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Therapeutic silencing of miR-10b inhibits metastasis in a mouse mammary tumor model

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MicroRNAs (miRNAs) are increasingly implicated in the regulation of metastasis. Despite their potential as targets for anti-metastatic therapy, miRNAs have only been silenced in normal tissues of rodents and nonhuman primates. Therefore, the development of effective approaches for sequence-specific inhibition of miRNAs in tumors remains a scientific and clinical challenge. Here we show that systemic treatment of tumor-bearing mice with miR-10b antagomirs—a class of chemically modified anti-miRNA oligonucleotide—suppresses breast cancer metastasis. Both *in vitro* and *in vivo*, silencing of miR-10b with antagomirs significantly decreases miR-10b levels and increases the levels of a functionally important miR-10b target, Hoxd10. Administration of miR-10b antagomirs to mice bearing highly metastatic cells does not reduce primary mammary tumor growth but markedly suppresses formation of lung metastases in a sequence-specific manner. The miR-10b antagomir, which is well tolerated by normal animals, appears to be a promising candidate for the development of new anti-metastasis agents.

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Ninety percent of cancer-related mortality is caused by metastases formed by disseminated primary tumor cells at distant anatomic sites[1]. Although surgery, radiation therapy and chemotherapy can control many primary tumors effectively, these treatments have limited utility in curbing the metastatic spread of cancer cells and resulting metastasis formation[2]. Critical regulators of the metastatic process, including proteins and miRNAs, are under intensive investigation[2–4]. Understanding the actions of these regulatory molecules provides the basis for molecularly targeted therapeutics. Candidate antimetastasis therapeutic approaches that target tyrosine kinase pathways, the transforming growth factor–β pathway, tumor angiogenesis and the tumor microenvironment have shown efficacy in preclinical studies[5]. Some have been tested in clinical trials: the monoclonal anti-HER2 antibody trastuzumab (Herceptin), when combined with adjuvant chemotherapy, improved metastasis-free survival in women with surgically resected HER2-positive breast cancer[6,7]; bevacizumab (Avastin), a neutralizing antibody against vascular endothelial growth factor, showed measureable but limited benefit in prolonging the time to disease progression in patients with metastatic renal-cell cancer[8] or metastatic colorectal cancer[9]. However, current treatment options rarely cure metastatic cancer. Similarly, there is a lack of prophylactic therapies that are capable of blocking dissemination from primary tumors and preventing future metastasis formation.

Emerging evidence suggests that cancer initiation and progression involve miRNAs—noncoding RNA molecules that act as negative regulators of gene expression. These small cellular RNAs bind to partially complementary sequences at the 3′ untranslated region (UTR) of specific target mRNA molecules, leading to the degradation of the target mRNAs or to the inhibition of their translation, or both[10,11].

Recently, several miRNAs have been found to regulate metastasis[12–17]. For example, we reported that miR-10b is highly expressed in metastatic cancer cells propagated as cell lines as well as in metastatic breast tumors from patients[12]. Its expression is induced by Twist, a transcription factor that orchestrates epithelial-mesenchymal transitions and imparts multiple traits of high-grade malignancy to carcinoma cells[18,19]. miR-10b inhibits translation of the mRNA encoding the homeobox D10 (HOXD10) protein, leading to an increased expression of *RHOC*, a well-characterized pro-metastatic gene[12]. Others reported that ectopic expression of the *BRMS1* (breast cancer metastasis suppressor-1) gene, a negative regulator of Twist expression, leads to decreased expression of miR-10b and RHOC, as well as increased expression of HOXD10 in highly metastatic breast cancer cells[20]. Notably, overexpression of miR-10b in otherwise nonmetastatic breast cancer cells confers invasive and metastatic abilities on these cells when they are growing as xenografts *in vivo*[12].

miR-10b expression levels in unfractionated bulk populations of early-stage tumors removed from breast cancer patients do not predict future metastatic relapse[21]. However, such miRNA expression analyses were carried out on the heterogeneous cell populations present within early primary tumors, in which the invasive and metastatic cells may constitute only a rare subpopulation of the total tumor mass. Activation of Twist and the resulting induction of miR-10b expression may often occur at relatively late stages of primary tumor progression.

miR-10b has indeed been found to be positively associated with high-grade malignancy. This association held true for various cancer types[22]. miR-10b is one of the most significantly upregulated miRNAs in human pancreatic adenocarcinomas[23] and glioblastomas[24], two types of highly metastatic and/or invasive cancers. miR-10b is also

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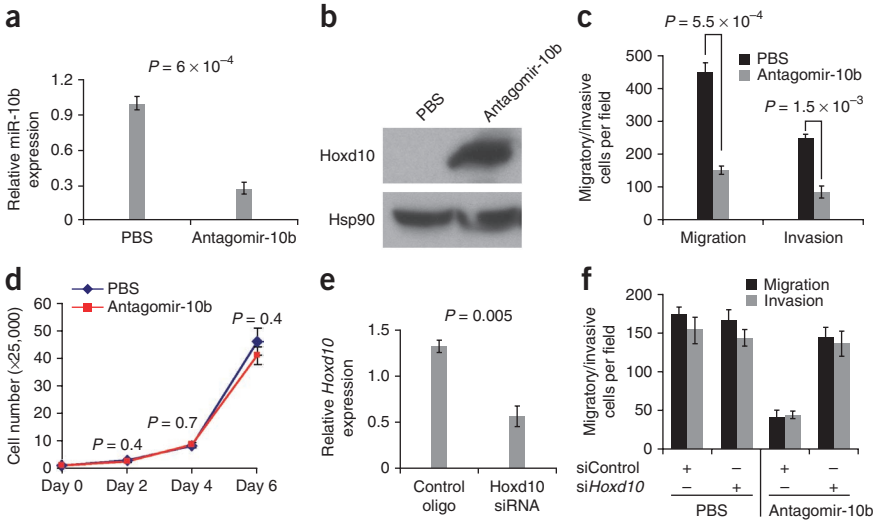

