Gd-metallofullerenol nanomaterial as non-toxic breast cancer stem cell-specific inhibitor

Ying Liu1,*, Chunying Chen1,*, Pengxu Qian2,*, Xuefei Lu2, Baoyun Sun1, Xiao Zhang2, Liming Wang1, Xingfa Gao1, Han Li1, Zhiyun Chen1, Jinglong Tang1, Weijie Zhang2, Jinquan Dong1, Ru Bai1, Peter E. Lobie2,3, Qingfa Wu2, Suling Liu2, Huafeng Zhang2, Feng Zhao1, Max S. Wicha4, Tao Zhu2 & Yuliang Zhao1

The contemporary use of nanomedicines for cancer treatment has been largely limited to serving as carriers for existing therapeutic agents. Here, we provide definitive evidence that, the metallofullerenol nanomaterial Gd@C$_{82}$(OH)$_{22}$, while essentially not toxic to normal mammary epithelial cells, possesses intrinsic inhibitory activity against triple-negative breast cancer cells. Gd@C$_{82}$(OH)$_{22}$ blocks epithelial-to-mesenchymal transition with resultant efficient elimination of breast cancer stem cells (CSCs) resulting in abrogation of tumour initiation and metastasis. In normoxic conditions, Gd@C$_{82}$(OH)$_{22}$ mediates these effects by blocking TGF-$\beta$ signalling. Moreover, under hypoxic conditions found in the tumour microenvironment, cellular uptake of Gd@C$_{82}$(OH)$_{22}$ is facilitated where it functions as a bi-potent inhibitor of HIF-1$\alpha$ and TGF-$\beta$ activities, enhancing CSC elimination. These studies indicate that nanomaterials can be engineered to directly target CSCs. Thus, Gd-metallofullerenol is identified as a kind of non-toxic CSC specific inhibitors with significant therapeutic potential.
C ompared with classic small-molecule drugs, nanomaterial-based nanomedicines are distinguished by their nanosizes and nanosurfaces that facilitate their interactions with biological systems at the nano/bio interface. Nanomedicines hold great promise in medical applications especially in cancer therapeutics. Currently, the predominant use of nanomaterials has been as carriers of conventional drugs, oligonucleotides or bioactive molecules where the nanomaterials may improve their bioavailability. However, little evidence exists that nanomaterials themselves might possess intrinsic anticancer properties. We have previously reported the fullerene-based nanomaterial Gd@C_{82}(OH)_{22}, which is characterized by a rare earth atom gadolinium encapsulated by a cage consisting of 82 carbon atoms. The surface of the carbon cage is modified with 22 hydroxyl groups to form Gd@C_{82}(OH)_{22} with a virus-like morphological nanosurface. With a size of ~1 nm, Gd@C_{82}(OH)_{22} nanoparticles may aggregate by hydrogen bond interaction in a solution to form larger particles with sizes ranging from 20 to 120 nm, depending on the concentration and microenvironmental pH. One of the most fascinating features of the Gd@C_{82}(OH)_{22} nanoparticle is its strikingly low cytotoxicity and systemic-toxicity despite a remarkable anticancer capacity in a variety of solid cancers. However, the mechanisms by which Gd@C_{82}(OH)_{22} nanoparticles mediate this cancer target specificity remain undefined.

Metastasis, chemotherapeutic resistance and recurrence are the major hurdles to successful treatment of cancer. There is increasing evidence that these obstacles to clinically efficacious treatment may be mediated by a subpopulation of tumour cells that display stem cell properties. Although a number of approaches are being developed to target cancer stem cells (CSCs), as of yet, no single approach has proven efficacious. Intra-tumoral heterogeneity as well as potential toxicity to normal tissues are important concerns that limit CSC-targeted therapeutics. Herein, we utilized two claudin-low triple-negative breast cancer (oestrogen receptor (ER), progesterone receptor (PR), no human epidermal growth factor receptor 2 (HER2) overexpression); TNBC) cell lines (MDA-MB-231 and BT549) that are enriched for features associated with epithelial-to-mesenchymal transition (EMT) and breast cancer stem cell phenotypes. TNBC stands for a promiscuous stem cell phenotype14–16. TNBC provides the first definite evidence that a specific nanomaterial can selectively target CSC populations.

Results

Gd@C_{82}(OH)_{22} treatment reverses the EMT phenotype. Gd@C_{82}(OH)_{22} and C_{60}(OH)_{22} nanoparticles synthesized as previously described have been well characterized. As shown in Fig. 1a–A, Gd@C_{82}(OH)_{22} possesses a lower C_{2v} symmetry compared with C_{60}(OH)_{22}. The carbon cage of Gd@C_{82}(OH)_{22} receives electrons from the endohedral Gd atom, being in an anionic state. As a result of its lower symmetry, the C_{60} cage exhibits an inhomogeneous charge distribution. Accordingly, Gd@C_{82}(OH)_{22} and C_{60}(OH)_{22} were expected to react differently in the experimental hydroxylation processes, yielding fullerenes with distinct geometries and physical properties. Indeed, according to theoretical predictions the most thermodynamically stable structure of C_{60}(OH)_{22} is that with all hydroxyl groups added aggregated to the equatorial region of the C_{60} cage. In contrast, the theoretically most stable structure of Gd@C_{82}(OH)_{22} is that with hydroxyls around the C_{62} cage in a more homogenous and scattered manner (Fig. 1a–B,C). Such different geometries and the associated physical properties may serve as an origin of differential biological effects of Gd@C_{82}(OH)_{22} and C_{60}(OH)_{22}.

Despite pronounced antitumour effects reported in vivo, we observed that this nanoparticle exerted no significant effect on total TNBC cell proliferation or apoptosis in vitro. We treated triple-negative MDA-MB-231 human breast cancer cells with Gd@C_{82}(OH)_{22}, C_{60}(OH)_{22}, GdCl_{3} or PBS for extended periods. The ER-positive (ER+) MCF-7 cell line and immortalized but non-transformed MCF-10A human mammary epithelial cells were used as controls. Gd@C_{82}(OH)_{22} and C_{60}(OH)_{22} tended to aggregate in aqueous solutions (pH 7.0) and formed dispersed nanoparticles, respectively, with an average diameter of 100 nm. No significant alteration in cell proliferation, as determined by the CCK-8 assay, was observed in any of the cell lines tested (days 3–21) (Supplementary Fig. 1a,c,e). Flow cytometric analysis with annexin V and PI double staining confirmed that Gd@C_{82}(OH)_{22} exerted no appreciable effect on cell apoptosis/necrosis (Supplementary Fig. 1b,d,f,h).

