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Dual-targeting Wnt and uPA Receptors Using Peptide Conjugated Ultra-small Nanoparticle Drug Carriers Inhibited Cancer Stem-Cell Phenotype in Chemo-Resistant Breast Cancer

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Abstract

Heterogeneous tumor cells, high incidence of tumor recurrence, and decrease in overall survival are the major challenges for the treatment of chemo-resistant breast cancer. Results of our study showed differential chemotherapeutic responses among breast cancer patient derived xenograft (PDX) tumors established from the same patients. All doxorubicin(Dox)-resistant tumors expressed higher levels of cancer stem-like cell biomarkers, including CD44, Wnt and its receptor LRP5/6, relative to Dox-sensitive tumors. To effectively treat resistant tumors, we developed an ultra-small magnetic iron oxide nanoparticle (IONP) drug carrier conjugated with peptides that dually targeted to Wnt/LRP5/6 and urokinase plasminogen activator receptor (uPAR). Our results showed that simultaneous binding to LRP5/6 and uPAR by the dual receptor targeted IONPs was required to inhibit breast cancer cell invasion. Molecular analysis revealed that the dual receptor targeted IONPs significantly inhibited Wnt/ β -catenin signaling and cancer stem-like phenotype of tumor cells, with marked reduction of Wnt ligand, CD44 and uPAR. Systemic administration of the dual targeted IONPs led to nanoparticle-drug delivery into PDX tumors, resulting in stronger tumor growth inhibition compared to non-targeted or single-targeted IONP-Dox in a human breast cancer PDX model. Therefore, co-targeting Wnt/LRP and uPAR using IONP drug carriers is a promising therapeutic approach for effective drug delivery to chemo-resistant breast cancer.

Graphical Abstract

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Keywords

Multiplexed peptide conjugated nanoparticle; Wnt/LRP receptors; uPAR; cancer stem cells; and chemo-resistant breast cancer

Introduction

Breast cancer is the most commonly diagnosed form of cancer in women worldwide [1]. Chemotherapy remains the main therapeutic option for the treatment of breast cancer patients with metastatic diseases or that have the triple negative breast cancer subtype, which lacks expression of estrogen receptor, progesterone receptor, and HER2. Unfortunately, a high percentage of those cancer patients are resistant to chemotherapy. The presence of residual chemo-resistant tumors following neoadjuvant therapy is one of the most important prognostic factors for tumor recurrence and short survival regardless of hormone receptor status and pathological stages of the disease [2]. Therefore, the development of effective therapies for overcoming chemo-resistance is a critical unmet need.

Breast cancer tissues have been found to contain a sub-population of cancer stem-like cells (CSCs) or progenitor cells, which are identified as CD44^{high}/CD24^{low} and are an important cell population to investigate when developing therapies for chemo-resistant breast cancers [3,4]. Breast cancers that are enriched with the CSC population appear to be more drug resistant and are associated with a poorer prognosis [5–7]. Because of their self-renewal capacity, failure to eliminate CSCs may mediate a patient's relapse. These recurrent tumors not only are resistant to the initial treatment but also exhibit a more aggressive phenotype than that of the original tumor. Therefore, more potent therapeutic agents are needed to treat drug-resistant breast cancer.

The Wnt/β-catenin pathway is involved in the regulation of proliferation and migration of tumor cells, the epithelial-mesenchymal transition, and the maintenance of stemness in cancer stem cells [8–11]. When the Wnt/β-catenin pathway is inactive, cytosolic β-catenin is bound to a complex consisting of adenomatous polyposis coli (APC) and Axin, which promotes phosphorylation of β-catenin by casein kinase-1 (CK1) and glycogen synthase kinase 3 (GSK3). Phosphorylated β-catenin is then ubiquitinated and degraded [12]. Upon Wnt ligand binding to co-receptors LRP5/6 and Frizzled, the Wnt/β-catenin pathway is activated and β-catenin is freed from the APC/Axin destruction complex and accumulates in the nucleus where it binds to transcription factors to activate gene transcription (Figure 1). However, it has been shown that Axin is a multifunctional signal molecule that interacts with

the Wnt-induced Frizzled-LRP6-Dishevelled complex to mediate GSK3 phosphorylation of LRP6, initiating activation of the Wnt signaling [13].

Phase 1 clinical trials to develop targeted therapies for the Frizzled receptor, such as Vantictumab, have been completed in solid tumors [14,15]. There are currently no LRP5/6 targeted therapies, though LRP6 has shown to have an important role in Wnt pathway activation in breast cancer [16]. Upregulation of LRP6 has been found in a variety of solid tumors and, when overexpressed, LRP6 has been shown to promote tumorigenicity, cell invasion, and metastasis [17,18]. Studies in breast cancers have also shown that inhibition of Wnt/ β -catenin signaling by blocking LRP6 resulted in the reduction in the ability of cancer cells to self-renew and for tumor cells to undergo epithelial to mesenchymal transition, resulting in suppression of tumor growth [16,19–21].

The urokinase plasminogen activator system, consisting of urokinase plasminogen activator (uPA) and its receptor uPAR, is also a target of interest in breast cancer. Binding of uPA to uPAR leads to the activation of plasminogen to plasmin, and then proteases, such as matrix metalloproteinases, which promote tumor cell invasion and metastases. Expression of uPA and uPAR have been identified as biomarkers associated with the development of recurrent and/or metastatic breast cancers [22]. Inhibition of uPAR decreased cell viability, migration, and cell invasion of breast cancer cells [23]. Overexpression of uPAR induced cancer stem-like properties in breast cancer and led to the activation the Wnt/ β -catenin pathway [24,25]. It has been shown that uPAR can bind to β -catenin in the cytoplasm and enter the nucleus to facilitate β -catenin-mediated gene transcription while inhibition of uPAR reduced the level of expression of Wnt/ β -catenin signal activated genes [25]. On the other hand, activation of Wnt/ β -catenin signal pathway also increased the level of uPAR expression [26].

To develop a new therapy targeting chemo-resistant and stem-like breast cancer cells that have a high level of Wnt receptors and activated Wnt signaling, we designed a Wnt receptor LRP5/6 binding peptide (iWnt) based on the LRP5/6 binding region of Dickkopf-related protein-1 (DKK1). Previous studies showed that the binding of DKK1 to LRP5/6 inhibited the activation of Wnt/ β -catenin pathway [27]. A DKK1 derived peptide should be a good mediator for both inhibition of Wnt signaling and delivery of nanoparticle drug carriers into tumor cells since the formation of the complex of DKK1, LRP6 and Kremen-2 induced endocytosis [28]. Therefore, the iWnt peptide conjugated nanoparticle was expected to block WNT/ β -catenin signaling and allow targeted drug delivery into cancer stem-like cells.

To improve tumor cell targeting, facilitate internalization for drug delivery, and enhance the effect on inhibition of Wnt signaling in cancer stem-like cells, we developed a dual Wnt/ LRP5/6 and uPAR targeted nanoparticle by conjugating both iWnt and uPAR targeting ATF₂₄ peptides with three histidine residues (His₃) to nitrilotriacetic acid-copper (NTA-Cu) modified and amphiphilic polymer coated iron oxide nanoparticles (IONPs). We then evaluated the effect of conventional chemotherapy drugs and targeted nanoparticle drug carriers in human chemo-resistant breast cancer tissue derived tumor xenograft (PDX) models in nude mice. Here we report that chemo-resistant breast cancer PDX tumors exhibit heterogeneity in response to the chemotherapeutic drug, doxorubicin (Dox). We found that Dox-resistant breast cancers have increased levels of Wnt receptor LRP5/6 and uPAR

expression compared to drug-sensitive tumors. Treatment of human breast cancer cells *in vitro* with the dual Wnt/LRP and uPAR-targeted nanoparticles reduced CD44^{high}/CD24^{low} cancer stem cell population, and inhibited the epithelial to mesenchymal transition, resulting in decreased cell invasion. We further found that systemic delivery of the dual-targeted nanoparticles carrying Dox led to targeted delivery and inhibited the Wnt/ β -catenin pathway, cancer stem cell phenotype, and tumor growth in the chemo-resistant breast cancer PDX models. Our results demonstrated that the NTA-Cu modified ultra-small IONP provides a drug delivery platform for the development of targeted nanoparticles using peptide-based targeting ligand and/or therapeutic peptides. The dual receptor targeted nanoparticle drug carrier developed in this study has the potential to provide new molecular targeted nanoparticle drug delivery systems for the treatment of chemo-resistant breast cancer.

