

Topical delivery of siRNA-based spherical nucleic acid nanoparticle conjugates for gene regulation

Dan Zheng^{a,b}, David A. Giljohann^{a,b}, David L. Chen^c, Matthew D. Massich^{a,b}, Xiao-Qi Wang^c, Hristo Iordanov^c, Chad A. Mirkin^{a,b,1}, and Amy S. Paller^{c,1}

^aDepartment of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208; ^bInternational Institute for Nanotechnology, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208; and ^cDepartment of Dermatology, Feinberg School of Medicine, Northwestern University, 676 North St. Clair Street, Suite 1600, Chicago, IL 60611

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Topical application of nucleic acids offers many potential therapeutic advantages for suppressing genes in the skin, and potentially for systemic gene delivery. However, the epidermal barrier typically precludes entry of gene-suppressing therapy unless the barrier is disrupted. We now show that spherical nucleic acid nanoparticle conjugates (SNA-NCs), gold cores surrounded by a dense shell of highly oriented, covalently immobilized siRNA, freely penetrate almost 100% of keratinocytes in vitro, mouse skin, and human epidermis within hours after application. Significantly, these structures can be delivered in a commercial moisturizer or phosphate-buffered saline, and do not require barrier disruption or transfection agents, such as liposomes, peptides, or viruses. SNA-NCs targeting epidermal growth factor receptor (EGFR), an important gene for epidermal homeostasis, are >100-fold more potent and suppress longer than siRNA delivered with commercial lipid agents in cultured keratinocytes. Topical delivery of 1.5 μ M EGFR siRNA (50 nM SNA-NCs) for 3 wk to hairless mouse skin almost completely abolishes EGFR expression, suppresses downstream ERK phosphorylation, and reduces epidermal thickness by almost 40%. Similarly, EGFR mRNA in human skin equivalents is reduced by 52% after 60 h of treatment with 25 nM EGFR SNA-NCs. Treated skin shows no clinical or histological evidence of toxicity. No cytokine activation in mouse blood or tissue samples is observed, and after 3 wk of topical skin treatment, the SNA structures are virtually undetectable in internal organs. SNA conjugates may be promising agents for personalized, topically delivered gene therapy of cutaneous tumors, skin inflammation, and dominant negative genetic skin disorders.

transdermal delivery | nanocluster | antisense | nanotechnology | dermatology

The recent development of small molecule inhibitors and antibodies that target components of signaling pathways has revolutionized the treatment of cancers, inflammatory diseases, and genetic disorders, including those that largely manifest in skin (1–3). These protein-based therapeutics, however, are costly, have limited targeting ability, and, when delivered through traditional intravenous or gastrointestinal routes, can lead to systemic toxicity (4, 5). Topical delivery is particularly attractive for the therapy of skin disorders. However, proteins larger than a few hundred daltons cannot easily enter the skin, and high concentrations of proteins must be applied for a cutaneous effect (6). An alternative to protein-based pathway inhibition involves the blocking and/or degradation of precursor mRNA before translation into protein. Gene silencing leads to down-regulation of protein expression and functions with greater specificity than inhibitors of protein function (7). In fact, targeted gene suppression by antisense DNA and siRNA has shown promising preclinical results, and/or is currently in clinical trials for a variety of diseases, including many forms of cancer (e.g., melanoma, neuroblastoma, and pancreatic adenocarcinoma), genetic disorders, and macular degeneration (8). For gene suppression in skin, a topical delivery

route is optimal, given the easy accessibility of skin and the reduced risk of systemic side effects. However, penetration of oligonucleotides through the epidermal barrier has remained a major technological challenge that must be overcome before the therapeutic potential of oligonucleotides in skin can be realized (9–11).

Recently, we introduced spherical nucleic acid nanoparticle conjugates (SNA-NCs, inorganic gold nanoparticles densely coated with highly oriented oligonucleotides) (Scheme S1) as agents capable of simultaneous transfection and gene regulation (12–15). In contrast with conventional approaches to gene regulation (16–20), these nanoparticle conjugates require neither cationic transfection materials nor additional modifications to drive cellular entry. SNA-NCs enter almost 100% of cells in the more than 50 cell lines and primary cells tested to date, as well as cultured tissues and whole organs (21, 22). These unusual constructs exhibit remarkable stability, nuclease-degradation resistance, and decreased immunogenicity as a result of their high-density oligonucleotide shells (23, 24). To date, they have shown no toxicity at the cellular level at concentrations required for effective gene knockdown. Therefore, we hypothesized that siRNA-based SNA gold nanoparticle conjugates might be ideal topical delivery agents to treat diseases of the skin.

Results

To evaluate the potential for targeted gene suppression by siRNA-based SNA-NCs through noninvasive topical delivery, we first investigated their uptake in primary normal human keratinocytes (hKCs), which are typically difficult to transfect (25). hKCs were incubated with Cy3 fluorophore-labeled SNA-NCs (Table S1), and entry was studied by confocal fluorescence microscopy. Within 2 h, 100% of the observed hKCs showed strong cytoplasmic fluorescence (Fig. 1A). The number of gold nanoparticles in cells (and tissues) can be quantified by inductively coupled plasma mass spectrometry (ICP-MS). Incubation of 0.025–0.1 nM SNA-NCs (0.75–3.0 nM siRNA) for 48 h with hKCs leads to uptake of approximately $1.4 \cdot 10^3$ SNA-NCs per cell, which is approximately five times greater than that in either HaCaT (spontaneously immortalized hKCs) or HeLa cells (Fig. 1B). Although the reason for this high KC uptake is still

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¹To whom correspondence may be addressed. E-mail: apaller@northwestern.edu or chadnano@northwestern.edu.

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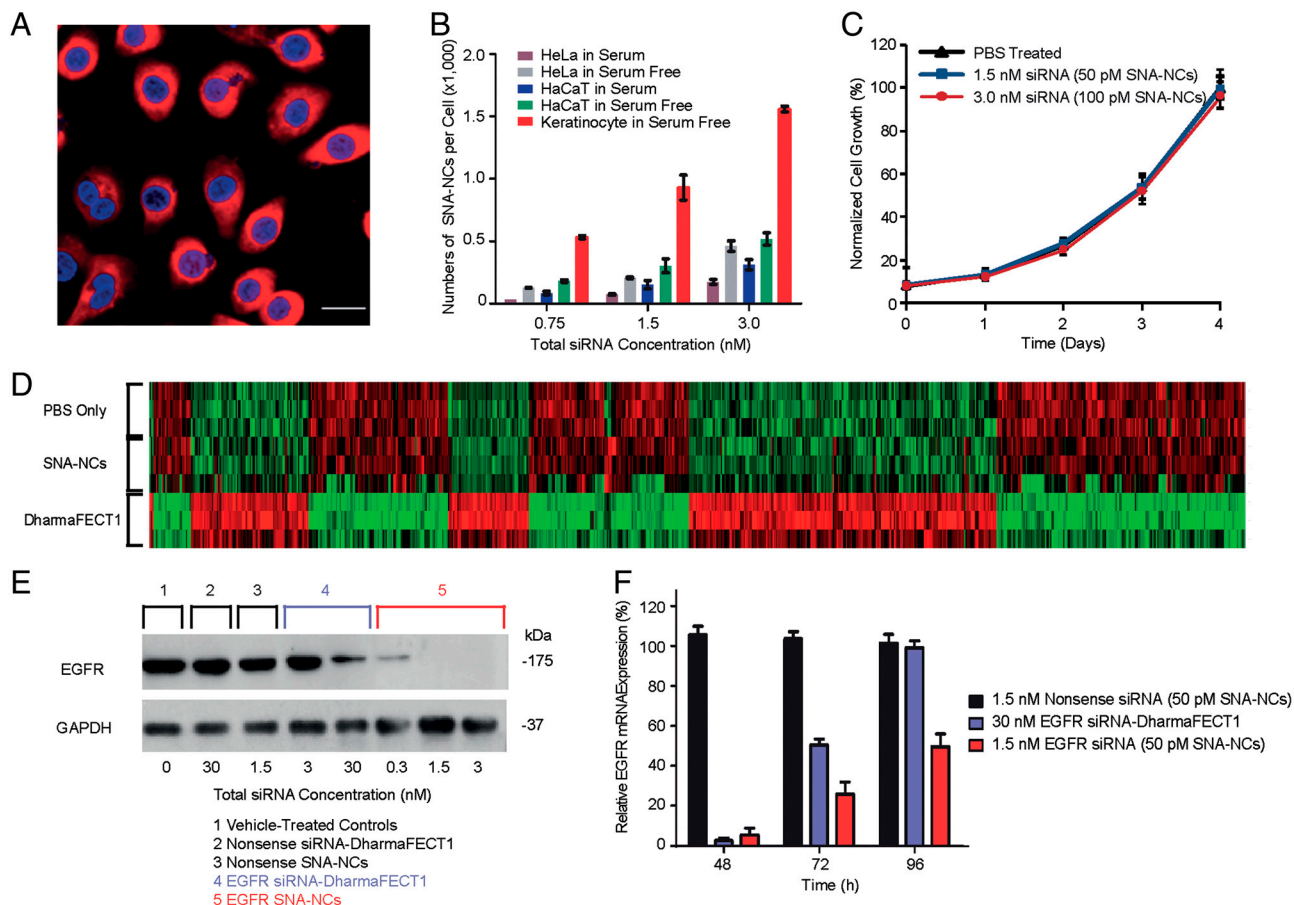


