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ABSTRACT: The presence of dormant, microscopic cancerous lesions poses a major obstacle for the treatment of metastatic and recurrent cancers. While it is well-established that microRNAs play a major role in tumorigenesis, their involvement in tumor dormancy has yet to be fully elucidated. We established and comprehensively characterized pairs of dormant and fast-growing human osteosarcoma models. Using these pairs of mouse tumor models, we identified three novel regulators of osteosarcoma dormancy: miR-34a, miR-93, and miR-200c. This report shows that loss of these microRNAs occurs during the switch from dormant avascular into fast-growing angiogenic phenotype. We validated their downregulation in patients’ tumor samples compared to normal bone, making them attractive candidates for osteosarcoma therapy. Successful delivery of microRNAs is a challenge; hence, we synthesized an aminated polyglycerol dendritic nanocarrier, dPG-NH2, and designed dPG-NH2-microRNA polyplexes to target cancer. Reconstitution of these microRNAs using dPG-NH2 polyplexes into Saos-2 and MG-63 cells, which generate fast-growing osteosarcomas, reduced the levels of their target genes, MET proto-oncogene, hypoxia-inducible factor 1α, and moesin, critical to cancer angiogenesis and cancer cells’ migration. We further demonstrate that these microRNAs attenuate the angiogenic capabilities of fast-growing osteosarcomas in vitro and in vivo. Treatment with each of these microRNAs using dPG-NH2 significantly prolonged the dormancy period of fast-growing osteosarcomas in vivo. Taken together, these findings suggest that nanocarrier-mediated delivery of microRNAs involved in osteosarcoma tumor–host interactions can induce a dormant-like state.

KEYWORDS: microRNA, polymeric nanomedicine, hyperbranched polymer, dendrimer, polyplex, tumor dormancy, osteosarcoma

Tumor dormancy, a state in which tumors exist without expanding in mass beyond 1–3 mm, has important implications for the early detection and treatment of cancer. Dormant tumors are highly prevalent in the general
population. In 1993, a study by Black and Welch demonstrated that the prevalence of dormant tumors in autopsies of otherwise healthy individuals ranged from 40 to 100%, depending on the cancer type. However, dormant tumors can be present not only as one of the early stages of tumor development, but also as micrometastases and as recurrent disease following treatment. Although great efforts have been made to shed light on the tumor dormancy phenomenon, its underlying molecular and cellular mechanisms remain unclear.

Several mechanisms have been proposed for the escape of tumors from dormancy including immunoescape, an exit from cell cycle arrest and the induction of angiogenesis. Failure of a tumor to undergo the "angiogenic switch" and recruit new vasculature results in a nonangiogenic and nonprogressing dormant tumor.

Osteosarcoma is the most common primary bone cancer and the sixth leading cancer in children under the age of 15. The current standard of care for osteosarcoma patients is surgery, neoadjuvant and adjuvant chemotherapy, which results in a 5-year survival rate of 60–80%. However, many of these patients will suffer from recurrent disease, either due to minimal residual disease or the presence of dormant micrometastatic lesions at the time of diagnosis. Understanding the early stages by which osteosarcomas develop from dormant occult into large vascularized tumors could facilitate the development of new diagnostic and therapeutic tools for this malignancy.

Among the emerging players in regulation of physiological and pathological processes are microRNAs (miRNAs), small non-coding RNA molecules which regulate gene expression at the post-transcriptional level. Despite the great advancements in molecularly targeted therapies in the last two decades, resistance to these medications often arises due to the activation of compensatory mechanisms that maintain the function of alternative targets. miRNAs have the potential to overcome these compensatory mechanisms, as a single miRNA can regulate entire networks of genes. Numerous studies have pointed out miRNAs as potential therapeutic agents and targets for cancer. Therefore, we hypothesized that specific miRNAs may have a role in the switch from dormancy to progressive disease. We further postulated that unravelling novel dormancy associated miRNAs will potentially lead to the development of novel diagnostic and therapeutic tools for malignancies. Several studies have demonstrated dysregulation of miRNAs in osteosarcoma tumorigenesis, metastasis and resistance to chemotherapy. Among these miRNAs are known regulators of other malignancies, including oncomiR-21, miR-34a, and miR-16. Two recent studies demonstrated the involvement of specific miRNAs in tumor dormancy. Nonetheless, additional research investigating the molecular mechanisms governing osteosarcoma dormancy is necessary, in order to translate these insights into therapeutic applications. Furthermore, the potential therapeutic properties of nonself oligonucleotides, e.g., miRNAs, suffer from...
limitations such as degradability in plasma, immunogenicity, lack of membrane-crossing abilities of the oligonucleotides, and inability to escape from the endosome in order to reach their cytoplasmic targets.

To overcome these challenges, we have developed dendritic polyglycerolamine (dPG-NH₂), a novel polyglycerol-based nanocarrier to deliver oligonucleotides to tumors in vivo. This cationic hyperbranched polymer facilitates an efficient delivery of oligonucleotides by improving their stability, intracellular trafficking, silencing efficacy and accumulation at the tumor site due to the enhanced permeability and retention (EPR) effect. Furthermore, this novel nanocarrier demonstrated low cytotoxicity, an efficient delivery of active siRNA into cells and a successful silencing of a luciferase gene, ectopically overexpressed in a human glioblastoma cell line. Here, we exploited dPG-NH₂-miRNA polyplexes to deliver the selected miRNAs for osteosarcoma therapy. This study identifies, validates, and evaluates the therapeutic potential of three distinct miRNAs, miR-34a, miR-93, and miR-200c, when polyplexed to dPG-NH₂ nanocarrier in order to regulate osteosarcoma dormancy and progression.

RESULTS

Osteosarcoma Cells Generating Dormant Tumors Display Impaired Angiogenic Potential in Vivo. Using Saos-2 human osteosarcoma cells line, defined as non-tumorigenic by the ATCC, we have developed a model of osteosarcoma dormancy. When inoculated subcutaneously (sc) into mice, Saos-2 cells generate small masses that remain dormant (Saos-2-D for “dormant”) for a prolonged period of time until they spontaneously escape by adopting an angiogenic phenotype and grow in mass. From these “escaped” tumors, we isolated a new tumor cell line, which upon reinoculation into mice, rapidly recruits blood vessels and forms established vascularized tumors (Saos-2-E for “escape”) (Figure 1A). To explore the mechanisms underlying the switch from dormancy, we employed this pair of cell lines and an additional pair of nontumorigenic MG-63 human osteosarcoma cells and its
counterpart MG-63-E transfected with Ras, which generates fast-growing tumors.

Three weeks following tumor cell inoculation, when tumor volumes were 1–3 mm³, Saos-2-D tumors recruited new blood vessels, while Saos-2-E tumors showed no evidence for angiogenesis, as shown by flipping the skin of tumor-bearing mice (Figure 1B). CD31 staining showed that microvessels density and the number of vessels with lumen (i.e., functional vessels) of 1–3 mm³ Saos-2-E tumors were significantly higher compared with those of size-matched Saos-2-D tumors (Figure 1C,D). Despite some CD31-positive staining in Saos-2-D tumors, the lack of functional vessels prevented the oxygen and nutritional supply necessary for tumor growth and therefore, these tumors remained harmless to the host and asymptomatic. In contrast, vessels with lumens formed in Saos-2-E tumors were functional and, therefore, supported rapid tumor growth. Moreover, Saos-2-D and Saos-2-E tumors both contained proliferating cells, but almost no apoptotic cells (Figure 1C).