Results

Chemo-resistant breast cancer cells have upregulated levels of biomarkers associated with cancer stem cells

To identify cell surface molecular targets for the development of novel targeted therapies for chemo-resistant breast cancer, we established orthotopic human breast cancer PDX models derived from surgically resected residual chemo-resistant breast cancer tissues in patients following neoadjuvant therapy. The PDX tumors were passaged in nude mice and *in vivo* studies were conducted using the passage number as indicated in Figure 2. Dox treatment was started once PDX tumors reached to tumor volumes around 50–100 mm³. Following treatment with 5 mg/kg of Dox weekly for 5 treatments, we observed differential responses of the PDX tumors derived from the same breast cancer patients. Within the group of 6 mice that were treated with the same dose and schedule of Dox, PDX tumors showed significant differences in growth inhibition with drug sensitive tumors being 70 to 90% smaller than the resistant tumors. We selected representative tumor tissues that had significant tumor growth inhibition or stable tumor volume following the treatment to be Dox-sensitive, while those that had progressive tumor growth were determined to be Dox-resistant (Figure 2A). This heterogeneous response was observed both within the same patient's xenograft tumors at different passages and among different patient xenograft tumors. Figure 2 shows examples of results in the PDX tumor bearing mice that were selected from Western blot analysis. We found that Dox-resistant tumors had an increase in expression of Wnt/ β -catenin pathway receptor LRP5/6 and ligand Wnt1 as well as a high level of uPAR expression compared to Dox-sensitive tumors (Figure 2B). In addition, a significant increase in Wnt10b ligand, which has been shown to induce Wnt/ β -catenin activity and is significantly correlated with larger tumor size and poor survival in TNBC [29], was found in the Dox-resistant tumor tissues. A high level of cancer stem-like marker, CD44, was also detected in Dox-resistant tumors compared to no treatment control or Dox-sensitive tumors (Figure 2C–E).

Discovery of the expression of Wnt/ β -catenin pathway receptor LRP5/6 and uPAR in Dox resistant breast PDX tumors provided the opportunity to develop Wnt/LRP and uPAR-targeted nanoparticle drug delivery systems that can assist in overcoming drug-resistance in breast cancer.

Development and characterization of Wnt/LRP and uPAR single targeted and dual targeted IONPs

To develop a Wnt receptor targeted IONP for drug delivery and potential inhibition of the Wnt/ β -catenin pathway, we designed a LRP antagonist peptide mimetic that contains the 38 to 44 amino acid sequence of the LRP binding region of Dickkopf-related protein-1 (DKK1). As described previously [30], DKK1 binds to the E1 domain β -propeller of LRP5/6 through a conserved NXI sequence (Figure 3A). The inhibitor of Wnt (iWnt) DKK1 peptide mimetic has 7 amino acids from the human DKK1 sequence, including the NXI sequence, a 2-amino acid linker, and 3 histidine residuals that serve as a His-tag for conjugating the peptide to the NTA-Cu modified nanoparticle.

Results of our previous studies demonstrated that the full length amino terminal fragment (ATF, 135 aa) of uPA conjugated nanoparticles exhibited a high degree of target specificity into orthotopic tumors following systemic delivery in breast cancer mouse models for targeted tumor imaging and drug delivery [31]. The binding of ATF peptide conjugated nanoparticles to uPAR further mediates internalization of the nanoparticles [31]. In this study, we used a short peptide of the amino-terminal fragment of uPA from aa 3 to 26 (ATF₂₄) that contains the critical uPAR binding region fused with a 3-Histidine tag for nanoparticle conjugation and a unique cysteine at the amino-terminus for near-infrared (NIR) dye NIR-830-maleimide labeling (Figure 3B). The I-TASSER server [32,33] was used to generate and predict the peptide structure based on the amino acid sequence (Figure 3B). The use of the short peptides as uPAR targeting ligands should make it possible to produce targeting peptides in a large scale by chemical synthesis and to increase the density of targeting ligands conjugated to the nanoparticle.

iWnt and ATF₂₄ His-tagged peptides were conjugated to amphiphilic polymer coated IONPs (5 nm core size) modified with NTA-Cu using protocols described in the Materials and Methods. The molar ratios of IONP to peptide used for preparing the ligand conjugated IONPs were 1:20 for single targeted iWnt or ATF₂₄ and 1:10:10 for the dual-targeted (iWnt and ATF₂₄) configurations as shown in Figure 3C. Targeted IONPs encapsulated with Dox were produced using our established protocol [34] (Figure 3C). Examination of the hydrodynamic size of iWnt and ATF₂₄ peptide conjugated IONPs by dynamic light scattering (DLS) revealed that a polymer-coated IONP was 11.27 nm and conjugation of iWnt and ATF₂₄ increased its size to 23.74 nm (Figure 3D and Figure S1). Loading the iWNT-ATF₂₄-IONPs with Dox led to a slight increase in size to 25.8 nm (Figure 3D). Peptide conjugated nanoparticles have zeta potential of -30 mV (no Dox) or -34 mV (with Dox). Dox loading in single and dual targeted IONPs was 14% (w/w) of the IONP-Dox (Table S1).

Characterization of iWnt and iWnt-ATF₂₄ IONPs *in vitro*

First, we performed a pull-down assay using NTA-Ni conjugated agarose beads to determine binding specificity of iWnt and/or ATF₂₄ peptide conjugated IONPs in tumor cell lysates. Results indicated that iWnt and ATF₂₄ peptides bound to their respective receptors (iWnt to LRP5/6, or ATF₂₄ to uPAR) when compared to control shown in Figure 4A. Interestingly, iWnt peptide conjugated beads appeared to pull-down uPAR as well, which would indicate

the possibility of the interaction between LRP5/6 and uPAR. Our result that ATF₂₄ conjugated beads only pulled-down uPAR but iWnt-beads pulled-down both uPAR and Wnt/LRP suggested that a high portion of Wnt/LRP interacted with uPAR. Since the level of uPAR was much higher than that Wnt/LRP receptor in tumor cells as shown in the input sample in Figure 4A, considerable uPAR might not be associated with Wnt/LRP.

Specific binding and internalization of the iWnt and ATF₂₄ conjugated IONPs to human breast cancer cells was determined using human breast cancer MDA-MB-231 cells *in vitro*. Following incubation of the non-targeted-IONPs, single or dual-targeted IONPs for 6 hours, Prussian blue staining for iron, shown in Figure 4B, revealed higher levels of IONP in the cells incubated with targeted IONPs compared to that of non-targeted IONPs. However, there was no apparent difference between Wnt/LRP or uPAR single or dual targeted IONP treated cells.

The iWnt peptide was designed as a mimetic of the LRP5/6 inhibitor, DKK1. To demonstrate that iWnt-conjugated nanoparticle mimics DKK1 and binds to the DKK1 interaction site of the LRP, a competition assay was performed using a small molecule inhibitor of DKK1, NCI8642. NCI8642 has been shown to specifically bind to LRP5/6 at the DKK1 binding site [35]. In this assay, iWnt-IONPs or dual targeted iWnt-ATF₂₄-IONPs were labeled with a NIR 830 dye and incubated with MDA-MB-231 cells for 6 hours. By detection of NIR fluorescence signal in the cells, we found NIR fluorescence signals decreased with increasing NCI8642 concentrations, suggesting NCI8642 competing with iWnt-IONPs in binding tumor cells (Figure 4C). Quantification of fluorescence shows a significant decrease in binding of the iWnt-IONPs and dual targeted iWnt-ATF₂₄-IONPs at 50 μ M and 100 μ M of NCI8642 compared to no treatment control shown in Figure 4D. NCI8642 also reduced the binding of dual targeted iWnt-ATF₂₄-IONPs to cells. These results indicate that the interaction of iWnt with LRP5/6 was important for high affinity binding of dual targeted IONPs to tumor cells and addition of the ATF₂₄ did not hinder iWnt binding to LRP5/6.

