Supporting Information

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SI Materials and Methods

Materials. The 5, 20, 50, and 100 nm citrate-reduced nanoparticles were purchased from Ted Pella. They were used without any further modifications (see Figs. S1 and S2 for characterization).

Tetrachloroauric acid trihydrate, trisodium citrate, and sodium borohydride were from Sigma-Aldrich. [^{3}H] Thymidine was from Perkin-Elmer. Media and PBS was purchased from Mediatech. Primary antibodies were purchased from Cell Signaling (phospho-p42/44, total p42/44, Vimentin, E-Cadherin (E-Cad), phospho-stress-activated protein kinases (SAPK)/Jun N-terminal kinase (Jnk), total-SAPK/Jnk, phospho-p38, total-p38), N-Cadherin was purchased from BD Transduction Laboratories, and Snail and Twist from Santa Cruz Biotechnology. Phalloidin was purchased from Invitrogen. α-Tubulin was purchased from AbCam. Secondary antibodies were purchased from Santa Cruz Biotechnology. Scintillation mixture was purchased through Fisher Scientific. The Human Angiogenesis Proteome Profiler array kit was purchased through R&D Systems. *N*-Luciferin was purchased through Gold Bio Technologies.

Fabrication of 20 nm gold nanoparticles for animal model. Briefly, glassware and stir bar were rinsed extensively with distilled water, then Milli-Q water. Flasks were scratch-free. In a 250 mL flask, 2.5 mL of a 10 mM solution of tetrachloroauric acid trihydrate (HAuCL4) in 90 mL water was brought to a roiling boil with vigorous stirring. Once boiling, 7.5 mL of 1% solution sodium citrate (preheated to ca. 70 °C) was added rapidly. After ~30 s, the pale yellow solution became dark, then turned a deep red color. This solution was left to boil for an additional 10 min, at which point it was removed from heat and allowed to cool to room temperature while stirring. The gold nanoparticles (AuNPs) were characterized by transmission electron microscopy (TEM) after drop-coating 100 μL of the sample on a carbon-coated copper grid. The size of the nanoparticles was determined from analysis of the TEM images and dynamic light scattering (DLS) (Malvern Zetasizer Nano ZS). Zeta potential measurements were done using a clear zeta disposable capillary (Malvern DTS1061). All of the nanoparticles were concentrated by centrifugation before in vitro and in vivo studies. For the 50 nm and 100 nm AuNPs, they were centrifuged for 15 min at 8,000 × *g* at 10 °C. The 5 nm and 20 nm AuNPs were centrifuged for 45 and 15 min, respectively, at 13,200 rpm at 10 °C. Centrifugations were performed in a Beckman L7-55 Ultracentrifuge using 50.2 Ti rotor. Gold content in the pellet was quantified by Instrumental Neutron Activation Analysis (INAA).

Cell culture. The human ovarian cancer cell lines OVCAR5 and SKOV3-ip were grown in DMEM and McCoy's 5A medium, respectively. The human ovarian cancer cell line A2780 was grown in Roswell Park Memorial Institute (RPMI) medium 1640 (high glucose). All of the media was supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antibiotics (Penicillin/Streptomycin), and the cell lines were maintained at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air. Ovarian surface epithelial (OSE) cells were grown in MCDB105/Medium 199 (Sigma) supplemented with 15% FBS and 1% antibiotics (Penicillin/Streptomycin).

Cell proliferation assay. Ovarian cells (2×10^4) were seeded in 24-well plates and cultured overnight. The next day, the cells were replenished with starving media and were treated with various concentrations of AuNPs of various sizes for different periods of time as indicated. Following treatment, 1 μCi [^{3}H]thymidine was added; 4 h later, cells were washed with chilled PBS, fixed with 100% cold methanol, and collected for measurement of trichloroacetic acid (TCA)-precipitable radioactivity. Experiments were repeated at least three times each time in triplicate.

Cellular apoptosis assay. Cells were seeded in 60 mm dishes at 3×10^5 and allowed to grow overnight in standard conditions. The next day the growth media was removed and replaced with starving media. After starving the cells for 24 h, they were then treated with different doses of 20 nm AuNPs for 48 h in starving conditions. Annexin FITC–propidium iodide staining flow cytometry was performed per manufacturer's protocol (BioVision) after 48 h of treatment with 20 nm AuNPs.

Human Angiogenesis Array. A2780 cells were seeded at 4×10^5 in 60 mm dishes and allowed to grow under normal conditions overnight, and the assay was performed according to the manufacturer's instructions. The following day, the growth media was removed and replaced with starving media; the cells were allowed to grow overnight. The next day, the starving media was replenished and the cells were treated with 20 μg/mL of 20 nm AuNPs. After 48 h, the media was collected and centrifuged at 14,000 × *g* for 10 min. The resulting pellet was discarded, and the supernatant was collected for further analysis. Cells were lysed in radioimmunoprecipitation assay buffer (RIPA) (Boston Bio Products) containing HALT (Thermo Scientific) and collected into clean tubes. They were pelleted in a microcentrifuge at 14,000 × *g* for 10 min, and the supernatant was transferred into a clean test tube. An assay of human angiogenic cytokine expression was performed according to the manufacturer's instructions on the media and lysed cells (ARY007, R&D Systems).