Figure 1 Antagomir-10b can be directly delivered to tumor cells *in vitro* and can inhibit cell motility and invasiveness. (**a**) Real-time RT-PCR of miR-10b in cultured 4T1 cells treated with PBS or antagomir-10b. (**b**) Immunoblotting of Hoxd10 in 4T1 cells treated with PBS or antagomir-10b. Full-length blots and molecular weight markers are presented in **Supplementary Figure 6**. (**c**) Transwell migration assay and Matrigel invasion assay of 4T1 cells treated with PBS or antagomir-10b. (**d**) Growth curves of 4T1 cells treated with PBS or antagomir-10b. (**e**) Real-time RT-PCR of *Hoxd10* in cultured 4T1 cells transfected with *Hoxd10* siRNA or control oligonucleotides. (**f**) Transwell migration assay and Matrigel invasion assay of control siRNA- or *Hoxd10* siRNA-transfected 4T1 cells that are treated with either antagomir-10b or the vehicle (PBS). A representative experiment is shown in triplicate along with s.e.m. in **a**, and **c**–**f**.

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upregulated in metastatic hepatocellular carcinomas relative to those that are not metastatic[25]. In human gliomas, miR-10b levels correlate with tumor grade and invasiveness as well as levels of RHOC[26]. This indicates that overexpressed miR-10b may play a causal role in inducing metastatic behavior, but it is unclear whether this miRNA is required for metastasis formation by cancer cells that are naturally highly malignant and, if so, whether it represents a target for the development of antimetastasis therapies—topics that are addressed in the present report.

The development of agents that are directed against miRNAs and are efficacious *in vivo* requires the delivery of these molecules at pharmacologically effective levels. Inhibition of miRNAs can be achieved by antisense oligonucleotides. *In vivo,* the pharmacokinetics and pharmacodynamics of such agents can be improved by chemical modifications designed to enhance their stability and specificity[27]. Several types of antisense-based miRNA inhibitors, including antagomirs, locked nucleic acid oligonucleotides and various types of 2′-*O*-modifed oligonucleotides have proven to be successful for silencing a liver-specific miRNA, miR-122, both in mice[28,29] and in nonhuman primates[30]. However, effective systemic delivery of miRNA antagonists to the neoplastic cells within tumors has not been documented and thus has remained an attractive but untested approach to the development of anticancer agents. Accordingly, we synthesized antagomirs to examine the effects of miR-10b silencing in a 4T1 mouse mammary carcinoma metastasis model[19,31].

RESULTS

Antagomir-mediated silencing of miR-10b in cultured tumor cells

Antagomirs are chemically engineered antisense RNA oligonucleotides against cognate miRNAs[28]. They differ from normal RNAs because of 2′-*O*-methylation of their ribose moieties, partial replacement of phosphodiester bonds by phosphorothioate linkages, and a cholesterol moiety conjugated to the 3′ end[27,28]. Intravenous injection of such antagomirs markedly reduces corresponding miRNA levels in most normal murine tissues except the brain, and the silencing effect can last for over 3 weeks following systemic administration[28]. Whereas the miRNA inhibition by other types of antisense miRNA antagonists reflects either miRNA degradation or competitive inhibition of miRNA detection[32], the decrease in miRNA level after antagomir treatment is likely to reflect miRNA degradation, based on both northern blot analysis under stringent denaturing conditions and successful detection of the degradation products[28].

We first evaluated the ability of the miR-10b antagomir (antagomir-10b) to silence its target miRNA in cultured 4T1 mouse mammary tumor cells, which exhibit high expression levels of both Twist and miR-10b[12,19]. This cell line was isolated as a subpopulation of cells from a mouse mammary tumor with high tumorigenic and metastatic ability, whereas its three isogenic relatives (67NR, 168FARN and 4TO7) are tumorigenic, but are either poorly metastatic or nonmetastatic[19,31].

We treated cultured 4T1 cells with 50 μg/ml antagomir-10b, equivalent to the concentrations used in previous *in vitro* investigations of other antagomirs[33]. Because of the antagomir-induced degradation of its cognate miRNA[27,28], we measured mature miR-10b levels in cellular extracts using a TaqMan RT-qPCR assay, which has been shown to be able to distinguish between similar miRNAs that differ by only a single nucleotide (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042142.pdf). When compared with the vehicle control, cells cultured in the presence of antagomir-10b consistently displayed an ~75% reduction in miR-10b levels (**Fig. 1a**). This coincided with a pronounced induction of the Hoxd10 protein (**Fig. 1b**), whose mRNA is targeted by miR-10b[12]. Hence, antagomir-10b could be readily delivered to cultured cells and could silence miR-10b without use of special transfection procedures.