iWnt-ATF₂₄-IONPs decreased the activation of Wnt/ β -catenin pathway and cell invasion in human breast cancer cells *in vitro*

To determine the effects of the binding of iWnt-IONPs, ATF₂₄-IONP, or dual targeted iWnt-ATF₂₄-IONPs to Wnt receptor and/or uPAR on the canonical Wnt/ β -catenin pathway and uPAR expression, MDA-MB-231 cells were incubated with iWnt-IONPs, ATF₂₄-IONP, and iWnt-ATF₂₄-IONPs. Western blot analysis of cell lysates showed that treatment of cells with the unconjugated IONPs decreased the level of active β -catenin for 68% compared to no treatment control, while not affecting total β -catenin expression. However, cells treated with iWnt-IONPs or dual targeted iWnt-ATF₂₄-IONPs, had markedly lower levels of active β -catenin compared to no-treatment control (85% and 91% reduction, respectively), or non-targeted IONPs (51% and 70% decrease) (Figure 5A). ATF₂₄-IONP treatment did not further inhibit the level of active β -catenin compared with non-targeted IONP. Although IONP treatment alone affected the Wnt signals, the binding of Wnt/LRP or dual receptor targeted IONPs to LRP5/6 further enhanced the specific inhibitory effect on Wnt signaling. Supporting this, only iWnt/LRP or dual receptor targeted IONP treated cells showed

increased levels of E-cadherin, a downstream signal molecule for reduced Wnt signaling (Figure 5A). Several molecular targets in the Wnt/LRP pathway were also examined. The level of Axin, a dual function molecule in the Wnt signaling pathway, was markedly inhibited in the cells treated with the dual receptor targeted IONPs for 95% of the no treatment control, but was not affected by the treatment of either ATF₂₄-IONP or iWnt-IONPs (Figure 5A). Treatment with non-targeted IONP led to decreases in the levels of total GSK 3 β for 66% and p-GSK 3 β for 93% in tumor cells. Single receptor targeted IONP showed 35 to 64% of decreases of the total GSK3 β . In contrast, dual targeted IONP treated cells had very low levels of GSK 3 β and p-GSK3 β with 93 and 99% decreases respectively compared to on treatment control. It is intriguing that the levels of two key molecules, Axin and GSK3 β , in the β -catenin destruction complex, were markedly decreased in dual receptor targeted IONP treated cells, but the level of active β -catenin was still very low. The mechanism of this paradox observation regarding how β -catenin is degraded or inhibited in the absence of Axin and GSK3 β has yet to be elucidated. Supporting the effect of inhibition of Wnt/ β -catenin signaling, dual receptor targeted-IONP treated cells showed a decreased level of another Wnt/LRP regulated transcription factor, Snail, and upregulation of E-cadherin (Figure 5A).

The MDA-MB-231 cell line has a mesenchymal phenotype with limited expression of epithelial marker E-cadherin [36]. To determine whether the changes in the Wnt/ β -catenin pathway following the IONP treatment affect tumor cell invasion, cells were incubated with 20 picomolar (pmol) of non-targeted-IONP, iWnt-IONP, ATF₂₄-IONP or iWnt-ATF₂₄-IONPs for 48 hours. The treated cells were then collected and seeded in Boyden chamber wells for an invasion assay. We found that only cells treated with dual-targeted iWnt-ATF₂₄-IONPs had a significant reduction (43.1 %) in cell invasion compared to no treatment control (Figure 5B). Although changes in the levels of β -catenin and E-cadherin were found in cells treated with iWnt-IONPs, the invasive phenotype of those cells was not altered. Treatment of cells with ATF₂₄-IONP and non-targeted IONPs also did not inhibit cell invasion. Together these results suggest that dual receptor targeting is necessary for inhibition of Wnt/LRP signaling and, consequently, inhibition of cell invasion.

Targeted IONP treatment reduced the levels of cancer stem cell associated biomarkers without affecting cell proliferation and cell cycle status

Activation of the Wnt/ β -catenin pathway is important for the maintenance of stemness properties of cells in normal tissue and in many types of human tumors. The Wnt/ β -catenin pathway has been shown to be upregulated in cancer stem cells, such as CD44^{high}/CD24^{low} cell population [37]. Activation of the Wnt/LRP pathway also increases uPAR expression, which is often used as an indicator of whether the Wnt pathway has been stimulated [38]. A high level of uPAR expression is found in the cancer stem-like cell population [39]. uPAR signaling has also been observed to induce stem cell-like characteristics in breast cancer cells, including CD44^{high}/CD24^{low} phenotype [24]. To determine whether the binding of Wnt receptor targeted IONPs to LRP5/6 and modulation of Wnt signaling affect the cancer stem cell population and uPAR expression, we examined the levels of protein expression of CD44, CD24, and uPAR in MDA-MB-231 cells following incubation with single-targeted iWnt-IONP, ATF₂₄-IONP or dual targeted iWnt-ATF₂₄-IONPs by Western blot analysis as

shown Figure 6. Within 24 hours following treatment, there was a notable decrease in uPAR in all treatment groups and by 48 hours there was a complete abrogation of uPAR expression in groups treated with the single-targeted iWnt-IONP and ATF₂₄-IONP as well as the dual targeted iWnt-ATF₂₄-IONP compared to those control groups received no treatment and non-targeted IONP (Figure 6A). Forty-eight hours following the treatment, there was a distinct decrease in CD44 in the Wnt/LRP or uPAR targeted-IONP treated cells. There was no detectable level of CD44 in the dual receptor targeted cells. Before and within 24 hours following treatment, CD24 was not detectable in tumor cells (Figure 6A). However, 48 hours following treatment, a marked increase in the level of CD24 expression was observed in all treatment groups. While non-targeted IONP induced an upregulation of CD24, those cells also retained a high level of CD44. Cells treated with single Wnt/LRP or uPAR targeted IONP had intermediate levels of CD24 and a low level of CD44. In contrast, cells treated with dual receptor targeted IONPs had only expressed a high level of CD24 but lacked CD44 expression, suggesting that dual targeted IONPs have a stronger effect on the inhibition of cancer stem-like cells.

Next, we investigated the effect of the single-targeted iWnt-IONP and ATF₂₄-IONP as well as the dual targeted iWnt-ATF₂₄-IONP on cell proliferation. After 48 hours of treatment with non-targeted-IONP, iWnt-IONP, ATF₂₄-IONP or iWnt-ATF₂₄-IONPs, there was no significant difference in the total cell numbers of MDA-MB-231 cells compared to untreated control cells (Figure 6B). We further conducted flow cytometry analysis to determine if changes in the CD44 positive cell population affected the cell cycle status of the total cell population. We found that 48 hours following treatment, there was no observable difference in the percentage of cells in different phases of the cell cycle amongst different treatment groups (Figure 6C and Figure S2).

Results of this study indicated that the binding of the dual Wnt/LRP and uPAR targeted IONP to tumor cells strongly inhibited the proliferation of CD44^{high}/CD24^{low} cancer stem-like cell population, but had a minimal effect on the cell population that has a CD44^{low}/CD24^{high} phenotype. Therefore, selective proliferation of CD44^{low}/CD24^{high} cells *in vitro* caused no significant change in the cell number or in cell cycling among the cells treated with single and dual receptor targeted IONPs. This result also suggested that uPAR and Wnt/LRP targeted IONPs acted upon the same cell population in the tumor cells.

Inhibition of Wnt/LRP signaling and uPAR using single or dual receptor targeted IONP carrying Dox had a similar effect on tumor cell viability as the conventional Dox *in vitro*

Next, we tested the effect of Wnt/LRP and uPAR targeted IONPs carrying Dox on MDA-MB-231 cells *in vitro*. Amphiphilic polymer coated IONPs have a hydrophobic space between the core and coating polymer, which allows for encapsulation of hydrophobic chemotherapeutic agents, such as Dox, and have pH-dependent drug release after being internalized into cells [34].