Western Blot Analysis. Cell lysates (20 μg) were electrophoresed through 10% denaturing polyacrylamide gels (BioRad) and transferred to a polyvinylidene difluoride membrane (Millipore). The blots were probed with primary antibodies, and bound antibody was detected using enhanced chemiluminescence according to the manufacturer's protocol. Primary antibody dilution was a 1:500 for p42/44 (phospho and total), SAPK/Jnk (phospho and total), p38 (phospho and total), E-Cad, *N*-Cad, and Vimentin. The dilution factor for Snail and Twist was 1:250 and 1:5,000 for α-Tubulin. Secondary antibody dilution factors were 1:10,000 (3).

Transmission Electron Microscopy. Cells were treated with AuNPs for 24 h in starving conditions. After the incubation, cells were washed thrice in PBS and cell pellets collected after trypsinization and centrifugation at 14,000 × *g* for 10 min. The resultant cell pellets were further washed thrice with PBS and fixed in Trumps fixative (1% glutaraldehyde and 4% formaldehyde in 0.1 M phosphate buffer, pH 7.2) and processed for TEM sectioning. Micrographs were taken on a TECNAI 12 operating at 120 kV. TEM samples from the extracted mice tumors were prepared as follows; on day 22, the mice ($n = 5$) were killed and the tumors were collected, weighed, measured, and placed immediately in Trumps fixative solution and stored on ice. The tumors were briefly removed from the Trumps solution and sliced thinly for TEM mounting. Micrographs were taken on a TECNAI 12 operating at 120 KV.

Primer Sequences for Quantitative RT-PCR. TGF-β, forward: GCCTTTCCTGCTTCTCATGG, reverse: TCCTTGCGGAAGTCAATGTAC; PDGF, forward: GATACCTCGCCCATGTTCTG, reverse: CAAAGAATCCTCACTCCCTACG; hepatocyte growth factor, forward: GCTATACTCTTGACCCTCACAC, reverse: GTAGCCTTCTCCTTGACCTTG; urokinase plasminogen

activator, forward: GGGAGATGAAGTTTGAGGTGG, reverse: AGATGGTCTGTATAGTCCGGG; basic fibroblast growth factor (bFGF), forward: ACCCTCACATCAAGCTACAAC, reverse: AAAAGAAACACTCATCCGTAACAC; Serpin E1, forward: GTGGACTTTTCAGAGGTGGAG, reverse: GAAGTAGAGGGCATTCACCAG; and GAPDH, forward: ACATCGCTCAGACACCATG, reverse: TGTAGTTGAGGTCAATGAAGGG.

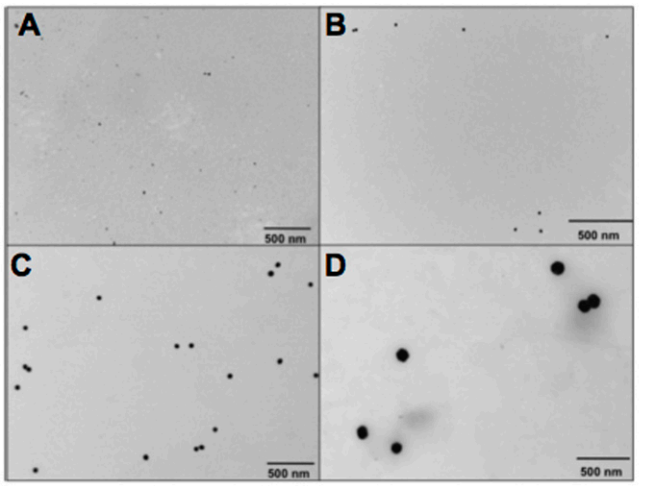
Confocal Microscopy. The treatment was taken off after 48 h and acid-stripped on ice for 1 min with stripping buffer (14.6 g NaCl, 2.5 mL acetic acid in 500 mL H_2O) to remove surface-bound nanoparticles. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% TritonX-100 in PBS, and blocked with 4% BSA in PBS for 1 h at RT followed by staining with E-Cad antibody (BD Biosciences; 1:100 dilution in 1% BSA containing PBS) for 25 min at RT, blocking with 5% goat serum in 1% BSA containing PBS for 30 min at RT and staining with Cy3-labeled goat anti-mouse secondary (Mayo Clinic Microscopy Core; 1:500 dilution in 1% BSA containing PBS) and Phalloidin-Alexa488 conjugate (Invitrogen; 1:400 dilution in 1% BSA containing PBS) for 30 min at RT. The cells were then mounted with Vector Shield containing DAPI.