Interestingly, Gd@C_{82}(OH)_{22}-treated MDA-MB-231 cells exhibited a less elongated morphology at day 14 compared with the PBS-treated cells. The Gd@C_{82}(OH)_{22} promoted conversion of the spindle-like mesenchymal phenotype into a ‘cobble stone’ like epithelial phenotype was clearly observed after treatment for 21 days (Supplementary Fig. 2a). A limited conversion to an epithelial phenotype was observed in C_{60}(OH)_{22}-treated MDA-MB-231 cells, whereas the morphology of GdCl_{3}- or PBS-treated cells remained unaltered (Fig. 1b, A–D). In comparison, no morphological alteration was observed in MCF-7 cells (Supplementary Fig. 3a) or MCF-10A cells (Supplementary Fig. 3c) treated with the same agents. Consistently, decreased branching colonies were only observed in MDA-MB-231 cells cultured on 2-dimensional, and in 3-dimensional matrigel with Gd@C_{82}(OH)_{22} treatment (Supplementary Fig. 2a–B,F). Gd@C_{82}(OH)_{22} modulation of cell morphology was concentration-dependent (Supplementary Fig. 4a). Colony scattering assays revealed a significantly larger proportion of compact cells and a smaller proportion of scattered cells on Gd@C_{82}(OH)_{22} treatment (Supplementary Fig. 2a–J and Supplementary Fig. 2b). To determine the generality of this observation, we examined an additional TNBC cell line BT549. Consistently, an epithelial like conversion was only observed in BT549 cells treated with Gd@C_{82}(OH)_{22} but not with other compounds or control (Supplementary Fig. 5a). Increased expression of epithelial makers (E-CADHERIN, γ-CATENIN) and reduced expression of mesenchymal markers (VIMENTIN, FIBRONECTIN-1) at both the mRNA (Fig. 1c) and protein levels (Fig. 1b,E–T,d) were observed in MDA-MB-231 cells treated with Gd@C_{82}(OH)_{22} but not with the other compounds. Gd@C_{82}(OH)_{22} treatment also resulted in increased expression of epithelial markers in mesenchymal like BT549 cells (Supplementary Fig. 5b) and epithelial like MCF-7 cells (Supplementary Fig. 3b) but not MCF-10A cells (Supplementary Fig. 3d). Functionally, MDA-MB-231 (Supplementary Fig. 5e,f) and BT549 cells (Supplementary Fig. 5c,d) treated with Gd@C_{82}(OH)_{22} displayed reduced wound closure (Supplementary Fig. 4b) as well as reduced migratory and invasive capacities.

Strikingly, after the removal of Gd@C_{82}(OH)_{22} for 14 days (Supplementary Fig. 6), MDA-MB-231 cells maintained an epithelial morphology either in adherent culture or in 2D/3D culture for at least five more days (Supplementary Fig. 6a). Furthermore, MDA-MB-231 cells maintained reduced cell motility (Supplementary Fig. 6e,f) and a similar expression
pattern of epithelial/mesenchymal markers (Supplementary Fig. 6d) following Gd@C82(OH)22 withdrawal. These studies demonstrate the sustained and potentially irreversible effects of Gd@C82(OH)22 treatment on TNBC cells.

Gd@C82(OH)22 abrogates cell growth and metastasis. The effects of Gd@C82(OH)22 treatment on TNBC cell behaviour in vivo were investigated by two complimentary approaches. In the first approach (approach I, early treatment), 1 × 10⁶ MDA-MB-231 cells were injected s.c. into female BALB/c nude mice, followed by treatment (PBS, Gd@C82(OH)22 (2.5 μmol kg⁻¹), C60(OH)22 (2.5 μmol kg⁻¹) or GdCl₃ (2.5 μmol kg⁻¹)) once daily (Fig. 2a). Alternatively, in the second approach (approach II, terminal treatment), MDA-MB-231 cells were allowed to grow for 12 days to produce a tumour volume of ~100 mm³ and followed by once daily treatment (Supplementary Fig. 7a). Primary tumours (Supplementary Tables 1 and 2) and organ weights (the liver, spleen, kidney and lung) were measured on killing.

Gd@C82(OH)22 significantly inhibited tumour growth by 450% in both approaches (Fig. 2b and Supplementary Fig. 7b). In the first approach (early treatment), the accumulation of Gd in tumour tissues was 3.11 ± 0.73 ng g⁻¹ tumour wet weight. Pathological inspection indicated that primary tumours derived from TNBC cells treated with Gd@C82(OH)22 were significantly smaller and less invasive compared to control samples treated with PBS, C60(OH)22 or GdCl₃.
**Approach I**

**Primary tumour**

- Tumour cell injection s.c.
- Administration i.p. once a day
- Sacrifice and harvest

**Lung metastasis**

- PBS
- Gd@C_{82}(OH)_{22}
- C_{60}(OH)_{22}
- GdCl_{3}

**Liver metastasis**

- PBS
- Gd@C_{82}(OH)_{22}
- C_{60}(OH)_{22}
- GdCl_{3}

**Days post tumour cell injection**

**Tumour volume (mm^3)**

- 0
- 100
- 200
- 300
- 400
- 500
- 600
- 700

**Log2-fold changes of EMT marker**

- E-cadherin
- γ-catenin
- Vimentin
- Fibronectin-1

**Metastatic burden**

- Lung
- Liver

**Metastatic foci per lung**

- PBS
- Gd@C_{82}(OH)_{22}
- C_{60}(OH)_{22}
- GdCl_{3}

**Administration i.p. once a day**

- Tumour cell injection i.v.

**Sacrifice and harvest**

**Lung metastasis**

- PBS
- Gd@C_{82}(OH)_{22}
- C_{60}(OH)_{22}
- GdCl_{3}

**Liver metastasis**

- PBS
- Gd@C_{82}(OH)_{22}
- C_{60}(OH)_{22}
- GdCl_{3}
from MDA-MB-231 cells treated with PBS, C_{60}(OH)_{22} or GdCl_{3} were poorly encapsulated and highly invasive with tumour emboli observed in lymphatic vessels. In contrast, tumours derived from the Gd@C_{82}(OH)_{22}-treated group remained well confined and noninvasive (Fig. 2c). Pulmonary and hepatic micro-metastases were quantified by qPCR analysis of the relative expression of hHPRT/mGAPDH. Both pulmonary and hepatic micrometastases were significantly reduced in the Gd@C_{82}(OH)_{22}-treated groups compared with the PBS-, C_{60}(OH)_{22}- or GdCl_{3}-treated groups (Fig. 2d,e). Hence, Gd@C_{82}(OH)_{22} inhibited both local invasion and distant metastasis of MDA-MB-231 cells. Consistently, tumours derived from the Gd@C_{82}(OH)_{22}-treated groups expressed higher levels of epithelial markers and lower levels of mesenchymal markers at both the mRNA (Fig. 2f) and protein levels (Fig. 2g and Supplementary Fig. 7c) compared with the other treatment groups. Tumour cell proliferation and apoptosis were also examined. The tumours derived from Gd@C_{82}(OH)_{22}-treated groups exhibited significantly less Ki-67-labelled cells (Supplementary Fig. 7d–f), but no significant changes were observed in the number of active-caspase-3-labelled cells (Supplementary Fig. 7g–i) compared with control tumours.

Pulmonary metastasis was also analysed in nude mice injected with 1 \times 10^{6} MDA-MB-231 cells via the tail vein (Fig. 2h). The mice were treated with either PBS, Gd@C_{82}(OH)_{22}, C_{60}(OH)_{22} or GdCl_{3} once daily. Metastatic tumours were readily detectable in the lungs of mice treated with either PBS (6/6), GdCl_{3} (6/6) or C_{60}(OH)_{22} (5/6). In contrast, only one of six mice treated with Gd@C_{82}(OH)_{22} developed pulmonary metastases (Supplementary Table 3). The incidence of micrometastatic deposits in either lung or liver tissue was also significantly lower in the Gd@C_{82}(OH)_{22}-treated groups compared with control treated groups (Fig. 2i,j).

