary HPCa cells. Scale bar, 20 μ m.

a

miR-NC

phase

GFP

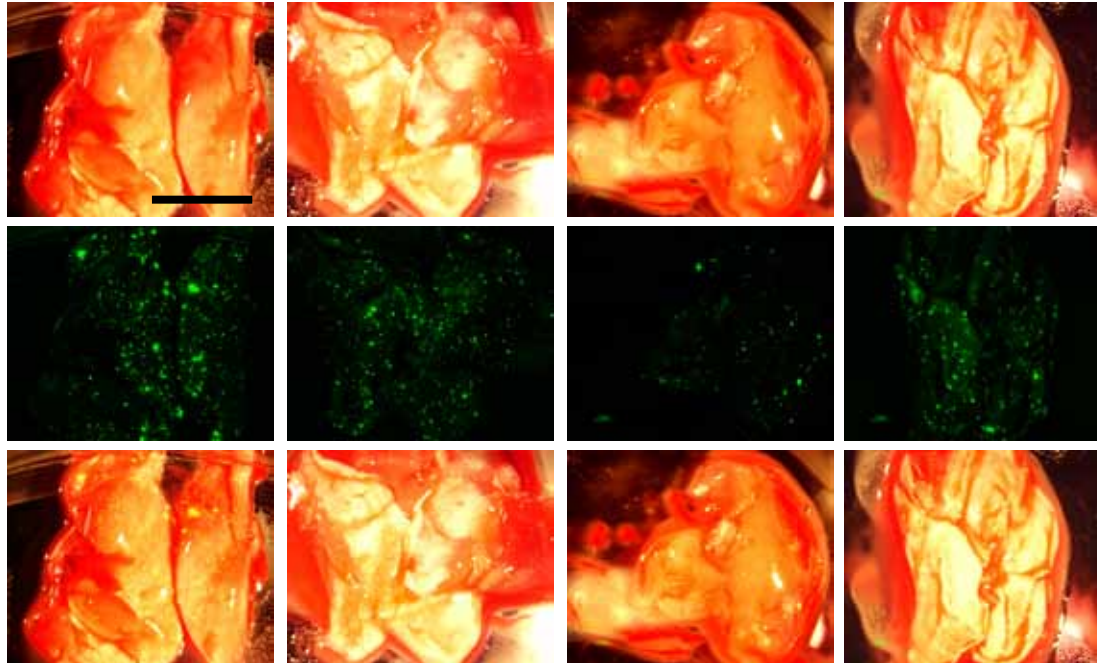
merge

1769 (1.3 g)

1768 (0.94 g)

258 (0.8 g)

1759 (0.8 g)

**Supplementary Figure 10. Systemic miR-34a inhibits orthotopic LAPC9 lung metastasis.**

Presented is the third therapeutic experiment described in the TEXT and ONLINE METHODS (see also Fig. 2b-d). Shown are representative lung images in the miR-34a treated group (b) compared to the miR-NC group (a). Animal tag number and tumor weight are indicated. Scale bar, 100 μ m.

b

miR-34a

phase

GFP

merge

251 (0.72 g)