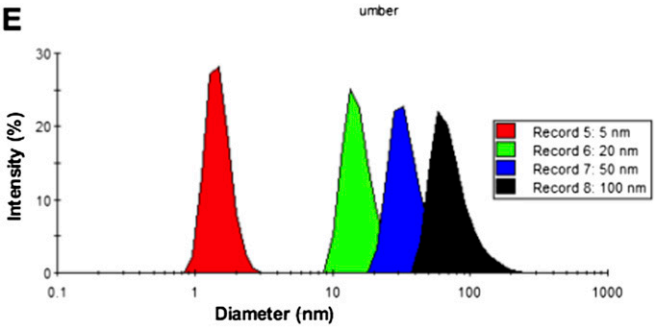
Measurement of gold content by INAA. Briefly samples were transferred with 100 μL of 18 MIliohm water into a precleaned, high-density polyethylene irradiation vials, lyophilized to constant dry weight, and mass recorded. Samples were then loaded in polyethylene transfer "rabbits" and irradiated for 90 s in a thermal flux density of ~$5 \times 1{,}013$ ncm^2/s.

TUNEL assay. Paraffin-embedded tumor tissues were cut into 4-μm sections. TUNEL assay was conducted using the in situ Cell Death Detection kit from Roche Applied Science following the manufacturer's instructions. In brief, tissue sections were deparaffinized by 30 min of incubation at 65 °C, followed by a series of washes in xylenes and ethanol. The tissues were permeabilized by a 30-min incubation at 95 °C in EDTA (pH 8.0) followed by 10-min exposure to Proteinase K at RT. The sections were then exposed to a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and nucleotides with FITC-labeled dUTP.

bFGF ELISA. Human recombinant bFGF and the Quantikine bFGF ELISA was obtained from R&D Systems (233-FB-025/CF and DFB50, respectively). We incubated 20 μg/mL of AuNPs (20, 50, and 100 nm) with 300 picograms (pg) of bFGF in PBS containing a protease inhibitor mixture (Sigma; P8465). After mixing for 30 min at RT, the samples were then centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was removed. The amount of unbound bFGF in the supernatant was then quantified using the bFGF ELISA following the manufacturer's protocol. For a positive control, we added 500 pg of bFGF in PBS (with protease inhibitor mixture), which ran alongside the nanoparticle samples.

Preclinical model of ovarian cancer. Female athymic nude mice (NCr-nu; 6–8 wk old) were purchased from the National Cancer Institute–Frederick Cancer Research and Development Center. All mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the US Department of Agriculture, US Department of Health and Human Services, and National Institutes of Health. All studies were approved and supervised by the Mayo Clinic Institutional Animal Care and Use Committee. On day 4 postinjection, bioluminescence was measured and the mice randomized into several groups before the initiation of treatment. In vivo optical imaging for luciferase was done 10 min after i.p. injection of 30 mg n-Luciferin (pharmaceutical grade) into each animal using a Xenogen–IVIS–cooled CCD optical system (Xenogen–IVIS). Mice were anesthetized with isoflourene before the administration of luciferen. All of the chemicals were pharmaceutical grade. Tumor growth was monitored using the Xenogen–IVIS twice a week as described above. For the mouse model using A2780 cells, 20 nm citrate-capped AuNPs (injected concentrations: 100, 200, or 400 μg) were injected i.p. to each mouse 3×/wk using a syringe fitted with a 28–30 needle. The mouse model using SKOV-ip3 cells were injected with 200 μg/mL of 20 nm citrate-capped AuNPs. The treatments for both mouse models continued for a period of 3 wk, with the first injection beginning 4 d after tumor inoculation (nine total treatments). Mice weights were recorded weekly (Fig. S8 *B* and *C*), and their health/behavior was monitored daily. Efficacies of the AuNP dosimetry groups were compared with the control groups, where mice were treated only with PBS. After the ninth treatment and assessing tumor growth/regression in these animals, mice were killed by CO_2 inhalation with tumors and tissue harvested for further analysis.





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AuNP	5 nm	20 nm	50 nm	100 nm
Zeta Potential (mV)	-46.9	-45.1	-46.6	-50.4

Fig. S1. Nanoparticles used in the in vitro studies are fairly monodisperse. TEM micrograph of (*A*) 5 nm, (*B*) 20 nm, (*C*) 50 nm, and (*D*) 100 nm AuNPs used for in vitro studies after drop-coating 100 μL of the sample on a carbon-coated copper grid. The nanoparticles were further characterized using (*E*) DLS and (*F*) zeta potential.

