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

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Abstract

MicroRNAs (miRNAs) are increasingly implicated in regulating metastasis. Despite progress in silencing miRNAs in normal tissues of rodents and non-human primates, the development of effective approaches for sequence-specific inhibition of miRNAs in fast-growing tumors remains a significant 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 oligonucleotides – suppresses breast cancer metastasis. Silencing of miR-10b both *in vitro* and *in vivo* with antagomirs significantly decreases miR-10b levels and increases 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 instead markedly suppresses formation of lung metastases. This metastasis-suppressing effect is sequence-specific. The miR-10b antagomir, which is well tolerated by normal animals, appears to be a promising candidate and a starting point for the development of new anti-metastasis agents.

Ninety percent of cancer-related mortality is caused by metastases, which result from the dissemination of primary tumor cells to distant anatomic sites1. 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 formation2. Critical regulators of the metastatic process, including proteins and microRNAs (miRNAs), are under intensive investigation at present2–4. Understanding the actions of

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AUTHOR CONTRIBUTIONS

R.A.W. supervised research. L.M., J.S., and E.M. designed experiments. J.S., B.B., and E.M. provided antagomirs and conditions. L.M., F.R., and E.P. performed most of the experiments. E.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 INTERESTS STATEMENT

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these regulatory molecules provides the basis for molecularly targeted therapeutics. Candidate anti-metastasis therapeutic approaches that target tyrosine kinase pathways, the TGF-β pathway, tumor angiogenesis, and the microenvironment, have showed efficacy in preclinical studies5. Some have been brought to clinical testing: the monoclonal anti-HER2 antibody trastuzumab, when combined with adjuvant chemotherapy, improved metastasis-free survival in women with surgically resected HER2-positive breast cancer6, 7; bevacizumab, a neutralizing antibody against vascular endothelial growth factor, showed measureable but limited benefit in prolonging the time of disease progression in patients with metastatic renal-cell cancer8 or metastatic colorectal cancer9. However, current treatment options rarely cure metastatic cancer. There is also 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, which are non-coding RNA molecules that act as negative regulators of gene expression. These small cellular RNAs bind to partially complementary sequences at the 3'UTR (3' untranslated region) of specific target mRNA molecules, leading to either degradation of target mRNAs or inhibition of their translation, or both10, 11.

Recently, several miRNAs have been found to regulate metastasis12–17. As an 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 patients12. Its expression is induced by Twist, a transcription factor that orchestrates epithelial-mesenchymal transitions and imparts multiple traits of high-grade malignancy to carcinoma cells18, 19. miR-10b inhibits translation of the mRNA encoding the homeobox D10 (HOXD10) protein, leading to increased expression of *RHOC*, a well-characterized pro-metastatic gene12. 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 cells20. Importantly, overexpression of miR-10b in otherwise-non-metastatic 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 relapse21; 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. Moreover, activation of Twist and resulting induction of miR-10b expression may often occur at relatively late stages of primary tumor progression.

miR-10b was indeed found to be positively associated with high-grade malignancy; this association held true for various cancer types22. miR-10b is one of the most significantly upregulated miRNAs in human pancreatic adenocarcinomas23 and glioblastomas24, two types of highly metastatic and/or invasive cancers. miR-10b is also upregulated in metastatic hepatocellular carcinomas (HCCs) relative to non-metastatic HCCs25. In human gliomas, miR-10b levels correlate with tumor grade and invasiveness as well as levels of RHOC26.

While indicating that overexpressed miR-10b may play a causal role in inducing metastatic behavior, 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 novel anti-metastasis 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; when acting *in vivo*, the pharmacokinetics and pharmacodynamics of such agents can be improved by chemical modifications designed to enhance their stability and specificity27. Several types of antisense-based miRNA inhibitors, including antagomirs, LNA (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 mice28, 29 and in non-human primates30. 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 novel anti-cancer agents. Accordingly, we synthesized antagomirs to examine the effects of miR-10b silencing in a 4T1 mouse mammary carcinoma metastasis model19, 31.

RESULTS

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

Antagomirs are chemically engineered antisense RNA oligonucleotides against cognate miRNAs28. 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' end27, 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 three weeks following systemic administration28. While the detection of miRNAs has been confounded in other settings by interference caused by other types of antisense miRNA antagonists32, the miRNA decrease 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 products28.