We performed Transwell migration and Matrigel invasion assays and found that 4T1 cells treated with antagomir-10b displayed a 65–70% decrease in both motility and invasiveness *in vitro* (**Fig. 1c**). In contrast, their *in vitro* proliferation was not affected by this treatment (**Fig. 1d**). In addition, we used a small interfering RNA (siRNA) to knockdown *Hoxd10*, which reduced *Hoxd10* mRNA levels by ~60% (**Fig. 1e**). We then treated 4T1 cells transfected with either control siRNA or *Hoxd10* siRNA with either PBS buffer or antagomir-10b and found that knockdown of *Hoxd10* sufficed to reverse the loss of motility and invasiveness observed in antagomir-10b–treated cells (**Fig. 1f**). Hence, derepressed Hoxd10 expression could explain the reduced cell motility and invasiveness after antagomir-10b treatment.

Pharmacological delivery and specificity of antagomir-10b

Next, we tested the therapeutic efficacy of the miR-10b antagomir when delivered systematically in a tumor metastasis model. To do so, we implanted the 4T1 cells into the orthotopic site—the mammary fat pad—of immunocompetent, syngeneic BALB/c hosts. Consistent

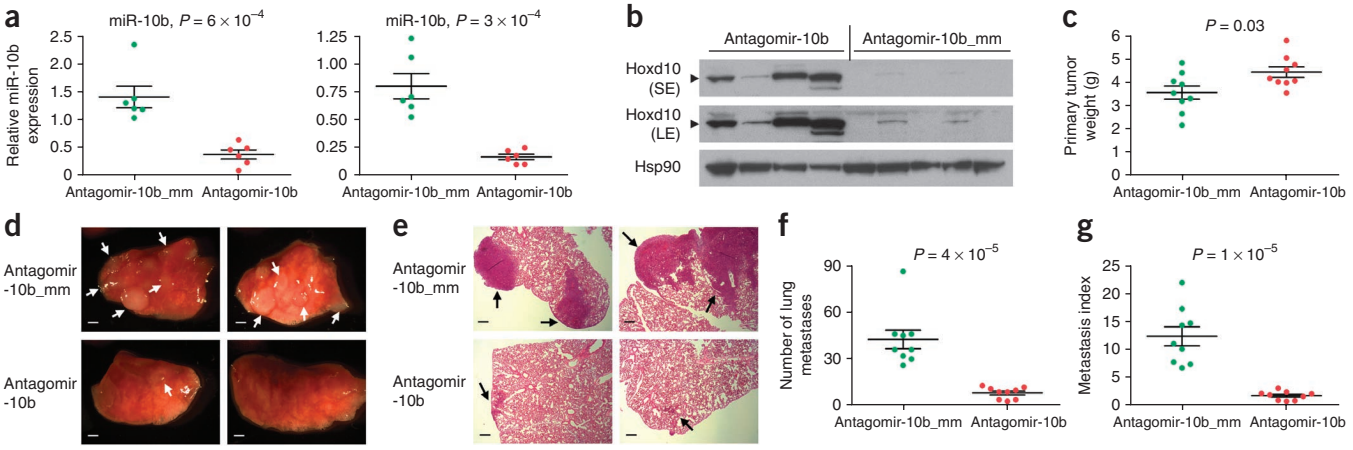

Figure 2 The metastasis-suppressing effect of antagomir-10b is sequence-specific. (**a**) Real-time RT-PCR of miR-10b in primary breast tumors (left panel) and livers (right panel) of 4T1 tumor-bearing mice treated with antagomir-10b or antagomir-10b_mm. Data are presented as mean ± s.e.m. ($n = 6$ mice in each group; each data point represents the mean expression value of triplicates of the sample from one mouse). (**b**) Immunoblotting of Hoxd10 in primary breast tumors of 4T1 tumor-bearing mice treated with antagomir-10b or antagomir-10b_mm. SE: short exposure; LE: long exposure. Full-length blots and molecular weight markers are presented in **Supplementary Figure 6**. (**c**) Primary tumor weight of 4T1 tumor-bearing mice treated with antagomir-10b or antagomir-10b_mm, at 4 weeks after orthotopic implantation. (**d**,**e**) Brightfield imaging (**d**, scale bars, 800 μm) and H&E staining (**e**, scale bars, 200 μm) of the lungs from 4T1 tumor-bearing mice treated with antagomir-10b or antagomir-10b_mm, at 4 weeks after orthotopic implantation. Arrows indicate lung metastases. (**f**,**g**) Number of visible lung metastases (**f**) and metastasis index (= metastasis number divided by primary tumor weight, **g**) in 4T1 tumor-bearing mice treated with antagomir-10b or antagomir-10b_mm, at 4 weeks after orthotopic implantation. Data in **c**, **f** and **g** are presented as mean ± s.e.m. ($n = 9$ mice per group).

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with previous reports[19,31], these cells formed primary breast tumors and metastasized to the lungs rapidly. Four weeks after tumor cell implantation, all recipients developed large primary tumors, and multiple visible metastatic nodules could be detected in the lungs with 100% incidence.

In light of the aggressiveness of these 4T1 cells, we began the antagomir treatment 2 d after cancer cell implantation, with the hope of blocking the early steps of the metastatic process, specifically invasion and intravasation. To determine the effective dosage, we referred to the previous *in vivo* testing of multiple antagomir species[27,28,34,35] and applied the following regimen (**Supplementary Fig. 1a**): twice-weekly intravenous doses of 50 mg/kg antagomir for 3 weeks, starting 2 d after tumor cell implantation. At day 28, we euthanized all mice for further analyses.

As gauged by real-time RT-PCR, miR-10b levels were markedly reduced in tissue samples from antagomir-10b–treated mice compared with those from PBS-treated mice: in the liver, which is known to be an organ most accessible to systemically introduced, small RNA-based agents, we observed a 71% reduction ($P = 3 \times 10^{-7}$) of the average miR-10b levels, whereas the average levels of miR-10b were reduced by 65% in the primary breast tumors ($P = 9.9 \times 10^{-5}$) (**Supplementary Fig. 1b**).