Fig. 1. Uptake, safety, and gene suppression efficacy of SNA-NCs in hKCs. (A) Uptake of Cy3-labeled nonsense SNA-NCs (red) was noted in the cytoplasm of approximately 100% of the primary hKCs after 24 h incubation. Blue, Hoechst 33343-stained nuclei. Scale bar, 20 μ m. (B) KCs exhibit dose-dependent SNA uptake that is higher than the uptake in the HeLa and HaCaT cell lines. (C) Cellular proliferation during 96 h of treatment with nonsense SNA-NCs is similar to that of control treatment with PBS only. (D) Heat map of cellular gene array study (Dataset S1). (E) Western blot showing EGFR protein levels. hKCs were treated for 48 h with SNA-NCs or DharmaFECT1-delivered siRNA, or with controls of PBS and/or nonsense SNA-NCs. After treatment, growth medium was replaced with fresh medium, and the cells were incubated for an additional 12 h. Cell lysates were then harvested for blotting. Note the greater suppression by 0.01 nM EGFR SNA-NCs (equivalent to 0.3 nM siRNA) as compared with the 30 nM EGFR siRNA delivered with DharmaFECT1 at 60 h. (F) EGFR mRNA suppression after 48 h treatment with EGFR SNA-NCs or DharmaFECT1-delivered EGFR siRNA (treatment terminated after 48 h). The decrease and eventual loss of gene suppression during the subsequent 48 h after termination of treatment was evaluated by RT-qPCR. By 48 h after removal of SNA-NCs from the medium, 50% of the maximum suppression is still observed in EGFR SNA-NC-treated KCs, whereas suppression by DharmaFECT1-delivered EGFR siRNA is no longer evident. All data are expressed as mean $\pm$ SD.

under investigation, these observations suggested SNA-NCs would be good candidates for topical delivery experiments.

With any gene regulation therapy or experiment, cell toxicity is a major concern. hKCs incubated with SNA-NCs are morphologically indistinguishable from untreated cells by routine microscopic evaluation. Proliferation assays also show no evidence of cytotoxicity after treatment of hKCs for 48 h with 0.05–0.1 nM SNA-NCs with nonsense siRNA sequences (Fig. 1C, Table S1). Finally, genome-wide expression profiling showed that only seven genes are up-regulated and none are down-regulated in hKCs treated with nonsense SNA-NCs, although the same cells treated with an equivalent amount of siRNA delivered through a cationic lipid-based transfection agent (DharmaFECT1®) exhibit up- or down-regulation of 427 genes (Fig. 1D and Dataset S1). These observations are consistent with previous studies in the HeLa cell line, which show that SNA-NCs enter cells in a stealth-like fashion and exhibit limited interference with normal cellular behavior (24, 26, 27).

Given the importance of EGFR signaling in epidermal cell proliferation and its recognized overexpression in many malignancies, EGFR was chosen as an initial target for gene suppression. EGFR knockdown in hKCs with the SNA-NCs is extremely effective. As little as 0.01 nM of SNA-NCs (0.3 nM of total

siRNA, Table S1) in 1 mL of cell culture medium down-regulates EGFR protein expression by more than 90% as compared to control experiments involving PBS-treated cells and cells treated with nonsense SNA-NCs (Fig. 1E, group 5, lane 1 vs. groups 1–3). Total depletion of protein expression, within the sensitivity of Western blots, can be effected with 0.05 nM EGFR SNA-NCs in cell culture medium (1.5 nM of total siRNA) (Fig. 1E, group 5, lane 2). Significantly, knockdown of EGFR with SNA-NCs in hKCs can be achieved with 100-fold less total siRNA than that required to observe an effect with DharmaFECT1® (Fig. 1E, group 5, lane 1 vs. group 4, lane 2). Interestingly, target knockdown with SNA-NCs is also more persistent than with DharmaFECT1-delivered siRNAs (Fig. 1F). Here, at 48 h, greater than 90% mRNA knockdown is seen in both groups; however, suppression is completely lost at 96 h with DharmaFECT1-delivered siRNA, whereas SNA-NC-treated cells still exhibit 50% knockdown. This persistence is likely caused by the known resistance of SNA constructs to nuclease degradation, and therefore increased intracellular stability of the nucleic acid sequences on the SNA construct surface (23).

Because the SNA-NCs easily enter hKCs in *in vitro* experiments, we explored their ability to penetrate both mouse skin and human skin models. The SKH1-E hairless strain was chosen for studies because of its ease in use (eliminating the need for depi-

lation), immunocompetency, and increased normal epidermal thickness (to better simulate human skin) in comparison with most mouse strains (28). Studies were also conducted in hair-bearing C57BL/6J mice (shaved 24 h before application to minimize epidermal barrier disruption). Cy5 fluorophore-labeled SNA-NCs in PBS were first mixed with a commonly used petrolatum-based moisturizer, Aquaphor® (1:1, vol/vol), and then topically applied to mouse dorsal skin. In both strains, SNA-NCs penetrated through the stratum corneum and into the epidermis and dermis as early as 3 h after a single application (Fig. 2A). To evaluate the clearance of SNA-NCs from the skin after topical application, SKH1-E hairless mice were treated daily for 3 d with 50 nM nonsense SNA-NCs, and the gold content was quantified by ICP-MS in mouse skin during the subsequent 10 d. At the end of the experiment, only 2% of the initial gold was present (Fig. 2B), demonstrating the clearance of the SNA constructs from skin.

To take an initial step toward determining the potential toxicity of the SNA-NCs in vivo, we topically applied nonsense SNA-NCs in Aquaphor to the skin of shaved C57BL/6J mice. SNA-NCs were delivered daily for 10 d at four different concentrations (500 nM, 250 nM, 100 nM, and 50 nM; $n = 5$ per concentration; these values refer to the final SNA concentrations in Aquaphor). Animals were killed and skin and internal organs were harvested and studied histologically. Analogous experiments were done with hairless mice ($n = 8$) for 4 wk using 50 nM SNA-NCs, the effective knockdown dose (see below and Fig. 3). Significantly, no cutaneous inflammation, ulceration, scaling, or color alteration was ever observed in all experiments. In addition, blinded histological assessment showed no abnormalities in the liver, lungs, kidneys, or skin of mice treated for the 10 d or 4 wk. ICP-MS showed low gold accumulation in the internal organs of hairless mice that were treated for 3 wk with 50 nM SNA in Aquaphor; indeed, gold content was undetectable in the kidneys and lungs, and it was very low in the liver (0.0003%) and spleen (0.00015%) when normalized to the total amount of applied gold. ELISA assays showed no significant increase of tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), interferon- α (IFN- α), interferon- β (IFN- β), and chemokine (C-X-C motif) ligand 10 (CXCL10; IP-10) in the serum of hairless mice treated three times weekly for 3 wk with 50 nM EGFR SNA-NCs (Fig. 2C). Similarly, no immune response was observed locally in the treated skin tissue as evidenced by lack of cytokine expression and activation of protein kinase R (PKR) pathway (Fig. 2D, and Figs. S1 and S2). Finally, these skin samples were also subjected to a microarray assay. At 6 h after application, only one off-target gene (cystatin A1) was found to be differentially expressed by >1.5-fold in skin treated with EGFR SNA-NCs in comparison with Aquaphor, and at 3 wk after initiation of treatment, only two off-target genes were differentially expressed (lactotransferrin and cathelicidin antimicrobial peptide). Off-target gene expression between the nonsense SNA-NCs and EGFR SNA-NCs was not significantly different after 3 wk.

To test SNA-NC efficacy in suppressing EGFR in mouse skin, 50 nM SNA-NCs in Aquaphor were applied three times per week (Table S1). After 3 wk of application, EGFR mRNA expression was reduced significantly (by 65%) (Fig. 3A), and protein levels were almost completely suppressed (Fig. 3B). EGFR expression in comparison with untreated skin was not diminished by Aquaphor alone, free EGFR siRNAs, or nonsense SNA-NCs. The specificity of EGFR knockdown was further confirmed by the concomitant 74% decrease in phosphorylation of downstream ERK 1/2, although total ERK 1/2 expression remained the same (Fig. 3B). The knockdown effect of EGFR SNA-NCs was also confirmed phenotypically with computerized morphometric analyses of histological sections of mouse skin treated with EGFR SNA-NCs, which showed an almost 40% reduction in thickness as compared to skin treated with either nonsense SNA-NCs or the Aquaphor