This finding rules out the possibility of “cellular dormancy” as the underlying cause for the in vivo dormant phenotype.21 Support to the increased in vivo angiogenic potential of Saos-2-E cells and to the differences in blood vessels’ functionality between the pair of cells was found in the matrigel plug angiogenesis assay.22 Three weeks following inoculation of the matrigel/cell mixture, plugs were assessed for neovascularization. The ability of Saos-2-E cells to recruit functional vessels into the plug was significantly increased compared to Saos-2-D cells, as demonstrated by the high present of contrast agent and hemoglobin concentration within plugs containing Saos-2-E cells (Figure 1E,F).

**Saos-2 Dormancy Model Imitates the Clinical Setting of Osteosarcoma.** To rule-out the possibility that the pattern of differential tumor growth in this model is a product of artificial subcutaneous inoculation, Saos-2 cells were inoculated orthotopically into the tibia and systemically through the tail vein for metastases establishment. Saos-2-E tumors escaped 60 days following orthotopic inoculation, whereas Saos-2-D
tumors remained dormant for 140 days (Figure 2A). These growth patterns were similar to those resulting from subcutaneous inoculation. However, while no differences were demonstrated in growth patterns of Saos-2-E tumors, the escape of Saos-2-D tumors following orthotopic inoculation was significantly faster (140 days) than following subcutaneous inoculation (240 days). This supports our hypothesis and the general notion that orthotopic tumors, while inoculated in the appropriate microenvironment, gain increased support from their stroma compared to subcutaneously inoculated tumors. It is possible that this increased stromal support affected Saos-2-D tumors to a greater extent, since they require more time to “educate” and “reprogram” the microenvironment and are less self-sufficient compared to Saos-2-E tumors. In addition, while the subcutaneous microenvironment is relatively poorly vascularized, the tibia has a rich blood supply with sizable feeding vessels. Therefore, when inoculated into the tibia, Saos-2-D cells are able to recruit blood vessels and to form large angiogenic tumors more rapidly than in the subcutaneous tissue. Micro-CT imaging performed 100 days after inoculation showed that Saos-2-D tumors had a significantly larger volume than Saos-2-E tumors (Figure 4).
following inoculation showed areas of calcification inside and outside the tibial shaft in tibias inoculated with Saos-2-E cells, a phenomenon typical to osteoid-forming osteosarcoma (Figure 2B). Moreover, at this time point, mice bearing Saos-2-E tumors developed calcified pulmonary metastases, the most common manifestation of metastatic osteosarcoma (Figure 2B). Similar clinical manifestations were observed following escape of Saos-2-D tumors (Figure S1A). Histological analysis 100 days post inoculation revealed that tibias inoculated with Saos-2-D cells remained intact and showed presence of dormant microscopic lesions that did not metastasize to the lungs. Conversely, Saos-2-E tumors invaded and destroyed the cortical bone and spread into the lungs (Figure 2C).

Similar growth kinetics of dormant and fast-growing tumors was maintained in the experimental lung metastasis model. Fifty days following systemic intravenous injection of Saos-2-E cells, rapid weight loss and overt metastatic colonization were observed. In contrast, mice injected with Saos-2-D cells experienced no weight loss and had no apparent lung colonies. Only 200 days following inoculation, dormant lung colonies escaped and became symptomatic (Figure 2D,E, Figure S1B). Moreover, at this time point, mice bearing Saos-2-E tumors showed small tumor foci, multiple tumor colonies were observed in lungs of mice inoculated with Saos-2-E cells (Figure 2F).

**Osteosarcoma Cells Generating Dormant or Fast-Growing Tumors Have Similar Growth, Invasion, Migration, and DNA Replication Kinetics.** We next evaluated the *in vitro* tumorigenic potential of Saos-2-D and Saos-2-E cells. We found no significant differences in their proliferation rates (Figure 3A). The ability to invade through matrigel or endothelial monolayer and migrate toward serum between adjacent origins (Figure 3B,C). However, differences observed in both cell lines (Figure 3B,C). This suggests that Saos-2-E cells are more capable of overcoming stress conditions, like hypoxia and nutrient deprivation, which arise during tumor development. To further evaluate the response of Saos-2-D cells to hypoxia, changes in hypoxia inducible factor α (HIF1α) and its downstream target vascular endothelial growth factor (VEGF) following hypoxia were analyzed by qRT-PCR. While in Saos-2-D cells HIF1α levels were reduced and VEGF levels were not changed following hypoxia, a significant increase in their levels was observed in Saos-2-E cells (Figure 3D). Therefore, Saos-2-D cells have an improved capacity to respond to hypoxic conditions in the tumor microenvironment, a trait that might explain the above-mentioned increased angiogenic potential of Saos-2-E tumors at a microscopic size.

In recent years, a number of studies have shown that in early stages of cancer development, cells are proliferating but fail to support normal DNA replication. Hence, the cells are undergoing replication stress leading to genomic instability and tumorigenicity.26,27 To test whether the difference in the tumorigenic potential between Saos-2-D and Saos-2-E cells might result from differences in their replication dynamics, we used the DNA combing technique which enables to analyze the replication of single DNA molecules.28 In two independent experiments, there was no significant change either in the replication rate of the cells (Figure 3E,F), or in the distance between adjacent origins (Figure 3G). Therefore, the differences observed *in vivo* cannot be attributed to differences in DNA replication dynamics.

**Dormancy-Associated miRNA Are Downregulated in Human Osteosarcoma Specimens.** To explore the basic mechanisms that govern the switch from a dormant to a fast-growing phenotype, Saos-2-D and Saos-2-E cells were analyzed for their miRNA profile (Figure 4A, Figure S2 and Table S1). Our focus was on miRNAs that were downregulated in fast-growing versus dormant-tumor-forming cells (Figure 4A). Out of those miRNAs, miR-200c, miR-34a, and miR-93 (representing a mild, intermediate, or substantial differential expression, respectively) were selected for further evaluation. Expression levels of these miRNAs were further evaluated in size-matched (1–3 mm³) tumors, showing that miR-34a, miR-93, and miR-200c are upregulated in Saos-2-D tumors compared to Saos-2-E tumors also in the *in vivo* setting (Figure 4B).