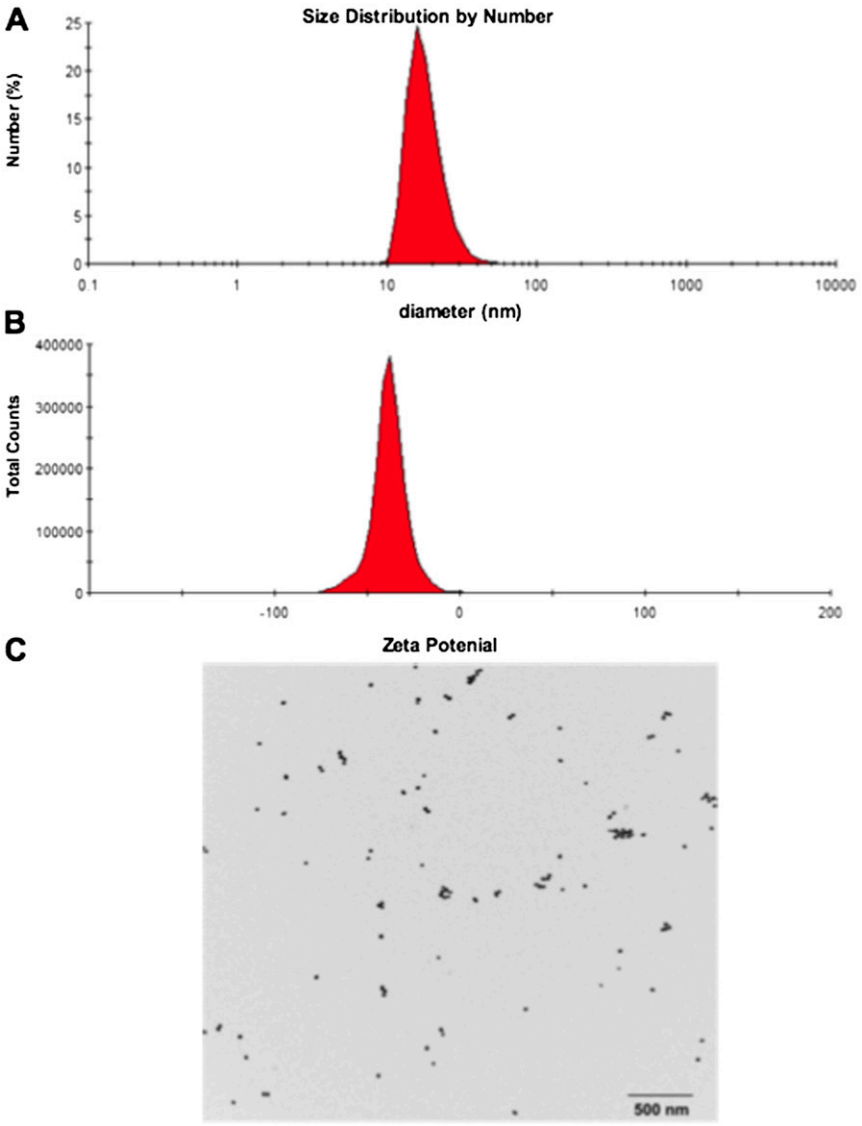

Fig. S2. Characterization of the synthesized 20 nm AuNPs used for the in vivo studies. (*A*) DLS of the AuNP reveals that it has a similar diameter of the purchased AuNPs. (*B*) Zeta potential of the AuNP shows circa –45 mV. (*C*) TEM micrograph of the 20 nm AuNP shows they are fairly monodisperse.