We first evaluated the ability of the miR-10b antagomir (antagomir-10b) to silence this miRNA in cultured 4T1 mouse mammary tumor cells, which exhibit high expression levels of both Twist and miR-10b12, 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 non-metastatic or poorly so19, 31.

We treated cultured 4T1 cells with 50 μg/ml antagomir-10b, this concentration being equivalent to those used in previous *in vitro* investigations of other antagomirs33. Because of the antagomir-induced degradation of its cognate miRNA27, 28, we measured mature miR-10b levels in cellular extracts using a TaqMan RT-qPCR assay, which has proven 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 approximately 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-10b12. 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 antagomir-10b-treated 4T1 cells 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 knock down *Hoxd10*, which reduced *Hoxd10* mRNA levels by approximately 60% (Fig. 1e). We then treated control siRNA- or *Hoxd10* siRNA-transfected 4T1 cells with either control 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 following antagomir-10b treatment.

Pharmacological delivery and specificity of the miR-10b antagomir

We wished to test 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 with previous reports19, 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 two days 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 species27, 28, 34, 35. For example, efficient miR-122 silencing *in vivo* by antagomir-122 was achieved by three doses of 40–80 mg/kg27. Accordingly, we undertook the following dosage regimen (Supplementary Fig. 1a): twice-weekly intravenous doses of 50 mg/kg antagomir for three weeks, started two days after tumor cell implantation. At day 28, we euthanized all mice and undertook several 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 average miR-10b levels, while the average levels of miR-10b were reduced by 65% ($P = 9.9 \times 10^{-5}$) in the primary breast tumors (Supplementary Fig. 1b).

To exclude the possibility of non-specific effects, we synthesized a mutant miR-10b antagomir (termed antagomir-10b_mm) that harbors 12 mismatches with the complementary sequence of miR-10b and does not match any sequence in the mouse genome. Relative to the actions of this mismatch control, the mice injected with antagomir-10b exhibited a 75% reduction ($P = 6 \times 10^{-4}$) of miR-10b levels in primary breast tumors and a 79% reduction ($P = 3 \times 10^{-4}$) of miR-10b levels in their liver (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 responses indicated that the miR-10b antagomir, which could be 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 miRNA27. 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: as shown in Supplementary Fig. 2, antagomir-10b treatment did not affect the levels of the closely related miR-10a or two unrelated miRNAs, miR-9 and miR-21, both of which are upregulated in clinical breast cancers36. 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 administration of the miR-10b antagomir

Reflecting the *in vitro* results (Fig. 1d) and our previous observation that miR-10b does not affect tumor cell proliferation12, we observed no significant difference in primary tumor size borne by the mice treated as controls with PBS and those tumors of mice treated with antagomir-10b (Supplementary Fig. 1d). In stark contrast, an 86% decrease ($P = 5 \times 10^{-5}$) in the number of macroscopically visible pulmonary metastases was achieved by antagomir-10b treatment: 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% decrease ($P = 1 \times 10^{-4}$) in the metastasis index (metastasis number divided by primary tumor weight, 1.36±0.45) in comparison to the PBS group (8.65±1.25) (Supplementary 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, while being present at low levels in normal adult tissues. For example, its expression level in the 4T1 mammary tumor was approximately 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.

Use of a miRNA sponge to specifically silence miR-10b in primary 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-10b37. We observed a 62% reduction in miR-10b detection following 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, just as it masks this miRNA from detection.

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 the miR-10b antagomir on late stages of the metastatic process

In order 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 PBS group 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 following 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 miR-10b 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%, while 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 an 8%–9% increase in liver and spleen size compared to PBS (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 (ALT) and aspartate aminotransferase (AST) liver enzymes were slightly elevated (Supplementary Fig. 5b,c), while cholesterol and blood urea nitrogen (BUN) 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 anti-metastasis therapy is possible via targeting of a Twist-induced, metastasis-promoting miRNA. The metastasis-suppressing effect of antagomir-10b on 4T1 tumor cells *in vivo* phenocopies that of a *Twist* siRNA expressed constitutively in the 4T1 cells19, suggesting that miR-10b is a functionally important Twist target. Since Twist is a pleiotropically acting transcription factor, it remains possible that silencing of another Twist target might also inhibit metastasis. We cannot exclude that other Twist-inducible

genes, as is the case with miR-10b, are also essential to the pro-metastatic functions of this transcription factor.

