RESEARCH ARTICLE

MATERIALS SCIENCE

Palladium nanoplates scotch breast cancer lung metastasis by constraining epithelial-mesenchymal transition

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Metastasis accounts for majority of cancer deaths in many tumor types including breast cancer. Epithelial-mesenchymal transition (EMT) is the driving force for the occurrence and progression of metastasis, however, no targeted strategies to block the EMT program are currently available to combat metastasis. Diverse engineered nanomaterials (ENMs) have been reported to exert promising anti-cancer effects, however, no ENMs have been designed to target EMT. Palladium (Pd) nanomaterials, a type of ENM have received substantial concern in nanomedicine due to their favorable photothermal performance for cancer therapeutics. Herein, Pd nanoplates (PdPL) were found to be preferentially biodistributed to both primary tumors and metastatic tumors. Importantly, PdPL showed a significant inhibition of lung metastasis with and without near-infrared (NIR) irradiation. Mechanistic investigations revealed that EMT was significantly compromised in breast cancer cells upon the PdPL treatment, which was partially due to the inhibition of the transforming growth factor-beta (TGF-β) signaling. Strikingly, the PdPL was found to directly interact with TGF-β proteins to diminish TGF-β functions in activating its downstream signaling, as evidenced by the reduced phosphorylation of Smad2. Notably, TGF-β-independent pathways were also involved in undermining EMT and other important biological processes that are necessary for metastasis. Additionally, NIR irradiation elicited synergistic effects on PdPL-induced inhibition of primary tumors and metastasis. In summary, these results revealed that the PdPL remarkably curbed metastasis by inhibiting EMT signaling, thereby
indicating the promising potential of PdPL as a therapeutic agent for treating breast cancer metastases.

**Keywords:** palladium nanoplates; tumor targeting; lung metastasis; epithelial-mesenchymal transition; TGF-β.

**INTRODUCTION**

While primary tumors cause significant morbidity in cancer patients, metastases to distant organs are the major reason for adverse disease outcomes among cancer patients. For example, metastatic tumors account for over 90% mortality among breast cancer patients [1]. Metastasis is a consecutive process, which involves the increased shedding of tumor cells from the primary tumor in a direction parallel to the growth of primary tumors, thereby leading to an ongoing delivery of tumor cells to distant organs [2], which essentially determines the prognosis of the patient. Metastasis is a multistep and dynamic cascade that begins from a primary tumor and requires the circulation of metastasizing cells through the lymph or blood, followed by the lodgment of tumor cells in the target organ(s). Metastasis involves a number of complex biological processes, in which epithelial-mesenchymal transition (EMT) largely dictates the invasion/spread and subsequent re-localization of cancer cells in distant organs [3]. EMT is a fine-tuned program controlling the transformation of epithelial cells into mesenchymal-like cells with more fibroblast properties, thereby increasing the invasion and migration capabilities of tumor cells [4]. Among a plethora of upstream regulators of EMT, the transforming growth factor-β (TGF-β) plays a crucial function in driving EMT during metastasis through the successive regulation of the genes responsible for cellular contact, plasticity, motility, stemness,
TGF-β ligands are known to bind with their transmembrane receptors (TGF-βR) to phosphorylate adapter and regulatory proteins, mainly Smad2 and Smad3 (Smad2/3), to orchestrate gene expression and contribute to EMT [6]. Research is being carried out to discover drugs that can block TGF-β signaling [6].

Nanomedicine continues to bring unprecedented advantages in the development of cancer therapeutics [7]. Engineered nanomaterials have been extensively studied for the inhibition and elimination of primary tumor growth; however, studies on the use of ENM-based strategies to selectively target metastases remain rather limited [8]. ENM-based anti-cancer approaches include nanocarrier-assisted chemo/gene delivery, photothermal therapy, and immunotherapy [9]. Although these treatments effectively kill cancer cells, several factors such as drug leakage, multidrug resistance, limited tissue penetration depth of near-infrared (NIR) light, and immune tolerance limit the effectiveness of these treatment, and ENM-based selective anti-metastatic therapies are unavailable. However, the potential of ENMs per se as therapeutic agents in cancer treatment has attracted increased attention. For instance, ENMs including cuprous oxide, zinc oxide, and silver nanoparticles are themselves cytotoxic, but they do not selectively kill cancer cells [10-12]. Additionally, ENMs such as gold nanoparticles (AuNPs) and Gd@C_{82}(OH)_{22} are also known to restrict tumor cell proliferation [13,14]; however, they do not show selectivity towards metastatic tumors. In a recent study, we reported the potential of palladium (Pd)-based ENMs (such as PdPL) as a therapeutic agent in cancer treatment owing to their outstanding photothermal and photoacoustic properties, and desirable bio-/cyto-compatibility [15,16]. Importantly, in comparison with other ENMs, PdPL displays greater photostability, and is more prone to localize to the cancer tissue [17,18]. However, regardless of these encouraging progresses, research on the effect of ENMs on EMT to diminish metastasis has rarely been
conducted. Therefore, it is important to conduct studies on the effect of ENMs on EMT to target metastasis.