To determine uptake of Dox alone or Dox encapsulated within non-targeted-IONPs, iWnt-IONP, ATF₂₄-IONP, or dual-targeted iWnt-ATF₂₄-IONP, we treated MDA-MB-231 cells for 4 hours and then determined Dox uptake by observing the fluorescent signal of Dox. No observable difference between free Dox and encapsulated Dox within the different IONP-

Dox was detected (Figure 7A). Next, we treated MDA-MB-231 cells for 72 hours with Dox equivalent concentration of 170 nM either as Dox alone or Dox encapsulated within non-targeted-IONPs, iWnt-IONP, ATF₂₄-IONP, or dual-targeted iWnt-ATF₂₄-IONP. The treatment led to a 41.3%, 42.8%, 47.1%, 45.1%, and 45.6% of reduction in cell viability, respectively, compared to no treatment control (Figure 7B). There was no difference in tumor cell killing among different treatment groups. Together, these results showed that encapsulated Dox within receptor-targeted IONPs can be delivered into tumor cells to generate a similar cytotoxic effect as free Dox. It is likely that prolonged culture of Dox and non-targeted IONP-Dox also led to drug internalization into tumor cells to induce cytotoxic effect. The effect of targeted delivery and therapeutic response for receptor targeted nanoparticle drug carriers was generally more prominent in *in vivo* studies compared with *in vitro* examination in cultured wells.

We then tested the effect of the receptor targeted IONPs carrying Dox on the Wnt/ β -catenin pathway, cancer stem-like cells (CD44^{high}/CD24^{low}) and the level of uPAR expression. Cells were treated for 6 hours and then cultured for an additional 42 hours. Results showed that iWnt-IONP-Dox, ATF₂₄-IONP-Dox, and iWnt-ATF₂₄-IONP-Dox could inhibit the Wnt/ β -catenin pathway through decreases in active β -catenin, total β -catenin, and uPAR expression (Figure 7C). In addition, iWnt-IONP-Dox, ATF₂₄-IONP-Dox, and iWnt-ATF₂₄-IONP-Dox treatment induced expression of the epithelial marker E-cadherin (Figure 7C). Interestingly, CD44^{low}/CD24^{high} cells were only observed in the dual targeted iWnt-ATF₂₄-IONP-Dox treated cells. There was no apparent change in the Wnt pathway co-receptor, LRP5/6, following treatment. Those changes in Wnt/LRP, CD44 and uPAR were similar to the receptor targeted IONPs that did not show tumor cell growth inhibition. It is likely that the alteration of Wnt, CD44, or uPAR by binding of Wnt/LRP and uPAR targeted IONP-Dox to breast cancer cells *in vitro* only had an impact on a small percentage of cancer stem-like cells but did not affect cell proliferation and viability of most cells as their growth was not dependent on the Wnt/LRP and uPAR signals *in vitro* since they did not have cancer stem-like properties. Although Wnt and uPAR pathways are known to be involved in cell invasion and migration, these effects may not be detectable in cell culture.

Targeted delivery of iWnt-IONPs, ATF₂₄-IONPs and iWnt-ATF₂₄-IONPs into orthotopic breast PDX tumors in nude mice following systemic administration

To determine target specificity and nanoparticle accumulation *in vivo*, we used near infrared (NIR) optical imaging to track the single or dual receptor targeted-IONPs labeled with NIR-830-maleimide dye by directly conjugating to the iWnt or ATF₂₄ peptides. Following the tail vein delivery of 800 pmol of iron-equivalent amount of single-targeted iWnt-IONP, or ATF₂₄-IONP, or dual-targeted iWnt-ATF₂₄-IONP into nude mice bearing breast PDX tumors, strong NIR fluorescence signals were detected in the tumors of the mice that received single or dual receptor targeted IONPs (Figure 8A and Figure S3).

To precisely locate optical signals that would indicate IONP accumulation, tumor and normal organs were collected from nanoparticle treated mice for *ex vivo* imaging following sacrifice. We found the highest accumulation of the single and dual-targeted IONPs in the PDX tumors compared to the normal organs. Low levels of signals were detected in the liver,

spleen, lung, heart, and kidney (Figure 8B). This data suggests that iWnt-IONP, ATF₂₄-IONP, and dual-targeted iWnt-ATF₂₄-IONP can be used as targeted drug delivery carriers for systemic administration of therapeutic agents into the breast PDX tumors in nude mice.

Targeted delivery of iWnt-ATF₂₄-IONP-Dox downregulated CD44, uPAR, and Wnt signaling, leading to decreased cell proliferation and tumor growth inhibition in an orthotopic chemo-resistant breast cancer PDX model

We examined the effect of the Wnt/LRP and uPAR receptor targeted IONPs carrying Dox *in vivo* on tumor cell proliferation, Wnt/ β -catenin pathway signaling, cancer stem-like cells (CD44^{high}/CD24^{low}), and levels of uPAR and E-cadherin expression. Using nude mice bearing the first passage of orthotopic human chemo-resistant breast cancer PDX tumors derived from a surgically resected residual tumor that was resistant to paclitaxel and cyclophosphamide, we found that systemic delivery of 5 mg/kg Dox equivalent dose of single or dual receptor targeted IONP-Dox inhibited tumor growth. Furthermore, the strongest effect on tumor growth inhibition was seen in the mice treated with the dual receptor targeted iWnt-ATF₂₄-IONP-Dox (student's t-test, *p 0.03) (Figure 9A). Histological analysis of residual tumors in different treatment groups following the targeted IONP-Dox treatment showed significant inhibition of cell proliferation, presented as a low level of Ki67 positive cells, in the tumors treated with dual targeted iWnt-ATF₂₄-IONP-Dox, compared with no treatment, non-targeted IONP-Dox and single receptor targeted ATF₂₄-IONP-Dox or iWnt-IONP-Dox (student's t-test: ***p 0.008, ****p 0.0001) (Figure 9B). H&E stained PDX tumor tissue sections also showed reduced tumor cell intensity in the tumors collected from the mice treated with dual receptor targeted IONP-Dox but not in the no treatment control and single receptor targeted IONP-Dox treated tumors (Figure 9C). Immunofluorescence staining of tumor tissue sections revealed significant downregulation of the levels of Wnt1 ligand, Axin, uPAR and CD44, and marked upregulation of E-cadherin only in the tumor tissues treated with the dual receptor targeted IONP-Dox (Figure 9C). Tumor tissues from the mice treated with iWnt-IONP-Dox and ATF₂₄-IONP-Dox had an increase in the levels of Wnt pathway proteins including Wnt1 and Axin (Figure 9C). Western blot analysis of tumor tissue lysates showed that in the residual tumor tissues, iWnt-ATF₂₄-IONP-Dox treated tumors had no detectable levels of CD44, uPAR, p-GSK3 β and Axin (Figure 9D). However, the levels of active β -catenin and GSK3 β were not decreased in the treatment resistant tumors (Figure 9D). Furthermore, a high level of CD24 was found in the tumors treated with dual targeted IONP-Dox, suggesting that the remaining tumor cells were CD44^{low}/CD24^{high}, a non-cancer stem cell population. In the tumors treated with single targeted IONP-Dox, the levels of CD44 and uPAR were reduced compared to no treatment control. However, there was no decrease in the level of Axin in single receptor targeted IONP-Dox treated tumors. Results from the PDX tumors treated with the dual targeted iWnt-ATF₂₄-IONP-Dox *in vivo* are consistent with results obtained from cell line studies *in vitro*, except for the levels of active β -catenin and total GSK3 β . In residual breast cancer tissues, there was no significant change in the level of active β -catenin in iWnt-IONP-Dox, ATF₂₄-IONP-Dox or iWnt-ATF₂₄-IONP-Dox treated tumors. There was a slight decrease in the level of total GSK3 β compared to the no treatment control. However, the level of p-GSK3 β was consistently absent in the dual targeted IONP treated tumor, which was similar to what was observed *in vitro* study on the tumor cell line. It is likely that highly

heterogeneous tumor cells in the breast PDX tumors including the presence of chemo-resistant tumor cells in residual tumors were the causes for the differences in expression levels of β -catenin and total GSK3 β between the breast PDX tumors *in vivo* and MDA-MB-231 cells *in vitro*.

Together these results indicate that the dual Wnt/LRP and uPAR targeted delivery of Dox can enhance therapeutic response in multi-drug resistant breast cancer through efficient delivery of Dox into tumor cells and inhibition of the Wnt/ β -catenin and uPAR pathways and cancer stem cell population.