Next, we set to validate the relevance of dormancy-associated miRNAs in osteosarcoma clinical specimens. Due to the clinical inaccessibility of dormant tumors, miRNA levels in primary and metastatic osteosarcoma specimens were compared with those of normal bones. qRT-PCR analysis for miRNA levels demonstrated that although expression levels of these three miRNAs vary between patients (Figure 4C, single patients displayed), there is an overall clear trend of miRNA downregulation in primary and metastatic osteosarcoma specimens (Figure 4C, Averages). To note, all samples in our study were of conventional osteosarcoma, defined by the College of American Pathologists as high grade tumors.29 Therefore, the observed variation in miRNA levels most likely does not depend upon tumor grade. To evaluate whether the expression signature correlates with tumor stage, which reflects tumor progression state, we compared the average miRNA levels of primary tumors (defined as stage II–III) and metastatic tumors (defined as stage IV). While no significant differences were found in miR-34a and miR-93 expression in primary versus metastatic samples, miR-200c was significantly higher in the metastatic specimens, suggesting its role in the metastatic spread of osteosarcoma (Figure 4C, Averages). Interestingly, miR-200c, with the mildest differential expression, showed the most remarkable downregulation in tumor samples compared to normal bone.

Having identified miR-200c as the miRNA which down-regulation was the most significant, its expression level was further evaluated using anti-miR-200c *in situ* hybridization (ISH) probe. In accordance with the qRT-PCR results, normal bone samples from clinical specimens expressed higher levels of miR-200c compared with primary and metastatic osteosarcoma specimens (Figure 4D). To test whether a similar miRNA expression pattern is found in Saos-2 metastases versus primary tumors, we isolated four new cell lines from spontaneous lung metastases generated from Saos-2-E tumors following intratracheal inoculation (see Figure 2B). When comparing miR-200c levels in Saos-2 cells to their level in hFOB human osteoblasts, *i.e.*, normal bone cells, a correlation to miR-200c levels in human specimens was found. miR-200c was downregulated compared to normal human osteoblasts in all types of Saos-2 cells (Figure 4E), but its level was higher in metastatic Saos-2-E (Saos-2-E-M) cells compared to Saos-2-E cells, similar to the trend seen in human specimens.

**miR-93 and miR-200c Revert Cells Generating Fast-Growing Tumors into a Dormant Nonangiogenic Phenotype.** Having shown that cells generating dormant and fast-growing tumors differ mainly in their ability to respond
to the tumor microenvironment in vivo, we set to evaluate the role of the suspected dormancy-associated miRNAs in osteosarcoma tumor growth and angiogenesis in vivo. miR-93 and miR-200c, representing either a high or a low differential expression, respectively, were constitutively overexpressed by retroviral infection in Saos-2-E cells, and phenotypic changes were monitored ex vivo and in vivo.

Overexpression of miR-93 and miR-200c prolonged the dormancy period of Saos-2-E tumors in immunodeficient mice. Saos-2-E and Saos-2-E control miR-infected cells established large angiogenic tumors within 70 days, whereas Saos-2-E miR-200c, Saos-2-E miR-93, and Saos-2-D tumors remained dormant for 120, 140, and 220 days, respectively (Figure 5A). These changes in the dormancy period correlate with the microarray results, as miR-200c, with the low differential expression, “escaped” faster from dormancy than miR-93 with the higher differential expression. Furthermore, the median survival of mice bearing Saos-2-E tumors (120 days) was significantly extended following overexpression of miR-200c or miR-93 (~210 days) (Figure 5B). During these dormancy periods, cells were detectable by noninvasive intravital CRI Maestro imaging system (Figure 5C). To note, reconstitution of miRNAs downregulated in Saos-2-D cells (i.e., miR-137, miR-147, and miR-205) did not affect the dormancy periods of these cells, defined as “Time to switch” (Figure S2). This suggests that the switch from dormancy in our model is predominantly governed by loss of specific miRNAs.

To evaluate the angiogenic activity of Saos-2-E tumors following miRNA overexpression, we used an ex vivo analysis of circulating endothelial progenitor cells (CEPs). CEPs, recruited from the bone marrow during tumor neovascularization, can be utilized as surrogate markers for angiogenic activity. Since viable CEPs contribute mainly to the early steps of tumor vascularization, we evaluated their level 14 days post-inoculation. While there was no difference in viable CEPs levels between nontumor bearing mice and mice bearing Saos-2-D tumors, their level was significantly higher in mice bearing Saos-2-E tumors, suggesting that fast-growing tumors are associated with increased systemic angiogenic activity (Figure 5D). In addition, introduction of miR-93 and miR-200c to Saos-2-E cells reverted the high levels of viable CEPs found in blood of mice bearing Saos-2-E tumors. This suggests that at least in the context of systemic angiogenesis, fast-growing tumors overexpressing miR-93 and miR-200c became similar to...
dormant tumors (Figure 5D). Immunostaining for microvessels in microscopic size-matched tumors showed that Saos-2-E tumors overexpressing miR-93 and miR-200c were almost avascular compared with Saos-2-E control miR tumors (Figure 5E).

Synthesis and Physicochemical Characterization of dPG-NH₂-miRNA Polyplexes. Retroviral overexpressing of selected miRNAs into Saos-2 cells generating fast-growing tumors showed that dormancy-associated miRNAs are good candidate for osteosarcoma therapy. To further explore their therapeutic potential, we decided to exploit the miRNA replacement approach, to reconstitute miRNAs under-expressed in fast-growing osteosarcomas using miRNA mimics. For this purpose, polyglycerol-amine (dPG-NH₂) (Figure 6A) was synthesized as previously described.²⁰ dPG-NH₂, synthesized for this study, bears 175 amines/mol polymer, thus enabling electrostatic complexation with the negatively charged miRNA (Figure 6B).

The ability of dPG-NH₂ to complex miRNAs was evaluated by incubating increasing amounts of polymer with a constant amount of miRNA. The efficacy of polyplex formation was assessed by electrophoresis mobility shift assay (EMSA), demonstrating complexation starting from an N/P ratio of 9 (Figure 6C). This polymer/miRNA ratio (equivalent to 2:1 molar ratio) was found to induce maximal miRNA activity and was therefore chosen for all consecutive experiments. The morphology of the polyplexes was evaluated by SEM, demonstrating a diameter of 100 nm (Figure 6D,E). dPG-NH₂-miRNA polyplexes were further analyzed for their hydrodynamic diameter and zeta potential using DLS. Mixture
of miRNA with dPG-NH₂ at pH 7.4 yielded polyplexes with positive charges (17.0 ± 8.68 mV), as expected. The hydrodynamic diameter of the polyplexes was 80.32 ± 23.79 nm with a polydispersity index of 0.092 (Figure 6E). This low polydispersity suggests that the polyplexes formed are highly stable. Moreover, the particle’s hydrodynamic diameter, measured by SEM and DLS, reflects an assembly of several dPG-NH₂-miRNAs, as previously reported by Fischer and colleagues.

To evaluate the physiological biocompatibility of dPG-NH₂-miRNA polyplexes, an ex vivo hemolysis assay was performed. Briefly, rat red blood cells (RBC) solution (2 wt %/wt) was incubated with serial dilutions of dPG-NH₂-miR-93 (2:1 molar ratio) and the absorbance of hemoglobin released from the RBCs was measured. The results show that dPG-NH₂-miRNA polyplexes were not hemolytic at concentrations up to 5 mg/mL. The positive control of sodium dodecyl sulfate (SDS) showed dose-dependent hemolysis and the negative control dextran showed no hemolysis (Figure 6F).