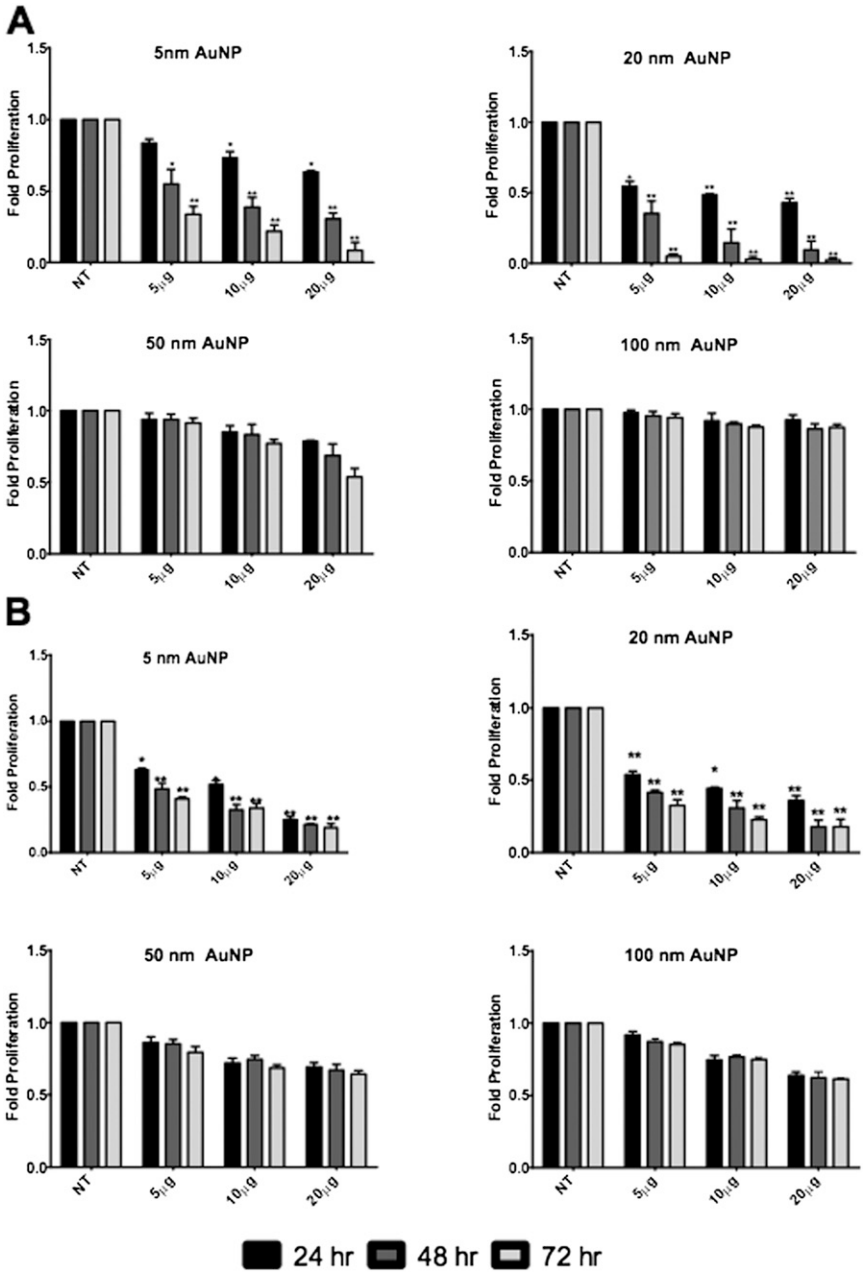

Fig. S3. Proliferation of (*A*) A2780 and (*B*) OVCAR5 upon incubation with 5, 20, 50, and 100 nm AuNPs over time and varied concentrations. $n = 3$; error bars are ±SD; $*P > 0.05$, $**P < 0.001$.

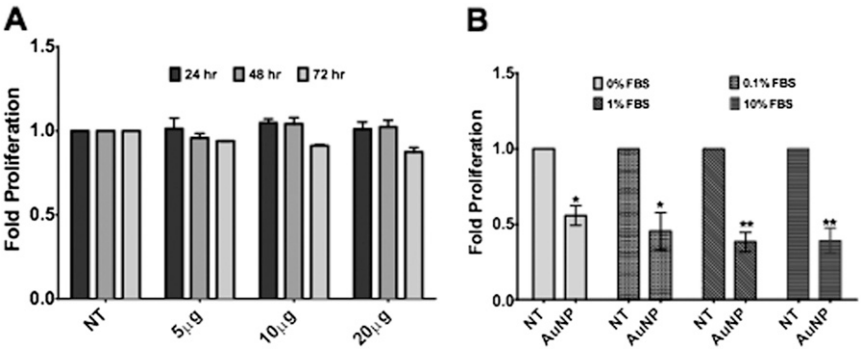

Fig. S4. Normal OSE cells are not affected after exposure to AuNPs. (*A*) Proliferation of OSE after incubation with 20 nm AuNPs after various exposure times and concentrations. (*B*) A2780 cells were incubated with 20 nm AuNPs (20 μg/mL) for 48 h with media containing various levels of FBS (0–10%). $n = 3$; error bars are ±SD; $*P > 0.05$, $**P < 0.001$.