**Gd@C_{82}(OH)_{22} inhibits TGF-β to reduce EMT under normoxia.** Recent evidence suggested that the tumour microenvironment regulates EMT that in turn generates CSCs. We therefore utilized Illumina next-generation sequencing and qPCR to examine the effects of the nanoparticles on the expression of EMT and CSC-related genes in MDA-MB-231 cells. Gd@C_{82}(OH)_{22}-responsive genes were mapped to KEGG pathways using the BLAST2GO software, which identified tumour-associated pathways (Fig. 3a and Supplementary Fig. 8). A heat map depicting the mRNA expression profiles is shown in Fig. 3b. As indicated, Gd@C_{82}(OH)_{22}, but not the other treatments, significantly reduced the expression of genes associated with the mesenchymal phenotype as well as those associated with the CSCs. The expression levels of TGF-β and HIF-1α were significantly diminished by Gd@C_{82}(OH)_{22}, as were the EMT promoting zinc-finger proteins of the SNAIL families, ZEB1/2 and basic helix-loop-helix factor E47 (ref. 16). In addition, TGF-β, SNAIL, ZEB1, TWIST1 and pro-angiogenic factors (VEGF, IL-6, IL-8, MMP2, MMP9) were also downregulated at both the mRNA and protein levels in both Gd@C_{82}(OH)_{22}-treated cells and tumours derived from Gd@C_{82}(OH)_{22}-treated mice compared with control groups (Fig. 3c–h and Supplementary Fig. 7j). A decrease in secreted TGF-β in response to prolonged treatment of Gd@C_{82}(OH)_{22} (Fig. 3i) was also observed. As expected, HIF-1α protein is undetectable in normoxia as oxygen exposure generates instability and rapid degradation (Fig. 3j). However, Gd@C_{82}(OH)_{22} treatment still leads to a reduction of hif-1α mRNA (Supplementary Fig. 3k). Interestingly, Gd@C_{82}(OH)_{22} also reduced the expression of CSC markers including cd44 and aldh1 as well as the CSC regulatory polycomb genes bmi1 and suz12 (Fig. 3b). We determined further whether the effects of Gd@C_{82}(OH)_{22} on EMT were mediated by its modulation of TGF-β expression. As shown in Fig. 4a and Supplementary Fig. 2c,d, exogenous TGF-β abrogated the effects of Gd@C_{82}(OH)_{22} on EMT. Reduced expression of E-CADHERIN and γ-CATENIN, and increased expression of VIMENTIN and FIBRONECTIN-1, at both the mRNA (Fig. 4b) and protein levels (Fig. 4a,c) was observed as a result. Consistently, the diminished cell motility due to Gd@C_{82}(OH)_{22} treatment was rescued by exogenous TGF-β (Fig. 4d). Moreover, the mRNA levels of tgf-β, hif-1α, snail, zeb1, twist1 and pro-angiogenic genes were also recovered to varying degrees as determined by qPCR (Fig. 4e–h). In summary, these results indicated that Gd@C_{82}(OH)_{22} blocks EMT under normoxic conditions to a large extent via abrogation of TGF-β signalling.

**Gd@C_{82}(OH)_{22} eliminates CSCs under normoxia.** EMT programs have been intrinsically associated with the acquisition of SC traits by normal and neoplastic cells. Heat map data also shows that Gd@C_{82}(OH)_{22} treatment decreased the expression of an array of stem cell markers (BM11, CSF1, KLF4, LIN28A, NANOG) as well as CD44 and ALDH1 (Fig. 3b). We therefore determined whether Gd@C_{82}(OH)_{22} could modulate CSC populations in TNBC. CSCs are enriched by growth as tumourspheres in vitro. MDA-MB-231 and BT549 cells were therefore cultured using ultra-low attachment dishes for 7–10 days to form tumourspheres. Dispersed cells were cultured on ultra-low-attachment dishes for 3 days in the presence or absence of Gd@C_{82}(OH)_{22} or the Paclitaxel. MDA-MB-231 and BT549 cells were also grown in adherent cultures in parallel. The proliferation of both MDA-MB-231 cells and BT549 cells in monolayer culture was markedly inhibited by Paclitaxel in a time- and dose-dependent manner as expected, whereas neither Gd@C_{82}(OH)_{22} nor control agents affected cell growth (Fig. 5a and Supplementary Fig. 9a). However, the proliferation of stem cell-like cells derived from MDA-MB-231 and BT549 cells was rapidly inhibited by Gd@C_{82}(OH)_{22} in suspension cultures. In comparison, treatment with Paclitaxel, PBS or GdCl_{3} did not...

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**Figure 2 | Gd@C_{82}(OH)_{22} abrogates tumour growth and metastasis in vivo.** (a) Schematic diagram of approach I (early treatment) in a mouse xenograft model. A total of 1 \times 10^{6} MDA-MB-231 cells were injected s.c. and mice were treated either PBS or 2.5 μmol kg^{-1} Gd@C_{82}(OH)_{22}, C_{60}(OH)_{22} or GdCl_{3} i.p., once a day, for 21 days. (b) Tumour growth curves in approach I mice were plotted (mean ± s.e.m., n = 5 each). *P < 0.05 (two-way ANOVA, Bonferroni’s post-hoc test). (c) Sections from primary tumours and lungs were examined. Tumour cell invasion is shown in the top and middle panel and pulmonary micro-metastases are shown in the bottom panel. The arrows indicate metastatic tumour foci. (d) Pulmonary micro-metastases count (mean ± s.e.m., n = 10 each). *P < 0.05 (one-way ANOVA, Tukey’s post-hoc test). (e) Relative expression of hHPRT/mGAPDH in the lung and liver tissues (mean ± s.e.m., n = 3 each). *P < 0.05, **P < 0.01 (one-way analysis of variance (ANOVA), Tukey’s post-hoc test). (f) mRNA levels of EMT markers in tumour tissues were analysed by qPCR (mean ± s.e.m., n = 3 each). *P < 0.05 (one-way analysis of variance (ANOVA), Tukey’s post-hoc test). (g) Protein levels of E-CADHERIN (E-CAD), γ-CATENIN (γ-CAT), VIMENTIN (VIM) and FIBRONECTIN-1 (FN-1) in tumour tissues were detected by western blot. (h) Experimental design of the xenograft model with tumour cell tail vein injection (i.v.). A total of 1 \times 10^{6} MDA-MB-231 cells were injected i.v., and mice were treated at day 0 for 21 days. (i) Pulmonary and hepatic micro-metastases of tumour cells. The arrows indicate metastatic tumour foci. (j) The lung micro-metastatic numbers were quantified (mean ± s.e.m., n = 6 each). *P < 0.05 (one-way ANOVA, Tukey’s post-hoc test).
**Pathways in cancer**
- Small cell lung cancer
- Renal cell carcinoma
- Chronic myeloid leukemia
- Bladder cancer
- Huntington's disease
- Ribosome
- Pyrimidine metabolism
- NOD-1-like receptor signaling pathway
- Arginine and proline metabolism
- Mismatch repair

**Panel A**
- **PBS**
- **Gd@C82(OH)22**
- **C60(OH)22**
- **GdCl3**

**Panel B**
- **Vim**
- **fn1**
- **e-cad**
- **cdh2**
- **ocn**
- **ctnna1**
- **ctnmb1**
- **hif-1a**
- **Snai1**
- **Snai2**
- **Twist1**
- **zeb1**
- **tcf3**
- **tgf-β1**
- **tafap5**
- **n-6**
- **n-8**
- **mmp-2**
- **mmp-9**
- **bmi1**
- **cd11b**
- **kif1a**
- **a2m**
- **nanog**
- **pigs2**
- **cdk4**
- **cdk2**
- **ash1**
- **suz12**
- **cdk6**
- **met**
- **ccnd1**
- **ctgf**
- **cdkn1**