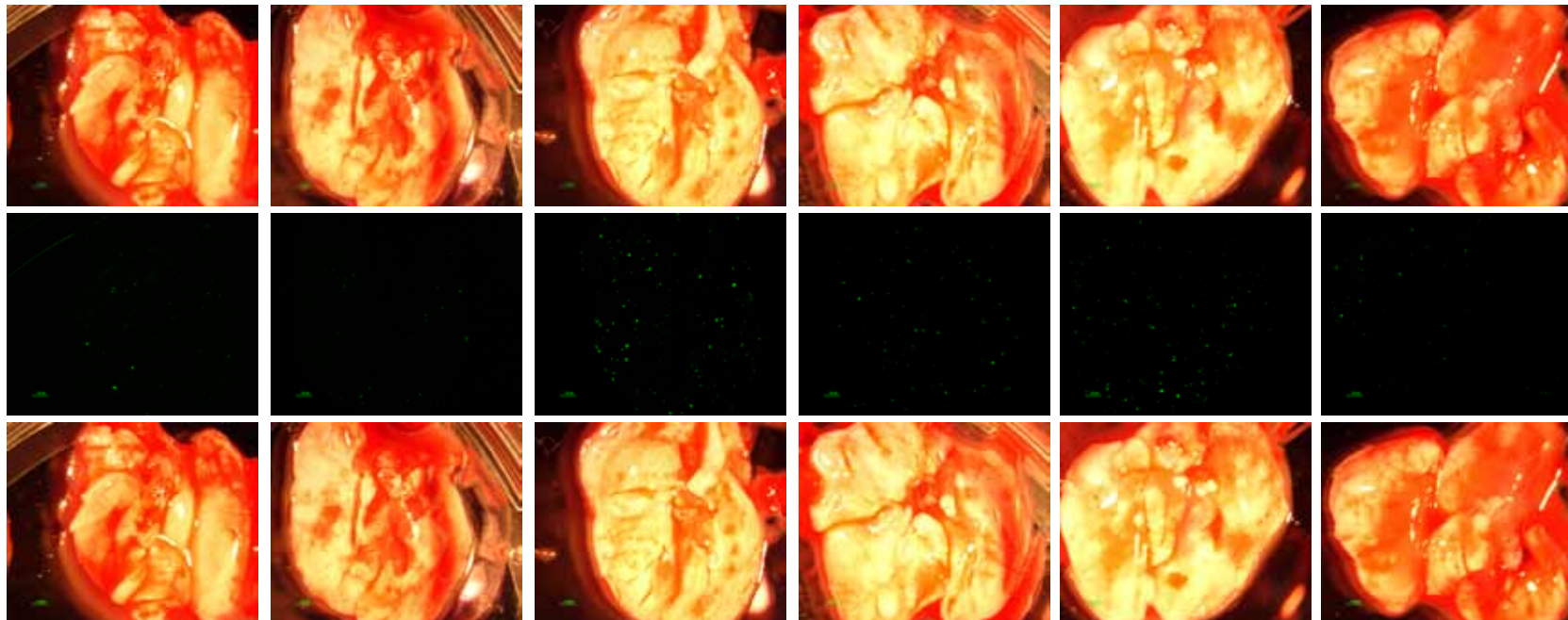
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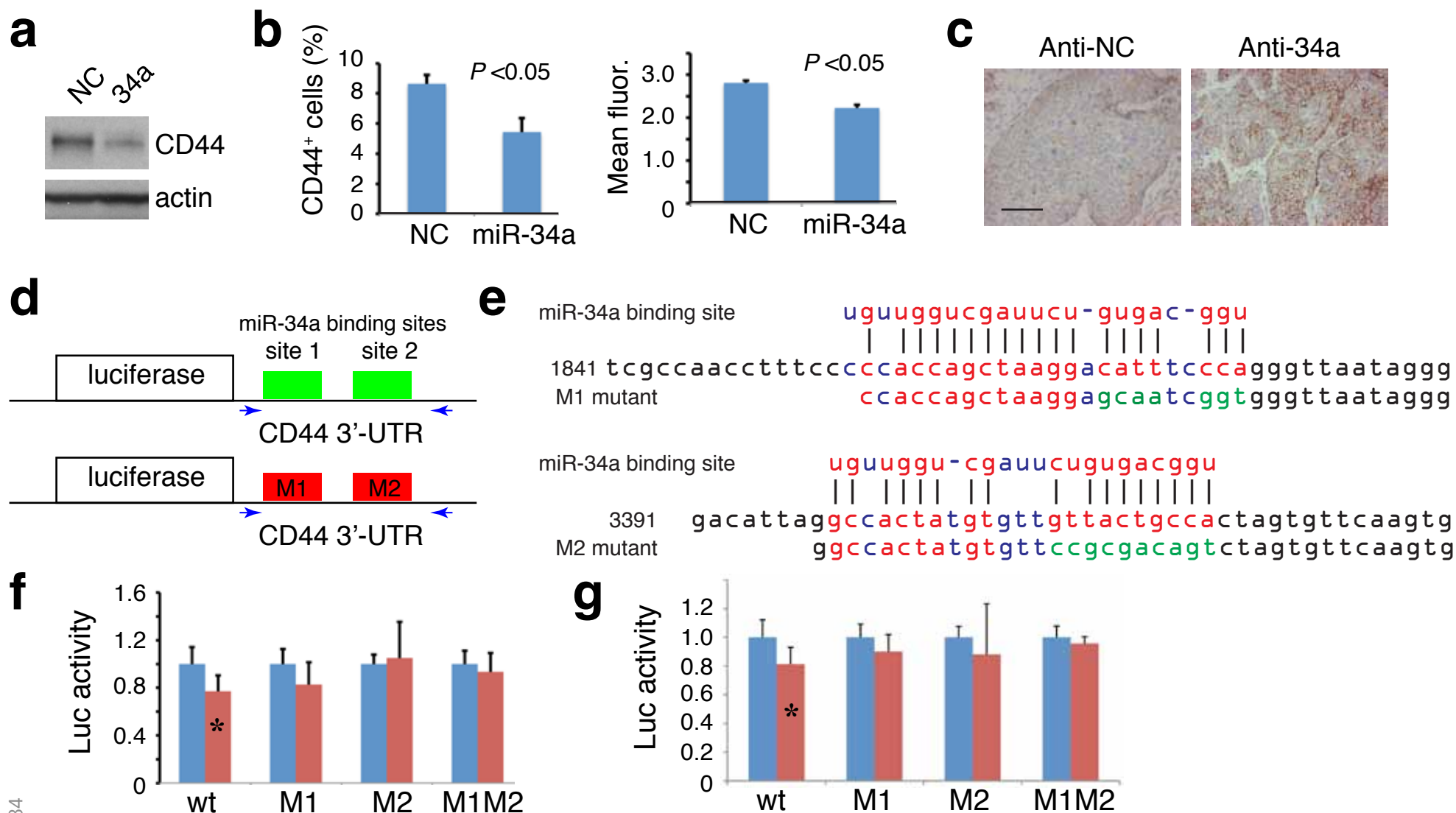
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257 (0.99 g)