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: 1) metastatic tumor tissues express far higher levels of miR-10b than normal tissues; 2) when administered at the same dose and frequency as used in the metastasis study, the miR-10b antagomir has minimal effects in normal animals.

miR-10a shares the same 'seed' sequence as miR-10b and functions as a pro-metastatic miRNA in pancreatic cancer cells38. 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 non-metastatic 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 cells39.

Unmodified antisense oligonucleotides are degraded quickly after systemic administration and have little effect on the miRNA being targeted28. This illustrates the need for chemical modifications of oligonucleotides in order 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 seven days after the last treatment. This is striking because, unlike normal tissues, these 4T1 tumor cells are actively dividing cells. Since the tissue half-life of antagomirs is 3–3.5 weeks (unpublished observations of J.S., B.B. and E.M.), and since 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. Such cells with metastatic powers may already exist at early stages of tumorigenesis40, 41. Perhaps more commonly, they arise later in the course of multi-step tumor progression42. In either case, the clinical utility of inhibiting metastatic dissemination by such cells is limited at present, since 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 anti-metastasis 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. Since a single miRNA can potentially target many mRNAs, and since 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 fashion43–46. 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 are available in the online version of the paper.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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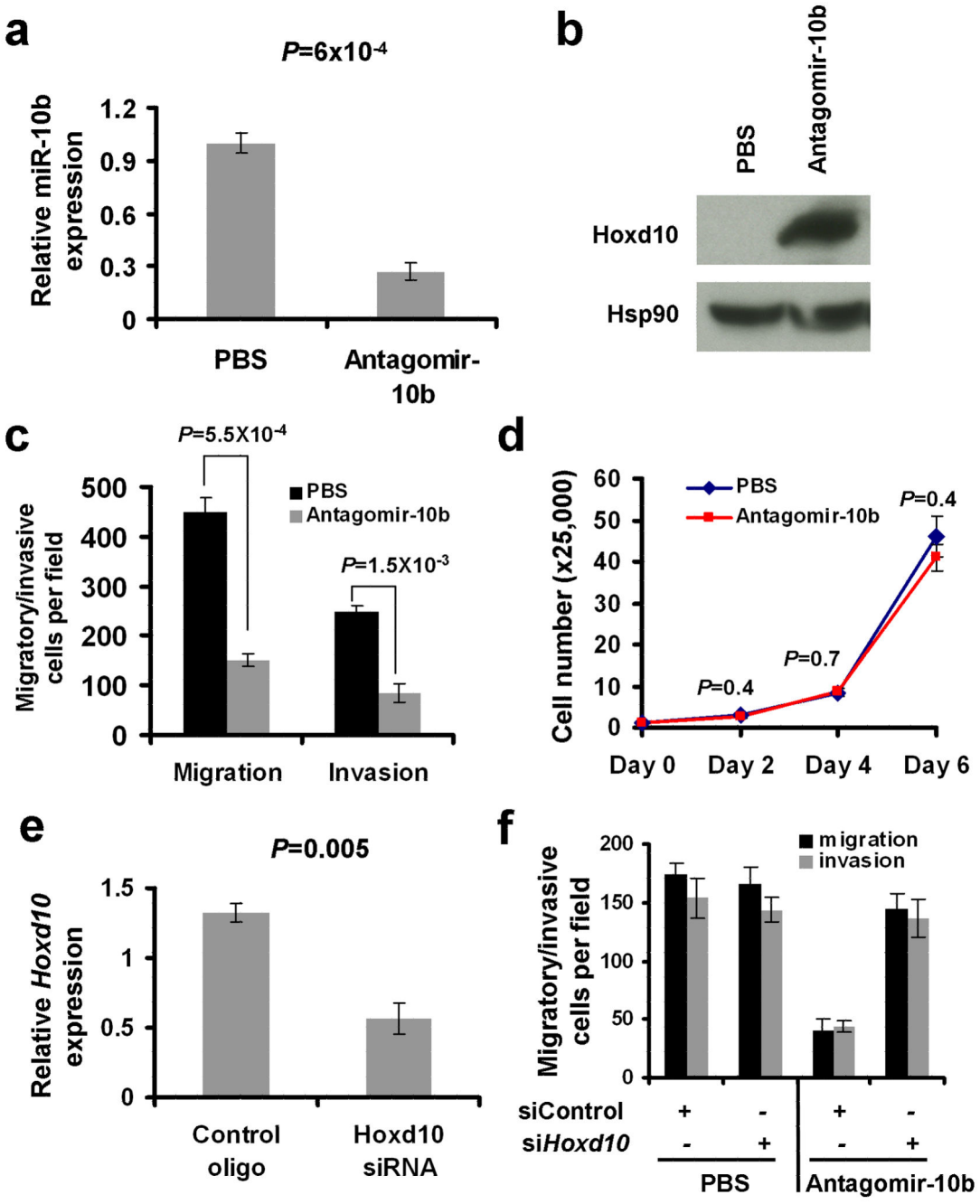