**Panel C**
- **HIF-1α**
- **TGF-β**
- **SNAIL**
- **ZEB1**
- **TWIST1**
- **E47**
- **β-Actin**

**Panel D**
- **Gd@C82(OH)22**
- **C60(OH)22**
- **GdCl3**

**Panel E**
- **PBS**
- **Gd@C82(OH)22**
- **C60(OH)22**
- **GdCl3**

**Panel F**
- **The concentration of IL-6 (pg ml⁻¹)**
- **The concentration of IL-8 (pg ml⁻¹)**

**Panel G**
- **The concentration of TGF-β (pg ml⁻¹)**

**Panel H**
- **The concentration of IL-6 (pg ml⁻¹)**
- **The concentration of IL-8 (pg ml⁻¹)**

**Panel I**
- **MDA-MB-231**
- **[TGF-β] (pg ml⁻¹)**

**Panel J**
- **MDA-MB-231**
- **HIF-1α**
- **HIF-1β**
- **TGF-β**
- **SNAIL**
- **ZEB1**
- **TWIST1**
- **E47**
- **β-Actin**

**Panel K**
- **Gd@C82(OH)22**
- **C60(OH)22**
- **GdCl3**

**Panel L**
- **MDA-MB-231**
- **[TGF-β] (pg ml⁻¹)**

**Supplementary Figures**
- Pathway analysis of cancer-related genes.
- MDA-MB-231 cell line analysis with TGF-β and Gd@C82(OH)22.
- Comparison of gene expression levels with and without treatment.

**Graphs**
- Log2-fold changes in gene expression.
- Concentration of cytokines over time.

**Legend**
- Red: increased expression
- Green: decreased expression
- Blue: unchanged expression

**References**
- TGF-β: Transforming Growth Factor-beta
- ZEB1: Zinc Finger E-box Binding Homeobox 1
- TWIST1: Transcriptional Regulatory Effector 1
- E47: Early growth response factor 1
- β-Actin: Actin, beta

**Statistical Analysis**
- Significance levels indicated by asterisks:
  - *: p < 0.05
  - **: p < 0.01
  - ***: p < 0.001
  - ****: p < 0.0001
affect tumoursphere formation under the utilized conditions (Fig. 5b and Supplementary Fig. 9b). Surprisingly, C60(OH)22 treatment promoted the proliferation of stem cell-like cells derived from MDA-MB-231 and BT549 cells.

We performed tumoursphere formation assays to further determine the influence of Gd@C82(OH)22 on the CSC population and properties in MDA-MB-231 and BT549 cells. Cells were treated with Gd@C82(OH)22 or controls for 21 days, or Paclitaxel for 7 days. Gd@C82(OH)22 potently diminished the number and size of tumourspheres formed in both TNBC cell lines compared with PBS treatment (Fig. 5c,d and Supplementary Fig. 9c,d). As expected, Paclitaxel promoted tumoursphere formation due to its selective toxicity to non-CSCs resulting in a relative enrichment of CSC and tumourspheres. Unexpectedly, C60(OH)22 significantly promoted tumoursphere formation and increased expression of SC markers compared with controls (Fig. 5e). Serial passaging experiments are the accepted methodology for the isolation of SC markers compared with controls (Fig. 5e). Heat map depicting the mRNA expression profile of selected genes. Red squares correspond to increased expression, while green squares correspond to decreased expression of mRNA levels. MDA-MB-231 cells were subcutaneously injected into the mice at day 0. Mice were treated with either PBS or 2.5 \( \times \)10^6 to 500 cells and treated with Gd@C82(OH)22 or controls and determined tumour formation. As summarized in Fig. 6d, in a period of 100 days after tumour cell injection, the group of mice injected with 5,000 or 500 cells did not form any tumours with Gd@C82(OH)22 treatment. In contrast, inoculation of the equivalent numbers of cells in PBS-, C60(OH)22- and GdCl3-treated groups resulted in significant tumour formation (Supplementary Table 4) (Table 1). The tumours were collected to isolate primary tumour cells for the ALDEFLUOR assay and tumoursphere formation. The proportion of ALDH + cells in the Gd@C82(OH)22-treated group was significantly less than PBS-treated group (0.65% versus 2.19%) (Fig. 6e), further verifying the capacity of Gd@C82(OH)22 to diminish the CSC population in vivo. Furthermore, the number and size of tumourspheres generated from (Fig. 6e,f), and the expression levels of SC markers, cells derived from Gd@C82(OH)22-treated tumours were significantly reduced compared with PBS controls (Fig. 5f). Collectively, these observations indicate that Gd@C82(OH)22 treatment significantly reduces the tumour-initiating CSC population in TNBC.

Gd@C82(OH)22 inhibits HIF-1α and TGF-β under hypoxia. In solid tumours, decreased oxygen and nutrient supply creates a hypoxic microenvironment in the central region of tumours. Hypoxia has previously been shown to increase the CSC population in a process mediated by HIF-1α, a major response gene to tissue hypoxia.25,31 HIF-1α was readily detectable in tumour sections by immunofluorescence. We further demonstrated that Gd@C82(OH)22 effectively inhibited the expression of HIF-1α and TGF-β in tumours derived from MDA-MB-231 cells treated with Gd@C82(OH)22 compared with controls in mouse xenografts (Fig. 7a). Interestingly, HIF-1α expression was significantly increased in the inner portions of tumour sections compared with the tumour periphery. In contrast, there was no detectable difference in the intensity of TGF-β staining between the periphery and inner portions of consecutive sections in the control tumours or those treated with the nanoparticles. As accumulating evidence suggests that...
intratumoral hypoxia and TGF-β promote tumour metastasis, we determined whether Gd@C₈₂(OH)₂₂ repression of HIF-1α or TGF-β signalling is responsible for inhibition of EMT by this compound.

Using inductively coupled plasma mass spectrometry (ICP-MS), we observed that Gd@C₈₂(OH)₂₂ nanoparticles can be rapidly internalized by MDA-MB-231 cells under hypoxic conditions. In comparison, the cellular uptake of Gd@C₈₂(OH)₂₂ nanoparticles was observed to be lower in the absence of hypoxia. This suggests that hypoxia enhances the internalization of Gd@C₈₂(OH)₂₂ nanoparticles, which may be beneficial for targeted therapy.