259 (0.98 g)

260 (0.94 g)





Supplementary Figure 11. miR-34a directly targets CD44.

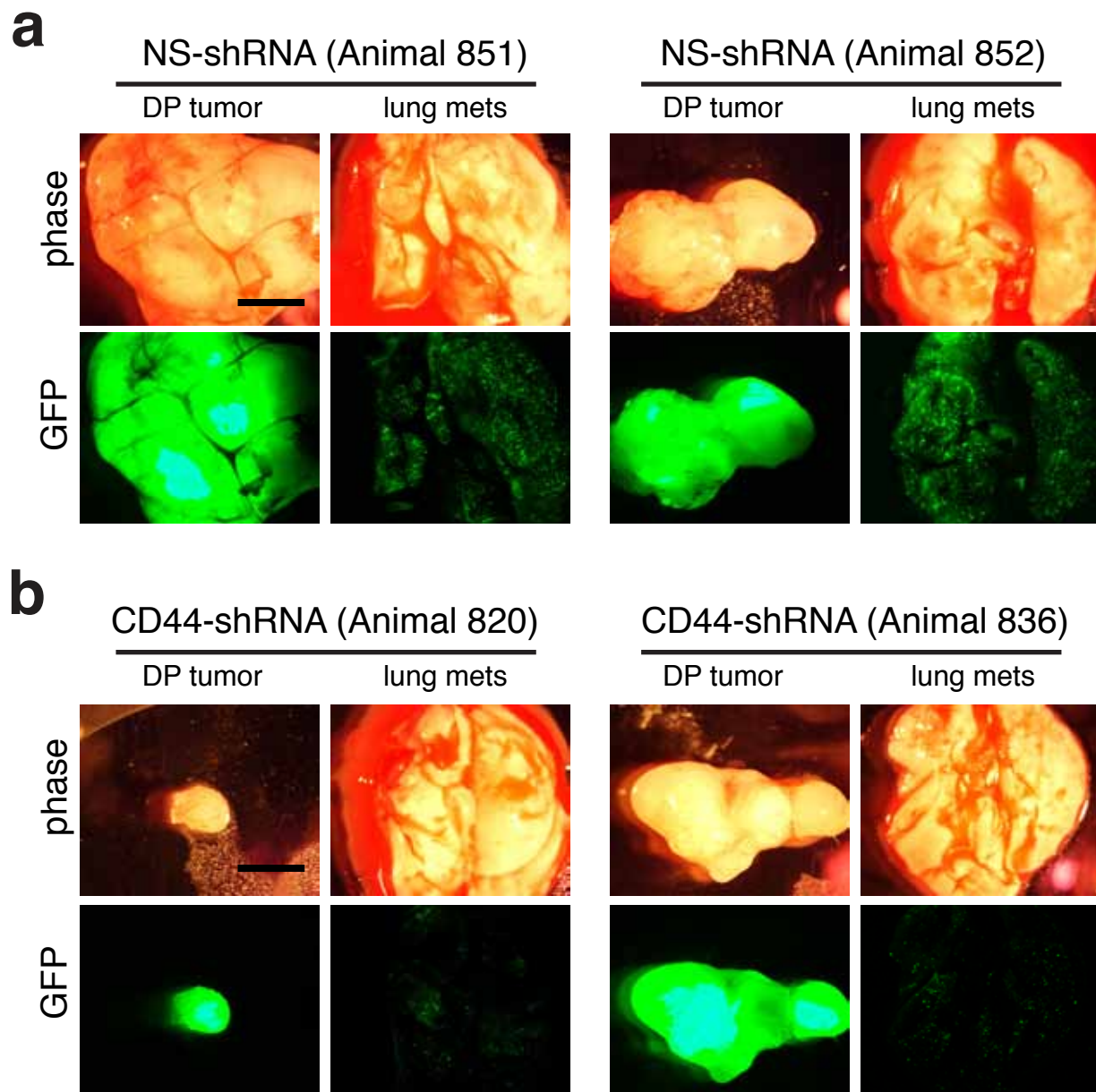
(a) PC3 cells transfected with miR-NC or miR-34a oligos (33 nM, 48 h) were harvested and used in Western blotting of CD44 or β -actin.