Figure 1.

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**.

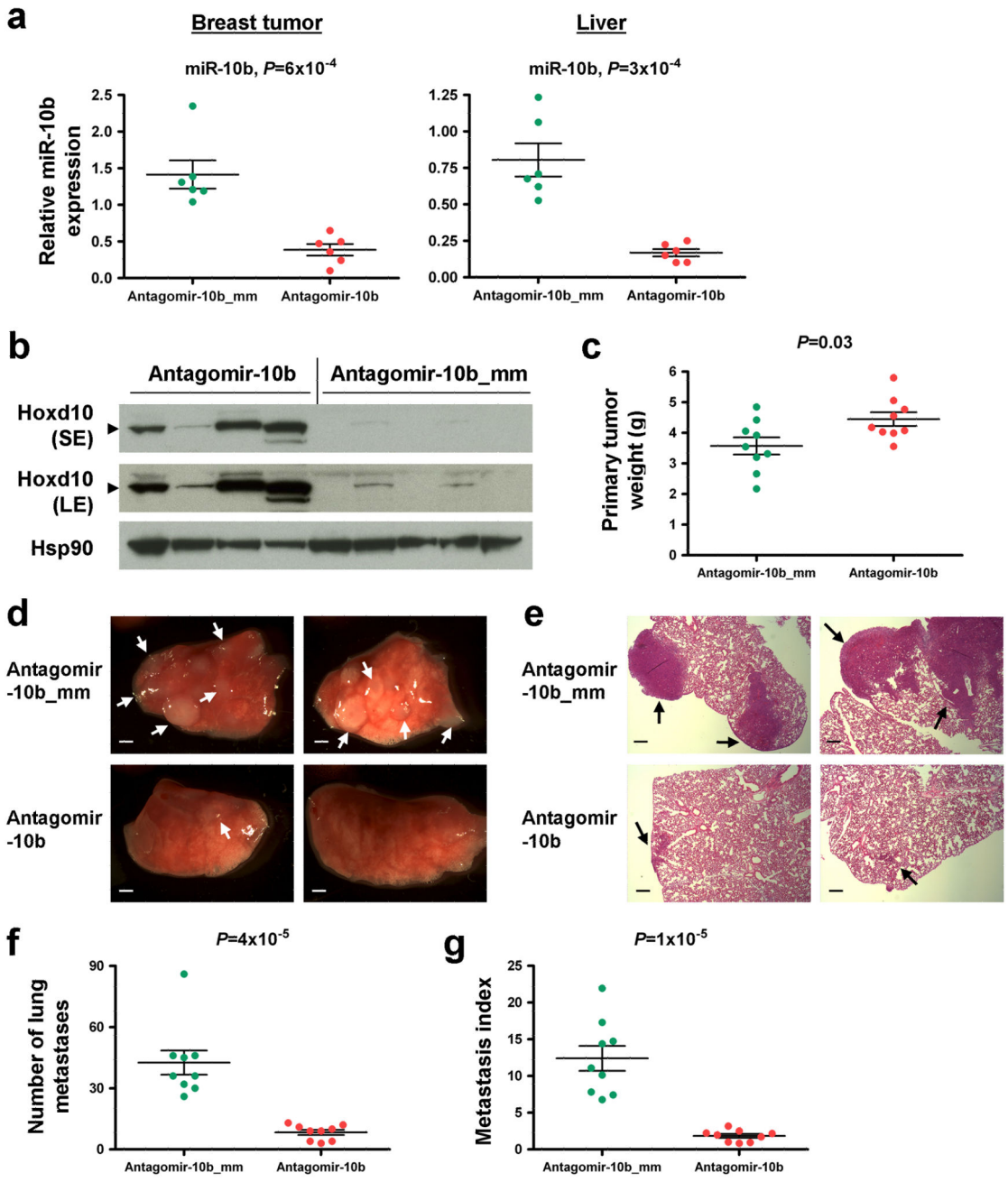

Figure 2.