Discussion

Increasing evidence supports the significant role of cancer stem-like cells in resistance to chemotherapeutics [40,41]. It is well recognized that therapeutic strategies acting upon tumor cells as well as cancer stem-like cell populations have the potential to overcome drug resistance and improve therapeutic responses in highly heterogeneous human cancer. Results of this study suggest a new targeted drug delivery approach aimed at inhibition of Wnt/LRP signaling and cancer stem cell properties while delivering a potent chemotherapeutic drug, Dox, into tumor cells.

Various chemotherapeutic drugs have been used to treat breast cancer patients as neoadjuvant therapy to reduce tumor recurrence or as the main therapeutic option for metastatic breast cancer. Unfortunately, chemo-resistance has been the major obstacle for effective treatment of breast cancer. For those patients with large residual tumors after chemotherapy, they have a high incidence of development of local and distant recurrent tumors and an extremely poor prognosis. Therefore, new therapeutic approaches targeting aggressive and drug resistant breast tumor cells offer the opportunity to improve the prognosis of the breast cancer patients with drug resistant tumors. Our research demonstrates the ability to target chemo-resistant breast cancer with a novel nanoparticle co-directed against Wnt/LRP and uPAR, both of which we found to be over-expressed in these cancers. Such a targeted therapy has potential to overcome the chemo-resistance found in a number of breast cancers and could provide therapeutic approaches for pre-operative neoadjuvant therapy as well as for the treatment of metastatic breast cancer.

Current developments in the field of nanotechnology offer promising platforms for effective treatment of chemo-resistant breast cancer. Theranostic nanoparticles are able to deliver therapeutic agents into the cancer cells, assist in monitoring the tumor's response to treatment, and conduct image-guided identification of residual tumor lesions for surgical removal. This work developed theranostic magnetic iron oxide nanoparticles (IONPs) targeting Wnt receptor LRP5/6 and uPAR that carry a clinically relevant chemotherapeutic agent (Dox) [34]. Although we examined targeted nanoparticle drug delivery using non-invasive optical imaging, IONP-based drug carriers are also MRI contrast agents that can be utilized for detection of IONP-drug delivery by MRI, which has been demonstrated in our previous studies [31,34].

Studies have shown that there is an interplay between the Wnt/ β -catenin pathway and uPAR [25]. The interaction between these two pathways has been observed but not extensively studied. uPAR and Wnt/LRP signaling are upstream of GSK-3 β and Snail, a transcription factor that promotes epithelial mesenchymal transition [42,43]. When uPAR and Wnt signaling is activated, GSK-3 β is inhibited and β -catenin can promote transcription of Wnt target genes such as CD44, Axin, and uPAR. In addition, when GSK-3 β is inhibited, the expression of Snail leads to the inhibition of epithelial marker E-cadherin and promotes more mesenchymal phenotype [44,45]. In this study, we showed that uPAR, Wnt/LRP, and dual Wnt/LRP targeted IONPs allowed targeted delivery of chemotherapeutics into breast cancer cells *in vitro* and human chemo-resistant breast PDX tumors *in vivo*. Although single-receptor targeted iWnt-IONP and ATF₂₄-IONPs can lead to intermediate levels of reduction in Wnt/ β -catenin signaling and in the CD44^{high}/CD24^{low} cancer stem cells in breast cancer cells *in vitro*, dual Wnt/LRP and uPAR receptor targeted IONP treated tumor cells showed a marked increase in inhibition of the Wnt/ β -catenin signal pathway, including the lowest level of β -catenin and lack of detectable levels of uPAR, CD44, Axin, p-GSK3 β /GSK3 β , and Snail. Despite these noted changes in the Wnt/ β -catenin pathway, the binding of single or dual receptor targeted IONPs in the absence of the chemotherapeutic Dox did not affect cell cycle status nor cell proliferation in cultured human breast cancer MDA-MB-231 cells. Interestingly, only the dual receptor targeted IONPs inhibited cell invasion of the tumor cells. It has been shown that tumor cells with activated Wnt signaling can maintain cancer stemness and undergo the epithelial to mesenchymal (EMT) transition without a reduction in cell proliferation [19]. Thus, blocking Wnt and/or uPAR signaling using the peptide conjugated nanoparticles may only inhibit EMT and has no apparent effect on inhibition of cell proliferation. This should be a favorable property of a targeted drug delivery nanoparticle since the effect of many cancer therapeutic agents requires DNA replication and cell proliferation.

In comparison to the levels of signaling molecules in the Wnt pathway (LRP5/6, β -catenin, Axin and GSK3 β) and downstream Wnt pathway regulated genes (CD44, uPAR, Snail), we found that a marked difference between single receptor targeted and dual receptor targeted IONP-treated tumor cells was lack of Axin in the dual receptor targeted IONP treated tumor cells. This observation was further confirmed in the PDX tumor tissues following treatment with dual Wnt/LRP and uPAR targeted theranostic IONP carrying Dox. Axin was discovered to act as a negative regulator of the Wnt pathway that formed the β -catenin degradation complex with GSK3 β , APC and CK1 to induce GSK3 β -dependent phosphorylation and degradation of β -catenin [12]. However, several studies also showed that Axin has multiple functions in cells [13]. For example, the binding of Wnt ligand to Frizzled receptor-LRP6 complex leads to the recruitment of Disheveled and then Axin-GSK3 β complex to promote LRP phosphorylation, initiating WNT/ β -catenin signaling [13]. Recruiting Axin to the membrane by LRP5/6 further resulted in degradation of Axin, which prevents β -catenin degradation and activation of β -catenin regulated gene transcription. Simultaneous binding of iWnt-ATF₂₄-IONP to uPAR and LRP5/6 completely abolished the level of Axin while mediating Axin/GSK3 β independent downregulation of β -catenin and its downstream signaling. These results suggest a potential new mechanism of regulation of Axin and β -catenin when the Wnt and uPAR are blocked simultaneously.

It is likely that the combined effect of inhibition of uPAR, CD44, and Snail leads to a reduction of invasiveness in breast cancer cells treated with dual Wnt/LRP and uPAR targeted IONPs. It is also clear that blocking Wnt/LRP and uPAR by iWnt-ATF₂₄-IONP alone is not sufficient for the development of cancer therapeutic agents. However, this dual receptor targeted IONP could be an appropriate drug carrier for targeted delivery of therapeutic agents into tumor cells with activated Wnt signaling and cancer stem-like cells. In this study, we developed iWnt-ATF₂₄-IONP carrying Dox that served as a multifunctional therapeutic agent and evaluated its effect on Wnt signaling and tumor growth in an orthotopic human chemo-resistant breast cancer PDX model in nude mice. Early passage chemo-resistant breast PDX tumors resemble primary breast cancer pathologically, being highly heterogeneous and enriched with tumor stroma. Systemic delivery of various IONP-Dox in the PDX tumor bearing nude mice supported stronger tumor growth inhibition in the dual receptor targeted IONP-Dox treated mice compared to non-targeted IONP-Dox, single Wnt/LRP or uPAR targeted IONP-Dox treated mice. Unlike the effect of the receptor targeted IONPs on cell proliferation in cultured cells, the finding of significant inhibition of cell proliferation in the tumors treated with the dual receptor targeted IONP-Dox, but not with the single receptor targeted IONP-Dox, suggested that the combination of targeted Dox delivery and Wnt/LRP signaling inhibition enhanced therapeutic responses in tumor cells. Additionally, the effect of inhibition of cell invasion as a result of downregulation of Wnt/LRP signaling may also contribute to the tumor growth inhibition. In the residual PDX tumors obtained after nanoparticle treatment, the effects of inhibition of Wnt/LRP signaling on downregulation of CD44, uPAR, and Axin and upregulation of E-cadherin and CD24 were consistent with those observed in the MDA-MB-231 breast cancer cells *in vitro*. However, the expression level of β -catenin in these chemo-resistant breast cancer cells was not reduced. Since breast PDX tumors contain highly heterogeneous tumor cells, it is possible that the growth of the tumor cell population with downregulated β -catenin in response to the targeted IONP-Dox therapy was significantly inhibited in the PDX tumors. Tumor cells that retained β -catenin activity survived the treatment but had low cell proliferation.