(b) Purified LAPC9 cells were transfected with miR-NC or miR-34a oligos (33 nM; 72 h) and then used in flow cytometric analysis of CD44. Shown are CD44⁺ cells (%; $n = 3$) and the mean fluorescence intensity of CD44 expression.

(c) Two representative IHC images of CD44 staining in LAPC9 tumors derived from cells transfected with anti-34a or anti-NC. Scale bar, 10 μ m.

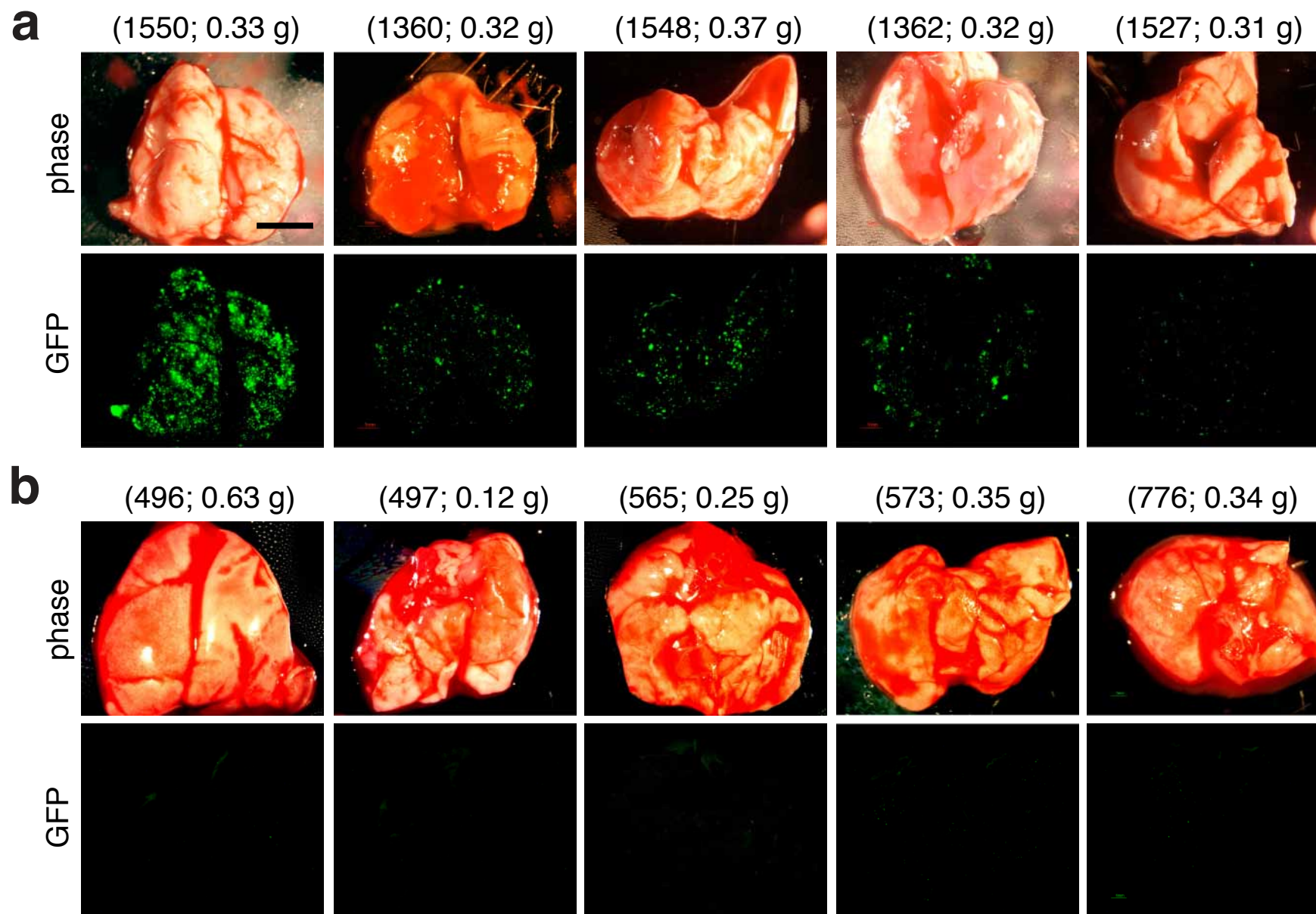
(d,e) Schematic of the 2.55-kb wt CD44 3'-UTR containing the two miR-34a binding sites and the two mutants (i.e., M1 and M2) that were cloned downstream the luciferase cDNA in the 3'UTR/pMIR plasmid (d). Shown in e are the actual mutated sequences (in green).