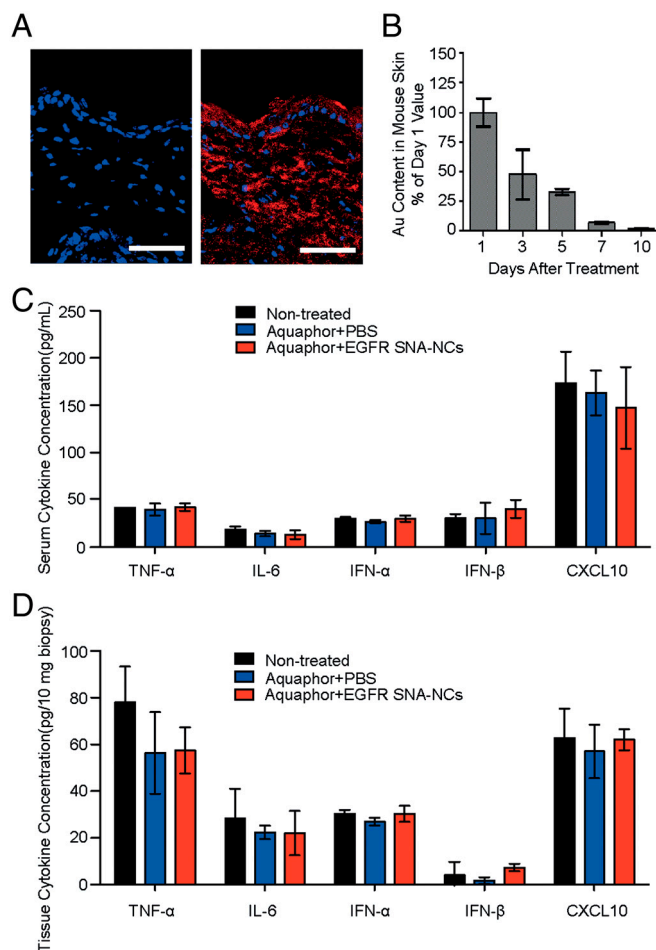


Fig. 2. Penetration and clearance of SNA-NCs in hairless mouse skin. (A) Mouse (SKH1-E) skin treated topically with 1:1 Aquaphor only (Left) or with 50 nM Cy5-labeled (red) SNA-NCs dispersed in the 1:1 Aquaphor (Right); 3 h after application, the SNA-NCs are seen in the cytoplasm of epidermal cells and the dermis as well. Blue, DAPI-stained nuclei. Scale bars, 100 μ m. (B) Mouse skin was treated daily for 3 d with 50 nM nonsense SNA-NCs; samples were harvested at 24 h to 10 d after treatment discontinuation and analyzed by ICP-MS for gold content. The gold content in mouse skin progressively decreases after cessation of topical treatment; 10 d after the final treatment, only 2% of the original gold content remains ($n = 3$ at each time point). (C) ELISA cytokine assays for TNF- α , IFN- α , IFN- β , IL-6, and CXCL10 in serum show no stimulation of innate immune responses after 3 wk of thrice-weekly treatment (every other day but Sunday; $n = 3$ per treatment group). (D) EGFR SNA-NC treatment does not induce the cytokine expression locally in the treated skin site after 3 wk of treatment. Results are presented in pg per 10 mg biopsy.

control. Representative examples of mouse skin from these studies are seen in Fig. 3C. Moreover, the number of basal epidermal nuclei, which were immunostained for Ki-67 (a proliferation marker), was reduced, demonstrating the ability of EGFR SNA-NCs to inhibit cellular proliferation in the epidermis (Fig. 3D). Skin samples from hairless mice treated with Aquaphor, nonsense SNA-NCs in Aquaphor, and EGFR SNA-NCs in Aquaphor were also subjected to microarray analysis. Taken together, our studies provide evidence that the SNA-NCs are able to knock down a specific gene target in vivo and lead to a biological effect with minimal off-target effects.

We then evaluated SNA-NC penetration through human skin using Franz cell chambers (28). Flux of nonsense SNA-NCs through human skin in Franz cell chambers was maximal between 8 and 24 h after topical application of nonsense SNA-NCs, regardless of solvent (SNA-NCs in PBS, glycerol, or Aquaphor)

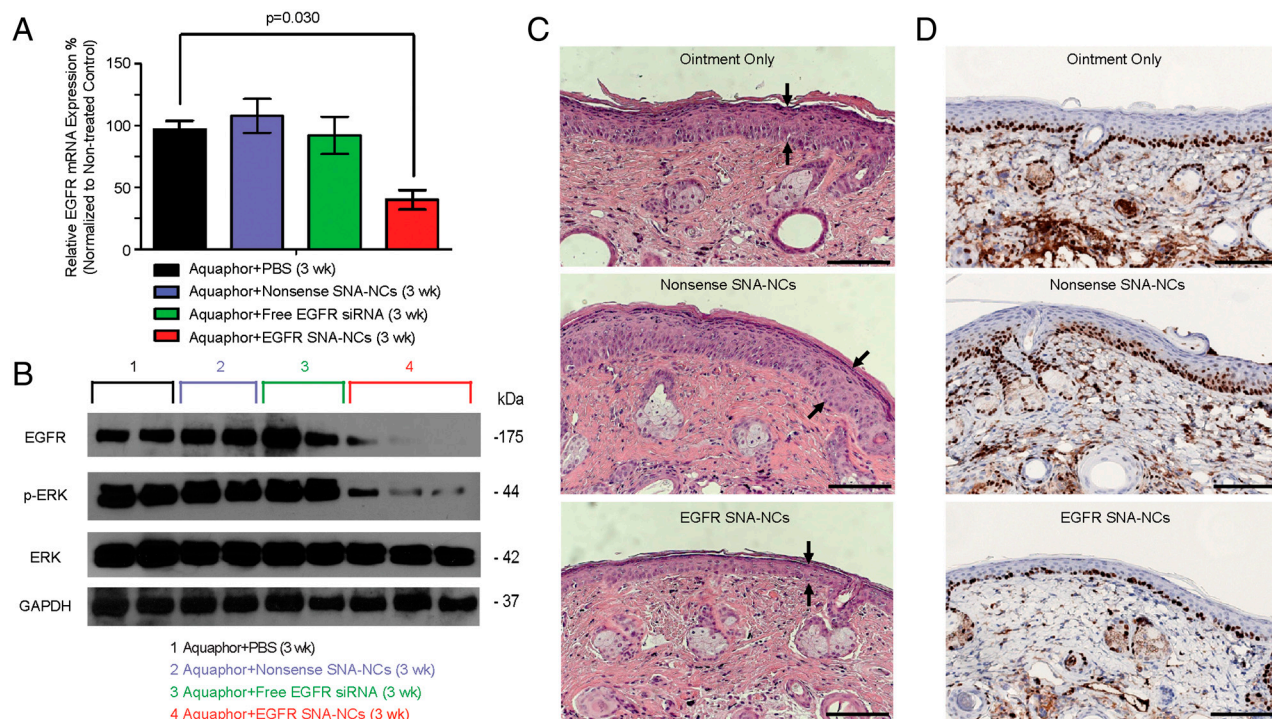


Fig. 3. Targeted gene knockdown by SNA-NCs correlates with biological effects in vivo. (A) Studies were performed to assess EGFR knockdown after 3 wk of treatment with EGFR SNA-NCs (three times per week). RT-qPCR analysis showed a 65% reduction in mRNA expression. Hairless mice, untreated or similarly treated with PBS: Aquaphor, nonsense SNA-NCs, or free siRNAs, showed no significant EGFR mRNA knockdown ($n = 5$ mice in each group at each time point). (B) The protein expression of EGFR was nearly eliminated in the EGFR SNA-NC—treated group, whereas the downstream phosphorylation of ERK was inhibited by 74%; total ERK expression remained constant. (C) The mean thickness of EGFR SNA-NC—treated skin was 40% less than that of control-treated skin ($P < 0.001$), as measured by computerized morphometry. Epidermal thickness was measured from the top of the stratum granulosum to the basement membrane (arrows) at three equidistant sites. (D) EGFR SNA-NC—treated skin samples show approximately 40% reduction in the percentage of basal layer KCs that stain positively for Ki-67 antigen, a marker of cellular proliferation, in comparison with controls. Scale bars, 100 μ m. Data in all figures are expressed as mean $\pm$ SD.

(Fig. S3). We also assessed penetration and knockdown efficacy of SNA-NCs in human skin equivalents, given that human skin is known to be thicker and more difficult to penetrate than mouse skin. Human skin equivalents (3D organotypic raft cultures) simulate in vivo human epidermis, including an intact lipid and well-differentiated protein epidermal barrier (29). Given the excellent penetration and knockdown in mouse skin with 50 nM EGFR SNA-NCs, the applied concentration was reduced to 25 nM for testing in human skin equivalents. In as little as 2 h after topical treatment, SNA-NCs were found in the basal layer of the epidermis (Fig. 4A), and the uptake and penetration through human skin equivalents increased in a time-dependent manner (Fig. 4A–C). EGFR SNA-NCs (25 nM) knocked down EGFR mRNA expression by 52% (Fig. 4D) and EGFR protein expression by 75% (Fig. 4E) compared to nonsense SNA-NC—treated skin equivalents, even though only 4.7% of the applied SNA-NCs entered the epidermis based on ICP-MS quantification. Light microscopic images of skin equivalents treated with EGFR SNA-NCs appeared similar to skin equivalents treated with nonsense SNA-NCs in PBS or control nontreated skin equivalents, as expected after 60 h of treatment.

Discussion

In this work we have discovered that SNA-NC constructs have the unusual ability to suppress gene expression. Historically, researchers have relied on physicochemical methods to facilitate epidermal penetration and cellular delivery of negatively charged nucleic acids (11, 30–33). However, these methods require high concentrations of free siRNAs, can have unacceptable toxicity in in vivo models, and are limited by the instability of conventional siRNAs. In contrast, SNA-NCs exhibit long-lasting knockdown ability at picomolar to nanomolar concentrations with no appar-

ent toxicity. We attribute this high efficacy to the dense monolayer of siRNAs on the nanoparticle surface. Our mechanistic studies with SNA-NCs show that the ion cloud associated with the high-density oligonucleotide shell, combined with steric inhibition at the surface of the particles, inhibits enzymatic nucleic acid degradation and results in an increased stability of siRNAs that comprise the SNA-NC (13, 14, 23).