Taken together, the results of this study suggest that dual Wnt/LRP and uPAR-targeted delivery of therapeutic agents offers a multifunctional drug carrier for effective cancer therapy that can overcome resistance to standard chemotherapeutics through the combinatorial effect of chemotherapy with inhibition of the Wnt/ β -catenin signal pathway, cancer stem cell population, and cell invasive properties. The development of targeted nanoparticle drug carriers using multiplexed peptide-based targeting ligands could provide a new approach for the targeting and delivery of therapeutic peptides and chemotherapy drugs into cancer stem-like cell population.

Materials and Methods

Production of Wnt/LRP and/or uPAR targeted IONPs

The designs of LRP targeting peptide derived from DKK1, iWnt, and uPAR targeting peptide derived from human uPA, ATF₂₄, are shown in Figure 3. His-tagged iWnt and ATF₂₄ peptides were chemically synthesized by Pepmic Co (Suzhou, Jiangsu, China). To retain a

high affinity binding of the short peptides when conjugating to nanoparticles, we used NTA-Cu--conjugated and amphiphilic polymer-coated IONPs with a core size of 5 nm (Ocean NanoTech, LLC, San Diego, CA, USA). To prepare NTA-Cu-IONP conjugates, the carboxylic groups available on the surface of IONPs were first activated via 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysulfosuccinimide (Sulfo-NHS) coupling, followed by reaction with NTA-Cu complex (Figure 3). The mixture was further incubated at room temperature for 4 hours. The conjugates were then separated from unbound NTA-Cu and byproducts using a PD-10 size-exclusion column. His tagged peptides were conjugated to NTA-Cu on the IONP surface to retain a high binding affinity. ATF₂₄, iWnt, or a combination of ATF₂₄ and iWnt were incubated with NTA-Cu-IONPs at a molar ratio of peptide to IONP of 20:1 for single-targeted and a 10:10:1 peptides to IONP for dual-targeted in 10 mM Borate buffer (pH 8.5) to produce iWnt-IONPs, ATF₂₄-IONPs, or iWnt-ATF₂₄-IONPs. The reaction was carried out for 1.5 hours at 4°C. The final peptide-IONPs were purified using a Nanosep 100 K column for 5 min at 3000 RPM. Some targeted IONPs were labeled with NIR-830 dye for optical imaging. NIR-830–maleimide dye [46] was incubated with iWnt or ATF₂₄ peptide with C-terminal cysteine. NIR-830 labeled peptides were then mixed with NTA-Cu-IONP as described as the above.

Encapsulation of Doxorubicin to IONPs

Dox dissolved in DMSO (10 mg/ml stock solution) was added into the peptide-IONP conjugates at a ratio of 1 mg of Dox: 3 mg of iron equivalent IONP in 10 mM borate buffer (pH 8.5). The loading of Dox was carried out for 3 hours at 4°C. The final peptide-IONPs loaded with Dox were purified using a Nanosep 100 K column.

We characterized the IONPs carrying Dox to determine the encapsulation efficiency and drug-loading of the different IONP formulations. We found that 97% of input Dox could be encapsulated onto the IONPs, resulting in 14% (w/w) of Dox loading in different IONP-Dox theranostic nanoparticles. Detailed results on efficiencies of encapsulation and Dox loading for each type of IONP-Dox are included in the supporting information (Table S1).

His-tag bead Pulldown Assay

Using Pierce His Protein Interaction Pull Down Kit by ThermoFisher Scientific (Waltham, MA), 30 µg of iWnt or ATF₂₄ 3x-his-tagged peptides was added to 100 µl of Cobalt resin beads and rotated for 3 hours at room temperature. 250 µg of protein from MDA-MB-231 cell lysate was then added to the bead: peptide mixture and incubated at 4 °C for 1 hour. The bead: peptide: protein mixture was then washed 5 times. Protein was then eluted using an imidazole elution buffer. Western blot analysis was conducted on eluted proteins to determine interacting proteins.

Specific binding and uptake of iWnt-ATF₂₄-IONP by breast cancer cells

The MDA-MB-231 human breast cancer cell line was cultured in an 8-well chamber slide with a density of 50,000 cells/well for 24 hours, 4 µg/mL of iron equivalent IONP solution of iWnt-IONPs, ATF₂₄-IONPs, iWnt-ATF₂₄-IONPs or nontargeted IONPs was then added. Cells were incubated with the IONPs for 6 hours and then washed three times with cold PBS

to remove unbound nanoparticles. Cells were then fixed with 4% paraformaldehyde in PBS, and Prussian blue staining was used to determine the presence of IONPs in the cells.

Prussian blue staining

Fixed cells were incubated with a mixture of 10% potassium ferrocyanide(II) trihydrate and 10% HCl solution for 3 hours at 37°C. After being washed three times with distilled water, cells were counterstained with nuclear fast red solution for 5 min. Following consecutive dehydrations with 70% and 100% EtOH and two rinses in xylene, the slides were mounted. Result of Prussian blue staining was examined under a light microscopy.

Competition assay to determine specific binding of iWnt-IONP and iWnt-ATF₂₄-IONP to LRP5/6 in breast cancer cells

MDA-MB-231 cells were cultured in a 24-well plate at a density of 100,000 cells/well. After 24 hours of plating, cells were treated with 50 µM or 100 µM of NCI8642 (Gallocyanine) purchased from Santa Cruz Biotechnology (Santa Cruz, CA) for 1 hour at room temperature. Cells were then washed twice with PBS. 20 pmol of NIR-830-labeled iWnt-IONP and iWnt-ATF₂₄-IONP were added for 2 hours at 4 °C. Cells were then washed twice with PBS and stored at 4 °C. Images were taken and analyzed using fluorescence microscopy (Keyence, USA).

Cell proliferation assay

MDA-MB-231 cells were cultured in a 96-well plate at a density of 6,000 cells/well. After 24 hours of plating, different targeted IONP or IONP conjugates were diluted in the culture medium and added to cell culture. For examination of the effect of IONP carrying Dox, 0.1 µg/mL of Dox equivalent concentration of IONP-Dox or targeted IONPs was added into cell cultures. Unconjugated Dox was used as a treatment control. After incubating for 6 hours when IONP bound to and entered into tumor cells, cells were washed three times with cold PBS to remove unbound IONPs. A 100 µL of fresh medium was then added to the plate. Cells were incubated for an additional 72° hours, and viability of the cells was determined by the Alamar blue assay (Life Technologies, NY, USA). Cells treated with culture medium alone were used as the no-treatment control. Results shown are the mean value of three repeat studies.

Immunofluorescence labeling

Frozen tissue sections of tumor and normal tissues were used for immunofluorescence labeling. The following antibodies were purchased from Santa Cruz: goat anti-Wnt1 (G-19; no. sc6280, 1:200), rabbit anti-Axin (H-19; no. sc14029, 1:500), rabbit anti-uPAR (FL-290; no. sc10815, 1:200), and goat anti-HCAM (CD44) (N-18; no.sc7051, 1:200). The following antibodies were purchased from eBioscience (Cambridge, MA): mouse/rat anti-Ki67 (no. 14-5698-82, 1:200). Mouse anti-E-cadherin (no. 610181, 1:200) was purchased from BD Biosciences. Alexa Fluor 555 dye (red fluorescence, dilution 1:500) labeled secondary antibodies (Invitrogen) were used to detect biomarker-positive cells. Images were taken using fluorescence microscopy (Keyence, USA).

Invasion chamber assay

Cells were pretreated with 4 µg/mL of iron equivalent IONP solution of iWnt-IONPs, ATF₂₄-IONPs, iWnt-ATF₂₄-IONPs or nontargeted IONPs for 6 hours. Treatment was then removed by replacement with culture medium with 2% serum for 24 hours. Cells were then plated in serum-free media in BD BioCoat Matrigel Invasion Chambers (BD Biosciences) (5×10^4 cells/ml) with 0.75 ml of chemoattractant (culture media containing 10% FBS) in the wells for 24 hours. Non-invading cells were removed from the interior surface of the membrane by scrubbing gently with a dry cotton-tipped swab. Each insert was then transferred into 100% methanol for 10 minutes followed by Crystal Violet staining for 20 minutes. Membranes were washed in water and allowed to air dry completely before being separated from the chamber. Membranes were mounted on slides with Permount permanent mounting medium (Fisher Scientific, Waltham, MA). Multiple photographs of each sample were taken at $\times 20$ magnification, with triplicates performed per treatment group. The number of cells was counted in each field; the sum of the fields was calculated for each sample. Experiments were performed three times with reproducible results.