(f,g) LNCaP (d) or LNCaP C4-2 (e) cells were co-transfected with wt or mutant luciferase construct together with NC (blue bars) or miR-34a (red bars) oligos. Each condition was run in 6 replicates and the experiment was repeated 2-3 times. The results were expressed as luciferase activity relative to the wt group after normalizing to the Renilla luciferase (internal control). Bars represent the mean $\pm$ SEM (* $P < 0.01$).



Supplementary Figure 12. CD44 knockdown inhibits LAPC4 lung metastasis.

Purified LAPC4 cells infected with either non-silencing (NS) pGIPz control lentiviral vector or pGIPz-CD44shRNA (see Supplementary Fig. 1d) were implanted in the DP of male NOD-SCID mice (euthanized at 76 d). Shown are the images of DP tumors and the lungs from two representative animals in each group ($n = 7$). The CD44-shRNA animals (b) had both smaller DP tumors and less lung metastasis (GFP⁺ foci) than in NS-shRNA animals. Scale bar, 100 μ m.

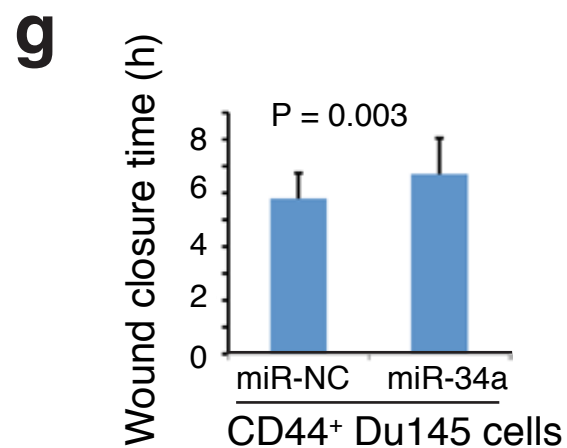
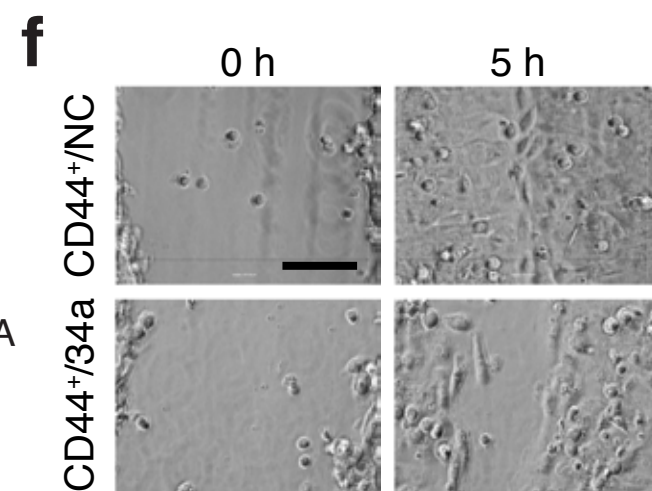
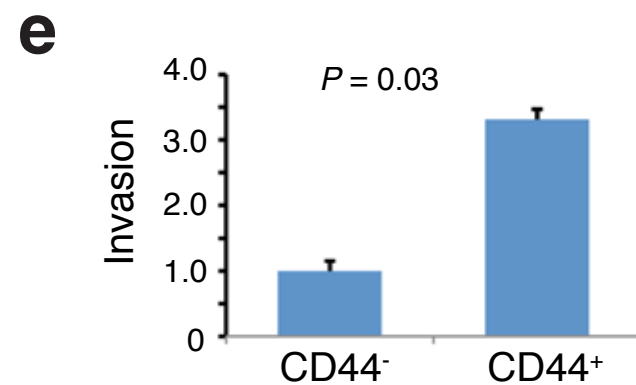
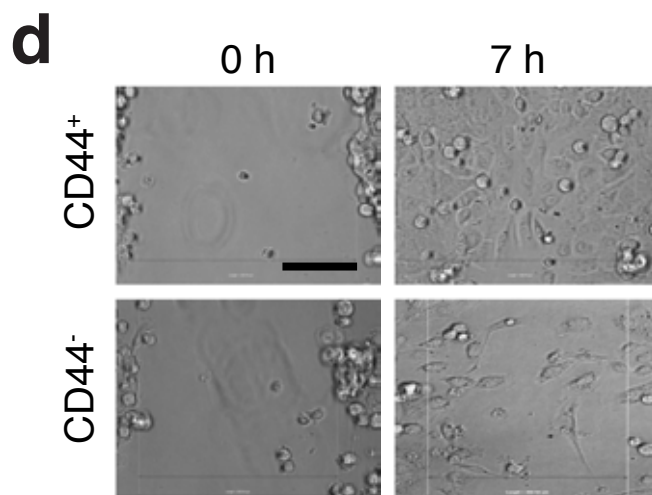
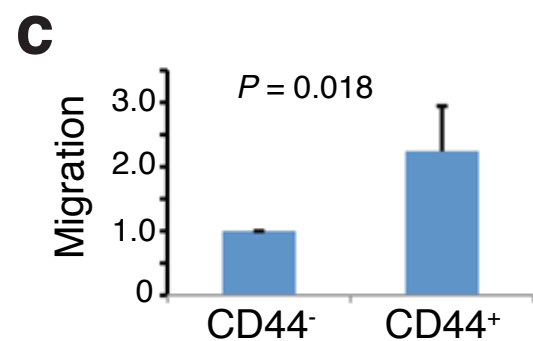
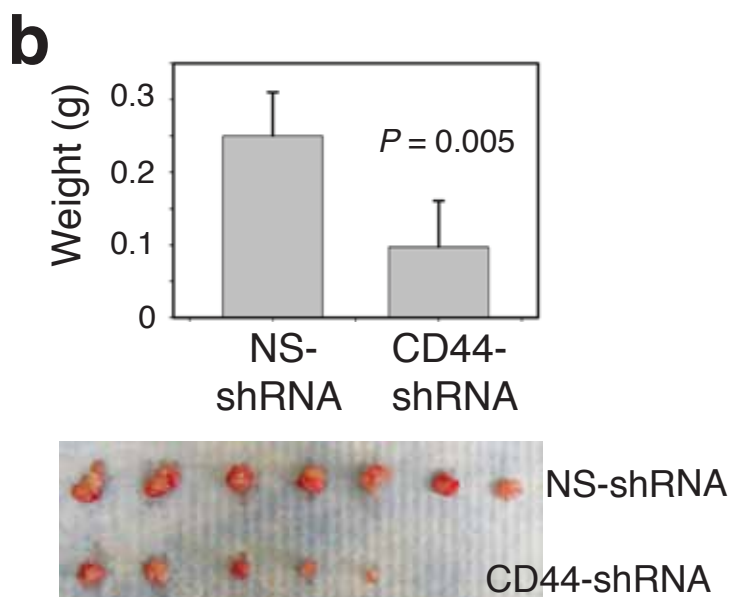
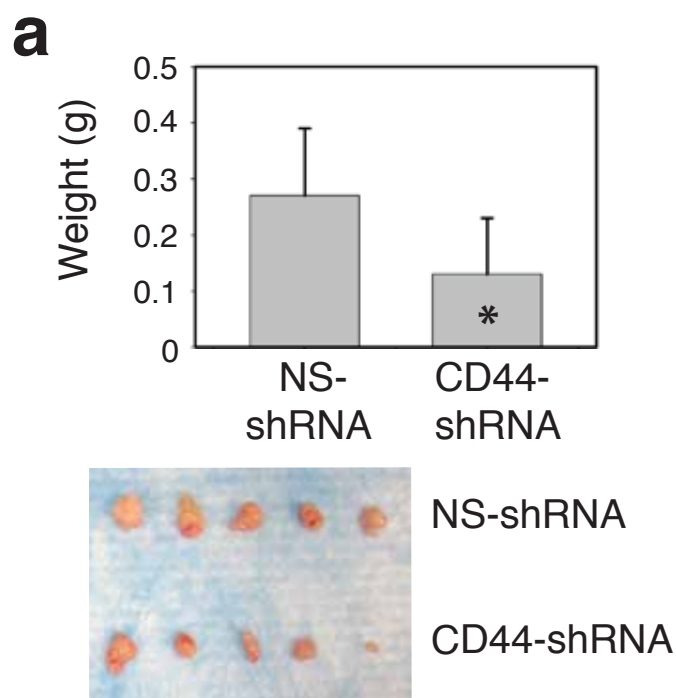