Herein, we primarily assessed the efficacy of PdPL to treat metastasis using different cancer models. We found that PdPL showed remarkable tropesis to target metastatic cancer cells, and strikingly suppressed lung metastasis. Mechanistic investigation revealed that PdPL partially inhibited the metastatic propensity of cancer cells by constraining EMT which was dependent on the blockade of TGF-β signaling. Thus, PdPL represents a novel agent against cancer metastases.

RESULTS AND DISCUSSION

Synthesis and characterization of PdPL

Hexagonal PdPL was prepared by reducing Pd(acac)$_2$ using carbon oxide (Fig. S1), as described in our previous reports [19]. Transmission electron microscopy (TEM) analysis revealed that the obtained PdPL was monodispersed with an average particle diameter of approximately 11 nm (Fig. 1a). Furthermore, atomic force microscopy (AFM) showed that the thickness of the as-prepared PdPL was approximately 1.8 nm (Fig. 1b). Additionally, the as-prepared PdPL showed a strong surface plasmon resonance absorption in the NIR region, i.e., 808 nm (Fig. 1c), thereby indicating that they could be used in NIR-mediated phototherapy. To confirm this, we monitored the temperature of the PdPL solutions at different concentrations (6.25-100 μg/mL) over time under irradiation at 808 nm with NIR laser (1 W/cm$^2$, 600 s). The temperature was observed to have increased rapidly to 61.4 °C for the 100-μg/mL PdPL aqueous dispersion under NIR irradiation (Fig. 1d). The excellent photothermal performance of the PdPL was further verified using the infrared thermal imaging analysis (Fig. 1e). The PdPL showed high photothermal stability, and no agglomeration or aggregation was observed after NIR irradiation (Fig. S2) [19].
Additionally, no visible change was observed in the photothermal efficacy of the PdPL after irradiation for 4 cycles (5-min irradiation followed by a 5-min cooling interval) (Fig. S2a).

A crucial technical limitation of NIR irradiation is its shallow penetration into tissues, which is typically limited to 3.4 cm [20]. To determine if the NIR laser was able to penetrate the chest shield in mice to target metastatic nodules in the lungs (a major target organ for cancer metastases), we assessed the photothermal efficacy of the PdPL under the chest shields that were dissected from mice (Fig. S3). The photothermal performance remained strong under these conditions, as a large increase in temperature (characterized by ΔT/°C) was observed in the PdPL solutions at all concentrations, with a maximum increase of 16.6 ΔT/°C at 100 μg/mL (Fig. 1f). These data indicated that the NIR irradiation adequately penetrated the murine chest, and the induced temperature elevation was sufficient to trigger the ablation of tumor nodules in the lung.