Conventional RNA and DNA are also limited in their ability to cross the cellular membrane, even in monolayer cultures, without the use of viral vectors, lipid and polymer agents, or techniques such as electroporation. In contrast, SNA-NCs in PBS are taken up by approximately 100% of KCs without ancillary agents. Although the increased uptake in serum-free media suggests that a factor in serum suppresses uptake, the mechanism for better uptake in a primary cell (the hKCs) compared to immortalized cell lines (HaCaT and HeLa cells) remains unclear. Previous studies in HeLa and C166 endothelial cells have shown that the high-density SNA-NC shell recruits scavenger receptors from cells to facilitate endocytosis and is responsible for the superior cell entry of SNA-NCs in comparison to free siRNA of an identical sequence (22). How oligonucleotide density and charge and nanoparticle core size affect uptake, and how nanoparticles exit cells, are currently being investigated.

Proof that siRNA could successfully suppress gene expression in vivo was recently achieved in a mouse model (32) and a patient (11) with a genetic condition of thickened soles (pachyonychia congenita) through siRNA injection, which bypassed the epidermal barrier. Although the small treated areas showed a marked reduction in skin thickening, the pain and the impracticality associated with these injections made the approach untenable. Other mechanical approaches, such as ultrasound and laser, have been used to facilitate penetration through the mouse stratum

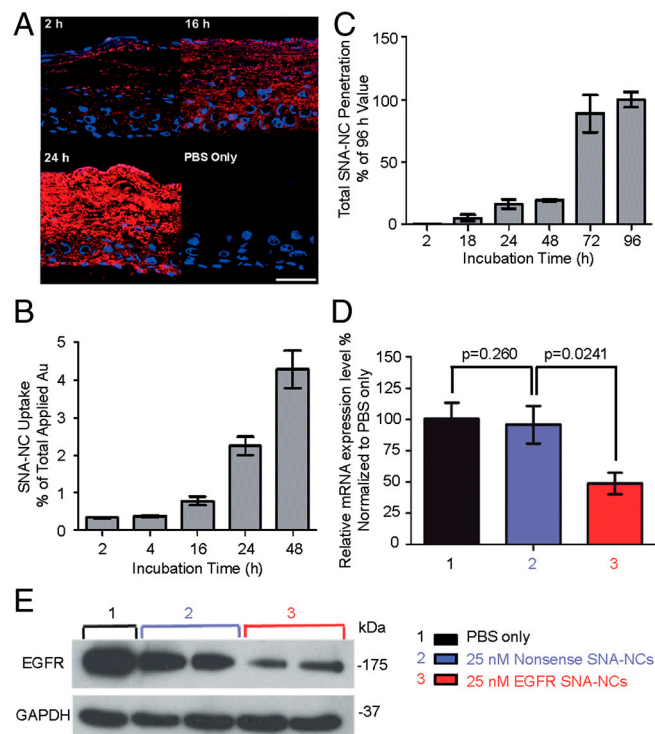


Fig. 4. Penetration and gene knockdown in human skin equivalents. (A) Skin equivalents (EpiDerm; MatTek) treated with a single application of 25 nM Cy5-labeled (red) SNA-NCs or PBS for up to 48 h. Blue, Hoechst 33343-stained nuclei. Note the presence of SNA-NCs throughout the stratum corneum and nucleated epidermis. Scale bar, 50 μ m. (B) ICP-MS measurements of gold show time-dependent uptake of the SNA-NCs in the epidermis. (C) The amount of gold in the cell culture medium, representing the number of particles passing through skin equivalents as measured by ICP-MS, increases with time. EGFR mRNA expression measured by RT-qPCR (D) and EGFR protein expression measured by immunoblotting (E) in skin equivalents treated with a single application of 25 nM EGFR SNA-NCs for 60 h demonstrate effective gene knockdown in human skin. Each study was performed at least three times in triplicate. Data are expressed as mean $\pm$ SD.

corneum and drive siRNA into skin, but require specialized equipment (30, 31, 33), limit the area of delivery, and can potentially harm the skin. Recent investigations have demonstrated uptake of siRNAs and DNAs into the epidermis in mice with no stratum corneum (e.g., open wounds or mucosae) or with atopic dermatitis, an inflammatory skin disorder with an ineffective epidermal barrier (33, 34). Indeed, traversing the intact multilayered epidermis in vivo for intracellular delivery is particularly challenging. Stratum corneum-penetrating peptides have been some of the most promising topical constructs. Topical administration of hybrid MITF siRNA—peptide conjugates reduced MITF mRNA by 50% in mouse skin and reduced pigmentation in Chinese human subjects with facial melisma when the constructs were applied for 12 wk to their skin (35). Most recently, a skin-permeating and cell-entering peptide was shown to enhance siRNA delivery, leading to 43% knockdown of GAPDH protein in shaved BALB/c mouse skin at the maximal concentration tested, 10 μ M (36). Our studies show that SNA-NCs, in contrast, virtually abolish EGFR protein expression in hairless mouse skin with its intact stratum corneum at siRNA concentrations of 1.5 μ M (50 nM SNA-NCs), emphasizing the promise of this delivery system. We speculate that the delay in observation of significant EGFR knockdown beyond 2 wk likely reflects the predominant expression of EGFR in proliferating basal KCs and the 2 wk turnover of mouse epidermis, but could also suggest that accumulation of siRNA through continued delivery is required.

As immune systems in biological organisms are capable of mounting responses against foreign nucleic acids, a growing concern for RNA interference (RNAi) technology is the potential induction of inflammatory cytokines by synthetic siRNAs both in vitro and in vivo. KCs and skin dendritic cells are immune cells, capable of the production of inflammatory cytokines such as TNF- α , IL-6, and type I interferons, as well as chemokines (such as CXCL10), further increasing the immune risks of topically applied siRNAs. Remarkably, our data from blood serum and mouse skin show that siRNAs, when densely conjugated to the nanoparticles in the form of SNA-NCs, are minimally stimulatory and have far fewer off-target effects than the free siRNAs of the same sequence introduced by traditional methods (24). Furthermore, our studies show rapid clearance from skin, minimal accumulation in viscera, and no evidence of histological changes in internal organs after topical delivery. The low immunogenicity and few off-target effects, coupled with low toxicity and high efficacy, point to a significant advantage for using SNA-NC technology to introduce siRNAs.

Taken together, the data presented here provide compelling evidence of RNAi-mediated gene suppression in vivo after topical application to intact skin of siRNAs delivered without the need for additional physical measures, keratolytics, or peptides to enhance penetration. The ability to penetrate into the epidermis in sufficient concentrations to cause morphological change through gene knockdown suggests significant therapeutic potential for treating skin diseases. In fact, the generation of siRNAs is much faster and more cost-effective than the discovery of new small molecule or antibody therapies, and may eventually allow for personalized, genotype-based therapy. The SNA-NC structure is highly tailorable, and the gold core can serve as a scaffold for multicomponent functionalization, not just with siRNAs, but with targeting antibodies or peptides, and small molecule drugs as well (22). As such, these SNA conjugates are part of the growing arsenal of nanomaterials that are showing promise as alternatives to conventional molecular formats for the development of novel and effective therapies for a wide variety of diseases (22, 37–40).

Materials and Methods

Cell Culture, Human Skin Equivalents, and Mouse Skin. Human epidermal keratinocytes were isolated from neonatal foreskin, as previously described, and cultured in supplemented keratinocyte serum-free medium (M154CF; Cascade Biologics) with 0.07 mM CaCl_2 (41). Human cervical cancer HeLa cells and human keratinocyte line HaCaT cells were maintained in DMEM (Gibco®; Invitrogen) with 10% heat-inactivated fetal bovine serum. Human skin equivalents were generated by the Skin Disease Research Center (SDRC) at Northwestern University (NU) and used when they were fully differentiated as intact skin (42). Studies were also performed with EpiDerm skin model samples (MatTek), grown in medium provided by MatTek. All cultures were maintained at 37°C in 5% CO_2 . SKH-1E hairless (Charles River Labs) and C57BL/6J (Jackson Laboratory) immunocompetent mice were used for penetration, toxicity, and efficacy studies at 6 wk with institutional Animal Care and Use Committee approval.

Visualization of Nanoparticle Uptake in Vitro and in Vivo. For in vitro uptake analysis, hKCs grown on chamber coverglass (Nalge Nunc International) were incubated with 50 pM Cy3-labeled SNA-NCs for 2–24 h and the uptake of nanoparticles was visualized by a LSM 510 confocal microscope (Zeiss). Hoechst 33342 (Invitrogen) was used to stain the nucleus of hKCs. For penetration studies, 50 nM Cy5-labeled SNA-NCs in PBS or PBS only were added to the top of the EpiDerm 3D skin samples at different time points. EpiDerm samples were then washed and harvested for hematoxylin and eosin staining or DAPI nuclear stain (Sigma) for fluorescent imaging after OCT embedding. In vivo studies, the 50 nM Cy5-labeled SNA-NCs in PBS:Aquaphor (Beiersdorf) mixture (1:1) was applied to the skin of SKH-1E and C57BL/6J mice for up to 24 h before skin samples were harvested and sectioned in OCT for fluorescent imaging.