**Figure 4** | Gd@C₈₂(OH)₂₂ nanoparticles impeded EMT by the abrogation of TGF-β expression under normoxia. MDA-MB-231 cells were cultured with 20 ng ml⁻¹ TGF-β supplement for 24 h after treatment with PBS or Gd@C₈₂(OH)₂₂ for 21 days. (a) Fluorescent visualization of F-ACTIN cytoskeleton was observed (A–D). Scale bar, 12.5 μm. E–T is the immunofluorescence staining of E-CADHERIN (E-CAD), γ-CATENIN (γ-CAT), VIMENTIN (VIM) and FIBRONECTIN-1 (FN-1). Scale bar, 25 μm. (b) mRNA levels of E-cadherin, γ-catenin, Vimentin and Fibronectin-1 were analysed by qPCR (mean ± s.e.m., n = 3 each). *P < 0.05 (two-way ANOVA, Bonferroni’s post-hoc test). (c) The protein expression of E-CADHERIN (E-CAD), γ-CATENIN (γ-CAT), VIMENTIN (VIM) and FIBRONECTIN-1 (FN-1) were detected by western blot. (d) Cell migration and invasion were examined using trans-well cell culture chambers and Matrigel-coated ones (mean ± s.e.m., n = 6 each). *P < 0.05, **P < 0.01 (two-way ANOVA, Bonferroni’s post-hoc test). (e) mRNA levels of tgf-β, hif-1α, snail, zeb1, twist1 and e47 were analysed by qPCR (mean ± s.e.m., n = 3 each). (f) mRNA levels of il-6, il-8, mmp-2, mmp-9 and vegf were analysed by qPCR (mean ± s.e.m., n = 3 each). (g,h) ELISA analysis of IL-6 (g) and IL-8 (h) to determine their concentrations in the cell culture medium. All the data are represented as mean ± s.e.m. (n = 3 each) with *P < 0.05 and **P < 0.01 (two-way ANOVA, Bonferroni’s post-hoc test).
nanoparticles under normoxia was significantly lower compared with that observed under hypoxia in the first 10 days of treatment. The average Gd concentration in cells cultured under hypoxia for 10 days was similar to that under normoxia for 21 days (Fig. 7b). In hypoxic conditions, Gd@C_{82}(OH)_{22}-treated cells exhibited a compact, cobblestone-like epithelial phenotype, whereas PBS-, C_{60}(OH)_{22}- or GdCl_{3}-treated cells retained a mesenchymal spindle-like morphology (Fig. 7c, Supplementary Fig. 2e,f and Supplementary Fig. 11a). These results demonstrated that hypoxia facilitates Gd@C_{82}(OH)_{22} uptake resulting in repression of the EMT phenotype. The altered expression of epithelial and mesenchymal markers at the mRNA (Fig. 7d) and
protein levels (Fig. 7c,e, IHC & WB) in Gd@C82(OH)22-treated cells compared with control cells under hypoxia further support the phenotypic changes observed. Functionally, cell migration and invasion were markedly inhibited by Gd@C82(OH)22 compared with controls (Fig. 7f, Supplementary Fig. 11b,c). We further demonstrated that under hypoxic conditions Gd@C82(OH)22 significantly reduced the expression of TGF-β, HIF-1α, SNAIL, ZEB1, TWIST1 and pro-angiogenic factors (VEGF, IL-6, IL-8, MMP2, MMP9) at the mRNA (Fig. 7g,h) and protein levels (Fig. 7i and Supplementary Fig. 11d–f) when compared with controls. In contrast, Gd@C82(OH)22 treatment did not affect total cell proliferation or apoptosis or necrosis under hypoxia indicating a lack of cytotoxicity towards the bulk cell population.

**Table 1 | The effect of Gd@C82(OH)22 treatments on MDA-MB-231 in vivo tumorigenesis.**

| Tumour/injected cell number | 5 × 10^6 | 5 × 10^5 | 5 × 10^4 | 5 × 10^3 | 5 × 10^2 |
|----------------------------|----------|----------|----------|----------|----------|
| PBS                        | 6/6      | 5/6      | 5/6      | 4/6      | 1/6      |
| Gd@C82(OH)22               | 6/6      | 5/6      | 2/6      | 0/6      | 0/6      |
| C60(OH)22                  | 6/6      | 5/6      | 3/6      | 3/6      | 2/6      |
| GdCl3                      | 6/6      | 6/6      | 5/6      | 1/6      | 1/6      |

**Different numbers of MDA-MB-231 cells were injected s.c. and mice were treated with PBS, Gd@C82(OH)22, C60(OH)22 or GdCl3 daily. The numbers represent the number of mice with tumorigenesis/the number of mice in every group.**

Figure 6 | Gd@C82(OH)22 nanoparticles effectively eliminate CSCs by the abrogation of TGF-β under normoxia. (a) After treated with PBS, Gd@C82(OH)22, C60(OH)22 or GdCl3 for 21 days and 20 ng ml⁻¹ TGF-β for 24 h, MDA-MB-231 tumourspheres were photographed and quantitated (b) (mean ± s.e.m., n = 3 each) (scale bar, 100 μm). To tumourspheres of 70–150 μm, **P<0.01; to tumourspheres of >150 μm, ##P<0.01 (two-way ANOVA, Bonferroni’s post-hoc test). (c) mRNA levels of CSC markers were analysed by qPCR (mean ± s.e.m., n = 3 each). *P<0.05 (two-way ANOVA, Bonferroni’s post-hoc test). (d) Tumour injection of MDA-MB-231 cells by limiting dilutions. Different numbers (5 × 10^6, 5 × 10^5, 5 × 10^4, 5 × 10^3, 5 × 10^2) of MDA-MB-231 cells were injected into the right back flanks of the mice s.c. Mice were monitored and treated daily. (e) Tumoursphere formation of cells isolated from tumour tissues (mean ± s.e.m., n = 3 each). **P<0.01 (one-way ANOVA, Tukey’s post-hoc test). (f) The tumourspheres were counted.
cell population (Supplementary Fig. 12). To further determine whether the effects of Gd@C_{82}(OH)_{22} are mediated by repression of HIF-1α and TGF-β, we transiently transfected MDA-MB-231 cells with a HIF-1α expressing plasmid in the presence or absence of exogenous TGF-β. Cells were maintained under hypoxia with 50 μM Gd@C_{82}(OH)_{22} or PBS treatment for 10 days. As shown in
Fig. 8a–c and Supplementary Fig. 2g,h, forced expression of HIF-1α or TGF-β supplementation partially reversed Gd@C₈₂(OH)₂₂ repression of cell migration and invasion. The expression levels of TGF-β, HIF-1α and a series of EMT markers, which were diminished by Gd@C₈₂(OH)₂₂ treatment, were all significantly recovered by HIF-1α overexpression and/or TGF-β treatment (Fig. 8d–h). These results suggest that Gd@C₈₂(OH)₂₂ nanoparticles block EMT in hypoxia through abrogation of TGF-β and HIF-1α expression.

Gd@C₈₂(OH)₂₂ eliminates CSCs under hypoxia. We next utilized the ALDEFLUOR and tumoursphere assays to determine the efficacy of Gd@C₈₂(OH)₂₂ on CSC populations of MDA-MB-231 cells grown under hypoxic conditions. MDA-MB-231 cells were treated with PBS, Gd@C₈₂(OH)₂₂, C₆₀(OH)₂₂ or GdCl₃ under hypoxia or normoxia for 3, 6 and 10 days. Consistent with recent reports that intratumoral hypoxia increases the CSC populations31,33, MDA-MB-231 cells under hypoxia exhibited a significantly increased ALDH expressing cell population (Fig. 9a) compared with cells cultured in normoxia at all time points. Gd@C₈₂(OH)₂₂ treatment of MDA-MB-231 under hypoxia for 10 days markedly reduced the percentage of ALDH + cells by more than 11-fold, whereas Gd@C₈₂(OH)₂₂ treatment under normoxia up to 10 days did not alter the percentage of ALDH + cells. In contrast, C₆₀(OH)₂₂ treatment under both hypoxia and normoxia for 10 days significantly increased the percentage of ALDH + cells (Fig. 9a). To demonstrate that ALDH is a reliable marker for CSCs under hypoxia, we counted total cell numbers when the cells were collected for ALDEFLUOR assay. A total of 1 × 10⁶ cells from either control group or Gd@C₈₂(OH)₂₂ group were utilized for the ALDEFLUOR assay. The ratio of the total cell number and the percentage of ALDH + cells from each group, the absolute number of ALDH + cells was quantitated (Control group: (14.74 ± 0.94) × 10⁶, Gd@C₈₂(OH)₂₂ group: (1.22 ± 0.43) × 10⁶), indicating decreased CSC numbers rendered by Gd@C₈₂(OH)₂₂ under hypoxia. Consistently, Gd@C₈₂(OH)₂₂ treatment under hypoxia for 10 days markedly inhibited tumoursphere formation and mRNA expression of CSC markers (Fig. 9d), whereas C₆₀(OH)₂₂ treatment increased tumoursphere formation (Fig. 9b,c).