To exclude the possibility of nonspecific effects, we synthesized a mutant miR-10b antagomir (termed antagomir-10b_mm) that harbors 12 mismatches within the complementary sequence to miR-10b and does not match any sequence in the mouse genome. Relative to the effect of this mismatch control, the mice injected with antagomir-10b exhibited a 75% reduction of miR-10b levels in primary breast tumors ($P = 6 \times 10^{-4}$) and a 79% reduction of miR-10b levels in their liver ($P = 3 \times 10^{-4}$) (**Fig. 2a**).

Importantly, the levels of the Hoxd10 protein expressed in the primary tumors were markedly increased by antagomir-10b treatment. Unlike antagomir-10b–treated mice, which showed abundant, derepressed Hoxd10 protein expression in primary tumors (**Fig. 2b** and **Supplementary Fig. 1c**), mice treated with antagomir-10b_mm showed low to undetectable Hoxd10 expression in their primary tumors (**Fig. 2b**)—levels comparable to those observed in PBS-treated mice (**Supplementary Fig. 1c**). These results indicated that the miR-10b antagomir, which was readily taken up by the cells in rapidly growing tumors, acts in a sequence-specific manner.

Antagomirs have been shown to be able to discriminate between single-nucleotide mismatches of the targeted miRNA[27]. Accordingly, we examined the levels of other miRNAs in the antagomir-exposed tumors. These analyses indicated that the actions of antagomir-10b are highly specific. Antagomir-10b treatment did not affect the levels of the closely related miR-10a or two unrelated miRNAs, miR-9 and miR-21 (**Supplementary Fig. 2**), both of which are upregulated in clinical breast cancers[36]. Because miR-10a differs from miR-10b by only one nucleotide and is not affected by antagomir-10b treatment, it is highly unlikely that antagomir-10b has a direct effect on any other miRNAs beyond miR-10b. We cannot, however, exclude the possibility that antagomir-10b, by reducing levels of miR-10b, modulates other miRNAs through indirect mechanisms.

Effects on metastasis of systemic antagomir-10b delivery

Reflecting the *in vitro* results (**Fig. 1d**) and our previous observation that miR-10b does not affect tumor cell proliferation[12], we observed no significant difference in primary tumor size between mice treated with PBS or antagomir-10b (**Supplementary Fig. 1d**). In contrast, an 86% decrease in the number of macroscopically visible pulmonary metastases was achieved by antagomir-10b treatment ($P = 5 \times 10^{-5}$). Examination of the lungs revealed an average of 28.6 ± 3.78 visible lesions in mice injected with PBS (**Supplementary Fig. 1e**,**f**), whereas mice treated with antagomir-10b exhibited an average of 4.1 ± 1.6 macroscopic lung metastases (**Supplementary Fig. 1e**,**f**). Presented differently, antagomir-10b–treated mice exhibited an 84% lower ($P = 1 \times 10^{-4}$) metastasis index (metastasis number divided by primary tumor weight, 1.36 ± 0.45) in comparison to the PBS group (8.65 ± 1.25) (**Supplementary**

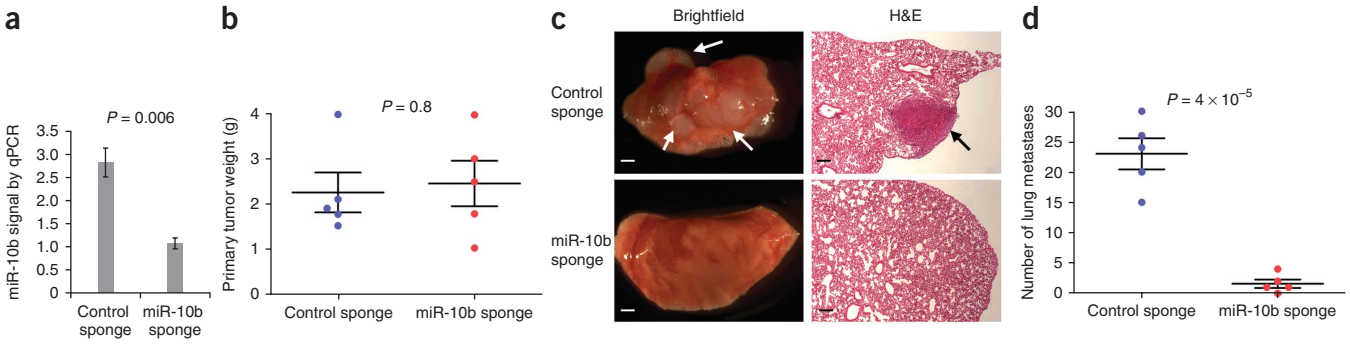

Figure 3 'Sponge'-mediated silencing of miR-10b in tumor cells is sufficient to inhibit metastasis. (**a**) Real-time RT-PCR of miR-10b in 4T1 cells infected with the miR-10b sponge or control sponge. A representative experiment is shown in triplicate along with s.e.m. (**b**) Weight of primary mouse mammary tumors formed by 4T1 cells infected with the miR-10b sponge or control sponge. (**c**,**d**) Brightfield imaging and H&E staining of the lungs (**c**) and number of visible lung metastases (**d**) in mice bearing 4T1 cells infected with the miR-10b sponge or control sponge, at 4 weeks after orthotopic implantation. Arrows indicate lung metastases. Scale bars, 800 μm for brightfield imaging; 200 μm for H&E staining. Data in **b** and **d** are presented as mean ± s.e.m. (n = 5 mice in each group).