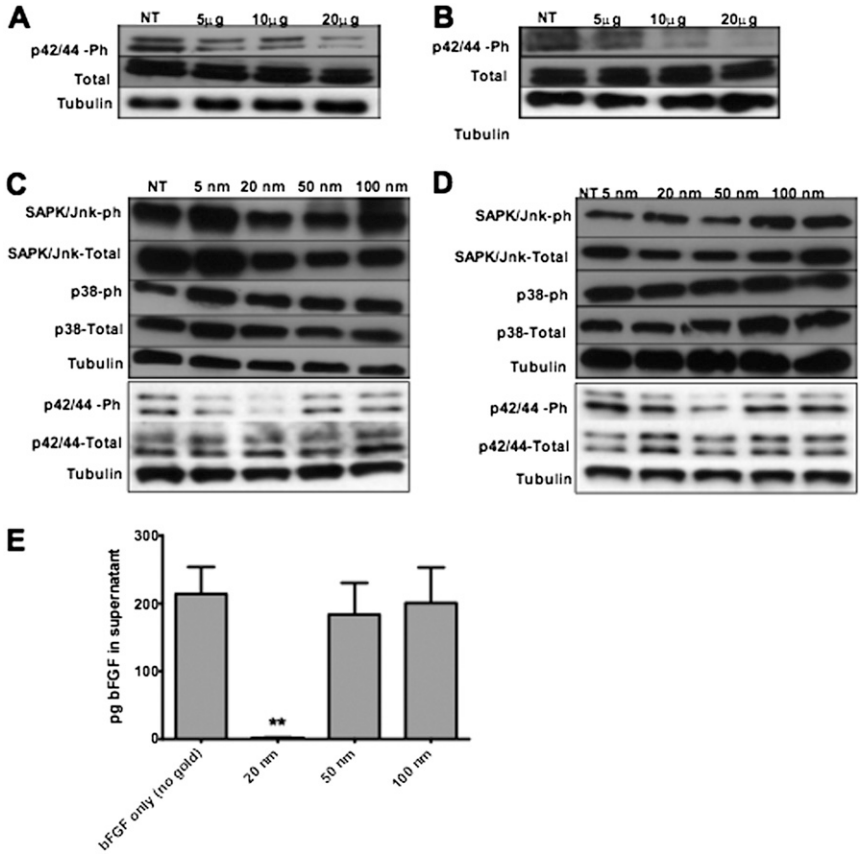

Fig. S5. Determining the effect of AuNPs on the MAPK pathways in ovarian cancer cells. Serum-starved (*A*) OVCAR5 and (*B*) A2780 cells were incubated with 20 nm AuNPs (20 μg/mL) for 48 h, and the cell lysates were immunoblotted with antibodies to p42/44-phospho (p42/44-Ph) and total p42/44 levels in the cell extracts. Serum-starved (*C*) SKOV3-ip and (*D*) A2780 cells were incubated with various sizes of AuNPs (5 nm, 20 nm, 50 nm, and 100 nm) at a concentration of 20 μg/mL for 48 h, and the cell lysates were immunoblotted with antibodies to p38 (phospho and total), SAPK/Jnk (phospho and total), and p42/44 (phospho and total). α-Tubulin was used as a loading control. (*E*) AuNPs of 20, 50, and 100 nm (20 μg/mL) were preincubated with 300 pg of bFGF for 30 min at RT. The supernatant was removed, and an ELISA for bFGF was run and analyzed. (Scale bar, 500 nm.) $n = 3$; error bars are ±SD; $*P > 0.05$, $**P < 0.001$.

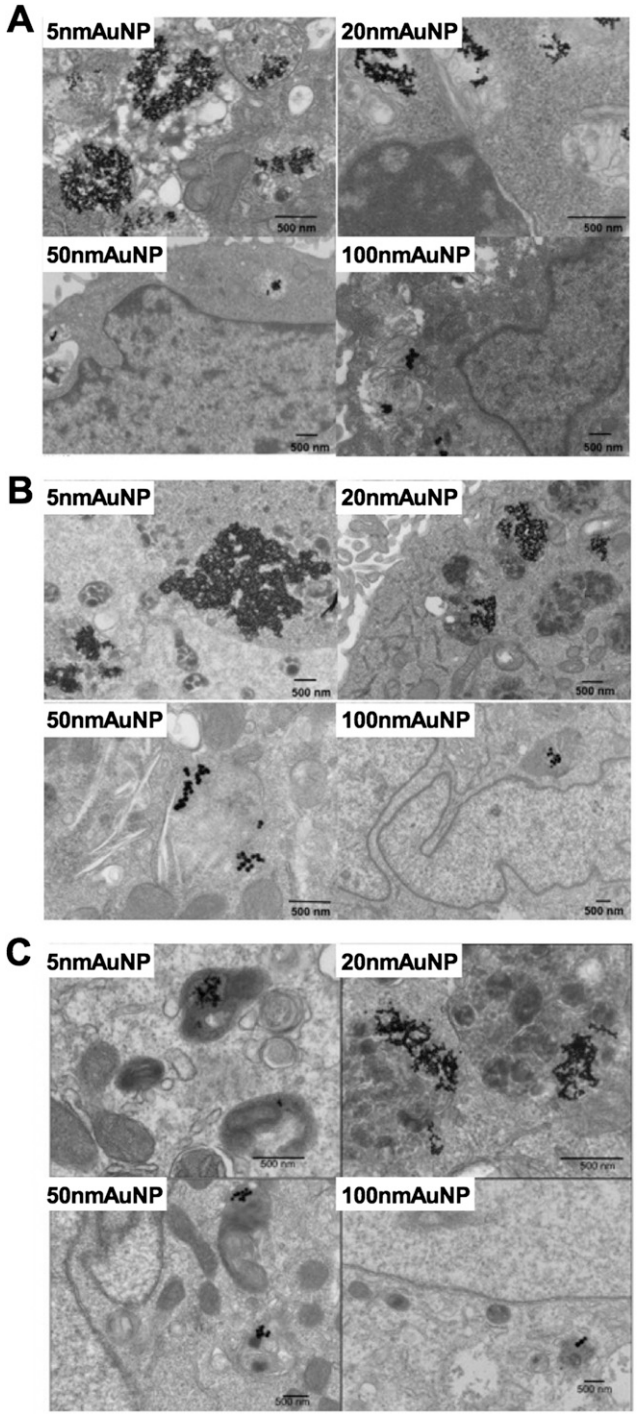

Fig. S6. TEM images at 500 nm magnification showing internalization of the various sizes (5, 20, 50, and 100 nm) of AuNPs uptake into (*A*) SKOV3-ip, (*B*) OVCAR5, and (*C*) A2780 cells after 48 h of treatment (20 μg/mL).