**Significant PdPL accumulation in primary tumors and metastatic tumors**

The tumor-targeting propensity of the PdPL was evaluated using our previously established mouse model of breast cancer lung metastasis from orthotopic tumors (Fig. 2a) [21]. In this mouse model, 4T1-derived subline LG12 (4T1-LG12) breast cancer cells showed highly efficient and extremely selective metastasis to the lungs [21]. Four weeks after orthotopic transplantation of 4T1-LG12 cells, after the lung metastases were well-established, 10 mg/kg body weight of PdPL was injected intravenously (i.v.) to the animals. Mice were sacrificed after 6, 12, 24, and 48 h injection, followed by the collection of primary tumors, metastatic lung nodules, and lung tissues adjacent to metastatic loci, and other organs (heart, liver, spleen, kidneys, and brain). The Pd content, measured by the inductively coupled plasma mass spectrometry (ICP-MS), in each organ confirmed that the PdPL mainly accumulated in the liver and spleen, as shown in Fig. 2b,
which was consistent with previous observations [22]. In support of the above-mentioned
findings, an agglomerated PdPL was also clearly observed in the liver specimens from treated
mice (Fig. S4). Notably, the PdPL was remarkably localized in tumors including the primary and
metastatic tumors (Figs 2c-e), thus showing PdPL amounts comparable to that observed in the
liver and spleen after normalization (Fig. 2b). Additionally, a time-dependent increase in the
accumulation of the PdPL in the primary tumors was observed (Figs 2b-c), as the PdPL amount
at 24 h and 48 h was about 3- and 5-fold higher than that at 6 h (P<0.001), respectively. A
similar accumulation of the PdPL was also observed in the metastatic tumors, however, the mass
decreased at 48 h after peaking at 24 h (Figs 2b-c). In contrast, no significant PdPL accumulation
was observed in the adjacent normal lung tissue (Fig. 2c). These findings confirmed the
outstanding tendency of the PdPL to localize at the primary tumors and metastatic tumors. It is
important to note that our findings significantly differed from previous reports, where the ENMs
were reported to target primary tumors dependent on selective targeting modifications, such as
antibodies, small molecules, and tumor-targeting peptides [23-25]. For solid tumors, the
accumulation of the PdPL mainly depends on the enhanced permeability and retention (EPR)
effect, through which the accumulation of the PdPL within tumors is much greater than that in
normal tissues due to their leaky vasculature and poor lymphatic drainage [26,27]. The
aggressive growth of metastatic tumors in the lung damages the architecture of the alveoli,
thereby leading to an easy exchange of substances in and out of the tumor microenvironment
[28,29]. Under this condition, a mechanism similar to that occurring in the primary tumors was
proposed to be responsible for the retention of the nanoparticle in the metastatic tumors.
Moreover, the pronounced tropesis of the PdPL to intrude metastatic tumors in the lung can be
ascribed to its two-dimensional (2D) nanostructure, in analogy with the molecular basis, as we
recently discovered for other 2D nanomaterials, such as graphene oxide [30]. In support of this finding, we found that molybdenum disulfide/graphene oxide (MoS$_2$/GO), a 2D nanocomposite, displayed selectively target the lungs, owing to the easy capture of the lung capillary vessels for the GO-protein complexes, as described in our previous report [30]. Therefore, our findings revealed that the PdPL could effectively target primary tumors, and metastatic nodules in the lung.

**Suppression of lung metastasis by PdPL in various murine models**

The anti-metastasis efficiency of the PdPL was assessed using various mouse models (Fig. 2a). First, the effects of the PdPL administration on the metastatic propensity of the $i.v.$ injected 4T1-LG12 cells ($2 \times 10^4$ cells/mouse) were assessed with and without NIR irradiation (2 W/cm$^2$, 5 min/each side) (Fig. 2a$_2$). The bioluminescence data showed that lung metastases were observed in all groups 2 weeks after the $i.v.$ injection of the 4T1-LG12 cells (Fig. 3a), which was in agreement with our previous report [21]. Mice were randomly divided into four groups including the control group: (equal volume of phosphate-buffered saline [PBS]); NIR group: NIR irradiation (2 W/cm$^2$, 5 min/each side); PdPL group: PdPL injection (10 mg/kg body weight); and NIR+PdPL group: PdPL injection (10 mg/kg body weight) and NIR irradiation (2 W/cm$^2$, 5 min/each side). The term 5-min/each side refers to irradiation of the back and chest of the mouse for 5 min. During the 15-day treatment period, we found that in comparison with the untreated groups, the metastatic tumor growth of the PdPL- and NIR+PdPL-treated groups was restrained immediately after PdPL administration, particularly in combination with NIR irradiation (Fig. 3, P<0.001, P<0.05), with a dose (2 W/cm$^2$, 5 min/each side), which was consistent with most previous reports [31,32]. It is important to note that NIR irradiation alone did not inhibit
metastasis formation or growth (Fig. 3).

To corroborate these observations, histological examination with hematoxylin-eosin (H&E) staining was performed (Fig. 3c). Multiple lung nodules were observed in the untreated mice and the NIR-treated mice; however, a few nodules were found in the lungs of the PdPL-treated mice, and almost no modules were found in the lungs of NIR+PdPL-treated mice (Fig. 3c). Nodule counts confirmed these differences, as the PdPL treatment led to a 51% reduction in nodule number, while the NIR+PdPL treatment caused 82% reduction, compared to the untreated controls (Fig. 3d, P<0.05). Additionally, the H&E staining also revealed that the PdPL and NIR+PdPL treatment caused no overt damage to the normal lung tissues and other organs (heart, liver, spleen, and kidneys) (Figs 3c and S5).