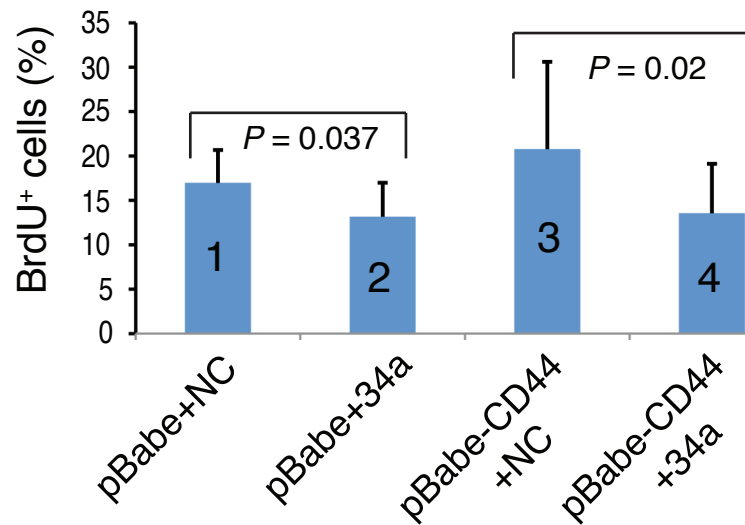
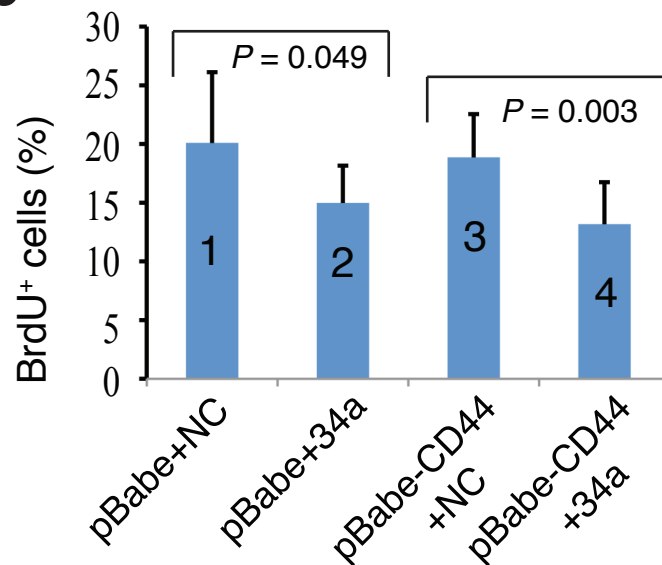
Supplementary Figure 13. CD44 knockdown inhibits PC3 cell lung metastasis.

Shown are phase and GFP images of five representative lungs in the animals bearing orthotopic PC3 tumors derived from cells infected with pGIPz control (a) or pGIPz-CD44shRNA (b) lentiviral vectors ($n = 8$ for each group; animals sacrificed at 40 d). Scale bar, 100 μm .

Supplementary Figure 14. Effects of CD44 knockdown or miR-34a overexpression on Du145 cells.

- (a) Shown are the weights (above; mean $\pm$ s.d, $*P < 0.05$) and images (below; incidence, 5/5 for both groups) of subcutaneous tumors derived from Du145 cells infected with NS-shRNA or CD44-shRNA (MOI 20, 72 h; harvested at 56 d).
- (b) Shown are the weights (above; mean $\pm$ s.d) and images (below) of orthotopic tumors derived from Du145 cells infected with NS-shRNA or CD44-shRNA (harvested at 41 d). Tumor incidence for the NS-shRNA and CD44-shRNA group was 7/7 and 5/8, respectively.
- (c) Shown are the relative migratory abilities of purified CD44⁺ and CD44⁻ Du145 cells plated on the top of the Boyden chamber without Matrigel.
- (d) Shown are two representative static images of CD44⁺ (out of a total of 17 movies) and CD44⁻ (out of a total of 14 movies) Du145 cells at the beginning of recording and at 7 h post wounding. In the movie images (scale bar, 20 μ m) shown, the CD44⁺ but not CD44⁻ Du145 cells had closed the wound by 7 h.
- (e) Shown are the relative invasive capacities of purified CD44⁺ and CD44⁻ Du145 cells plated on the top of the Boyden chamber with Matrigel.
- (f) Shown are two representative static images of CD44⁺ Du145 cells transfected with miR-NC (out of a total of 25 movies) or miR-34a oligos (out of a total of 29 movies) at the beginning of recording and at 5 h post wounding. Scale bar, 20 μ m.
- (g) Quantitative presentation of results in f.



a**b**

Supplementary Figure 15. CD44 overexpression does not relieve miR-34a-mediated inhibition of proliferation.

BrdU immunostaining in PPC-1 (**a**) or LNCaP (**b**) cells first infected with pBabe-CD44 (which encodes human CD44 cDNA that lacks the two miR-34a binding sites at the 3'-UTR) or its empty control vector (pBabe) and then (48 h later) transfected with miR-NC or miR-34a oligos. Presented are the % BrdU⁺ cells from counting a total of 400–500 cells in 2-3 experiments. In both cell types, miR-34a oligos reduced BrdU⁺ percentages in cells infected with pBabe (conditions 1 and 2) or pBabe-CD44 (conditions 3 and 4). There were no differences between conditions 4 and 2 or between conditions 3 and 1 ($P > 0.1$).