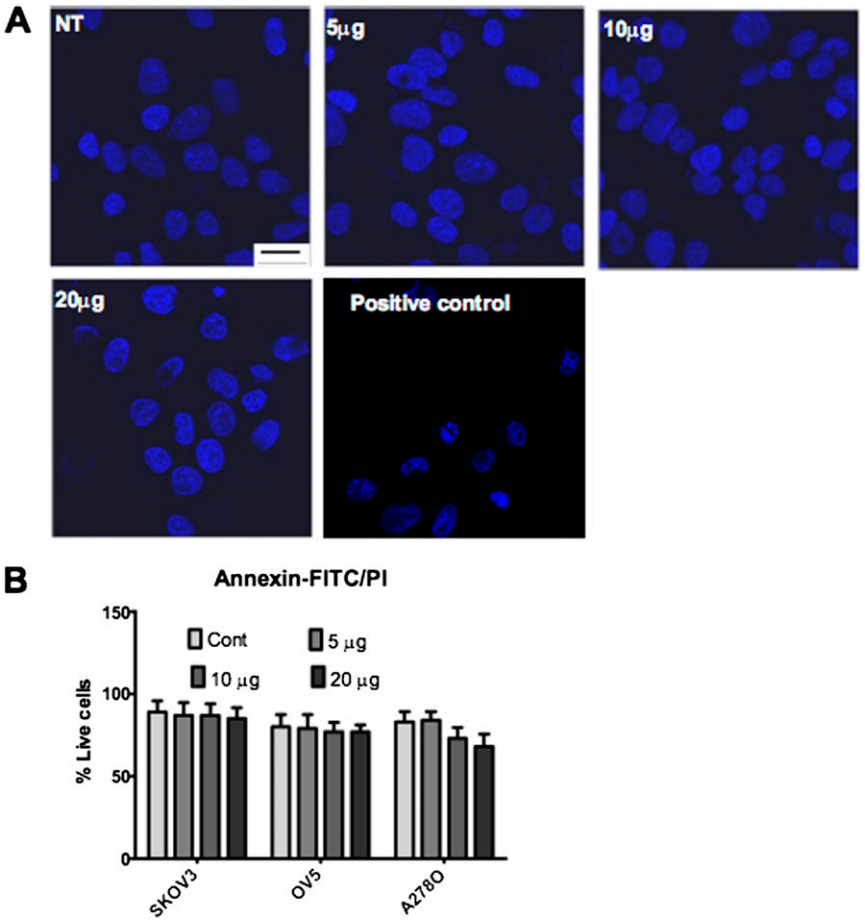

Fig. S7. Effect of 20 nm AuNPs on cellular apoptosis. (*A*) SKOV3-ip cells were exposed to various doses of 20 nm AuNPs in starving conditions for 48 h. No apparent change in the nuclear morphology (DNA condensation and convolution) was noted excluding a proapoptotic role of the nanoparticle on these cells. For the positive control, SKOV3-ip cells were treated with 50 μM of cisplatin for 24 h. (*B*) Similar results were seen after staining with Annexin–FITC/propidium iodide (PI). Values are means ± SD. (Scale bar, 20 mm.)