To substantiate the above-mentioned findings, we investigated the suppressive effect of the PdPL on the lung metastasis from orthotopic primary breast tumors in a murine model (Fig. 2a). Briefly, mice were injected with 4T1-LG12 cells (1×10⁴ cells/mouse) at the 4th mammary fat pad. 10 mg/kg body weight of PdPL was injected through the intravenous tail once, until the volume of the primary tumors reached 100 mm³ (approximately 3 weeks). NIR irradiation was executed for the PdPL+NIR group (2 W/cm², 5 min/mouse). The control group was subjected to the same volume of injection with PBS simultaneously. As shown in Figs S6a-d, the PdPL+NIR administration almost completely ablated primary tumors, and also restrained metastatic tumor growth with a 76% reduction of the lung tumor nodules in the lungs, in comparison with the untreated control (P<0.001). Despite being milder than the PdPL+NIR treatment, the PdPL treatment significantly constrained 4T1-LG12 lung metastasis with a 53% reduction of the lung tumor nodules in comparison with the control group (Fig. S6d, P<0.05). Notably, the PdPL per se treatment also inhibited the primary tumor growth with approximately 24% reduction of the
tumor weight in comparison with the control group (Fig. S6c, P<0.05). Nonetheless, the body weight of these mice was not significantly altered during treatment (Fig. S6e). Furthermore, no significant systemic inflammation was found in these mice, as evidenced by the non-responsive induction of the inflammatory cytokines (Fig. S7). Moreover, no abnormal variations were observed in the complete blood count (CBC) and blood biochemical indices of the PdPL group, compared to those in the untreated mice with and without cancer cell implantation (Figs S8 and S9). In contrast to the reports of previous studies, where the ENMs relied on the ablation of primary tumors to inhibit metastases [9,33], the findings in this study suggested that the PdPL per se profoundly repressed breast cancer lung metastasis irrespective of complete elimination of primary tumors, and also indicated that the PdPL exhibited an ability to repress primary tumor growth.

To further understand the inhibitory effect of the PdPL on primary tumors, we established a breast cancer mouse model with subcutaneous 4T1-derived subline PD1 (4T1-PD1) breast cancer cells (2×10^5 cells/per mouse), which harbored no metastatic capability to distant sites. Similar to the above-mentioned results as described in the metastasis model (Fig. S6), Figure S10 shows a marked decline of the primary tumors with approximately 40% drop in the tumor weight upon PdPL treatment (10 mg/kg body weight) (P<0.05), compared to that in the control group. Furthermore, as shown in Fig. S11, histological examination with H&E staining indicated that the PdPL did not cause significant injuries to the heart, liver, spleen, lung, and kidney. Additionally, the levels of inflammatory cytokines, including interferon (IFN)-γ, interleukin (IL) 6, and tumor necrosis factor (TNF)-α, were not significantly induced in the PdPL-treated mice, compared to those in the control mice (Fig. S7). Furthermore, no abnormal changes were observed in the PdPL-treated mice for the blood tests including white blood cell (WBC),
platelets (PLT), red blood cells (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), platelet distribution width (PDW), hematocrit (HCT), hemoglobin (HGB), neutrophils (NE), and serum aspartate aminotransferase (AST) (Figs S8 and S9). Nonetheless, tumor implantation in both models triggered systemic inflammation, as evidenced by the significant increase in the WBC count (Fig. S8), which was in agreement with previous findings [34,35]. Additionally, previous research also demonstrated the high biocompatibility of Pd materials in diverse animal models with acute and chronic treatment [16,36]. In summary, the biosafety and biocompatibility of PdPL-based nanomaterials have been investigated comprehensively in many animal models, including mice, rats, and rabbits through different methods of administration including oral administration, intraperitoneal (i.p.) injection, and i.v. administration. Previous reports have demonstrated the favorable biocompatibility of PdPL-based nanomaterials towards tissues and organs after short and long periods [16]. Collectively, these data implied that PdPL could interact with tumor cells either by the interaction of the PdPL accumulated at the tumor site depending on the EPR effect, or by other mechanisms such as active uptake by tumor cells and tumor-infiltrating immune cells [37,38]. We studied the effects of PdPL on tumor cells as described below.

We studied the effect of the PdPL on the proliferation in various cell lines. As shown in Figs S12 and S13, the PdPL did not inhibit cell division at lower concentrations, however, a significant repression of proliferation was observed at the highest concentration, 100 μg/mL, at 48 h post treatment in all cells, including the 4T1-LG12, parental 4T1, 4T1-PD1, B16F0, B16F10, human bronchial epithelial (BEAS-2B), and human umbilical vein endothelial (HUVE) cells, as measured by the cell counting kit 8 (CCK-8) and Alamar blue (AB) assays. These
results suggest that the PdPL inhibited cell division at a high dose, which is in agreement with the *in vivo* findings of the inhibitory effect of accumulated PdPL on tumor growth at the tumor sites. Therefore, the anti-metastatic feature of the PdPL can be attributed to the repression of primary tumor cells and the impairment of the propensity of inborn metastasis induced by the PdPL.