**Dormancy-Associated miRNAs Downregulate Angiogenesis and Migration-Related Pathways.** Following a TargetScan and miRDB search for potential targets, we

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**Figure 7.** Dormancy-associated miRNAs target genes essential to cancer angiogenesis and migration. (A) Venn diagram representing the sets of miRNA target genes as predicted by miRDB and TargetScan alone and their overlap. (B) Relative expression of miR-34a, miR-93, and miR-200c target genes in dormant versus fast-growing tumor-generating cells, quantified by qRT-PCR. (C) Relative expression of cMET, HIF1α, and MSN in Saos-2-E and MG-63-E cells following transfection with dPG-NH₂-miRNA polyplexes, quantified by qRT-PCR. (D) Sprouting of endothelial cells from the aortic wall in the presence of conditioned media from Saos-2 or MG-63 cells reintroduced with miRNA mimics. Scale bar represents 100 μm. (E) Relative quantity of angiogenesis-related proteins secreted by Saos-2 cells transfected miRNA mimics. Data was obtained using an angiogenesis protein array for conditioned media, and is represented as mean pixel density, compared to conditioned media of cells introduced with control miR. (F) Changes in HIF1α and VEGF levels following 24 h hypoxia, quantified by qRT-PCR. Data represents mean ± SD.
obtained an extended list, out of which we found targets that have been previously implicated as regulators of tumorigenesis (Figure 7A). qRT-PCR showed that mRNA levels of three cancer-related target genes are significantly upregulated in Saos-2-E compared to Saos-2-D cells: MET proto-oncogene (cMET), targeted by miR-34a; hypoxia-inducible factor 1α (HIF1α), targeted by miR-93; and moesin (MSN), targeted by miR-200c (Figure 7B). This upregulation, although seemingly modest (1.5−2.5 fold change), can have paramount biological effect, as previously shown.33,34 Both cMET and HIF1α were shown to induce pro-angiogenic signaling pathways in cancers.35,36 MSN, a protein localized to filopodia, was reported to have a role in tumor cells migration and metastatic abilities.37 This data may account for the aforementioned differences in the angiogenic potential and the stress-induced migratory capabilities between Saos-2-D and Saos-2-E cells. To confirm that these genes are targets of our dormancy-associated miRNAs in osteosarcoma, we additionally exploited our above-mentioned MG-63 model. Target genes mRNA levels were determined following transfection of Saos-2-E and MG-63-E with dPG-NH2/miRNA mimics polypexes (200 nM dPG-NH2; 100 nM miRNA). A marked reduction of cMET was observed in both cell lines following reconstitution of miR-34a, miR-93, and miR-200c. HIF1α levels were downregulated by reconstitution of miR-93 and miR-200c. miR-200c also reduced MSN levels in both cell lines (Figure 7C).

The effect of these miRNAs on the angiogenic phenotype of osteosarcoma cells was further evaluated using the aortic ring assay. Sprouting of endothelial cells in the presence of conditioned media from Saos-2-E and MG-63-E cells was significantly reduced following transfection with miR-34a or miR-200c to the same extent as in the presence of conditioned media from cells generating dormant tumors, Saos-2-D or MG-63. Transfecting with miR-93 in Saos-2-E cells resulted in a similar inhibitory effect on endothelial cell sprouting, but did not affect MG-63-E-induced sprouting (Figure 7D).

Using an angiogenesis antibody array, we further evaluated the effect of miR-34a, miR-93, and miR-200c on the serum...
levels of a number of angiogenesis-related proteins in Saos-2-E cells. In general, an antiangiogenic effect was received, with a tendency of little to no effect on the secretion of pro-angiogenic proteins and a marked effect on the secretion of antiangiogenic proteins. miR-34a induced the secretion of the antiangiogenic proteins endostatin, transforming growth factor beta 1 (TGF-β1), TIMP metalloproteinase inhibitor 4 (TIMP-4) and thrombospondin-1 (TSP-1). miR-93 reduced the secretion of tissue factor and induced the secretion of endostatin, while miR-200c reduced tissue factor secretion and induced the secretion of endostatin, TGF-β1, TIMP-1, TIMP-4, and TSP-1 (Figure 7E).

Next, we evaluated the effect of miR-34a, miR-93, and miR-200c on the in vitro phenotype of Saos-2-E cells. Growth and migration rates of Saos-2-E cells were not affected by reconstitution of control miR or our miRNAs (Figure S3A,B). No effect on HUVEC migration toward conditioned media of cells reconstituted with these miRNAs was observed (Figure S3C). However, following starvation, the ability of Saos-2-E cells to migrate and to invade through an endothelial cell monolayer was significantly reduced by the dormancy-associated miRNAs (Figure S3D,E). These suggests that these miRNAs reduce the ability of Saos-2-E cells to overcome stress conditions.

To test this hypothesis, we evaluated the influence of our miRNAs on the response to an additional stress condition, a hypoxic microenvironment. Our miRNAs interrupted the ability of Saos-2-E cells to respond to hypoxia, either by reducing HIF1α levels (by miR-34a and miR-93), or by reducing VEGF levels (by miR-93 and miR-200c) (Figure 7F). HIF1α and VEGF downregulation by miR-93 polyplex during hypoxia corresponds with the reduction in HIF1α mRNA levels by miR-93 polyplex, and suggests an important role of this miRNA in osteosarcoma response to hypoxia. Moreover, the fact that miR-93 and miR-200c polyplexes did not affect VEGF secretion in normal conditions, as received by the angiogenesis array (Figure 7E), but significantly reduced VEGF levels following hypoxia emphasizes our notion that these dormancy-associated miRNAs have a role within the tumor microenvironment.

**DISCUSSION**

Since the discovery of the RNA interference (RNAi) machinery, numerous RNAi-based strategies for therapeutic gene silencing have been developed. RNAi delivery systems were designed to enable accumulation of oligonucleotides at the target tissue, facilitating their internalization into the cells reaching their cytoplasmic target. However, despite numerous attempts, most RNAi systems that reached clinical trials are targeted to the liver. Moreover, the great promise of RNAi therapeutics has been halted by limitations such as poor pharmacokinetic properties, immunogenicity, inability to cross cellular membranes and properly escape from the endosomes, and high toxicity.38–41 Therefore, greater efforts must be made to overcome these limitations in order to fully exploit the therapeutic potential of oligonucleotides.

miRNA-based therapies represent a relatively new field, as most previous efforts were focused on solutions for siRNA delivery. With the realization that silencing a single pathway in cancer results in the development of compensatory mechanisms, miRNAs have become attractive candidates as they regulate entire networks of genes. Therefore, advances in clinical translation of miRNA-based nanomedicines lag behind those of siRNA-based ones. Currently, there is only one anticancer miRNA formulation in clinical trial. In fact, the first cancer-targeted miRNA to enter clinical trials is a liposome-based miR-34a mimic (MRX34) used for patients with hepatocellular carcinoma.