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**) Bright field 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).

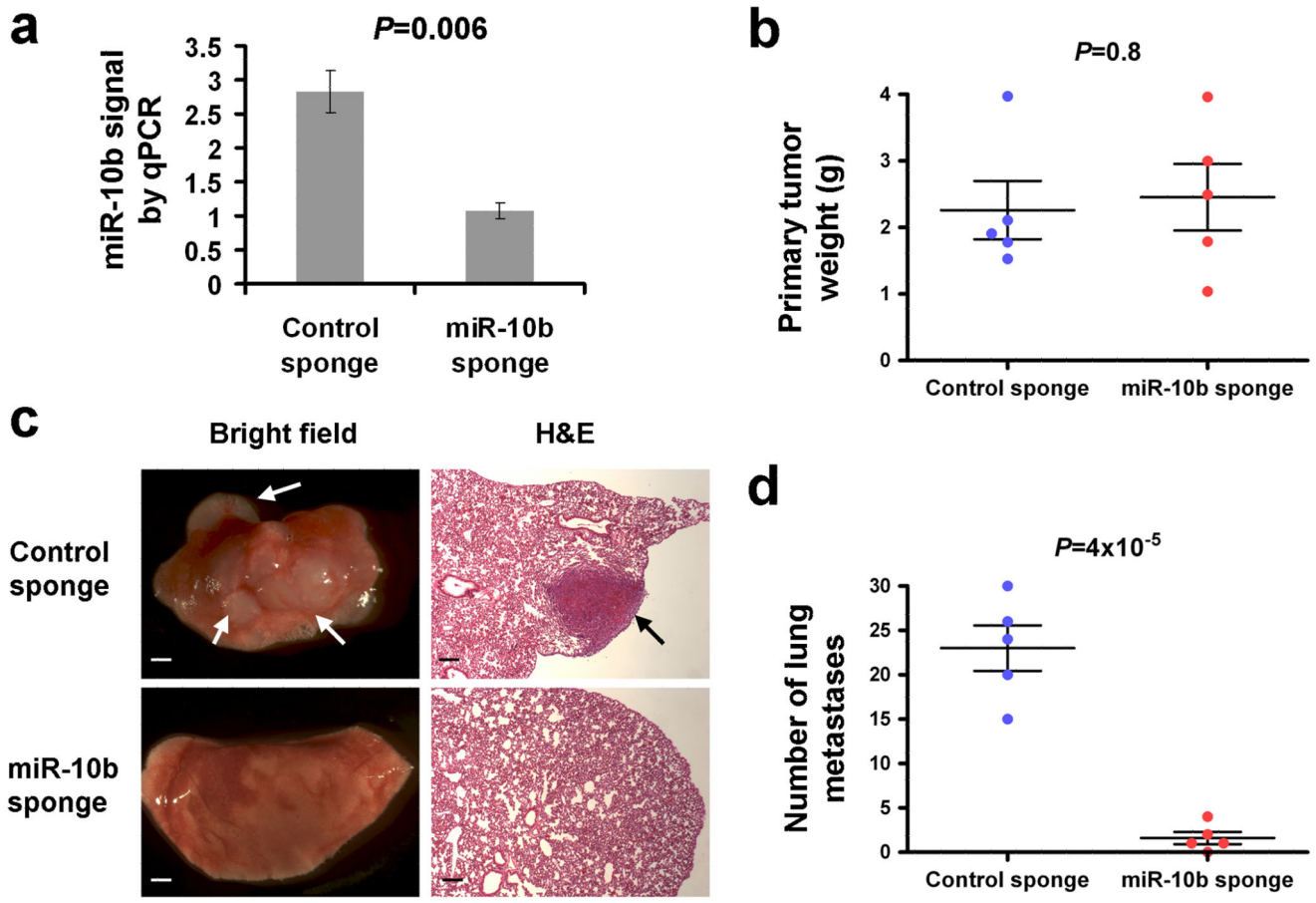

Figure 3.