Quantification of Nanoparticle Uptake, Penetration, and Clearance. Samples from cells, EpiDerm, and mouse skin were harvested for ICP-MS to measure

gold content (*SI Text*), which quantitatively represents nanoparticle uptake (43). Final values were normalized to the total amount of applied SNA-NCs.

Immune Response Studies. To evaluate immune responses to nanoparticle treatment, SKH1-E hairless mice were treated for 3 wk as described earlier. Blood was collected in HEMATO-CLAD Hematocrit Tubes (1-000-7500-HC/5; Drummond Scientific). Isolated serum samples were used for ELISA cytokine testing for TNF- α , IL-6, IFN- α , IFN- β , and CXCL10 (ELISA Kit KMC3011, KMC0061, and KMC4041; Invitrogen; Elisa Kit BMS6018 and BMS6027; eBioscience). RNA samples from treated mouse skin were analyzed by RT-qPCR to measure mRNA expression.

Cell Proliferation Assay and Gene Microarray Analysis. hKCs (3×10^3 cells/well) in 48-well plates were treated with 0.05 or 0.1 nM nonsense SNA-NCs or PBS for 48 h. Proliferation was assessed daily using a Guava EasyCyte flow cytometer (Millipore). Studies were performed four times in triplicate. Microarray expression analyses were performed at the NU Genomics Core Facility and analyzed at the Bioinformatics Core Facility (44) (*SI Text*).

mRNA Analysis by RT-qPCR. Total RNA was extracted and 1 μ g of RNA was reverse transcribed using qScript cDNA SuperMix (Quanta BioSciences). Real-time reverse-transcription PCR was performed on the cDNA with LightCycler@480 SYBR Green I Master on a LightCycler@480 system (Roche). The relative abundance of each mRNA transcript was normalized to GAPDH expression, and compared to untreated cells. Primers were purchased from Integrated DNA Technologies (*Table S2*). Each study was performed three times in triplicate.

Immunoblotting. Immunoblotting was performed as previously described (45) (*SI Text*).

Histology and Immunohistologic Analyses. Human skin equivalents and treated mouse skin, distant skin, and internal organs (liver, lung, and kidney) were harvested, embedded in paraffin, prepared for routine histology, and stained with hematoxylin and eosin. The thickness of mouse epidermis treated with EGFR SNA-NCs, nonsense SNA-NCs, or PBS, each mixed with Aquaphor, was measured (three equidistant sites per specimen) from the basement membrane to the top of the stratum granulosum using the Zeiss AxioPlan2 microscope at $20\times$ magnification and AxioVision software. Samples from treated mouse skin were also stained for Ki-67 using a rabbit monoclonal antibody (SP6; Cell Marque) as a marker for proliferation.

Statistical Analysis. Multifactor ANOVA was used to assess in vivo study results. Posthoc comparisons after ANOVA and significance testing for in vitro studies were performed by Student's *t* test. With all analyses, $P < 0.05$ was considered significant.

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Supporting Information

Zheng et al. 10.1073/pnas.1118425109

SI Text

SI Materials and Methods. siRNA SNA-NC synthesis and functionalization. RNA oligonucleotides (Scheme S1 and Table S2) were synthesized using TOM-RNA reagents (Glen Research) in a BioAutomation MerMade 6 synthesizer (AME Bioscience) and purified as previously described (1). siRNA duplexes were formed by hybridizing 15 nmol of sense and antisense RNA in 120 μ L of RNase-free Duplex Buffer (100 mM KOAc/30 mM HEPES pH 7.5; Integrated DNA Technologies) and subsequently incubated with 10 mL of 13 nm RNase-free Au NPs colloid solution (10 nM). The mixture was aged by increasing the salt concentration (from 0.1 to 0.3 M NaCl) and sonicating to increase the coverage of oligoribonucleotides on the surface of Au (2, 3). Oligoethylene glycol (OEG; Prochemia) was added (30 μ M final concentration) 24 h after duplex addition. After functionalization, the SNA-NCs were purified by centrifugation at (15,900 $\times$ g, 20 min) at 4 $^{\circ}$ C, and resuspended in RNase-free phosphate-buffered saline (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) to remove the excess siRNA (1). This process was repeated three times. The number of RNA duplexes per SNA-NCs was determined by using a fluorophore-labeled (Cy5) antisense strand. Briefly, the SNA-NCs were treated with 25 μ M potassium cyanide (KCN) to oxidize the gold core. Fluorescence was measured using a Jobin Yvon Fluorolog FL3-22 with excitation at 633 nm and measuring emission from 670 to 720 nm and compared to a standard curve. The number of duplexes per particle was determined as 33 ± 4 per 13 nm gold nanoparticle.

Sample preparation for ICP-MS. For in vitro experiments, hKCs, HaCaT, and HeLa cells were incubated with 50 pM nonsense SNA-NCs for 48 h, trypsinized, counted, and prepared for the ICP-MS as previously described (2). For in vivo experiments, skin samples were oven dried at 85 $^{\circ}$ C overnight, weighed, and dissolved overnight at 60 $^{\circ}$ C in neat nitric acid containing 2% HCl and 5 ppb indium, and prepared for ICP-MS analysis. Each experiment was done three times in triplicate. For clearance studies, SKH1-E mice were topically treated with 50 nM SNAs or PBS mixed in Aquaphor ointment (1:1, vol/vol) daily for 3 d. Any residual SNA-NCs remaining on the outer surface of mouse skin were removed on the day of harvest by rinsing with PBS and tape stripping mouse skin three times with Durapore Tape (3M) prior to harvesting for ICP-MS analysis per above. Samples were harvested at 1, 3, 5, 7, and 10 d from cessation of treatment ($n = 3$ for each time point and application).

EGFR knockdown conditions. In vitro, hKCs ($5 \cdot 10^3$ cells/well) in six-well plates were treated with EGFR siRNAs delivered by DharmaFECT1 (3–30 nM) or SNA-NCs (0.01–0.1 nM; equivalent to 0.3–3 nM free siRNA), nonsense siRNA in DharmaFECT1 or SNA-NCs, or PBS alone for 48 h. Treatment-containing medium was replaced and cells were incubated in fresh medium for an additional 12 h, at which time cells were harvested for Western blot analysis. Cells were treated similarly to harvest for mRNA analysis. Each in vitro experiment was performed three times in triplicate.

For studies in mouse skin ($n = 5$ for each condition), 50 μ L of 50 nM SNA-NCs mixed in Aquaphor were applied to the dorsal aspect three times weekly for 3 wk, using Tegaderm (3M) to protect the application site. Given the excellent results in mouse models with 50 nM EGFR SNA-NCs, we utilized 25 nM EGFR SNA-NCs, nonsense SNA-NCs, or PBS for studies with human skin equivalents. Skin equivalents were treated for 60 h and then

harvested for protein and mRNA, and intact sections for histopathological analysis.

Microarray analysis. For in vitro RNA microarray analysis, hKCs were treated with 0.05 nM nonsense SNA-NCs (equivalent to 1.5 nM siRNA duplexes) or 1.5 nM siRNA duplexes delivered in DharmaFECT1 for 48 h. Total RNA was extracted using TRIzol (Invitrogen), purified (RNeasy MinElute Cleanup Kit; Qiagen), and labeled (TargetAmp 1-Round Aminoallyl-aRNA Kit; Epicenter). RNA samples were also extracted for tissue microarrays from the skin of hairless mice after treatment with SNA-NCs for 6 h or 3 wk. Labeled RNA was hybridized to microarrays (Human HT-12 BeadChip; Illumina). Raw signal intensities of each probe were obtained using data analysis software (Beadstudio; Illumina) and imported to the Lumi package of Bioconductor for data transformation and normalization. Differentially expressed genes were identified using ANOVA model with empirical Bayesian variance estimation. The problem of multiple comparisons was corrected using the false discovery rate (FDR). Genes were identified as differentially expressed based on statistical significance (raw P value < 0.05), FDR < 5%, and >1.5-fold change (up or down) in expression level.

Immunoblotting. Whole cell lysates were prepared in Cell Lysis Buffer (Cell Signaling), and protein concentration was determined using a BCA Protein Assay Kit (Pierce). Proteins were separated by 10% SDS-PAGE, transferred to Hybond ECL membrane, and detected with anti-EGFR, anti-p44/42 MAPK (Erk 1/2), anti-phospho-p44/42 MAPK (Erk 1/2), anti-eIF2- α , anti-Phospho-eIF2- α (Ser51) (2232, 9102L, 9106L, 9722, 9721; Cell Signaling), and anti-GAPDH (sc-32233) antibodies using ECL Western Blotting Substrate (Pierce). For skin equivalent experiments, proteins were extracted and analyzed in a similar manner, except with RIPA buffer, nitrocellulose membrane (162-0115; BioRad), and SuperSignal West Pico Chemiluminescent Substrate (34080; Thermo). Relative protein expression in all Western blots was determined by densitometry analysis using Image J software (National Institutes of Health). All studies were repeated at least in triplicate.