We hereby show the rational design of RNAi nanomedicines starting from the establishment of novel tumor mouse models of osteosarcoma, through the discovery and validation of novel key miRNA players in osteosarcoma progression, up to the creation of new nanosized miRNA-mimics polyplexes. Using models of osteosarcoma dormancy and clinical specimens, we demonstrate that (i) tumor growth differences between cells generating dormant and fast-growing tumors are not attributed to differences in replications kinetics; (ii) miR-34a, miR-93, and miR-200c have a role in the switch of osteosarcoma from dormancy to progressive disease; (iii) miR-200c is involved in osteosarcoma progression; (iv) miR-34a, miR-93 and miR-200c levels are decreased in metastatic osteosarcoma, both in clinical samples and in metastases-derived cells compared to normal tissues and cells; and (v) these dormancy-associated miRNAs have therapeutic potential when polyplexed with a polymeric nanocarrier, as opposed to previous published studies that demonstrated their in vivo biological effect following ectopic overexpression.

We successfully delivered miR-93, miR-200c, and miR-34a to human osteosarcoma cells both in vitro and in vivo using dPG-NH₂, dPG-NH₂, dPG-NH₂ formed a stable complex with these preselected miRNAs, of circa 80 nm size and zeta potential of 17.0 ± 8.68 mV, at N/P 9, that showed the optimal activity. Our relatively wide distribution in zeta potential might result from the stochastic nature of surface activation of PG dendrimers with amine moieties and the uncontrolled process of self-assembly of the miRNA and the positively charged dendrimers. However, it is not uncommon for such polyplexes or their free nanocarriers to have such a wide zeta potential distribution.42,43 Despite the narrow polydispersity of the polyplex, received by DLS measurements, the SEM image showed heterogeneous particle distribution. This might result from the sample preparation following treatment withdrawal in all treatment groups (Figure 8E).
In this study, we suggest that miR-34a, miR-93, and miR-200c, upregulated in dormant tumors and healthy bones, prevent tumor progression by reducing the mRNA levels of genes critical to tumor angiogenesis and cancer progression in general. These three miRNAs are known tumorigenesis regulators: miR-34a and miR-200c were found to have tumor suppressive properties, whereas miR-93 was found to have either a promoting or suppressive properties in malignancies. miR-34a and miR-93 have been previously implicated as regulators of osteosarcoma tumorigenesis. This study shows a direct link between miR-200c and osteosarcoma progression and between all three miRNAs and the switch from tumor dormancy.

miR-34a is a known “tumor suppressor” miRNA. Several studies have shown downregulation of miR-34a in osteosarcoma. One of the predicted targets of miR-34a, cMET, was found to have a key role in osteosarcoma. We report here that cMET, an important contributor to angiogenesis and tumorigenesis, is a putative target of miR-34a in osteosarcoma and indeed was downregulated following introduction of miR-34a.

miR-93 was reported to have a differential role in osteosarcomagenesis. Although this study showed miR-93 overexpression in several osteosarcoma cell lines compared to osteoblasts, levels of miR-93 in MG-63 and Saos-2 were within normal values. This data supports our results, as both MG-63 and Saos-2 cell lines are defined as nontumorigenic by the ATCC. Moreover, they reported no difference in miR-93 levels between primary osteosarcoma tumors and paired normal tissues. Nevertheless, a larger clinical sample is required to fully understand the role of miR-93 in osteosarcoma. So far, no evidence for an antiangiogenic effect of miR-93 was found in cancer. We suggest here that the antiangiogenic properties of miR-93 in osteosarcoma are derived from inhibition of HIF1α expression. This factor upregulates expression of proteins that promote angiogenesis and other survival factors in several malignancies including osteosarcoma.

Members of the miR-200 family have been extensively reported to act as “tumor suppressors” in carcinomas, primarily by targeting epithelial to mesenchymal (EMT)-related genes. miR-200b was found to be upregulated in osteosarcoma cells following treatment with the anticancer agent diallyl disulfide; however, there is no direct evidence for a role of miR-200c in osteosarcoma progression. We show that miR-200c is downregulated in primary and metastatic osteosarcoma clinical samples. Interestingly, both metastatic clinical specimens and Saos-2 lung metastases express higher miR-200c levels compared to primary tumors. This suggests that loss of miR-200c enables metastasis, and that following metastatic colonization, miR-200c levels are restored. Similar findings were previously demonstrated in breast cancer clinical samples. In terms of therapeutic potential, restoring miR-200c levels might be more beneficial for the treatment of primary disease, as it may prevent metastatic spread. A clear antiangiogenic effect of miR-200c was demonstrated in our study, which can be attributed to downregulation of VEGF during hypoxia. Furthermore, we show here that one of the targets of miR-200c in osteosarcoma is MSN, a regulator of migration and metastases. This data is in line with the above-mentioned differential expression of miR-200c in primary and metastatic osteosarcoma. Interestingly, another member of the VEGF family, VEGF-C, was reported to promote cancer metastases via upregulation of MSN. This suggests that there
may be a link between the signaling cascades of VEGF and MSN in osteosarcoma.

It is important to state that the miRNAs evaluated in this manuscript represent only a part of the dormancy signature, as the additional differentially expressed miRNAs are potential dormancy regulators. The specific role of these miRNAs in tumor dormancy will be evaluated in future studies. Moreover, since a single miRNA can regulate entire networks of genes, it is clear that not only the aforementioned miRNA targets are involved in osteosarcoma progression. Therefore, the function of additional targets of these miRNAs in the switch of osteosarcoma from dormancy will need to be further explored mechanistically. This will be feasible using appropriate nanocarriers that efficiently and safely deliver preselected miRNAs to cancerous tissues.

CONCLUSIONS

The anticancer therapeutic benefit of miRNA-based therapies has been reported previously in numerous studies. Exploiting our well-characterized dendritic nanocarriers, we showed the therapeutic efficacy of dormancy-associated miRNAs. Recovering novel dormancy regulators can be harnessed to treat tumors since a single miRNA can regulate entire networks of genes, it is clear that not only the aforementioned miRNA targets are involved in osteosarcoma dormancy progression. Therefore, the function of additional targets of these miRNAs in the switch of osteosarcoma from dormancy will need to be further explored mechanistically. This will be feasible using appropriate nanocarriers that efficiently and safely deliver preselected miRNAs to cancerous tissues.

METHODS

Cell Culture. Saos-2 and MG-63 human osteosarcoma cell lines and human embryonic kidney 293T cells (HEK 293T) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Green fluorescent protein (GFP)-labeled Saos-2 cell line was established as previously described. MG-63-Ras cells were established by transfection with activated Ras as previously described, in order to generate an in vivo fast-growing angiogenic cell line (MG-63-E). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin, 2 mM L-glutamin (Biological Industries, Kibbutz Beit Haemek, Israel). Human umbilical vein endothelial cells (HUVEC) were isolated as previously described and cultured in M199 medium (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 20% FBS, 100 mg/mL penicillin, 100 U/mL streptomycin, 12.5 U/mL nystatin, 2 mM L-glutamin, 100 mg/mL heparin, and 50 mg/mL of endothelial cell growth supplement (Biomedical Technologies, Inc, MA, USA). Cells were grown at 37 °C in 5% CO2.

hFOB 1.19 human osteoblasts were obtained from the ATCC and cultured in 1:1 mixture of phenol-free Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (DMEM/F12) supplemented with 10% FBS, 2 mM L-glutamin and 0.3 mg/mL G418. Cells were grown at 34 °C in 5% CO2.