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Fig. 1g). We anticipate that metastatic cells are the main target of the miR-10b antagomir, because miR-10b is expressed at high levels in these cells, whereas it is present at low levels in normal adult tissues. For example, its expression level in the 4T1 mammary tumor was ~15-fold higher than observed in the normal liver (**Supplementary Fig. 3**).

We also plotted the number of lung metastases versus the miR-10b level expressed in the primary breast tumor in individual recipients (**Supplementary Fig. 1h**). This analysis revealed that levels of this miRNA in primary tumors correlated significantly with pulmonary metastasis numbers in both PBS-treated and antagomir-10b–treated groups of mice (R = 0.94; **Supplementary Fig. 1h**), providing further support for the association of miR-10b with high-grade malignancy.

When using the mismatched antagomir as the control, we observed a moderate 19.7% decrease (P = 0.03) in primary tumor size in the antagomir-10b_mm group relative to the antagomir-10b group (**Fig. 2c**). In contrast, both the number and size of lung metastases were remarkably reduced in the antagomir-10b–treated mice (**Fig. 2d**,**e**). Mice exposed to antagomir-10b or antagomir-10b_mm bore an average of 8.3 and 42.6 lung metastases, respectively (81% reduction, $P = 4 \times 10^{-5}$; **Fig. 2f**); this corresponded to metastasis indices of 1.85 and 12.4, respectively (85% reduction, $P = 1 \times 10^{-5}$; **Fig. 2g**). Taken together, systemic delivery of antagomir-10b has a potent and specific metastasis-suppressing effect on these mouse breast cancer cells without having a notable effect on their ability to grow as primary tumors.

Sponge-mediated miR-10b silencing in tumor cells

To further substantiate the idea that the miR-10b antagomir prevents metastasis largely by targeting the tumor cells, rather than by targeting the host microenvironment, we used an alternative strategy to silence miR-10b in 4T1 cells—a retroviral 'miRNA sponge'[17,37]. This construct encodes a *gfp* mRNA that contains in its 3′UTR multiple tandem binding sites for miR-10b[37]. We observed a 62% reduction in miR-10b levels after sponge expression (**Fig. 3a**), which represented either miRNA degradation or competitive inhibition of detection by sustained expression of the miR-10b sponge (or a combination of both mechanisms). However, even in the latter case, the continued presence of the sponge allows it to absorb miR-10b, thereby interfering with binding of miR-10b to its natural target mRNAs.

This knockdown did not affect the size of the primary mammary tumor formed by sponge-infected cells (**Fig. 3b**). It did, however, dramatically reduce the number of lung metastases (>90% reduction, $P = 4 \times 10^{-5}$; **Fig. 3c**,**d**), suggesting that silencing of miR-10b in primary tumor cells is sufficient to inhibit metastasis.

Effects of miR-10b antagomir on late metastatic stages

To determine whether antagomir-10b has any effect on tumor cells that have already disseminated, we performed tail vein injection of 4T1 cells. This route of transplantation circumvents the initial steps of the invasion-metastasis cascade by introducing cancer cells directly into the lung microvasculature. We then treated the recipient mice with antagomir-10b until they became moribund due to lung metastases (**Fig. 4a**). In this setting, despite expected reduction of miR-10b levels in mouse tissues (66% reduction, $P = 5 \times 10^{-5}$; **Fig. 4b**), both the PBS and antagomir group of mice developed similar numbers of lung metastases (P = 0.7; **Fig. 4c**,**d**), suggesting that antagomir-10b does not affect late stages of the metastatic process, specifically the steps after extravasation of disseminated cells into the foreign tissue parenchyma.

Toxicity assessment of the miR-10b antagomir

To assess potential toxicity of antagomir-10b treatment, we exposed normal mice to the antagomir using the same dosing regimen as described in the metastasis study (**Supplementary Fig. 1a**). As anticipated, intravenous delivery of antagomir-10b reduced miR-10b levels in liver tissues by 72%, whereas antagomir-10b_mm did not modulate miR-10b levels relative to PBS (**Fig. 5a**).

All three groups of mice tolerated the procedure well and exhibited normal behaviors, as determined by activity level and grooming behaviors throughout the study. Body weights as well as lung and heart weights were unaffected by antagomir-10b treatment (**Fig. 5b** and **Supplementary Fig. 4a**,**b**). Histopathological examination of the livers revealed no steatosis, portal or lobular inflammation, necrosis, fibrosis, or biliary change in any of the three groups (**Fig. 5c**). The only notable liver change was increased Kupffer cell macrophages in the antagomir-10b_mm group; however, for unknown reasons this effect was much milder in the antagomir-10b group (**Fig. 5c**). The numbers of white blood cells and lymphocytes in the antagomir-10b group of mice showed a slight decrease and were just below the normal range, when compared to both the PBS group and the mismatched antagomir group (**Fig. 5d**,**e**).