As HIF-1α and TGF-β have been identified as two critical stimulators of HIF-1α overexpression and TGF-β signalling in cancer cells36–39. The single cage molecule of a fullerenol has a diameter of <1 nm. When dissolved in aqueous solution, they form nanoparticles as polyanion nano-aggregates, and the nanoparticle size is reduced in more acidic solution40–42. It was reported that the smaller sized nanoparticles may allow deeper penetration into tumour tissues43. We observed that in the acidic hypoxic condition, the sizes of Gd@C₈₂(OH)₂₂ nanoparticles are reduced to around 40 nm (Supplementary Fig. 14), which would presumably allow enhanced penetration of the nanoparticles into tumour tissues.

The size variation with pH alterations can be understood by the deprotonation of the hydroxyl groups on the cage surface. It has been previously reported that a decrease in pH occurs when fullerol powder is added into water41. The Gd@C₈₂(OH)₂₂ cage surface has both attractive (C-O) and repulsive (C-O−) sites. Hence, the acidic protons are involved in attractive hydrogen bonding interactions with other Gd@C₈₂(OH)₂₂ molecules and this constitutes a driving force of nanoparticle formation40. Our measurement of its nanoparticle formation at the different pH solutions indicates that at pH 4.3 solution, the average size of Gd@C₈₂(OH)₂₂ nanoparticles is ~40 nm, at pH 5.1, ~116 nm and at pH 7.4, ~175 nm. When the pH value is further increased

**Discussion**

We provide definitive evidence that the Gd@C₈₂(OH)₂₂ is able to reverse the EMT program of cancer cells and efficiently deplete CSC populations. We suggest the major mechanism by which Gd@C₈₂(OH)₂₂ targets CSCs may be due to its specificity in abrogating CSC self-renewal and driving terminal differentiation of CSCs, effects not observed in the normal SC-like cells. These studies therefore identify a novel class of nanomaterial-based CSC specific inhibitors with minimal toxicity in normal tissues. Intratumoral heterogeneity and intolerable toxicity towards normal tissue are among the leading causes that limit the efficacy of contemporary CSC targeting approaches. Furthermore, toxicity is often even more pronounced with combinatorial strategies employed to increase the efficacy of such therapies44,45. Interestingly, neither appreciable toxicity towards normal mammary epithelial cells nor systemic somatic toxicity was observed when utilizing Gd@C₈₂(OH)₂₂ as a CSC inhibitor (Supplementary Fig. 13). Furthermore, the cellular uptake of Gd@C₈₂(OH)₂₂ is increased under hypoxic conditions in which it abrogates EMT and depletes CSC populations via the simultaneous inhibition of HIF-1α and TGF-β signalling (Fig. 10). The Gd@C₈₂(OH)₂₂ targeting of HIF-1α and TGF-β signalling may be achieved due to its high efficiency to scavenge reactive oxygen species22,23, which are known as potent stimulators of HIF-1α and TGF-β expression in cancer cells36–39.

Figure 7 | Gd@C₈₂(OH)₂₂ impaired EMT under hypoxia. A total of 1 × 10⁶ MDA-MB-231 cells were injected s.c. and mice were treated at day 0 for 21 days. (a) HIF-1α and TGF-β expression in the periphery and inner portions of tumours. Scale bar, 20 μm. (b) The amount of intracellular Gd after MDA-MB-231 cells were treated with 50 μM Gd@C₈₂(OH)₂₂ under hypoxia and normoxia were detected by ICP-MS (mean ± s.e.m., n = 3 each). *P < 0.05, **P < 0.01 (two-way ANOVA, Bonferroni's post-hoc test). (c) MDA-MB-231 cells were cultured under hypoxia and treated with PBS (A, E, I, M, Q), 50 μM Gd@C₈₂(OH)₂₂ (B, F, J, N, R), C₆₀(OH)₂₂ (C, G, K, O, S) and GdCl₃ (D, H, L, P, T) for 10 days. (A–D) F-ACTIN cytoskeleton of cells (scale bar, 12.5 μm). Immunofluorescence staining (e, E-T), qPCR (d) and western blot analysis (e) of E-CADHERIN (E-CAD), γ-CATENIN (γ-CAT), VIMENTIN (VIM) and FIBRONECTIN-1 (FN1) of MDA-MB-231 cells under hypoxia. (f) Cell migration and invasion were examined using trans-well cell culture chambers and Matrigel-coated ones (mean ± s.e.m., n = 6 each). **P < 0.01 (one-way ANOVA, Tukey's post-hoc test). mRNA levels of hif-1α, hif-1β, tgf-β, snail, zeb1, twist1, e47 (g), il-6, il-8, mmp-2, mmp-9 and vegf (h) were analysed by qPCR (mean ± s.e.m., n = 3 each). (i) Protein levels of HIF-1α, HIF-1β, TGF-β, SNAIL, ZEB1, TWIST1 and E47 were detected by western blot.
Figure 8 | Gd@C₈₂(OH)₂₂ impaired EMT by inhibition of TGF-β and HIF-1α expression under hypoxia. MDA-MB-231 cells were transfected with hif-1α expressing plasmid and/or treated with 20 ng ml⁻¹ TGF-β with further culture in the presence of Gd@C₈₂(OH)₂₂ or PBS under hypoxia for 10 days. (a; A-E) F-ACTIN cytoskeleton of cells (scale bar, 12.5 μm). Immunofluorescence staining (a, F-Y) and western blot analysis (b) of E-CADHERIN (E-CAD), γ-CATENIN (γ-CAT), VIMENTIN (VIM) and FIBRONECTIN-1 (FN-1). Scale bar, 25 μm. (c) The migratory and invasive cells were examined using trans-well cell culture chambers and Matrigel-coated ones (mean ± s.e.m., n = 3 each). *P < 0.05 and **P < 0.01 (two-way ANOVA, Bonferroni’s post-hoc test). mRNA levels of EMT markers (d), hif-1α, hif-1β, tgf-β, snail, zeb1, twist1, e47 (e), il-6, il-8, mmp-2, mmp-9 and vegf (f) were analysed by qPCR (mean ± s.e.m., n = 3 each). ELISA analysis for expression of IL-6 (g) and IL-8 (h) in the MDA-MB-231 cell culture medium was determined. All the data are represented as mean ± s.e.m. (n = 3 each) with *P < 0.05 and **P < 0.01 (two-way ANOVA, Bonferroni’s post-hoc test).
Figure 9 | Gd@C82(OH)22 efficiently eliminated breast CSC population through TGF-β and HIF-1α. MDA-MB-231 cells were maintained in PBS, Gd@C82(OH)22, C60(OH)22 or GdCl3 under hypoxia for 10 days for ALDEFLUOR (a) and tumoursphere formation assay (b) (scale bar, 100 μm) (mean ± s.e.m., n = 3 each). *P < 0.05 (two-way ANOVA, Bonferroni’s post-hoc test). (c) The tumourspheres count (mean ± s.e.m., n = 3 each). Tumourspheres of 70–150 μm, *P < 0.05 and **P < 0.01; to tumourspheres of >150 μm, #P < 0.05 and ##P < 0.01 (one-way ANOVA, Tukey’s post-hoc test). (d) The mRNA levels of CSC markers were analysed by qPCR (mean ± s.e.m., n = 3 each). **P < 0.01 (one-way ANOVA, Tukey’s post-hoc test). (e) Tumoursphere formation assay after MDA-MB-231 cells were transfected with HIF-1α expressing plasmid and/or supplemented with 20 ng ml⁻¹ TGF-β and cultured under hypoxia for 10 days (scale bar, 12.5 μm). (f) The tumourspheres count (mean ± s.e.m., n = 3 each). Tumourspheres of 70–150 μm, *P < 0.05 and **P < 0.01; to tumourspheres of >150 μm, #P < 0.05 and ##P < 0.01 (two-way ANOVA, Bonferroni’s post-hoc test). (g) The mRNA levels of CSC markers were analysed by qPCR (mean ± s.e.m., n = 3 each). *P < 0.05 (two-way ANOVA, Bonferroni’s post-hoc test).
to 8.5, the average size of Gd@C_{82}(OH)_{22} nanoparticles decreases to ~84 nm, and at pH 9.7, it is 38 nm, rather smaller (Supplementary Fig. 14).