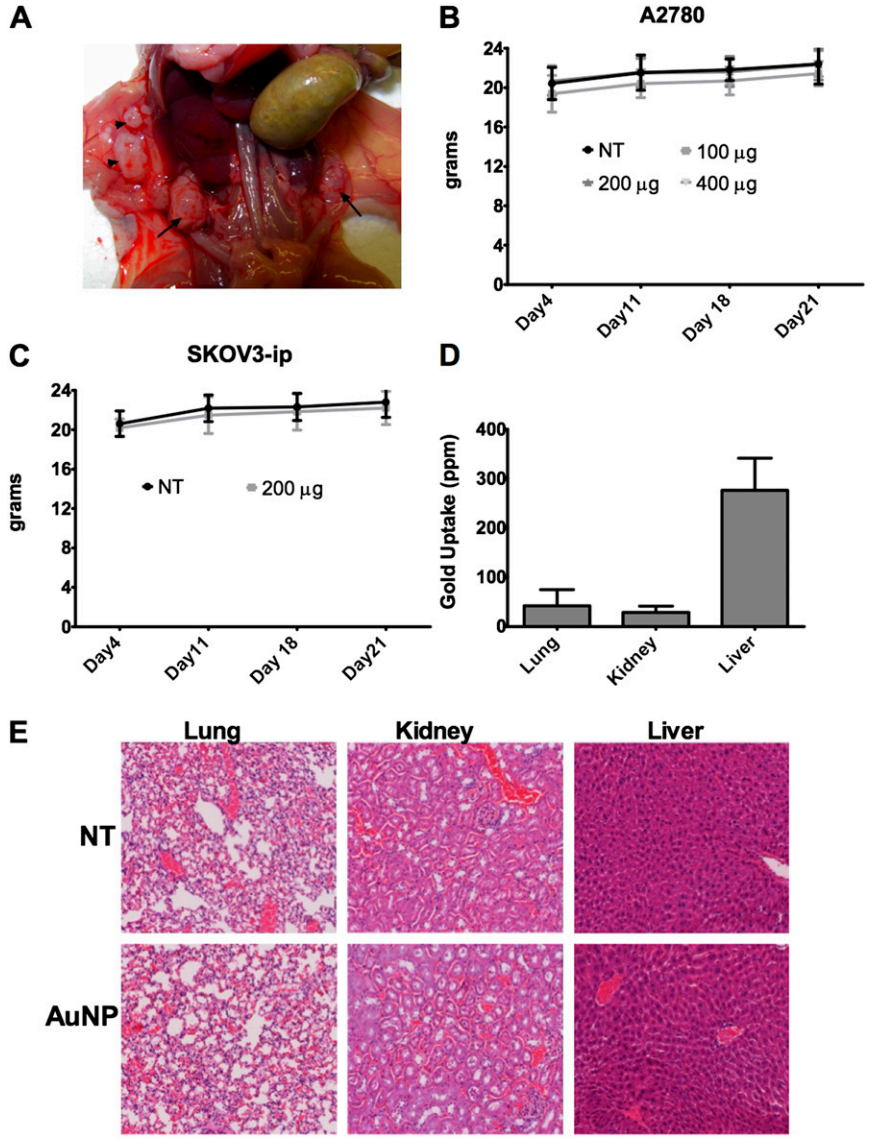

Fig. S8. (*A*) Representative image of tumors and nodules collected from SKOV3-ip tumor–bearing mice. Arrows point at the primary tumor in the ovary, whereas the arrowheads indicate the metastatic nodules in the peritoneum. (*B* and *C*) Mice weights were recording during the course of the study. The increase in weight over time indicates that the AuNPs were not toxic to the animals. (*D*) Lung, liver, and liver tissues were analyzed for gold using INAA ($n = 3$). (*E*) Immunohistochemistry (IHC) analysis of lung, kidney, and liver tissues of mice bearing A2780 tumors shows they are free of inflammation and do not indicate any signs of toxicity. Images shown are representative.

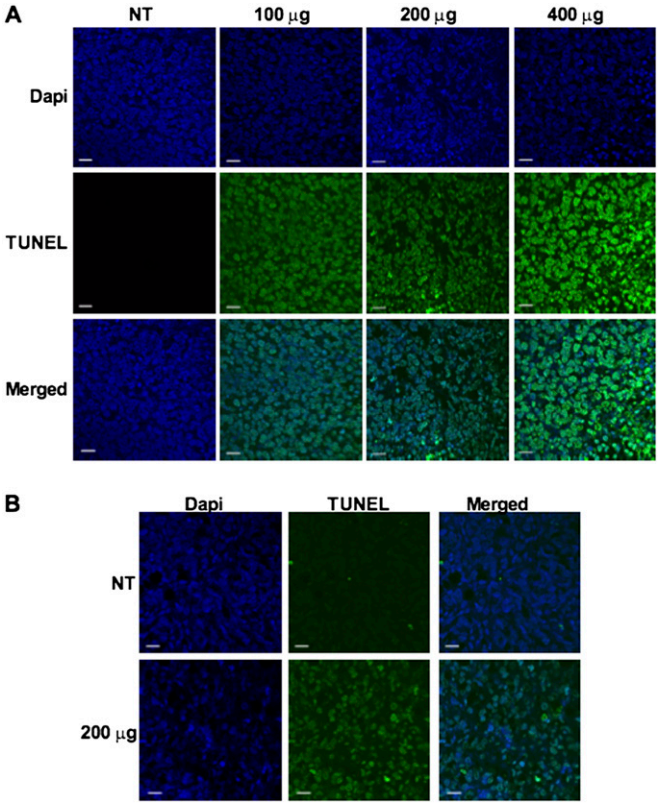

Fig. S9. TUNEL staining of tumor tissue from mice injected with (*A*) A2780 cells and (*B*) SKOV3-ip cells indicates AuNP treatment leads to cellular death. (Scale bar, 50 μm.) Representative images.