Both compounds, antagomir-10b and antagomir-10b_mm, caused a 8–9% increase in liver and spleen size compared to PBS

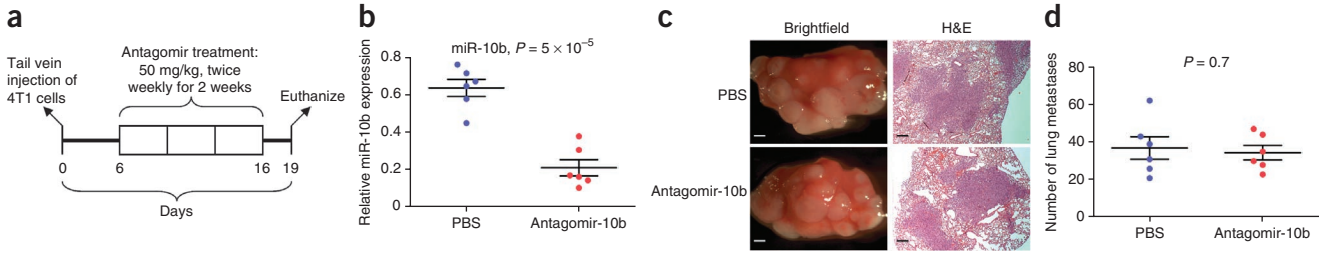

Figure 4 Antagomir-10b treatment does not affect late stages of the metastatic process. (**a**) Schematic representation of the antagomir administration schedule for mice with tail vein injection of 4T1 cells. (**b**) Real-time RT-PCR of miR-10b in livers of mice with tail vein injection of 4T1 cells and subsequent treatment with PBS or antagomir-10b. Data are presented as mean ± s.e.m. (n = 6 mice in each group; each data point represents the mean expression value of triplicates of the sample from one mouse). (**c**,**d**) Brightfield imaging and H&E staining of the lungs (**c**) and number of visible lung metastases (**d**) in PBS- or antagomir-10b–treated mice at day 19 after tail vein injection of 4T1 cells. Scale bars, 800 μm for brightfield imaging; 200 μm for H&E staining. Data in **d** are presented as mean ± s.e.m (n = 6 mice in each group).

(**Supplementary Fig. 4c**,**d**). Serum chemistry panels revealed unchanged albumin levels (**Supplementary Fig. 5a**). A slight change in several serum proteins and metabolites was observed in both compound groups, but all of them remained in the normal range. Serum levels of the alanine aminotransferase and aspartate aminotransferase liver enzymes were slightly elevated (**Supplementary Fig. 5b**,**c**), and cholesterol and blood urea nitrogen were slightly decreased (**Supplementary Fig. 5d**,**e**). Total bilirubin was the only parameter that showed a marked change, being increased by 2.3-fold upon antagomir-10b treatment (**Supplementary Fig. 5f**), but it nevertheless still remained in the normal range (0–0.9 mg/dl). All these effects were present in both wild-type and mutant antagomir groups (**Supplementary Fig. 5b–f**) and are therefore not specific to miR-10b silencing.

Taken together, when administered at the same dose and frequency as used in the metastasis study, the miR-10b antagomir showed minimal toxic effects in normal animals. The modest effects on liver and spleen size and the levels of several serum proteins and metabolites appear to be related to the chemistry of antagomirs rather than silencing of miR-10b.

DISCUSSION

Our findings demonstrate that targeting of a Twist-induced, metastasis-inducing miRNA is a viable antimetastasis strategy. The metastasis-suppressing effect of antagomir-10b on 4T1 tumor cells *in vivo* phenocopies that of a *Twist* siRNA expressed constitutively in the 4T1 cells[19], suggesting that miR-10b is a functionally important Twist target. Because Twist acts pleiotropically, it is possible that other Twist targets are also essential to the pro-metastatic functions of this transcription factor and that silencing them will also inhibit metastasis.

The actions of antagomir-10b are highly specific, leading to reduction of miR-10b, but not miR-10a or other miRNAs examined. Although systemic administration of the miR-10b antagomir leads to silencing of miR-10b in both metastatic tumor tissues and normal tissues, we reason that metastatic cells are the main target of this agent, because: (i) metastatic tumor tissues express far higher levels of miR-10b than normal tissues; (ii) when administered at the same dose and frequency as used in the metastasis study, the miR-10b antagomir has minimal effects in normal animals; (iii) specific silencing