**Alteration of the intrinsic metastatic propensity of cancer cells by PdPL**

We determined the underlying molecular mechanisms of the PdPL-induced suppression of the metastatic propensity of breast cancer cells (Fig. 4a). The cellular morphology of the 4T1-LG12 cells after PdPL treatment substantially changed from a spindle-like shape to a pebble-like shape, namely from a “mesenchymal” to an “epithelial” shape, and their length greatly decreased post treatment, compared to the untreated cells (Fig. 4b), thereby suggesting a large reduction of the invasive and metastatic features of the 4T1-LG12 cells, and the likely occurrence of mesenchymal-epithelial transition (MET). Thereafter, the PdPL-pretreated 4T1-LG12 cells were xenotransplanted into mice (Fig. 4a) to determine the influence of the PdPL on the metastatic behaviors of the 4T1-LG12 cells. As shown in Figs 4c-e, the lung metastasis of the 4T1-LG12 cells was considerably constrained (54% reduction of metastatic nodules; P<0.05) following PdPL pretreatment in comparison to the untreated cells. Additionally, bioluminescence imaging revealed a similar reduction in metastatic cancer cell mass in the mice transplanted with PdPL-pretreated cells (Fig. S14, P<0.001).

To understand the significant suppression of cancer metastasis in response to the PdPL pretreatment, we examined another model based on the B16F10 melanoma cell line, which metastasizes to the lung quickly after i.v. injection. In agreement with the findings of the 4T1-
LG12 cells, a significant decrease of tumor nodules (65% decrease) was found in the lungs of the mice that acquired the PdPL-pretreated B16F10 cells, compared to the mice that received control cells (Fig. S15, P<0.05). These results revealed that the PdPL considerably undermined the inherent metastatic propensity of cancer cells. In fact, some monoclonal antibodies (e.g., 2G7 and 1D11), acting as pan-TGF-β-neutralizing antibodies, have been used to block lung metastases [39,40]. Based on the above-mentioned results, it could be inferred that the PdPL could have functions similar to those of the TGF-β-neutralizing antibody. Therefore, the PdPL harbors a great potential to block TGF-β actions, suggesting that the PdPL is a promising therapeutic agent for treating TGF-β-dependent disorders.

**Impairment of the EMT signaling pathways by PdPL**

To understand the molecular basis for the reduction of the metastatic potential of tumor cells by the PdPL, we conducted close mechanistic investigations. First, we established that higher concentrations of PdPL (up to 100 μg/mL) were not overtly toxic to the 4T1 cells (Fig. S16). Our previous research also found little toxicity of PdPL in non-tumor normal cells, including NIH-3T3 (normal mouse fibroblasts) and QSG-7701 (normal human liver cells) [18]. These combined data demonstrated that PdPL elicited little toxicity to normal cells. Moreover, our data showed that PdPL could be readily taken up by the 4T1-LG12 cells (Fig. S17). Additionally, the ICP-MS analysis revealed an increase in cellular uptake of PdPL over the time course from 6 to 12 and 24 h in the 4T1-LG12 cells responding to PdPL at 50 μg/mL (Fig. 5a, P<0.05), which was similar to that in macrophages [41,42]. These results confirmed the massive accumulation of the PdPL in the 4T1-LG12 cells. To confirm this finding, we evaluated the endocytosis using breast cancer cells with lower metastatic potential and numerous metastatic tumor cells and epithelial cells,
including 4T1-LG12, 4T1, 4T1-PD1, B16F0, B16F10, BEAS-2B, and HUVE cells. As shown in Fig. S18, the ICP-MS analysis revealed that more PdPL materials were internalized by cells with high metastatic potential such as 4T1-LG12 and B16F10 cells, in comparison with their parental 4T1 and B16F0 cells with lower metastatic potential at 24 h (P<0.05 and P<0.001). Additionally, an active endocytosis of the PdPL was observed in other cells, but with much less uptake in comparison with the 4T1-LG12 cells at 24 h (P<0.05). Thus, our data indicated enhanced endocytosis of the PdPL by cancer cells with higher metastatic potential, thus highlighting the importance of PdPL targeting metastatic loci.

Since PdPL has a high photothermal efficacy when internalized by the 4T1-LG12 cells, NIR irradiation should be able to kill the cells. As shown in Fig. 5b, a dose-dependent phototoxicity was observed, which was consistent with the in vivo results, as described above (Figs 3 and S5-10). Given that metastasis is critically dependent on the adequate motility of the cells from the primary tumor, we analyzed the effect of the PdPL treatment on the migration/motility of the 4T1-LG12 cells. The Transwell migration assay revealed that the transmigration of the PdPL-treated 4T1-LG12 cells reduced by 65% in comparison with the untreated cells, as shown in Figs 5c and S19a (P<0.001). Similarly, the wound-healing assay showed a 73% decrease in the cell motility of the PdPL-treated 4T1-LG12 cells in comparison with the untreated cells (Figs 5d and S19b, P<0.001). These in vitro data indicated that PdPL inhibited the metastatic behaviors of cancer cells by diminishing their motility and migration.