Animal Studies. All animals were housed in Tel Aviv University’s animal facility and the experiments were approved by our institutional animal care and use committee (IACUC) and conducted in accordance with NIH guidelines.

For subcutaneous inoculation, 6–8 weeks old SCID male mice were inoculated into the right flank with 1 × 10⁶ Saos-2 cells (n = 5). Tumor progression was monitored by caliper measurement (width × length × 0.52) and by CRI Maestro noninvasive intravitral imaging system. Two weeks post inoculation, retro-orbital blood was collected for analysis of circulating endothelial progenitor (CEP) cells. Mice were euthanized when tumor size reached 1.5 cm³ or when mice became moribund.

For orthotopic intratibial tumor cell inoculation, 25 × 10⁶ Saos-2 cells were injected into the right proximal tibia of 4-week-old male SCID mice. Briefly, mice were anesthetized using ketamine (100 mg/kg) and xylazine (12 mg/kg) and treated with a depilatory cream (Veet) to expose the anterior surface of the right tibia and the knee joint. The knee of the mice was flexed to a 90° position and 50 μL of the cell suspension was injected into the right tibia using 27 G needle.

The needle was inserted into the proximal tuberosity of the tibia using a rotational (drilling) motion of the syringe. Tumor growth and metastatic spread were monitored weekly using TomoScope Synergy microCT scanner (CT imaging, Germany). Mice were euthanized when tumor size reached 1 cm³ or when mice became moribund and immediately perfused intracardially with PBS followed by 4% formaldehyde.

For the experimental lung metastasis model, 5 × 10⁶ Saos-2 cells were injected into the tail vein of 6–8 weeks old SCID male mice. Mice were monitored for weight change 3 times a week and euthanized when a rapid weight loss was observed (15–20% within a few days). Immediately following euthanasia, mice were perfused and deselected intracardially as mentioned above. Lungs were then imaged ex vivo for metastatic colonization using CRI Maestro imaging system.

Intravitral Noninvasive Imaging of GFP-Labeled Saos-2 Tumors. CRI Maestro noninvasive fluorescence imaging system was used to follow tumor progression of mice bearing subcutaneous GFP-labeled tumors. Mice were anesthetized using ketamine (100 mg/kg) and xylazine (12 mg/kg) and placed inside the imaging system. Data was acquired using a 360° individual projection collected every 1° to complete one rotation around the animal, with X-ray tube voltage of 40 kV. Cross-sectional images (DICOM format) were generated from the projected CT images using TomoScope image reconstruction software (CT imaging, Germany). Tumor volume was calculated from DICOM images using the manufacturer’s software.

In Vivo Matrigel Plug Angiogenesis Assay. To analyze the angiogenic potential of Saos-2 lines in vivo, we used the matrigel plug model system. A total of 1 × 10⁶ cells were mixed with growth factor-reduced liquified matrigel (BD Biosciences, NJ, USA) and injected sc (final volume, 0.5 mL) into the flanks of 6-week-old male SCID mice. Three weeks post matrigel inoculation, vascularization within the plugs was evaluated by microbubble contrast-enhanced ultrasound imaging. Mice were then euthanized and plugs were recovered and photographed. Hemoglobin content within the plug was measured using Drabkin’s reagent (Sigma-Aldrich, MO, USA).

Microbubble Contrast-Enhanced Ultrasound Imaging. Ultrasound was performed using a Vevo2100 from VisualSonics, Inc. (Toronto, Ontario, Canada) using the 55 MHz 7F8 probe. Mice were anesthetized and hair on the tumor area was removed using a depilatory cream. Nontargeted microbubbles (VisualSonics, Inc., Toronto, Ontario, Canada) were mixed with saline and injected into the tail vein of mice. 3D contrast enhanced cine loop of the tumor was acquired using the 3D acquisition motor immediately after injection.
Following microbubbles destruction, a second 3D contrast cine loop was taken. The difference in video intensity from subtraction of the pre-and postdestruction image frames was automatically displayed by the software as a colored (green) overlay on the grayscale images. Immunohistochemistry. FFPE samples of tumor nodules were sectioned at 5 μm thick. One section per sample was deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). Additional sections were stained by immunohistochemistry for microvessels using CD31 antibody (Dianova), proliferating cells using Ki67 (Abcam) and apoptotic cells using cleaved caspase 3 antibody (Epitomics). Briefly, slides were pretreated with 10 mM citrate, pH 6.0, for 20 min at 95 °C. All further steps were performed at room temperature (RT) in a hydrated chamber. Slides were covered with 3% H2O2 in methanol for 10 min to quench endogenous peroxidase activity, followed by incubation with 2% goat serum for 30 min to block nonspecific binding sites. Primary rat anti-mouse CD31 (1:10; Dianova, Hamburg, Germany) antibody was applied in 1% BSA at RT for 2 h. Rabbit anti-human Ki67 (1:100; Abcam, MA, USA) and rabbit monoclonal anti-caspase 3 (1:100; Epitomics, CA, USA) antibodies were applied in 1% BSA at RT for 1 h. A broad spectrum biotinylated secondary antibody was added for 1 h for CD31 or 30 min for Ki67 and caspase 3. Slides were then incubated with streptavidin–horseradish peroxidase conjugate for 30 min (Histostain, Life Technologies, CA, USA). Following washing, immunoperoxidase staining was developed using ImmPACT DAB diluent kit (Vector Laboratories, CA, USA) per the manufacturer’s instructions and counterstained with hematoxylin. Microvessel density (MVD) was calculated as previously described.71 Briefly, tumors were scanned for areas of high vessel density (i.e., “hot spots”) at low power. Then, individual microvessels were counted at a higher power (>200 field). Vessels with lumen were defined as vessels positively stained for CD31, with an open lumen that, in most cases, contained red blood cells and were counted at high power (>200 field).

Growth Curve. Saos-2 cells were plated into 24-well plates in triplicates (1 × 10^4 cells/well). Following incubation (37 °C; 5% CO2), cells were counted daily by Coulter Counter (Beckman Coulter) over a 4-day period.

Migration Assays. Cell migration assays were performed using 8 μm Transwell inserts (Costar Corp., Corning, NY, USA). Cells (1 × 10^5) were added to the upper chamber of the Transwell. Following 2 h incubation, cells were allowed to migrate for an additional 4 h to the lower chamber of Transwell inserts containing DMEM supplemented with 10% FBS or conditioned media. Cells were then fixed with ice-cold methanol and stained using hematoxylin 3 stain system (Thermo Fisher Scientific, MA, USA). The stained migrated cells were imaged using Nikon TE2000E inverted microscope integrated with Nikon DSS cooled CCD camera by 10× objective, brightfield illumination. Total area of migrated cells from the captured images per membrane was analyzed using NIH ImageJ software.