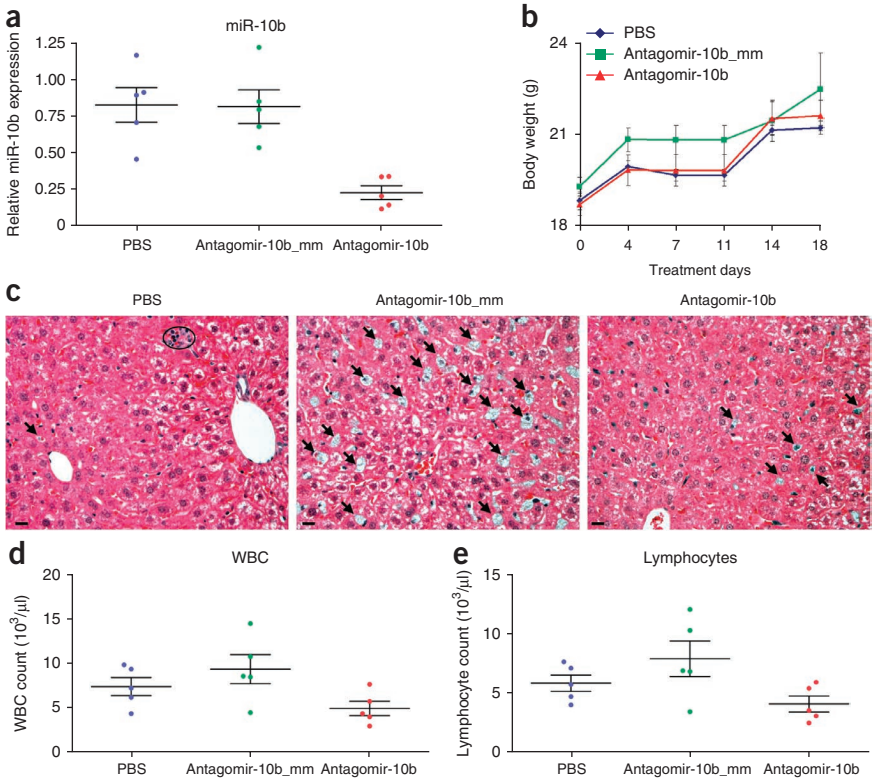

Figure 5 Toxicity assessment following intravenous delivery of antagomir-10b in normal mice. (**a**) Real-time RT-PCR of miR-10b in normal BALB/c mice after treatment with PBS or six doses of 50 mg/kg antagomir-10b or antagomir-10b_mm. Data are presented as mean ± s.e.m. (n = 6 mice in each group; each data point represents the mean expression value of triplicates of the sample from one mouse). (**b**) Total body weight was measured twice a week during the study. (**c**) H&E-stained sections of liver samples. Arrows indicate Kupffer cell macrophages. The circle indicates occasional lobular lymphocytes. Scale bars, 30 μm. (**d**,**e**) White blood cell (**d**) and lymphocyte (**e**) count. Data in **b**, **d** and **e** are presented as mean ± s.e.m. (n = 5 mice in each group).

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of miR-10b in tumor cells is sufficient to phenocopy the antimetastasis effect of systemic antagomir-10b administration.

miR-10a shares the same 'seed' sequence as miR-10b and functions as a pro-metastatic miRNA in pancreatic cancer cells[38]. However, miR-10a expression is not modulated by antagomir-10b treatment and does not compensate functionally for miR-10b loss. The genes of these two miRNAs are located on different chromosomes and their transcription is regulated independently. There is evidence that miR-10a and miR-10b have differential expression patterns, at least in breast cancer cells. For instance, miR-10b is one of the most significantly upregulated miRNAs in the 4T1 metastatic cell line compared to its nonmetastatic or poorly metastatic isogenic relatives (67NR, 168FARN and 4TO7)[39]; in contrast, miR-10a is downregulated in 4T1 and 4TO7 cells compared to 67NR and 168FARN cells[39].

Unmodified antisense oligonucleotides are degraded quickly after systemic administration and have little effect on the miRNA being targeted[28]. This illustrates the need for chemical modifications of oligonucleotides to improve their stability, resistance to RNase and pharmacologic properties. In the present study, the analyses of levels of miR-10b and Hoxd10 suggested that the antagomir effect was still sustained 7 d after the last treatment. This is noteworthy because, unlike normal tissues, these 4T1 tumor cells are actively dividing cells. Because the tissue half-life of antagomirs is 3–3.5 weeks (unpublished observations of J.S., B.B. and E.M.) and because we intravenously administered a relatively high dose of antagomirs repeatedly, it appears that by the time dosing is stopped, the tissue concentration of antagomirs has accumulated to a level well above what is necessary for pharmacological activity. In addition, the antagomir levels achieved in the general circulation could be more than sufficient to titer the targeted miRNA, even in the face of increasing numbers of tumor cells.

Taken together, the actions of the miR-10b antagomir provide a proof-of-principle that antagomirs can be efficiently delivered to rapidly growing tumor cells *in vivo* and can prevent metastasis formation by these highly malignant cells. It remains unclear precisely when cells that are capable of dissemination first arise within primary tumors. Cells with metastatic abilities may already exist at early stages of tumorigenesis[40,41]. Perhaps more commonly, they arise later in the course of multi-step tumor progression[42]. In either case, the clinical utility of inhibiting metastatic dissemination by such cells is limited at present, as dissemination may have already occurred when cancer is detected using current diagnostic methods.

With these reservations in mind, the miR-10b antagomir appears to represent a promising antimetastasis agent that does not act in a cytotoxic fashion on primary tumor cells but instead blocks their ability to launch metastases. We envision that the miR-10b antagomir is a starting point for the development of miRNA-based prophylactic therapies against future metastasis formation. Extensive analyses will be required to determine the long-term efficacy and safety of such agents in various experimental models.

Finally, the functions of miR-10b and the effects of antagomir-10b in other cancer types should be evaluated carefully. Because a single miRNA can potentially target many mRNAs and miRNA-targeted genes themselves may exert differential or even opposing effects in different cellular contexts, the functions of a particular miRNA are often tissue specific, being dependent on the expression pattern of its target mRNAs in a given cell type. In fact, several miRNAs have been reported to be capable of both promoting and suppressing tumorigenesis in a tissue type–dependent fashion[43–46]. For this reason, it remains unclear at present whether agents such as the presently described antagomir will have widespread utility in cancer treatment, or whether such agents will prove to be useful in only a limited set of the tumors encountered in the oncology clinic.

METHODS

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

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

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AUTHOR CONTRIBUTIONS

R.A.W. supervised research. L.M., J.S. and E.G.M. designed experiments. J.S., B.B. and E.G.M. provided antagomirs and conditions. L.M., F.R. and E.P. performed most of the experiments. E.G.M. led toxicity assessment at Regulus. J.T.-F. performed pathological analysis. G.W.B. contributed to graphics and statistical analysis. L.M. and R.A.W. wrote 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/naturebiotechnology/.