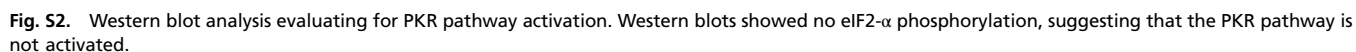
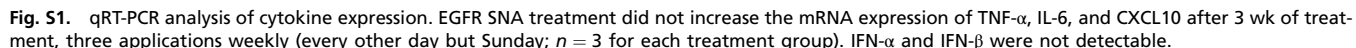
Franz cell assay. Human abdominal skin samples from abdominoplasties were obtained by the Skin Disease Research Center. The epidermis was separated from dermis after a 1 min incubation in 60 $^{\circ}$ C water, desiccated, and stored at -80° C before use. Frozen epidermis was reconstituted in PBS for Franz cell chamber assays (4). Particle penetration (gold content present in receptor cells) was quantified by ICP-MS. Each experiment was done three times in triplicate. Use of all human samples was approved by the Northwestern University Institutional Review Board.

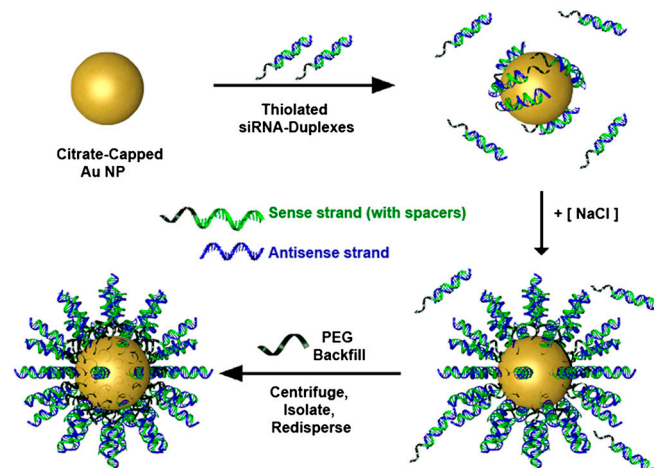
Toxicity in animals. To evaluate potential toxicity with the effective dosing in skin, 50 μ L of 50 nM nonsense SNA-NCs in Aquaphor, or Aquaphor alone, were applied to SKH-1E hairless mice three times a week for 4 wk ($n = 8$ for each condition). Similar experiments were conducted with C57BL/6J mice shaved 24 h before application, in which 50 μ L of 50–500 nM SNA-NCs or PBS, each mixed with Aquaphor, were applied daily for 10 d ($n = 5$ at each concentration). Gross and microscopic evaluations of viscera and skin were performed. Organ samples from skin, kidneys, liver, spleen, lymph nodes, brain, and lung were oven dried at 85 $^{\circ}$ C overnight, weighed, and dissolved overnight at 60 $^{\circ}$ C in neat nitric

PNAS

1. Giljohann DA, Seferos DS, Prigodich AE, Patel PC, Mirkin CA (2009) Gene regulation with polyvalent siRNA-nanoparticle conjugates. *J Am Chem Soc* 131:2072–2073.
2. Giljohann DA, et al. (2007) Oligonucleotide loading determines cellular uptake of DNA-modified gold nanoparticles. *Nano Lett* 7:3818–3821.

3. Hurst SJ, Lytton-Jean AKR, Mirkin CA (2006) Maximizing DNA loading on a range of gold nanoparticle sizes. *Anal Chem* 78:8313–8318.
4. Lee WR, Shen SC, Zhuo RZ, Wang KC, Fang JY (2009) Enhancement of topical small interfering RNA delivery and expression by low-fluence erbium: YAG laser pretreatment of skin. *Hum Gene Ther* 20:580–588.





Scheme S1. Synthesis of siRNA-based SNA-NCs. Hybridized siRNA duplexes are added to solutions of citrate-stabilized gold colloid and attached through thiol-gold chemistry. Addition of salt screens repulsive charges, resulting in densely functionalized nanoconjugates. The number of siRNAs/nanoparticle can be tightly controlled; tested SNA-NCs have approximately 30 siRNAs densely arrayed around a 13 nM core.

Other Supporting Information Files

[Dataset S1 \(DOCX\)](#)

hKCs treated with nonsense SNAs

Symbol	Description	Fold Change
DEFB103A	defensin, beta 103A	2.6507
CRCT1	cysteine-rich C-terminal 1	1.8347
ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	1.7372
HLA-G	major histocompatibility complex, class I, G	1.6734
C10orf116	chromosome 10 open reading frame 116	1.6335
FTLP2	ferritin, light polypeptide pseudogene 2	1.5861
KLK5	kallikrein-related peptidase 5	1.5354

hKCs treated with DharmaFECT1[®]-delivered nonsense siRNA

Symbol	Description	Fold Change
BAMBI	BMP and activin membrane-bound inhibitor homolog (Xenopus laevis)	5.1275
DDIT3	DNA-damage-inducible transcript 3	4.4485
ASS1	argininosuccinate synthase 1	3.8027
ASNS	asparagine synthetase (glutamine-hydrolyzing)	2.8389
NOV	nephroblastoma overexpressed gene	2.7168
EGR1	early growth response 1	2.6658
NDRG1	N-myc downstream regulated 1	2.6281
GADD45A	growth arrest and DNA-damage-inducible, alpha	2.6218
HBEGF	heparin-binding EGF-like growth factor	2.5489
LCN2	lipocalin 2	2.5427
ATF3	activating transcription factor 3	2.43
CBX4	chromobox homolog 4	2.4089

G0S2	G0/G1switch 2	2.3723
MIR205	microRNA 205	2.3711
NCRNA00219	non-protein coding RNA 219	2.3421
H1F0	H1 histone family, member 0	2.3086
CLDN7	claudin 7	2.3022
LOC728734	similar to NPIP-like protein ENSP00000283050	2.2983
CBS	cystathionine-beta-synthase	2.2831
FLJ35024	hypothetical LOC401491	2.242
C6orf48	chromosome 6 open reading frame 48	2.232
ADM	Adrenomedullin	2.2115
AKR1C4	aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4)	2.2064
NCRNA00275	non-protein coding RNA 275	2.1734
ADM2	adrenomedullin 2	2.1717
ABCC3	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	2.1711
AVPI1	arginine vasopressin-induced 1	2.1467
DUSP1	dual specificity phosphatase 1	2.1433
CIRBP	cold inducible RNA binding protein	2.108
LGALS7B	lectin, galactoside-binding, soluble, 7B	2.1074
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	2.0843
ADAP1	ArfGAP with dual PH domains 1	2.0833
DCI	dodecenoyl-CoA isomerase	2.0811
THNSL2	threonine synthase-like 2 (<i>S. cerevisiae</i>)	2.0802
NRP1	neuropilin 1	2.0787

CAPNS2	calpain, small subunit 2	2.0768
BEX2	brain expressed X-linked 2	2.0746
FAM113B	family with sequence similarity 113, member B	2.0699
CLDN1	claudin 1	2.0475
IRAK2	interleukin-1 receptor-associated kinase 2	2.0369
DLK2	delta-like 2 homolog (Drosophila)	2.034
AKR1C2	aldo-keto reductase family 1, member C2 (dihydrodiol dehydrogenase 2; bile acid binding protein; 3-alpha hydroxysteroid dehydrogenase, type III)	2.0074
DDIT4	DNA-damage-inducible transcript 4	2.007
MEG3	maternally expressed 3 (non-protein coding)	1.9981
LYPLAL1	lysophospholipase-like 1	1.998
GFPT1	glutamine--fructose-6-phosphate transaminase 1	1.9931
MSLN	mesothelin	1.9862
IFIT2	interferon-induced protein with tetratricopeptide repeats 2	1.9629
GJC2	gap junction protein, gamma 2, 47kDa	1.961
WASH1	WAS protein family homolog 1	1.9498
GCLC	glutamate-cysteine ligase, catalytic subunit	1.943
CEBPD	CCAAT/enhancer binding protein (C/EBP), delta	1.9406
NPIP	nuclear pore complex interacting protein	1.9364
GPT2	glutamic pyruvate transaminase (alanine aminotransferase) 2	1.9305
MOCOS	molybdenum cofactor sulfurase	1.9018
GRAMD1A	GRAM domain containing 1A	1.8923
MMP28	matrix metalloproteinase 28	1.8922
CCL20	chemokine (C-C motif) ligand 20	1.881

KCNG1	potassium voltage-gated channel, subfamily G, member 1	1.8794
HNRPDL	heterogeneous nuclear ribonucleoprotein D-like	1.8764
ALOX15B	arachidonate 15-lipoxygenase, type B	1.86
LARP6	La ribonucleoprotein domain family, member 6	1.8469
FOLR3	folate receptor 3 (gamma)	1.8463
C16orf74	chromosome 16 open reading frame 74	1.8456
NOTCH1	notch 1	1.8391
LOC729742	similar to hCG1812832	1.8357
GADD45B	growth arrest and DNA-damage-inducible, beta	1.8327
ACSS2	acyl-CoA synthetase short-chain family member 2	1.8273
MUC1	mucin 1, cell surface associated	1.8219
DSG3	desmoglein 3	1.8101
NFE2L1	nuclear factor (erythroid-derived 2)-like 1	1.8097
BTG1	B-cell translocation gene 1, anti-proliferative	1.8041
CCNB1IP1	cyclin B1 interacting protein 1	1.8039
C1QTNF1	C1q and tumor necrosis factor related protein 1	1.8016
HSD17B8	hydroxysteroid (17-beta) dehydrogenase 8	1.8005
CTH	cystathionase (cystathionine gamma-lyase)	1.7978
GDF15	growth differentiation factor 15	1.7935
ECH1	enoyl CoA hydratase 1, peroxisomal	1.7862
AARS	alanyl-tRNA synthetase	1.7661
FBXL5	F-box and leucine-rich repeat protein 5	1.7644
LGALS7	lectin, galactoside-binding, soluble, 7	1.7506
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	1.7486