Transendothelial Migration Assay. HUVEC (25 × 10^4 cells) were grown to confluency in the upper chamber of 24-well Transwell inserts precoated with 10 μg/mL fibronectin. Following 24 h incubation, HUVEC monolayer was washed and 5 × 10^3 GFP-labeled Saos-2 cells were added to the upper chamber. After 1 h of incubation, DMEM supplemented with 10% FBS was added to lower chamber. Cells were allowed to migrate to the lower compartment for an additional 6 h and then fixed using 3.7% formaldehyde. The fluorescence of the migrated cells was imaged using Nikon TE2000E inverted microscope integrated with Nikon DSS cooled CCD camera by 10× objective. Fluorescence signal was measured using NIH ImageJ software.

Molecular Combing. The method was performed as described in Bester et al.27 Briefly, unsynchronized cells were sequentially pulse labeled by two thymidine analogues: iododeoxyuridine (IdU) and chlorodeoxyuridine (CldU), 50 μM for 30 min each. At the end of the labeling period, the DNA was extracted, combed and visualized by fluorescent antibodies. The length of the replication signals and the distances between origins were measured in micrometers and converted to kilobases according to a constant and sequence-independent stretching factor (1 μm = 2 kb), as previously reported.28 For the replication rate analysis, a minimum of 140 signals were analyzed for each sample, containing both the IdU and CldU signals. The distance between origins was extracted by analyzing the distance between out- and ingoing forks. The analysis was performed on a minimum of 88 forks and higher. All combing experiments were performed twice and yielded similar results. Representative data from one set of the experiments are shown and expressed as mean ± SE values.

MicroRNA Arrays. Saos-2 cells generating dormant and fast-growing tumors were analyzed for their miRNA profile as previously described.72 miRNA relative levels were calculated based on the comparative threshold cycle (CT) method. Candidate miRNAs were selected according to a differential expression of over 15-fold between cells that generate dormant versus fast-growing tumors. Suspected target genes associated with regulation of angiogenesis and oncogenesis were predicted using TargetScan and miRDB databases.

Quantitative Real-Time PCR. Total RNA was isolated from cultured cells using EZ-RNA II total RNA isolation kit (Biological industries, Kibbutz Beit Haemek, Israel), according to the manufacturer’s protocol. For total RNA isolation from FFPE tissues, RecoverAll Total Nucleic Acid Isolation Kit (Ambion, TX, USA) was used, according to the manufacturer’s protocol. One microgram of RNA was reverse transcribed using miScript II RT kit (Qiagen, CA, USA). Expression levels of miR-34a, miR-93, and miR-200c and their target genes (NOTCH1, BCL2, MET, STAT3, ADAM9, HIF1α, ZEB1, PLECG1, MSN) were analyzed using SYBR green based real-time PCR. miRNAs expression levels were quantified with miScript Primer assays (Qiagen, CA, USA) and normalized to RNU6. miRNA target genes levels were quantified using custom qPCR primers (Syntezza Bioscience Ltd., Jerusalem, Israel) and normalized to GAPDH.

Formalin-Fixed Paraffin-Embedded (FFPE) Human Osteosarcoma Specimens. FFPE samples were obtained with the approval of the Institutional Review Board (IRB) and in compliance with all legal and ethical considerations for human subject research. A total of 34 FFPE samples were collected from Sheba Medical Center tissue archive: 15 samples of primary osteosarcoma, 9 samples of osteosarcoma lung metastases and 10 control samples from patients who underwent acute, traumatic injuries to the long bones. All histological slides were reviewed by the study pathologists. FFPE samples were analyzed for miRNA levels by qRT-PCR and for miRNA localization by in situ hybridization.

miRNA in Situ Hybridization (ISH) on FFPE Specimens. miRNA ISH was performed using digoxigenin-labeled miRCURY LNAISH probes (Exiqon, Vedbaek, Denmark), according to the manufacturer’s protocol with slight modifications. Briefly, 6 μm sections were collected from each FFPE sample. Sections were deparaffinized, rehydrated and then permeabilized with protease K (10 μg/mL, Roche Diagnostics, IN, USA) for 10 min at 37 °C. Sections were then applied with hybridization mix and hybridized overnight in a humidified chamber. Following hybridization, sections were washed with sodium chloride sodium citrate (SSC) buffer (Ventana Medical Systems, AZ, USA), blocked for 1 h in blocking solution (Roche), and then incubated for 2 h in anti-DIG-alkaline phosphatase (AP) antibody (Roche Diagnostics, IN, USA) at room temperature. Following further washing, sections were incubated with AP substrate until desired sensitivity was achieved and counterstained with fast nuclear red.

miRNA Stable Overexpression. Stable miRNA overexpression was performed using pCDH-CMV-hygromycin retroviral vectors containing the miRNA genomic region or scrambled DNA (Systems Biosciences). Forty-eight hours following transfection, retroviral particles containing supernatant were collected. Saos-2 cells were then infected with the viral particles and selected for stable expression using hygromycin.
Measurement of Circulating Endothelial Precursors (CEPs) by Flow Cytometry. Blood was obtained from anesthetized mice by retro-orbital sinus bleeding. Briefly, a capillary pipet containing anticoagulant (0.1 M EDTA) was inserted in the lateral canthus and blood was collected from the retro-orbital sinus. After collection, the pipet was removed and bleeding stopped when the eye returned to normal position. EDTA anticoagulated blood samples were used to obtain a complete blood count. Samples were kept cold (4 °C) at all times and subsequently counted. Blood samples (10 μL) were mixed with TURK solution (90 μL in DDW supplemented with 1% acetic acid). White blood cells were counted by a Z1 Coulter Particle Counter (Beckman Coulter) for assay normalization. CEPs and CECs were quantitated using flow cytometry, as described previously.9,10 Briefly, monoclonal antibodies were used to detect all populations with the following antigenic phenotypes: for CEPs, CD13+/VEGFR2+/CD117+/CD45−/dim; for detecting CECs, CD13+/VEGFR2+/CD45−/dim. Nuclear staining was used in some experiments to exclude platelets or cellular debris. 7-Aminoactinomycin D (7AAD) was used to distinguish apoptotic and dead cells from viable cells. After red cell lysis, cell suspensions were analyzed. After acquisition of at least 200,000 cells per sample, analyses were considered informative when an adequate number of events (i.e., >50, typically 50–150) were collected in the CEP or CEC enumeration gate in untreated control animals. Percentages of stained cells were determined and compared with appropriate negative controls. Positive staining was defined as being greater than nonspecific background staining. Flow cytometry studies were performed on Cyan ADP (Beckman Coulter) and analyzed with Summit (Beckman Coulter) software. All monoclonal antibodies were purchased from BD Biosciences or BioLegend and used for flow cytometry analysis in accordance with the manufacturer’s protocols.

Dendritic Polyglycerolamine (dPG-NH2) Synthesis. Dendritic polyglycerol with average molecular weight of 14.5 kDa (PDI = 1.8) was prepared following literature procedures.7,16,77 Synthesis of dPG-NH2 analogues was executed according to a three-step protocol as previously reported.76 To convert 90% of all hydroxyl groups on dPG into amine groups, the hydroxyl groups were first activated by methanesulfonyl chloride. Mesyl groups were substituted by amines in a successive step. Finally, reduction of the azides by triphenylphosphine in a successive step. Finally, reduction of the azides by Staudinger reduction resulted in formation of functionalized PG groups). (PG). The optimal molar ratio and incubated at room temperature for 20 min.