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ONLINE METHODS

Antagomir synthesis. Antagomirs were designed and synthesized as described previously[28].

The antagomir-10b sequence was:

5′-oCsoAsoCoAoAoAoUoUoCoGoGoUoUoCoUoAoCoAoGoGsoGsoUsoAs-Chol-3′.

The antagomir-10b_mm sequence was:

5′-oAsoCsoGoAoUoAoAoAoCoGoGoUoUoGoUoCoUoAoCoGsoUsoCsoAs-Chol-3′.

All nucleotides used in synthesis were 2′-*O*-Me-modified (lower case 'o'). Lower case 's' represents a phosphorothioate linkage. 'Chol' represents cholesterol linked through a hydroxyprolinol linkage.

Cell line. The 4T1 cell line was purchased from American Type Culture Collecction and cultured in RPMI medium supplemented with 10% FBS and 1% penicillin/streptomycin. For antagomir treatment, 4T1 cells were incubated with 50 μg/ml antagomir-10b.

RNA interference. SMARTpool Hoxd10 (Dharmacon) represents four pooled SMART-selected siRNA duplexes that target mouse *Hoxd10*. 4T1 Cells were transfected with 200 nM of the *Hoxd10* siRNA or control oligonucleotides using the Oligofectamine reagent (Invitrogen).

miRNA sponge. The miR-10b sponge was constructed using a method modified from previous reports[17,37]: annealed oligonucleotides for tandem miR-10b binding sites were ligated into the pcDNA5-CMV-d2eGFP vector (Invitrogen) digested with XhoI and ApaI. The *gfp* mRNA along with the miR-10b sponge sequence in the 3′UTR was then subcloned into the pBabe-puro vector digested with BamHI and SalI. The control sponge was described previously[17,37].

RNA isolation and miRNA quantification. Total RNA, inclusive of the small RNA fraction, was extracted from cultured cells or homogenized mouse tissues using the mirVana miRNA Isolation Kit (Ambion). Quantification of the mature form of miRNAs was performed using the TaqMan MicroRNA Assay Kit, according to the manufacturer's instructions (Applied Biosystems). The U6 small nuclear RNA was used as an internal control.

Real-time RT-PCR of mRNAs. Total RNA was reverse transcribed with an iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA was used for PCR using the SYBR-Green Master PCR Mix (Applied Biosystems) in triplicates. PCR and data collection were performed on iCycler (Bio-Rad). The expression levels of samples were determined using the standard curve method. Data were normalized to an internal control *Gapdh*. Primer sequences for mouse *Hoxd10* are: forward—AACAGATCTTGTCGAATAGAGCAAC; reverse—GGGCTGTTATTGTACTCTTGGGTTT. Primer sequences for *Gapdh* were described previously[19].

Immunoblotting. Western blot analysis was performed as described previously[12], using antibodies against Hoxd10 (1:200, Santa Cruz) and Hsp90 (1:3000, BD Biosciences).

***In vitro* growth curves.** Growth curves were determined as described previously[12].

***In vitro* migration and invasion assays.** Transwell migration assays and Matrigel invasion assays were performed as described previously[12].

Metastasis studies. Animal experiments were performed in accordance with a protocol approved by the MIT committee on Animal Care. Surgery (mammary fat pad implantation), necropsy and histological analysis were performed essentially as described[12]. Six- to eight-week-old female BALB/c mice (from Jackson Laboratory) were used for implantation of 4T1 cells (one million cells per mouse). The antagomir treatment started 2 d after orthotopic tumor cell implantation: PBS, antagomir-10b, or antagomir-10b_mm (50 mg/kg) was injected via tail vein, twice weekly for 3 weeks. All mice were euthanized 4 weeks after tumor cell implantation due to large primary tumor burdens. The mammary tumors were removed and weighed. Lung metastases were examined and counted under a dissecting microscope equipped with brightfield imaging. Tissue samples were fixed in 10% buffered formalin overnight, washed with PBS, transferred to 70% ethanol and then embedded in paraffin, sectioned and stained with hematoxylin & eosin. To assess the antagomir-10b effect on late stages of the metastatic process, we implanted BALB/c female mice with 5×10^5 4T1 cells via tail vein and started antagomir-10b treatment on day 6 post tumor cell transplantation, with the same dose and frequency as used in the orthotopic experiments; mice were moribund on day 19 owing to lung metastases and were euthanized.

Toxicity assessment. BALB/c mice, five animals per group, were dosed intravenously with PBS or 50 mg/kg of antagomir-10b or antagomir-10b_mm twice a week for 3 weeks (6 doses). Body weight was determined twice a week during the study. Animals were euthanized 24 h after the last dose and tissues were harvested. Blood was collected by retro-orbital bleeding just before euthanasia. An aliquot of whole blood was sent to Molecular Diagnostic Services, Inc., Rabbit & Rodent Diagnostic Associates (RRDA). The remainder was collected in EDTA-treated tubes. Plasma was obtained by removing blood cells through centrifugation and was run on an Olympus Bioanalyzer to determine blood chemistry values. Liver sections were examined by a pathologist (J.T.-F.) for all possible pathological conditions.

Statistical analysis. Data are presented as mean ± s.e.m. Student's *t* test (two-tailed) was used to compare two groups for independent samples, assuming equal variances on all experimental data sets. Spearman's rank correlation test was used to test correlation between a sequence of pairs of values; *R* stands for the correlation coefficient (a measure of the strength of the correlation) that varies from −1 (perfect inverse correlation) through 0 (no correlation) to +1 (perfect positive correlation).

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