Thereafter, to gain detailed mechanistic insights into the reduction of the metastatic potential of tumor cells by the PdPL, we carried out RNA sequencing (RNA-Seq) to exploit differentially expressed messenger RNAs (mRNAs), long non-coding RNAs (LncRNAs), and microRNAs (miRNAs) using RNAs extracted from parental 4T1 and 4T1-LG12 cells with and without PdPL
treatment and with and without NIR irradiation. To illustrate the overall gene expression profiles, the R script was used to generate heatmap plots. As shown in Fig. 5e, an overall decreased gradient of gene expression (shown in color from red to purple) on the left side (divided by two black arrowheads), and otherwise increased gradient expression (shown in color from purple to red) on the right side were observed in the both parental 4T1 cells and 4T1-LG12 cells treated with PdPL and PdPL+NIR, compared to untreated cells. To validate the RNA-Seq data, reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) analyses were carried out, and a strong correlation was observed between the RNA-Seq and RT-qPCR results (Fig. S20, P<0.001), thus verifying the reliability of our RNA-Seq findings. These data therefore uncovered significant gene expression changes in the 4T1 cells upon the PdPL treatment and even more dramatic gene expression changes in these cells upon the PdPL+NIR treatment, thereby implying fundamental gene expression changes in the 4T1 cells.

To confirm metastasis-related genes that were differentially expressed in the 4T1 cells in response to the PdPL treatment, we compared the differentially expressed genes to the gene set that had been documented to be involved in breast cancer metastases [14,43-45]. Overlapping analysis recognized 115 common genes in our differentially expressed genes upon the PdPL and PdPL+NIR treatment, in comparison with the untreated cells. Comprehensive analyses using the online tools DAVID 6.8 and Metascape, together with data mining based on the current literature, showed that nearly all these genes were involved in EMT. Figures S21 and S22 show some important EMT-associated genes responsible for tight junctions, cell proliferation, cell-matrix adhesion, cell differentiation, angiogenesis, and cell-cell communication function. In substantiating these changes, increased occludin and reduced β-catenin were verified at the protein level in the orthotopic tumors following the PdPL treatment, particularly the PdPL+NIR
treatment (Fig. 5f). Additionally, a consistent increase in the occludin and decrease in the β-catenin levels were demonstrated in the 4T1-LG12 cells in vitro upon PdPL treatment (Fig. 5g). It is generally believed that EMT is the driving force for cancer metastases, as the EMT program essentially remodels the cytoskeleton from the polar epithelial cell phenotype to an actin stress fiber dominated phenotype indicative of mesenchymal cells [5,46]. Specifically, the enhanced β-catenin signaling promotes metastasis in various cancers, due to the transcriptional activation of the genes responsible for EMT induction [47]. In contrast, the loss of occludin, which is necessary for the integrity of tight junctions between epithelial and endothelial cells, can result in the loss of cell-to-cell adhesion, thereby leading to reinforced EMT [48]. Therefore, the differential changes of these EMT-associated genes undermine the inherent EMT of 4T1 cells, namely the reprogramming to MET, in response to the PdPL treatment. Additionally, the transcriptome analysis also revealed that a number of proliferation-related genes were disturbed upon PdPL treatment, such as the cleavage stimulation factor subunit 3 (Cstf3), lymphocyte antigen 6 family member e (Ly6e), and glutaminase 2 (Gls2) (Figs 5e and S21), further supporting the PdPL-induced suppression of the tumor cell growth.

Blockade of TGF-β signaling by PdPL and damage to the metastatic potential of cancer cells

Further, we attempted to probe the upstream regulators that may be responsible for the compromised EMT induced by the PdPL. Since TGF-β plays a central role in governing EMT by regulating some target genes [6,49], we compared our RNA-Seq results with the previously reported TGF-β target genes [50,51], and a wealth of common genes were found in between. To substantiate these changes, the target genes were further assessed by RT-qPCR in the cells upon
the PdPL treatment. As shown in Fig. S23, blunted TGF-β signaling upon PdPL treatment was confirmed by the differentially expressed genes including Id1, Id3, Lcn2, and Ndrg1 in the 4T1 and 4T1-LG12 cells (P<0.05 and P<0.0001). As previously established, Smad2/3 phosphorylation is the most important surrogate to recognize TGF-β signaling activation [52,53]. Therefore, phosphorylated Smad2 (P-Smad2) was examined using western blotting in tumor specimens. As shown in Fig. 6a, a decrease in P-Smad2 concentration was found in the tumors from mice upon PdPL treatment, especially PdPL+NIR treatment, thereby indicating the significant inhibition of TGF-β signaling via Smad2/3 in the tumors upon PdPL treatment. However, it should be noted that TGF-β conducts both canonical Smad-dependent signaling and Smad-independent signaling to promote EMT. In fact, as described above, our data also recognized Smad-independent targeting of downstream of TGF-β, such as Ocln [54], and the PdPL-induced changes in these genes were assumed to contribute to the inhibition of EMT. To verify the disrupting influence of the PdPL on TGF-β signaling activation, recombinant TGF-β protein was used to treat cells after incubation with the PdPL. As shown in Figs 6b and S24, TGF-β1-induced phosphorylation of Smad2 was remarkably diminished by the PdPL in both the 4T1 and 4T1-LG12 cells in a dose-dependent manner. Hence, the above data revealed great disruption of PdPL to TGF-β signaling in 4T1 cells.