Electrophoresis Mobility Shift Assay (EMSA). The optimal ratio for the polyplex formation was studied by electrophoretic mobility shift assay (EMSA) as described previously.20 In brief, 100 pmol of miR-93 was incubated with dPG-NH2 at 0.5, 2, 4.5, 9, and 18 N/P ratio (equivalent to 0.12:1, 0.5:1, 1:1, 2:1, and 4:1 molar ratios of carrier to miRNA) for 20 min at RT. Mobility of free and nanocarrier-complexed miRNA was then analyzed by agarose gel electrophoresis. N/P ratios were calculated according to standard formulas reported in the literature.7,17

Scanning Electron Microscope (SEM). Polymer solution at 0.1 mg/mL concentration was mixed with miRNA solution at the indicated molar ratio and incubated at room temperature for 20 min. Samples were dropped on a Si wafer and air-dried for 30 min. The dry samples were coated with 4 nm layer of Cr. SEM images were taken by Quanta 200 FEG Environmental SEM (FEI, OR, USA) at high vacuum and 5.0 kV.

Dynamic Light Scattering (DLS) and Zeta Potential Determination. The mean hydrodynamic diameter of the dPG-NH2-miR-93 polyplexes and the zeta-potential measurements were performed using a ZetaSizer Nano ZS instrument with an integrated 4 mW He–Ne laser (λ = 633 nm; Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). dPG-NH2-miR-93 polyplexes were prepared by dissolving 0.25 or 0.05 mg of dendrimer and the indicated molar ratio of miR-93 in 1 mL of DDW for the hydrodynamic radius measurement or the zeta potential measurement, respectively. Samples were incubated for 20–30 min at room temperature, then PBS was added from x10 stock to a final buffer concentration of 15 mM, pH = 7.4, to the zeta potential sample. All measurements were performed at 25 °C using polystyrol/polystyrene (10 × 4 × 45 mm) cell for DLS analysis and folded capillary cell (DTS 1070) for zeta potential measurements. Results are representative of 3 repeats.

Hemolysis Assay. Rat red blood cells (RBC) solution (2 wt %/wt) was incubated with serial dilutions of dPG-NH2-miR-93 polyplexes (2:1 molar ratio) for 1 h at 37 °C. The highest polyplex concentration (5000 μg/mL) is 100-fold higher than the one used for the in vivo experiments (50 μg/mL), adjusted to dilute in mouse blood volume. Sodium dodecyl sulfate (SDS) was used as a positive control and dextran (Mw ~ 70 kDa) as a negative control. Following centrifugation, the supernatants were transferred to a new plate and absorbance was measured at 550 nm using a SpectraMax M5 plate reader (Molecular Devices, CA, USA). The results were expressed as percentage of hemoglobin released by 1 wt %/vol solution of Triton X100 (100% lysis).

miRNA Transfection. miRNAs (100 nM) (Table S2) were allowed to form a polyplex with PG-NH2 (200 nM; 2:1 molar ratio) in serum free medium for 20 min at room temperature. Saos-2 or MG-63 cells were added with PG-NH2-miRNA polyplexes and incubated for 48 h at 37 °C in 5% CO2. Cells were analyzed for miRNA levels and target genes miRNA levels using qRT-PCR.

Conditioned Media Preparation. The angiogenic properties of osteosarcoma cell lines were evaluated by the effect of their conditioned media on the sprouting of endothelial cells from the aortic wall. To generate conditioned media, Saos-2 cells (7.5 × 105) or MG-63 cells (1.2 × 105) were plated on 10 cm² tissue culture plates. Twenty-four hours later, cells were transfected with 100 nM miRNA complexed with 200 nM PG-NH2. Following 48 h incubation at 37 °C in 5% CO2, conditioned media were harvested and filtered through 0.45 μm syringe filter to remove cells and debris.

Aortic Ring Sprouting Assay. Aorta was resected from a BALB/c mouse, sliced to 1 mm pieces and placed in 48 wells plate precoated with matrigel (BD Bioscience, NJ, USA) basement membrane (250 μL/well; 10 mg/mL). Additional matrigel basement membrane (100 μL/well; 10 mg/mL) was added and allowed to polymerize at 37 °C for 30 min. Conditioned media from Saos-2 or MG-63 cells (300 μL) was then added. Sprouting of endothelial cells from the resected aorta slices was imaged following 7 days incubation in 37 °C using Nikon TE2000E inverted microscope integrated with Nikon DSS cooled CCD camera by 15X objective, brightfield illumination.

Quantification of Serum Levels of Angiogenesis-Related Factors. Saos-2 cells were transfected with 100 nM miRNA or control RNA and allowed to grow for 72 h. Conditioned media was then collected and concentrated using 3K NMWL Centricron centrifugal filters (Millipore, MA, USA) to a final volume of 500 μL. Angiogenesis array membranes were added with conditioned media and further processed according to the kit procedure. Data is represented as mean pixel density, compared to conditioned media of cells introduced with control miR.

Antitumor Activity of dPG-NH2-miRNA-Mimic Polyplexes. dPG-NH2, dendritic nanocarrier, synthesized and characterized as previously described,19,20 was used to deliver miR-34a mimic, miR-93 mimic or miR-200c mimic (Thermo Fisher scientific, MA, USA) to tumor-bearing mice. miRNA mimic sequences were modified for enhanced stability as previously described20 (Table S2). Briefly, mice bearing 40 mm³ Saos-2-E tumors were injected intratumorally eight times, every 3 days, with miRNA-mimic/negative control miRNA complexed with dPG-NH2 (4 mg/kg dPG-NH2 with 2 mg/kg miRNA) or saline/normal mouse (n = 5 mice/group). Animal were monitored twice a week for general health, body weight and tumor volume.

Sphere-Forming Assay. Six-well plates were coated with poly(2-hydroxyethyl methacrylate) (30 mg/mL in ethanol) and left overnight at room temperature until the ethanol evaporated. Saos-2 cells were
then plated (50,000 cells/well) in knockout DMEM (Life Technologies, CA, USA) supplemented with 20% FBS, 10% nonessential amino acids, 100 ng/mL human epidermal growth factor (Peprotech, NJ, USA), 100 ng/mL human basic fibroblast growth factor (Peprotech, NJ, USA) and 10 ng/mL stem cells factor (Peprotech, NJ, USA). Cells were cultured in 5% CO₂ at 37 °C for a week.

**Statistical Methods.** Data are expressed as mean ± standard deviation (SD) for *in vitro* assays or ± standard error of the mean (SEM) for *in vivo* assays. Statistical analysis for two sets of data was performed using an unpaired *t* test. For more than two data sets, one-way analysis of variance (ANOVA) was performed followed by a post-hoc test. Statistical significance of differences in overall survival was determined using log-rank test. Significance was defined as *P* < 0.05.

**ASSOCIATED CONTENT**

© Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.5b06189.

Additional experimental data as Figures S1–S4 and Tables S1 and S2 (PDF)

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**Notes**

The authors declare no competing financial interest.

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**Additional experimental data as Figures S1–S4 and Tables S1 and S2 (PDF)**


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