Western blot analyses

MDA-MB-231 cells were incubated with 20 pmol of single-targeted iWnt-IONP, ATF₂₄-IONP or iWnt-ATF₂₄-IONPs for 6 hours and then cells were placed in fresh complete media at 37 °C for 48 hours. Cells were lysed in an immunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich). Total protein extracts were run on SDS-PAGE gel and blotted onto polyvinylidene difluoride (PVDF) membrane. Blots were probed overnight. The following antibodies were purchased from Santa Cruz: rabbit anti-Wnt10b (H-70; no. sc25524, 1:500), rabbit anti-phospho-LRP6 (H-300; no. sc15399, 1:500), rabbit anti-Axin (H-19; no. sc14029, 1:500), rabbit anti-β-catenin (H-102; no. sc7199 1:500), rabbit anti-uPAR (FL-290; no. sc10815, 1:500), goat anti-HCAM (CD44) (N-18; no.sc7051, 1:500), and rabbit anti-CD24 (FL-80; no. sc11406, 1:500). The following antibodies were purchased from eBioscience (Cambridge, MA): mouse/rat anti-Ki67 (no. 14-5698-82, 1:500). Rabbit anti-non-phosphorylated (active) β-catenin (S33/S37/T41; no. D13A1, 1:500) was purchased from Cell Signaling Technologies. Mouse anti-E-cadherin (no. 610181, 1:500) was purchased from BD Biosciences. Mouse anti-β-actin was purchased from Sigma-Aldrich (AC-15, 1:10,000). All primary antibodies were diluted in Tris-buffered saline and Tween 20. Goat anti-mouse secondary IgG-HRP antibody (no. sc-2005, 1:3,000), goat anti-rabbit secondary IgG-HRP antibody (no. sc-2004, 1:3,000), and donkey anti-goat secondary IgG-HRP antibody (no. sc-2020, 1:3,000) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein bands were examined using a chemoluminescence detection system.

Cell cycle analysis

MDA-MB-231 cells were cultured in a 6-well plate at a density of 300,000 cells/well using serum-free DMEM media from Corning (Manassas, VA) for 48 hours. Cells were then treated with 4 µg/mL of iron equivalent IONP solution of iWnt-IONPs, ATF₂₄-IONPs, iWnt-ATF₂₄-IONPs or nontargeted IONPs for 6 hours. After removing IONP agents, cells were placed in fresh complete media at 37°C for 48 hours. Cells were then trypsinized and

washed with PBS. Cells were then fixed in ice-cold 70% ethanol and placed in -20°C overnight. Cells were then washed in PBS and re-suspended in PI/RNase buffer from BD/Bioscience (San Diego, CA) for 30 minutes at room temperature. Incorporation of PI was then measured using flow cytometry.

Dox Cell Uptake

MDA-MB-231 cells were cultured in an 8-well chamber slide overnight to reach 70% confluency. Cells were treated with $0.1\text{ }\mu\text{g/mL}$ of Dox equivalent concentration of IONP-Dox or targeted IONPs for 4 hours at 37°C , then washed three times with cold PBS to remove unbound nanoparticles. Cells were then fixed with 4% paraformaldehyde in PBS. To determine the presence of Dox in the cells, cells were excited at 488 nm. Images were taken and analyzed (Keyence, USA).

Establishment of orthotopic human chemo-resistant breast cancer PDX tumor models in nude mice

Fresh tumor tissues were collected from surgically resected tumors from breast cancer patients who completed neoadjuvant therapy and had large residual tumors using an Emory Institutional Review Board approved protocol (IRB#00071700). Breast cancer patient #1 had neoadjuvant therapy using Sorafenib in combination with Cisplatin followed by dose dense Paclitaxel. Breast cancer patient #6 had neoadjuvant therapy using Dox followed paclitaxel. Breast cancer patient #7 received the combination of Paclitaxel and Cyclophosphamide. Within 2 hours of surgical resection, tumor tissues were cut into 1–2 mm fragments and implanted into the mammary fat pad of immune deficient SCID mice (8 to 10 weeks old, female) using a surgical procedure approved by Emory Institutional Animal Care and Use Committee (IACUC). Excess tissues were frozen in liquid nitrogen and stored for further pathological analysis. After surgery, the tumor growth in the mice was monitored by a caliper weekly. Orthotopic tumors grew to 5 to 10 mm diameter in about 8 to 10 weeks. These PDX tumors then were harvested, and tumor fragments at 1 to 2 mm sizes were then implanted into the mammary fat pad of 6- to 8-week-old female nude mice for large-scale studies. Human breast PDX tumors have histological and pathological characteristics that resemble their corresponding primary breast cancer tissues. PDX tumors were heterogeneous in their tumor cells and stroma components between different PDX tumors as well as among the PDX tumors derived from the same patient.

***In vivo* targeting and imaging**

Tumor bearing mice were subjected to NIR optical imaging 48 and 72 hours after the tail vein injection of 800 pmol of NIR-830-labeled iWnt-IONPs, ATF₂₄-IONPs, or iWnt-ATF₂₄-IONPs into the tumor-bearing mice. NIR optical imaging was conducted using the IVIS Spectrum *in vivo* imaging system (Perkin Elmer, Waltham, MA, USA) or the Kodak FX In Vivo imaging system (Carestream Health, Inc, NY). Mice were sacrificed and tumors and normal organs were collected for *ex vivo* optical imaging. Optical images were captured using an excitation wavelength of 745 nm and emission 810-875 nm filter set (IVIS imaging) or an 800 nm excitation and 850 nm emission filter set with 3 min exposure time and a gamma value of 0.2 (Kodak imaging). Optical images were analyzed using the software provided by the IVIS or Kodak imaging system.

***In Vivo* effect of nanoparticle-Dox treatment**

IONP-Dox, iWnt-IONP-Dox, ATF₂₄-IONP-Dox, or iWnt-ATF₂₄-IONP-Dox was injected at 5 mg/kg of Dox equivalent dose via the tail vein into the nude mice bearing the first passage of breast cancer patient #7 PDX tumors every 3–4 days for a total of 3 treatments. Three days after final treatment, tumors were harvested and weighed. Western blot and immunofluorescence analysis were performed on tumor tissue lysates and frozen tissue sections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic of Wnt Pathway

A. Following Wnt ligand binds to co-receptors LRP5/6 and Frizzled, the Wnt/ β -catenin pathway is activated and β -catenin is freed from the APC/Axin destruction complex and translocates into the nucleus where it activates Wnt-target gene transcription. **B.** When Wnt ligand is absent or blocked by a Wnt/ β -catenin pathway inhibitor like DKK1, β -catenin is phosphorylated by the APC/Axin destruction complex, ubiquitinated, and degraded. This leads to a repression in Wnt target gene transcription.

Figure 2. Differential tumor response to Dox treatment of human breast PDX tumors and phenotypic characterization of residual tumors following treatment

Nude mice bearing orthotopic PDX tumors derived from breast cancer patient #1, #6 and #7 received 5 mg/kg of Dox dose via the tail vein injection once per week for 5 weeks. **A.** Representative tumor growth curves of selected tumors in mice from four treatment studies using PDX tumors from Patient #1 at Passage 4 and Passage 7, Patient #6 at Passage 1 and Patient #7 at passage 1. Within the PDX tumors from the same patient, there were tumors sensitive to Dox treatment and those that were resistant to the treatment. **B.** Tumor tissue lysates from Dox sensitive (Sens.) and Dox resistant (Res.) were immunoblotted with anti-LRP5/6, anti-Wnt-1 and anti-uPAR antibodies. β -actin was used as a loading control. Numbers of the intensity ratio shown in the Western blot were the ratio of the biomarker band relative to β -actin for each protein sample loading. **C.** Immunofluorescence labeling. Frozen tissue sections of the PDX tumors from Patient #1 passage 4 were labeled with anti-Wnt10b and anti-CD44 primary antibodies and Alexafluor-555 conjugated secondary antibody (red). Blue: Hoechst 33342 nuclear background staining. **D–E.** Quantification of the mean fluorescence intensity of images in C was performed by Image J software. Bar graph shows the mean fluorescent intensity of six tissue sections.