Finally, to understand the underlying mechanism of the disrupting effect of the PdPL on TGF-β signaling, the likely interaction between the PdPL and TGF-β protein was investigated. The active TGF-β protein, in the form of dimeric ligands through a disulfide bridge, was prepared and incubated with the PdPL at various concentrations, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. As shown in Fig. 6c, outstanding binding between the PdPL and TGF-β protein was observed, especially at higher
concentrations, as evidenced by less proteins around 25 kDa (in the form of dimers, denoted by the second black arrowhead) and greater proteins around the loading wells (in the form of PdPL/TGF-β complex, as indicated by the first black arrowhead). These data denoted the significant interaction between the PdPL and TGF-β. Notably, this interaction was not strong enough to break the disulfide bridge, as no monomer around 12.5 kDa was visualized (indicated by the third black arrowhead), in contrast to a pronounced band around 12.5 kDa for the TGF-β protein with the addition of DL-Dithiothreitol (DTT). DDT, as a robust detergent, is sufficient to break disulfide bridges between dimeric TGF-β proteins. These results indicated that the PdPL associated with the TGF-β protein, and therefore, undermined its downstream signaling. To substantiate the interaction between the PdPL and TGF-β protein, direct binding and affinity were assayed using surface plasmon resonance (SPR) measurements. As shown in Fig. 6d, a remarkable association was observed between the PdPL and TGF-β1 protein, and the resultant binding constant value (KD) was calculated to be $7.59 \times 10^{-7}$ M. This value is similar to that of the interaction between graphene nanosheets and bovine fibrinogen (BFG, $1.97 \times 10^{-7}$ M), immunoglobulin (Ig, $7.67 \times 10^{-7}$ M), transferrin (Tf, $3.66 \times 10^{-6}$ M), and bovine serum albumin (BSA, $6.41 \times 10^{-6}$ M), where graphene has been demonstrated to be a very active type of nanomaterial to readily absorb proteins [55,56]. Therefore, we added more evidence to demonstrate the interaction between TGF-β1 and the PdPL.

EMT is a complex biological process that drives tumor metastasis; however, this process has not been fully understood, and TGF-β is not the only modulator in this process. Thus, we speculated that the PdPL-induced EMT repression might also involve other mechanisms, in addition to the direct disturbance of PdPL in TGF-β signaling. Therefore, we analyzed other possible regulators of EMT-associated genes. The analysis of the differentially expressed
LncRNAs and miRNAs showed that some LncRNAs and miRNAs were predicted to target genes involved in EMT-related signaling pathways, such as miRNAs (e.g., novel_mir111 and novel_mir52) and LncRNAs (e.g., NONMMUT075484.1 and NONMMUT036870.2) (Figs S25 and S26). Although these findings are very intriguing, the PdPL may also target other proteins and RNAs to compromise EMT. Therefore, extensive study on the detailed regulation network response to PdPL is required.

CONCLUSION

By thoroughly evaluating the multifaceted advantages of Pd nanomaterials in anti-cancer applications, we discovered that PdPL showed a marked preferential inclination to attack both primary tumors and metastatic tumors. Importantly, PdPL harbored a robust inherent property against metastasis irrespective of the suppressive effect on the primary tumor site. Mechanistically, we revealed that PdPL could considerably scotch metastasis (at least in part) by constraining EMT which was dependent on the inhibition of TGF-β signaling. TGF-β-independent molecular mechanisms have also been proposed. Additionally, NIR irradiation enhanced the anti-metastatic effects of PdPL. Here, we provided new evidence on the PdPL-induced anti-metastatic effects by constraining EMT, and also opened a new path for the development of target-specific nanomedicine in cancer therapeutics. This work provides a promising therapeutic option to overcome the challenges associated with the treatment of metastases.

METHODS

Detailed methods could be found in the supplementary data.
SUPPLEMENTARY DATA

Supplementary data are available at NSR online.