'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**) Bright field 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 bright field 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).

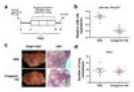

Figure 4.

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**) Bright field 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 bright field imaging; 200 μm for H&E staining. Data in **d** are presented as mean ± s.e.m. (n = 6 mice in each group).

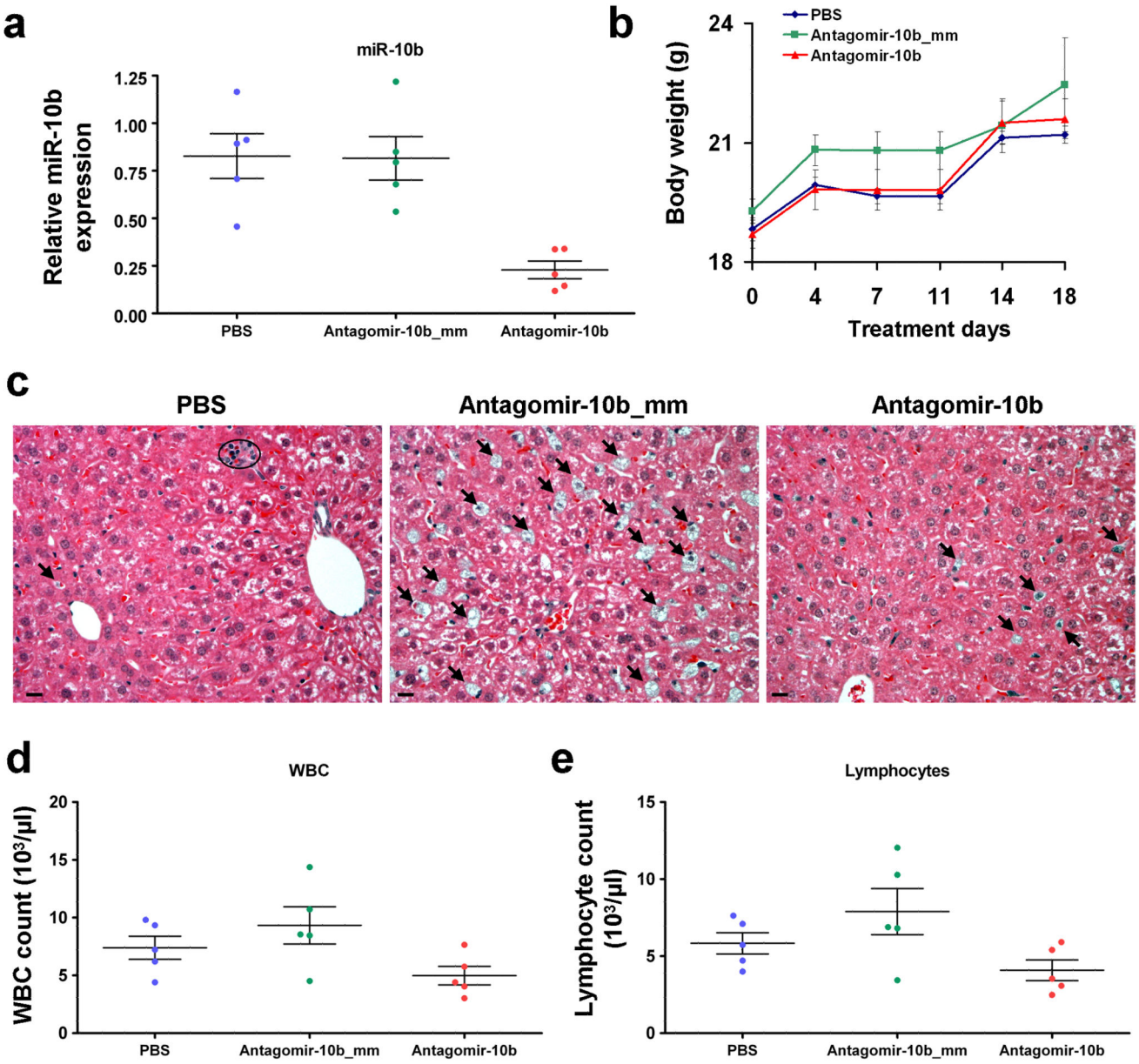

Figure 5.

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).