At pH 4.3, the highly acidic surrounding inhibits the deprotonation of the Gd@C_{82}(OH)_{22} surface hydroxyl groups, most hydroxyls present as C-OH with less C-O/C0 formation. Hence, the attractive hydrogen bonding interactions become weak, which thus inhibits the formation of the larger clusters. Compared with the acidic and alkalic surroundings, a neutral condition (at pH 7.4) presents no drive to inhibit the deprotonation process of Gd@C_{82}(OH)_{22}, it easily produces C-O/C0 groups to form hydrogen bonding with the attractive C-OH groups. Hence, we can understand why the size becomes the largest at the neutral pH value. The alkaline surroundings easily consume the H^{+} dissociated from hydroxyls, so, in the pH 9.7 solution, they mostly present as the repulsive C-O/C0 groups. The repulsive forces among Gd@C_{82}(O/C0)_{n} molecules inhibit their aggregation to form larger size nanoparticles.

Conversely, it was reported that the less negatively charged nanoparticle is internalized better by cells^{44,45}. Hence, we measured the zeta potential of Gd@C_{82}(OH)_{22} nanoparticles in solutions of different pH values. At pH 4.0 ~ 5.0 solutions, \( \zeta \) (negative value) is only half of that observed at pH 7.0 demonstrating that less negative charges are associated with Gd@C_{82}(OH)_{22} nanoparticles in more acidic surroundings, such as would be in a tumour microenvironment. Hence, the physiochemical properties of Gd@C_{82}(OH)_{22} may contribute to its selective uptake in areas of hypoxia where it blocks EMT and effectively targets CSC populations. As CSCs contribute to treatment resistance and facilitate tumour metastasis, Gd@C_{82}(OH)_{22} and/or related compounds may possess significant clinical utility.

A recent study has reported that treatment with anti-angiogenic agents increased intra-tumoral hypoxia in breast cancer xenografts and resulted in an increase in CSC populations^{31}. This increase in CSCs generated by tumour hypoxia was mediated by HIF-1α and may limit the efficacy of antiangiogenic agents. Gd@C_{82}(OH)_{22} has also been reported to be a potent inhibitor of tumour angiogenesis^{1,8}. As Gd@C_{82}(OH)_{22} specifically targets CSCs in hypoxia, the use of Gd@C_{82}(OH)_{22} might represent a novel strategy to abrogate tumour neo-angiogenesis without exacerbating the CSC population consequent to intra-tumoral hypoxia (Fig. 7a) by effectively targeting both bulk tumour cells and CSCs. Furthermore, the ability of Gd@C_{82}(OH)_{22} to accumulate in areas of tumour hypoxia may complement the well-known EPR (Enhanced Permeability and Retention) mechanism by which nanoparticles penetrate and accumulate in tumours via their leaky vasculature^{4,46}. Thus, the identification of fullerol nanomaterial with intrinsic CSC specificity represents a novel approach to target this crucial cancer cell population. The apparent absence of significant toxicity of these nanomaterials in normal tissue further highlights their therapeutic potential.

![Figure 10 | Schematic diagram of key pathways by which Gd@C_{82}(OH)_{22} nanoparticles inhibits tumour growth.](image-url)
Methods
Preparation of Gd@C_{82}(OH)_{22} and C_{60}(OH)_{22} nanoparticles. Gd@C_{82}(OH)_{22} nanoparticles were synthesized by the Kratschmer-Huffman method and extracted by a high-temperature and high-pressure method. Gd@C_{82} was separated and purified using high-performance liquid chromatography (HPLC, LC908-C60, Japan Analytical Industry), and identified by a matrix-assisted laser desorption time-of-flight mass spectrometer (MADLI-TOF-MS, Auto-Flex, Bruker, Germany). Gd@C_{82}(OH)_{22} was synthesized by the alkaline reaction and purified by Sephadex G-25 column chromatography (5 × 50 cm) with an eluent of neutralized water. C_{60}(OH)_{22} nanoparticles were prepared. In brief, 2 ml NaOH (2.22 g ml⁻¹) and 1 ml tetrabutyl ammonium hydroxide (TBAH) was mixed with 30 ml C_{60} (Sigma Aldrich) toluene (1.5 mg ml⁻¹). After the mixture was stirred for 24 h, solution was stirred continuously for another 12 h and then kept motionless for 2 h. The aqueous phase was washed three times. The precipitate was dispersed in ultrapure water after evaporating the methanol. Finally, the dispersion solution of the precipitate was purified using a Sephadex G-25 column (5 × 50 cm) using deionized water as the eluent.

Cell culture. All cell lines used in the studies were purchased from the American Type Culture Collection (Rockville, Maryland, USA). All cell strains were cryopreserved within three passages and no cell aliquot was cultured continuously for more than 6 months. No cross-contamination of other human cells was observed. The cell lines utilized are 100% matched with those of ATCC. Possible mycoplasma contamination of all cell lines in the laboratory is routinely and regularly monitored using mycoplasma detection set (M&C Gene technology). Contamination of all cell lines in the laboratory is routinely and regularly monitored using mycoplasma detection set (M&C Gene technology).

MDA-MB-231 and BT549 were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (Gibco), 100 U ml⁻¹ penicillin and 1 mg ml⁻¹ streptomycin (Invitrogen). MCF-10A was cultured in DMEM/F12 medium supplemented with 5% fetal bovine serum (Invitrogen). 10 ng ml⁻¹ insulin, 20 ng ml⁻¹ EGF, 100 ng ml⁻¹ cholera toxin, 500 ng ml⁻¹ hydrocortisone and 1% Antibiotic-Antimycotic (penicillin, streptomycin and amphotericin B). For normoxic culture, cell lines were cultured in the presence of PBS, Gd@C_{82}(OH)_{22}, C_{60}(OH)_{22} or GdCl₃ (50 µM) for 21 days at 37 °C in an incubator with 95% humidity (95% CO₂). For hypoxic culture, cells were maintained in stable and controlled hypoxic conditions (1.5 % O₂, 95 % N₂, and 5 % CO₂) for 10 days at 37 °C in a modular incubator chamber (Billups-Rothenberg). Partial pressure of oxygen (pO₂) was between 7 and 8 kPa in the culture plate. For rescue experiments, experiments were treated with 20 ng ml⁻¹ recombinant human TGF-β (Minneapolis, MN, USA) for 48 h.