Supplementary Table 1. Primary human prostate tumors (HPCa) used to purify CD44⁺ and CD44⁻ cells for qRT-PCR analysis

HPCa sample ^a	Age	Gleason	%CD44 ⁺ ^b	Purification ^c	Purity (%) ^d
HPCa60	54	8	8.7	MACS	CD44 ⁺ : ~50 CD44 ⁻ : N.A
HPCa62	59	7	2.4	MACS	CD44 ⁺ : ~50 CD44 ⁻ : 100
HPCa65	59	7	19.9	MACS	CD44 ⁺ : 67 CD44 ⁻ : 100
HPCa 66	58	6	15.0	FACS	CD44 ⁺ : N.A CD44 ⁻ : 94
HPCa72	58	7	10.2	MACS	CD44 ⁺ : 33 CD44 ⁻ : 100
HPCa74	59	7	16.2	MACS	CD44 ⁺ : 70 CD44 ⁻ : 100
HPCa76	64	7	0.02	MACS	CD44 ⁺ : ~10 CD44 ⁻ : 100
HPCa77	46	6	14.2	MACS	CD44 ⁺ : 45 CD44 ⁻ : 100
HPCa78	64	7	19.2	MACS	CD44 ⁺ : 45 CD44 ⁻ : 100
HPCa79	67	7	8.2	MACS	CD44 ⁺ : 15 CD44 ⁻ : 100
HPCa80	65	9	4.4	MACS	CD44 ⁺ : 13 CD44 ⁻ : 85
HPCa81	54	7	20.9	MACS	CD44 ⁺ : 64 CD44 ⁻ : 90
HPCa87*	57	9	N.D	MACS	CD44 ⁺ : 93 CD44 ⁻ : 100
HPCa89	55	9	24	MACS	CD44 ⁺ : 87 CD44 ⁻ : 90
HPCa91*	60	8	N.D	MACS	CD44 ⁺ : 95 CD44 ⁻ : 90
HPCa93	58	7	0.99	FACS	CD44 ⁺ : 87 CD44 ⁻ : 100
HPCa98	64	8	5.74	FACS	CD44 ⁺ : 79 CD44 ⁻ : 100
HPCa102	55	7	24.8	FACS	CD44 ⁺ : 99 CD44 ⁻ : 85

^aHuman primary tumors were obtained from the robotic (Da Vinci) surgery. The age and Gleason score of each tumor are indicated. *For HPCa87 and HPCa91, the first-generation xenograft tumors established in our lab were used in purifying CD44⁺ and CD44⁻ cells.

^bThe % of CD44⁺ HPCa cells was determined by flow analysis prior to sorting. N.D, not determined.

^cCD44⁺ and CD44⁻ cells were purified out using MACS (magnetic cell sorting) or FACS (fluorescence activated cell sorting). Four of the eighteen samples (shaded) were sorted using FACS as the MACS approach was more gentle on primary tumor cells.

^dThe purity of MACS-purified cells, determined by counting CD44⁺ cells under a fluorescence microscope, was variable for both CD44⁺ and CD44⁻ cell populations. The purity of FACS-purified cells, determined by post-sort flow analysis, was ~80-99% for CD44⁺ HPCa cells and 85-100% for CD44⁻ cells. N.A, not available.

Supplementary Table 2. Correlation of CD44 levels with miR-34 manipulations in PCa cells

Tumor systems	Comments
LAPC9	– LAPC9 cells transfected with miR-34a oligos exhibit reduced CD44 protein expression levels and CD44⁺ cells (Supplementary Fig. 11b). – LAPC9 tumors derived from cells transfected with anti-34a expressed higher levels of CD44 than tumors derived from the cells transfected with anti-NC (Supplementary Fig. 11c).
Du145	– Residual Du145 tumors from cells infected with MSCV-34a show reduced CD44 protein (Fig. 4a). – Du145 cells transfected with miR-34a oligos show time- and dose-dependent reduction in CD44 protein (Fig. 4b). – Du145 tumors derived from CD44⁺ Du145 cells transfected with anti-34a expressed higher levels of CD44 mRNA than tumors derived from the same cells transfected with anti-NC (Supplementary Fig. 4e).
PC3	– Residual orthotopic PC3 tumors in animals treated with miR-34a display reduced CD44 protein (Fig. 4a). – PC3 cells transfected with miR-34a oligos show reduced CD44 protein (Supplementary Fig. 11a).
PPC-1	– PPC-1 cells transfected with miR-34a oligos show reduced CD44 protein (Fig. 4b).