Figure 3. Design and preparation of Wnt/LRP and uPAR single targeted and dual targeted IONPs

A. Sequence of the Wnt receptor targeted peptide (iWnt) derived from amino acid 38 to 44 of DKK1, a LRP5/6 receptor inhibitor. Two lysine linkers and three histidine tags were added at the carboxyl terminal. **B.** Sequence of uPAR targeted peptide (ATF₂₄) derived from the amino terminal 33 to 56 amino acid sequence of human uPA, the uPAR ligand. The predicted peptide structures of iWnt and ATF₂₄ were generated using I-Tasser server (zhanglab.ccmb.med.umich.edu/I-TASSER/) [32,33]. **C.** Protocols used for the preparation of single or dual-targeted IONPs by conjugation of iWnt and/or ATF₂₄ peptides via NTA-Cu, and encapsulation of Dox into the hydrophobic layer of amphiphilic polymer coating of IONP. An electron micrograph shows uniform 5 nm core size of ultra-small IONPs. **D.** Hydrodynamic sizes and zeta potentials of IONPs, iWnt-ATF₂₄-IONPs, and iWnt-ATF₂₄-IONP-Dox were determined by the dynamic light scattering (DLS) method.

Figure 4. Determination of binding specificity of ATF₂₄-IONP, iWnt-IONP and iWnt-ATF₂₄ IONPs in human breast cancer cells *in vitro*

A. Agarose beads were used to conjugate the His-tagged iWnt and ATF₂₄ peptides and then pull-down LRP5/6 or uPAR from MDA-MB-231 cell lysates. Western blot showed the levels of pulled-down receptors in each group using anti-uPAR and LRP5/6 antibodies. Input: the amount of uPAR and LRP5/6 in the cell lysate as a value of 1. Numbers show relative amount pulling down by different peptide conjugated IONPs. **B.** Prussian blue staining of MDA-MB-231 cells following incubated with non-targeted IONP, ATF₂₄-IONP, iWnt-IONP, or iWnt-ATF₂₄-IONP at 4 μ g/mL of iron equivalent dose for 6 hours. Cells were counterstained with nuclear fast red. **C.** Specific binding of iWnt-IONP to LRP5/6 was confirmed using a binding competition assay. Cells were treated for 1 hour with increasing concentration of NCI8642, a LRP5/6 inhibitor, and then NIR-830-labeled iWnt-IONP and iWnt-ATF₂₄-IONP was added. Images were taken and quantified using fluorescence microscopy with a NIR filter. Scale bar is 100 μ m.

Figure 5. Effects of iWnt-ATF₂₄ IONPs on Wnt/β-catenin pathway and cell invasion

A. MDA-MB-231 cells were incubated with non-targeted IONP, ATF₂₄-IONP, iWnt-IONP, or iWnt-ATF₂₄-IONP for 6 hours. After removing the IONPs and cultured for 48 hours, cell lysates were immunoblotted with the following antibodies: anti-active β-catenin, anti-total β-catenin, anti-Axin, anti-phospho-GSK3β, anti-total GSK3β, anti-E-cadherin, and anti-Snail. β-actin was used as a loading control. Quantification of bands was the intensity ratio relative to β-actin of the same loading sample. **B.** Following incubation with IONP, ATF₂₄-IONP, iWnt-IONP, or iWnt-ATF₂₄-IONP, invasive potential of the MDA-MB-231 cells was determined using an invasion trans-well chamber assay. The percentage of invading cells was significantly reduced only in the iWnt-ATF₂₄-IONP treated cells compared to no treatment control cells (**p = 0.0007). Study was performed in triplicate and bar graph shows the mean and SD of studies.

Figure 6. Effects of single or dual receptor targeted IONPs on tumor cell populations expressing cancer stem cell biomarkers, cell cycle and proliferation

A. MDA-MB-231 cells were incubated with IONP, ATF₂₄-IONP, iWnt-IONP, or iWnt-ATF₂₄-IONP for 24 and 48 hours. Cells were then lysed and immunoblotted with anti-uPAR, anti-CD44, and anti-CD24 antibodies. β -actin was used as a loading control. **B.** Cell Proliferation Assay. 48 hours after cells were incubated with targeted and non-targeted IONPs, Alamar blue cell proliferation assay was performed in triplicate and bar figure showed the mean and SD of measurements. **C.** Cell cycle analysis using flow cytometry. Cell cycle analysis was performed on cells 48 hours after incubation with targeted and non-targeted IONPs.

Figure 7. Effects of iWnt-ATF₂₄ IONPs carrying Doxorubicin on cell viability and signal molecules in the Wnt/β-catenin pathway

A. Cell uptake of Doxorubicin. MDA-MB-231 cells were incubated with free Dox, IONP-Dox, ATF₂₄-IONP-Dox, iWnt-IONP-Dox, or iWnt-ATF₂₄-IONP-Dox (170 nM) for 4 hours. Uptake of Dox was measured using fluorescence microscopy (red fluorescence). **B.** Cell Proliferation assay. MDA-MB-231 cells were incubated with free Dox, IONP-Dox, ATF₂₄-IONP-Dox, iWnt-IONP-Dox, or iWnt-ATF₂₄-IONP-Dox (170 nM) for 6 hours. *In vitro* cell viability assay was conducted 72 hours after treatment. Study was performed in triplicate and bar figure showed the mean and standard deviation of values of each treatment. **C.** Western blot analysis of the cell lysates of MDA-MB-231 cells following the above treatment using anti-LRP5/6, anti-active β-catenin, anti-total β-catenin, anti-E-cadherin, anti-CD44, anti-CD24, and anti-uPAR antibodies. β-actin was used as a loading control. Numbers show quantification of bands relative to loading control (β-actin).

Figure 8. Detection of targeted delivery of ATF₂₄-IONP, iWnt-IONP, or iWnt-ATF₂₄-IONP into orthotopic breast PDX tumors in nude mice by optical imaging

A. The whole body NIR imaging of mice 48 hours after IONP administration. Optical images were overlaid with bright-field images of the mice. Red arrows indicate location of tumors. **B.** *Ex vivo* optical imaging of the tumors and organs. Optical images were overlaid with bright-field images to show location and size of tumor and organs. Tumor (Tu), Heart (Hr), Lungs (Lu), Liver (Li), Kidneys (Kid), Spleen (Spl). Fluorescence Intensity scale bars show the intensity of optical signals.

Figure 9. Evaluation of *in vivo* effects of targeted delivery of single and dual receptor targeted IONP-carrying Dox on tumor growth, signaling molecules, and cancer stem cell biomarkers in an orthotopic, drug-resistant breast PDX tumor model

Nude mice bearing orthotopic breast PDX tumors (Patient #7) received 5 mg/kg Dox equivalent concentration of different IONP-Dox theranostic nanoparticles via the tail vein injections 3 times over the course of 2 weeks. **A.** Bright field images of representing PDX tumors collected from different groups following the treatment. Mean and standard deviation of tumor volume (mm³) are shown. Student's t-test: No treatment control vs iWnt-ATF₂₄-IONP-Dox: $p < 0.03$. **B.** Effect of IONP-Dox, ATF₂₄-IONP-Dox, iWnt-IONP-Dox, or iWnt-ATF₂₄-IONP-Dox treatment on cell proliferation *in vivo*. Ki67-positive cells in tumor tissue sections were determined by immunofluorescence labeling using an anti-Ki67 antibody (red). Blue: Hoechst 33342 background staining. *** $p < 0.008$, **** $p < 0.0001$, Student's t-test. **C.** Histological characterization of the residual tumors by H&E staining and immunofluorescence using anti-Wnt1, anti-Axin, anti-E-cadherin, anti-CD44 and anti-uPAR primary antibodies and Alexafluor 555 conjugated secondary antibody (red). **D.** Tumor lysates were immunoblotted with anti-active β-catenin, anti-total β-catenin, anti-Axin, anti-phospho-GSK3β, anti-total GSK3β, anti-CD44, anti-CD24, and anti-uPAR antibodies. β-actin was used as a loading control. Quantification of bands relative to loading control.

Bands are from the same gel and organized based on treatment groups. Scale bar is 50 μm .
No Tx: no treatment control group.

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