Inductively coupled plasma mass spectrometry analysis. MDA-MB-231 cells were incubated with Gd@C_{82}(OH)_{22}, GdCl₃, or CuCl₂ for 20 min at 37 °C in a medium supplemented with 0.5% gelatin. For hypoxic culture, cells were maintained in stable and controlled hypoxic conditions (1.5 % O₂, 95 % N₂, and 5 % CO₂) for 10 days at 37 °C in a modular incubator chamber (Billups-Rothenberg). Partial pressure of oxygen (pO₂) was between 7 and 8 kPa in the culture plate. For rescue experiments, experiments were treated with 20 ng ml⁻¹ recombinant human TGF-β (Minneapolis, MN, USA) for 48 h.

Cell morphology. Two-dimensional (2D) and three-dimensional (3D) cell culture were used to observe cell morphology. Twenty-four-well plates were pre-coated with growth factor reduced Matrigel. In the 2D culture model, single-cell suspensions were directly plated (2 × 10⁴ cells/well) in complete culture medium on the 100% matrigel bed. In the 3D model, cells (2 × 10⁴ cells/well) were grown in complete culture medium containing 2% matrigel. After 5 days, all microscopic images were captured using Nikon TE 300 (Nikon, Japan).

Cells were fixed with 2.5% glutaraldehyde overnight and then incubated with 0.1% Triton-100 solution. The actin cytoskeleton in cells was visualized with 1 µg ml⁻¹ rhodamine-labelled Phalloidin (diluted in 1% Triton-100 solution). The nuclei were stained with 0.1% crystal violet for 10 min. Migrated or invaded cells were photographed and counted in five randomly chosen fields.

Wound-healing assay. Tumour cell migration was assessed using a wound-healing assay. In six-well plates, cells were cultured until they reached confluent. After culture in serum-free DMEM for 24 h, the monolayers were scratched with woundings by plastic tips and then were gently rinsed with PBS three times. Phase-contrast images were captured after further culture for 0, 12 and 30 h.

Cell proliferation assay. A cell count kit-8 (CCK-8) (Kumamoto Techno Research Park, Japan) was used to examine cell proliferation. CCK-8 includes WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4- disulfonylphenylazo)-2H-tetrazolium sodium) that can be reduced to highly water-soluble formazan dye, which is yellow. In brief, cells were incubated with 100 µl of culture medium in 96-well multiwell plates. Media were removed and 100 µl DMEM containing CCK-8 (10%) was added to each well. After 2 h incubation at 37 °C, the absorbance at 450 nm of each well was measured using a standard enzyme-linked immuno sorbent assay (ELISA)-format spectrophotometer.

Detection of apoptosis and necrosis. Apoptotic cells and necrotic cells were analyzed by double staining with Alexa Fluor 488 annexin V and propidium iodide (PI) (Invitrogen, USA), in which Alexa Fluor 488 annexin V bound to apoptotic cells with exposed phosphatidylserines (PS), while PI labelled necrotic cells with membrane damage. All cells (floating and adherent) were collected and washed once with cold PBS. Five microtubes Alexa Fluor 488 Annexin V was added to the cell suspension in the presence of 100 µl binding buffer and incubated for 20 min at room temperature. Cells were co-stained with 100 µl PI (1 µg ml⁻¹) and immediately analysed or sorted using BD flow cytometer (BD, USA). The percentage of apoptotic (annexin +/PI−) and necrotic (annexin +/PI+) cells was determined using software. Data represent the mean fluorescence obtained from a population of 10,000 cells.

ELISA analysis of TGF-β, IL-6 and IL-8 levels. Levels of TGF-β, IL-6 and IL-8 in culture supernatants for MDA-MB-231 cells were determined by a specific ELISA according to the manufacturer’s instructions, using matched antibody pairs and recombinant cytokines as standards (Cell Signaling Technology, USA). In brief, cells were cultured with PBS, Gd@C_{82}(OH)_{22}, CuCl₂ or GdCl₃ for 3, 7, 14, 21 and 28 days. Cells were washed and fresh media was added. After 24 h, culture supernatants were collected. Ninety-six-well multiwell plates were coated with the corresponding purified anti-human capture monoclonal antibody were used. Culture supernatants and serial dilutions of the standard were added to each well and incubated for 90 min at 37 °C. After four washes, bound samples were detected using the corresponding biotinylated anti-mouse antibody at 37 °C for 1 h. After another four washes, avidin-horseradish peroxidase solution was added, and plates were incubated at 37 °C for 30 min. After the final four washes, plates were kept at 37 °C for 20 min to react with the substrate solution. A 100 µl of blocking solution was added to stop the reaction, and the absorbance at 450 nm was then recorded. Results were expressed in pg ml⁻¹, and three independent experiments were performed.

Cell extraction and quantitative real-time PCR. Total RNA was extracted from cultured cell lines and primary tumours using Trizol reagent following the manufacturer’s guideline (Invitrogen, USA). mRNA was converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen, USA), and then expression levels of analysed genes were determined using the SYBR Premix Ex Taq Kit (Takara, Japan). The relative amount of gene transcripts was normalized to GAPDH. Primers are listed in Supplementary Table 5.

Western blotting. Monolayer cells were washed three times using ice-cold PBS and lysed using RIPA lysis buffer (40 mM Tris, 150 mM NaCl, 10 mM ethylene-diamine tetracetic acid, 1% glycerol, 1% Triton X-100, 10 mM glycophosphate, 1 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride) supplemented with complete protease inhibitor cocktail tablets (Roche, Switzerland). BCA protein assay (Thermo Fisher Scientific, Australia) was used to determine protein concentration.
Fifty micrograms of proteins were separated by SDS–polyacrylamide gel electro-
phoresis (SDS–PAGE) and transferred onto a polyvinylidene difluoride membrane (Bio-Rad, USA). After blocking for 60 min with 2% BSA, membranes were incu-
bated with the primary antibody overnight at 4 °C followed by incubation with the
Corresponding author: S. G. Kang.

### Retrospective analysis

**Identification of stem-like cells.** A total of 106 MDA-MB-231 cells were
injected. When the tumours grew to 100 mm3, the mice were treated for 21
days. Tumour volumes were calculated according to the following formula:

\[
V = \frac{4}{3} \pi \left( \frac{a \times b}{2} \right)^2
\]

where \(a\) and \(b\) are the longer and shorter diameters of the tumour, respectively.

**Histological analysis.** After surgical removal, tumours, lungs and livers were fixed overnight in 10% formalin neutral buffer, dehydrated in a series of graded ethanol solutions and embedded in paraffin. Baseline histological slides containing sections (4–5 μm thickness) were stained with haematoxylin/Eosin (HE) followed by dehydration
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Author contributions

Y.L., P.Q. and C.C designed and performed the experiments, collected and analysed the data and co-wrote the manuscript. Y.Z. and T.Z. conceived the principal idea, designed the experiments and wrote the manuscript. R.S. and J.D. provided Gd@C82(OH)22 nanoparticles. X.L., X.Z. and W.Z. performed the qPCR and western blot experiments. H.L., Z.G., L.W., R.B. and J.T. performed animal experiments. X.G. ran the density analysis of hypoxia of the tumour and wrote the manuscript. B.S. and J.D. provided Gd-metallofullerol nanomaterial as a pleiotropically acting microRNA, miR-31, inhibits breast cancer metastasis. Cell 137, 1032–1046 (2009).

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