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Figure 1. Synthesis and characterization of PdPL. (a) TEM analysis of the PdPL with an insert showing the size distribution. (b) AFM analysis of the PdPL lateral size distribution. (c) The absorption spectrum of the PdPL. (d) Temperature elevation of the PdPL solutions at different concentrations, irradiated by NIR (808 nm, 1 W/cm$^2$, 600 s). (e) IR thermal images of the PdPL
at different concentrations. (f) Temperature elevation of the PdPL through \textit{ex vivo} murine thorax shield, irradiated by NIR (808 nm, 1 W/cm\(^2\), 300 s).
Figure 2. Accumulation of PdPL in metastatic tumors. (a) Schematic illustration of the establishment of breast cancer metastasis models, including (a) orthotopic metastatic model and
(a2) tail-vein metastatic model. (b, c) Tissue distribution of the PdPL in mice with lung metastasis derived from orthotopic transplantation of 4T1-LG12 cells at different time points post PdPL (10 mg/kg body weight) injection (n=5). (d, e) Representative H&E staining images of the lungs from PdPL-treated mice. Green arrowheads denote the lung metastatic tumors. Dark blue arrowheads indicate the localized PdPL in the lungs.
Figure 3. PdPL elicited substantial suppression on 4T1-LG12 cell metastasis towards the lungs in various models. (a) Bioluminescent signals were recorded in the lungs in different groups via the spectral imaging systems over time (n=5). (b) Quantitative curves of the bioluminescent signals in the lungs in different groups. (c) H&E-stained images of lung tissue sections. Dark blue arrowheads point at tumor nodules in the lung sites. (d) Count of lung nodules at 15th day (n=5).
Figure 4. PdPL undermined the inherent metastatic propensity of 4T1-LG12 cells. (a) Schematic illustration of the establishment of lung metastasis model derived from the 4T1-LG12 cells upon PdPL pre-treatment for 24 h at 50 µg/mL. (b) Morphological alterations are displayed with phase-contrast images of the 4T1-LG12 cells upon PdPL treatment at 50 µg/mL for 24 h. (c) Count of lung nodules after injection of 4T1-LG12 cells with and without PdPL pre-treatment through the tail vein after 3 weeks (n=5), and (d) H&E-stained images of lung tissue sections and
(e) representative lung photographs from these mice. Dark blue arrowheads point at metastatic lesions in the lung tissue sections (d). White arrowheads indicate metastatic tumors in the lungs (e).
Figure 5. PdPL changes gene expression and restrains TGF-β signaling on cancer metastatic behaviors. (a) Quantitative analysis of the intracellular PdPL content by ICP-MS after different incubation times (n=4). (b) The phototoxic effect on the 4T1-LG12 cells in response to the
NIR+PdPL treatment at 50 μg/mL for 3 min (1 W/cm²) followed by additional 24-h incubation (n=4). Representative images of (c) Transwell migration/invasion assay and (d) wound-healing assay for the 4T1-LG12 cells with and without the PdPL treatment at 50 μg/mL for 24 h. The migration distances of the 4T1-LG12 cells were measured by the Image J software. (e) The heatmap plots of differentially expressed genes from RNA-Seq data of parental 4T1 and 4T1-LG12 cells responding to PdPL with or without NIR treatment for 24 h. (f) Western blot determination of Ocln (Occludin) and Ctnnb1 (β-catenin) in primary tumors from mice challenged by PdPL and PdPL+NIR. (g) Protein levels of Ocln and Ctnnb1 in the 4T1-LG12 cells upon PdPL treatment for 24 h.
Figure 6. PdPL targets TGF-β protein to diminish its signaling pathway. (a) Levels of P-Smad2 in primary tumors from mice after the PdPL and PdPL+NIR treatments. (b) Alterations of P-Smad2 in the 4T1-LG12 cells responding to recombinant TGF-β1 protein with and without the PdPL treatment. TGF-β1 at 10 ng/mL was first incubated with PdPL at various concentrations (from 0.04 to 1 mg/mL) at 37 °C for 30 min and then used to treat cells for 12 h. Cellular lysates were thereafter subjected to western blot analysis. (c) Binding assessment between PdPL and
TGF-β1. Recombinant TGF-β1 protein (50 ng) was incubated with PdPL at different concentrations (from 0.008 to 5 mg/mL) for 30 min at 37 °C, followed by SDS-PAGE and silver staining analysis. The band at the location of 12.5 kDa represents TGF-β1 monomer, and that at the location of 25 kDa denotes TGF-β1 dimer. Here, the sample loading buffer containing DTT was used to obtain TGF-β1 monomer. (d) Measurement of the TGF-β1-PdPL binding affinity through SPR affinity measurements (Biocore S200). Association and dissociation curves of the TGF-β1 protein at 15 μg/mL incubated with PdPL at different concentrations.