IL1B	interleukin 1, beta	1.7411
CDCP1	CUB domain containing protein 1	1.7406
ABCG1	ATP-binding cassette, sub-family G (WHITE), member 1	1.7381
MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	1.7325
BIRC3	baculoviral IAP repeat-containing 3	1.7325
CAMSAP1L1	calmodulin regulated spectrin-associated protein 1-like 1	1.7289
FOXQ1	forkhead box Q1	1.723
EVI5L	ecotropic viral integration site 5-like	1.7222
CYB5R1	cytochrome b5 reductase 1	1.7213
LOC100288911	hypothetical protein LOC100288911	1.7154
DEFB103A	defensin, beta 103A	1.7126
BCL2L2	BCL2-like 2	1.7096
MFSD3	major facilitator superfamily domain containing 3	1.7011
D2HGDH	D-2-hydroxyglutarate dehydrogenase	1.6879
IFNGR2	interferon gamma receptor 2 (interferon gamma transducer 1)	1.6848
COX7A2L	cytochrome c oxidase subunit VIIa polypeptide 2 like	1.6831
FHL2	four and a half LIM domains 2	1.6779
AHNAK2	AHNAK nucleoprotein 2	1.6697
MIF4GD	MIF4G domain containing	1.6678
C5orf32	chromosome 5 open reading frame 32	1.667
HK2	hexokinase 2	1.6631
BTG2	BTG family, member 2	1.6604
APLP2	amyloid beta (A4) precursor-like protein 2	1.6574
MARS	methionyl-tRNA synthetase	1.6531

BNC1	basenuclin 1	1.6508
BRWD1	bromodomain and WD repeat domain containing 1	1.645
NAPRT1	nicotinate phosphoribosyltransferase domain containing 1	1.643
FAM131A	family with sequence similarity 131, member A	1.6427
ICA1	islet cell autoantigen 1, 69kDa	1.6425
LOC441228	exportin, tRNA (nuclear export receptor for tRNAs) pseudogene	1.6402
ERCC1	excision repair cross-complementing rodent repair deficiency, complementation group 1 (includes overlapping antisense sequence)	1.6394
ECM2	extracellular matrix protein 2, female organ and adipocyte specific	1.6369
DST	dystonin	1.6367
NAT9	N-acetyltransferase 9 (GCN5-related, putative)	1.6316
C6orf105	chromosome 6 open reading frame 105	1.63
CHMP4A	chromatin modifying protein 4A	1.6279
ARHGEF2	Rho/Rac guanine nucleotide exchange factor (GEF) 2	1.6269
MFGE8	milk fat globule-EGF factor 8 protein	1.6246
C17orf90	chromosome 17 open reading frame 90	1.6214
GSTO2	glutathione S-transferase omega 2	1.6207
DTX2	deltex homolog 2 (Drosophila)	1.6127
FKBP2	FK506 binding protein 2, 13kDa	1.6118
LOC285741	tumor protein, translationally-controlled 1 pseudogene	1.6108
GPR56	G protein-coupled receptor 56	1.6036
ATF4	activating transcription factor 4 (tax-responsive enhancer element B67)	1.6025
CDC42EP5	CDC42 effector protein (Rho GTPase binding) 5	1.5995
DEDD2	death effector domain containing 2	1.5995

C20orf111	chromosome 20 open reading frame 111	1.5961
CBLC	Cas-Br-M (murine) ecotropic retroviral transforming sequence c	1.5953
NHP2L1	NHP2 non-histone chromosome protein 2-like 1 (S. cerevisiae)	1.5947
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	1.5935
GTF2IRD2B	GTF2I repeat domain containing 2B	1.5897
CD68	CD68 molecule	1.5883
KLHL12	kelch-like 12 (Drosophila)	1.5859
IL1RN	interleukin 1 receptor antagonist	1.5834
C6orf125	chromosome 6 open reading frame 125	1.5828
ARL2	ADP-ribosylation factor-like 2	1.5826
CLIP4	CAP-GLY domain containing linker protein family, member 4	1.5821
FYN	FYN oncogene related to SRC, FGR, YES	1.5776
CDK10	cyclin-dependent kinase 10	1.5764
FANCE	Fanconi anemia, complementation group E	1.5742
LOC613037	nuclear pore complex interacting protein pseudogene	1.5717
ELP2	elongation protein 2 homolog (S. cerevisiae)	1.5694
DPF2	D4, zinc and double PHD fingers family 2	1.5684
BRD2	bromodomain containing 2	1.5684
CXCL2	chemokine (C-X-C motif) ligand 2	1.5654
LEMD1	LEM domain containing 1	1.5653
EPAS1	endothelial PAS domain protein 1	1.5642
FAM167A	family with sequence similarity 167, member A	1.5635
DNAL4	dynein, axonemal, light chain 4	1.5621
LETMD1	LETM1 domain containing 1	1.5617

MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1.5607
H1FX	H1 histone family, member X	1.5586
LMO4	LIM domain only 4	1.5581
GLTSCR2	glioma tumor suppressor candidate region gene 2	1.5543
IL13RA2	interleukin 13 receptor, alpha 2	1.5535
LOC100132247	similar to Uncharacterized protein KIAA0220	1.553
MIB2	mindbomb homolog 2 (Drosophila)	1.5525
H2AFJ	H2A histone family, member J	1.5524
DNLZ	DNL-type zinc finger	1.5512
APBB3	amyloid beta (A4) precursor protein-binding, family B, member 3	1.5475
LARS	leucyl-tRNA synthetase	1.5469
MAN1B1	mannosidase, alpha, class 1B, member 1	1.546
FLNB	filamin B, beta	1.5439
TYMP	thymidine phosphorylase	1.5416
IARS	isoleucyl-tRNA synthetase	1.5411
ASAP2	ArfGAP with SH3 domain, ankyrin repeat and PH domain 2	1.5405
BCL2L13	BCL2-like 13 (apoptosis facilitator)	1.5359
ERRFI1	ERBB receptor feedback inhibitor 1	1.5351
MIR1978	microRNA 1978	1.535
MRPS33	mitochondrial ribosomal protein S33	1.5293
IL20RB	interleukin 20 receptor beta	1.529
CCNO	cyclin O	1.5282
EML2	echinoderm microtubule associated protein like 2	1.5278
MR1	major histocompatibility complex, class I-related	1.5264

KLHDC2	kelch domain containing 2	1.526
LOC399491	GPS, PLAT and transmembrane domain-containing protein	1.5252
CSNK1E	casein kinase 1, epsilon	1.5243
LHPP	phospholysine phosphohistidine inorganic pyrophosphate phosphatase	1.5237
COL17A1	collagen, type XVII, alpha 1	1.5231
KRT34	keratin 34	1.5228
ARFGAP3	ADP-ribosylation factor GTPase activating protein 3	1.5187
NRG1	neuregulin 1	1.5183
LOC729978	nuclear pore complex interacting protein pseudogene	1.5179
GTPBP6	GTP binding protein 6 (putative)	1.5172
FTLP2	ferritin, light polypeptide pseudogene 2	1.5157
MXD4	MAX dimerization protein 4	1.5153
WASH3P	WAS protein family homolog 3 pseudogene	1.5137
CASP4	caspase 4, apoptosis-related cysteine peptidase	1.5125
NCRNA00188	non-protein coding RNA 188	1.5121
CHAC1	ChaC, cation transport regulator homolog 1 (E. coli)	1.5119
HYAL1	hyaluronoglucosaminidase 1	1.5109
LY96	lymphocyte antigen 96	1.5106
MDK	midkine (neurite growth-promoting factor 2)	1.5104
AIG1	androgen-induced 1	1.5087
CLCN6	chloride channel 6	1.5073
FTH1	ferritin, heavy polypeptide 1	1.5071
LOC388796	hypothetical LOC388796	1.5068
ADAM8	ADAM metallopeptidase domain 8	1.5059

ACADVL	acyl-CoA dehydrogenase, very long chain	1.505
ACY1	aminoacylase 1	1.5029
C12orf57	chromosome 12 open reading frame 57	1.5026
DDT	D-dopachrome tautomerase	1.5017
MTSS1	metastasis suppressor 1	1.5017
EZH2	enhancer of zeste homolog 2 (Drosophila)	-1.5012
DEPDC1	DEP domain containing 1	-1.5031
BLZF1	basic leucine zipper nuclear factor 1	-1.5056
CKS2	CDC28 protein kinase regulatory subunit 2	-1.5083
EDN1	endothelin 1	-1.509
LAP3	leucine aminopeptidase 3	-1.5102
KIF18A	kinesin family member 18A	-1.5108
FEZ1	fasciculation and elongation protein zeta 1 (zygin I)	-1.5111
DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2	-1.5114
4-Mar	membrane-associated ring finger (C3HC4) 4	-1.5116
CLEC2D	C-type lectin domain family 2, member D	-1.5132
HN1L	hematological and neurological expressed 1-like	-1.5142
C3orf59	chromosome 3 open reading frame 59	-1.5149
CDK2AP1	cyclin-dependent kinase 2 associated protein 1	-1.515
CTSC	cathepsin C	-1.5175
LSM5	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-1.5181
LOC100133772	similar to MCT	-1.5191
LOC730107	similar to Glycine cleavage system H protein, mitochondrial	-1.5204
MRPL18	mitochondrial ribosomal protein L18	-1.5205

MTPN	myotrophin	-1.5232
LOC401098	hypothetical LOC401098	-1.5232
DLX1	distal-less homeobox 1	-1.5243
FCAR	Fc fragment of IgA, receptor for	-1.5264
FJX1	four jointed box 1 (Drosophila)	-1.5276
MLKL	mixed lineage kinase domain-like	-1.528
ARHGEF3	Rho guanine nucleotide exchange factor (GEF) 3	-1.5287
C6orf115	chromosome 6 open reading frame 115	-1.5313
FHOD1	formin homology 2 domain containing 1	-1.5316
MCART1	mitochondrial carrier triple repeat 1	-1.532
ERGIC2	ERGIC and golgi 2	-1.5361
EI24	etoposide induced 2.4 mRNA	-1.5364
EXO1	exonuclease 1	-1.5367
KPNA3	karyopherin alpha 3 (importin alpha 4)	-1.5368
C13orf27	chromosome 13 open reading frame 27	-1.5385
NIPAL1	NIPA-like domain containing 1	-1.5393
ATP1B1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	-1.5404
CDK2	cyclin-dependent kinase 2	-1.5405
HMGB2	high-mobility group box 2	-1.5411
C9orf80	chromosome 9 open reading frame 80	-1.5424
BCLAF1	BCL2-associated transcription factor 1	-1.5431
C2orf69	chromosome 2 open reading frame 69	-1.5454
HMGB1	high-mobility group box 1	-1.5471
ANXA8L2	annexin A8-like 2	-1.5482

ITPRIPL2	inositol 1,4,5-triphosphate receptor interacting protein-like 2	-1.552
AURKB	aurora kinase B	-1.5522
E2F3	E2F transcription factor 3	-1.5578
CENPA	centromere protein A	-1.5585
NAE1	NEDD8 activating enzyme E1 subunit 1	-1.5587
CCNF	cyclin F	-1.5592
NDC80	NDC80 homolog, kinetochore complex component (S. cerevisiae)	-1.5614
LOC389168	SET translocation pseudogene	-1.5627
NFATC2IP	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2 interacting protein	-1.5711
LOC647302	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae) pseudogene	-1.5784
NCL	nucleolin	-1.5786
CDK11A	cyclin-dependent kinase 11A	-1.5817
LMNB2	lamin B2	-1.5829
MAGT1	magnesium transporter 1	-1.5835
CBX6	chromobox homolog 6	-1.5855
IRS1	insulin receptor substrate 1	-1.5876
EFNB2	ephrin-B2	-1.5876
CDC5L	CDC5 cell division cycle 5-like (S. pombe)	-1.5884
GPR180	G protein-coupled receptor 180	-1.5925
NSL1	NSL1, MIND kinetochore complex component, homolog (S. cerevisiae)	-1.5937
BRX1	BRX1, biogenesis of ribosomes, homolog (S. cerevisiae)	-1.5947
C21orf45	chromosome 21 open reading frame 45	-1.5964
CDCA5	cell division cycle associated 5	-1.5967

AGRN	agrin	-1.5968
LOC255167	hypothetical LOC255167	-1.5968
DUT	deoxyuridine triphosphatase	-1.599
NDUFAB1	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa	-1.6026
NFIX	nuclear factor I/X (CCAAT-binding transcription factor)	-1.6028
IDI1	isopentenyl-diphosphate delta isomerase 1	-1.6034
CD82	CD82 molecule	-1.6043
CDC26	cell division cycle 26 homolog (S. cerevisiae)	-1.6064
LOC100133923	hypothetical protein LOC100133923	-1.6066
DPYSL3	dihydropyrimidinase-like 3	-1.6071
ASF1B	ASF1 anti-silencing function 1 homolog B (S. cerevisiae)	-1.6146
SNRNP25	small nuclear ribonucleoprotein 25kDa (U11/U12)	-1.6155
MRT04	mRNA turnover 4 homolog (S. cerevisiae)	-1.6164
L1TD1	LINE-1 type transposase domain containing 1	-1.6186
C15orf63	chromosome 15 open reading frame 63	-1.6191
CCDC58	coiled-coil domain containing 58	-1.6198
DIAPH3	diaphanous homolog 3 (Drosophila)	-1.6226
LMNB1	lamin B1	-1.6227
ANLN	anillin, actin binding protein	-1.623
DEM1	defects in morphology 1 homolog (S. cerevisiae)	-1.6232
INTS8	integrator complex subunit 8	-1.6251
FANCI	Fanconi anemia, complementation group I	-1.6252
CATSPER2	cation channel, sperm associated 2	-1.6254
CNN3	calponin 3, acidic	-1.6275

GEMIN6	gem (nuclear organelle) associated protein 6	-1.6286
NCAPD2	non-SMC condensin I complex, subunit D2	-1.6292
AMD1	adenosylmethionine decarboxylase 1	-1.6308
LLPH	LLP homolog, long-term synaptic facilitation (Aplysia)	-1.6341
DMC1	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)	-1.6404
DKK1	dickkopf homolog 1 (Xenopus laevis)	-1.6425
KIF20A	kinesin family member 20A	-1.6431
HIGD1A	HIG1 hypoxia inducible domain family, member 1A	-1.6436
C8orf45	chromosome 8 open reading frame 45	-1.6437
MPHOSPH10	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)	-1.6494
GRB2	growth factor receptor-bound protein 2	-1.6524
CENPW	centromere protein W	-1.6555
DHX15	DEAH (Asp-Glu-Ala-His) box polypeptide 15	-1.6572
DSCC1	defective in sister chromatid cohesion 1 homolog (S. cerevisiae)	-1.6576
CCNB1	cyclin B1	-1.6588
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	-1.6592
FAM111A	family with sequence similarity 111, member A	-1.6668
C14orf106	chromosome 14 open reading frame 106	-1.6747
LOC647150	ATP-binding cassette, sub-family E (OABP), member 1 pseudogene	-1.6794
ARL16	ADP-ribosylation factor-like 16	-1.6798
LYAR	Ly1 antibody reactive homolog (mouse)	-1.681
MCM5	minichromosome maintenance complex component 5	-1.6836
CTSB	cathepsin B	-1.689

CDCA3	cell division cycle associated 3	-1.6957
NCAPG	non-SMC condensin I complex, subunit G	-1.6968
HNRNPU	heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	-1.7021
KIAA1524	KIAA1524	-1.7068
KIAA1279	KIAA1279	-1.7071
CRYZ	crystallin, zeta (quinone reductase)	-1.7076
CCBE1	collagen and calcium binding EGF domains 1	-1.7082
C11orf82	chromosome 11 open reading frame 82	-1.7109
LOC100128510	hypothetical protein LOC100128510	-1.7148
KCNH6	potassium voltage-gated channel, subfamily H (eag-related), member 6	-1.7164
NR2F2	nuclear receptor subfamily 2, group F, member 2	-1.7182
LOC728903	hypothetical LOC728903	-1.7245
HNRNPH1	heterogeneous nuclear ribonucleoprotein H1 (H)	-1.7255
EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	-1.7266
KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	-1.7268
ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	-1.7275
NETO2	neuropilin (NRP) and tolloid (TLL)-like 2	-1.7275
DEPDC1B	DEP domain containing 1B	-1.7289
CENPE	centromere protein E, 312kDa	-1.7311
LOC100132585	similar to speedy homolog A	-1.7349
MFAP5	microfibrillar associated protein 5	-1.7363
GIN2	GIN complex subunit 2 (Psf2 homolog)	-1.7378
MRPL1	mitochondrial ribosomal protein L1	-1.7431

EIF1AX	eukaryotic translation initiation factor 1A, X-linked	-1.7507
AURKA	aurora kinase A	-1.7536
BUB1	budding uninhibited by benzimidazoles 1 homolog (yeast)	-1.7568
BMS1P5	BMS1 pseudogene 5	-1.7576
HELLS	helicase, lymphoid-specific	-1.7603
MOBKL2B	MOB1, Mps One Binder kinase activator-like 2B (yeast)	-1.7627
MSH6	mutS homolog 6 (E. coli)	-1.7629
HIATL2	hippocampus abundant transcript-like 2	-1.7634
EXOSC9	exosome component 9	-1.7644
CEP78	centrosomal protein 78kDa	-1.7666
BUB3	budding uninhibited by benzimidazoles 3 homolog (yeast)	-1.7714
LOC100216001	hypothetical LOC100216001	-1.7716
LOC100133692	similar to cell division cycle 2-like 1 (PITSLRE proteins)	-1.7816
FAM175A	family with sequence similarity 175, member A	-1.7861
IFI44	interferon-induced protein 44	-1.7862
KCTD12	potassium channel tetramerisation domain containing 12	-1.7902
HNRNPM	heterogeneous nuclear ribonucleoprotein M	-1.791
NT5DC2	5'-nucleotidase domain containing 2	-1.7915
KIF11	kinesin family member 11	-1.7956
HSPA14	heat shock 70kDa protein 14	-1.7959
HIST1H4C	histone cluster 1, H4c	-1.7961
FAM98A	family with sequence similarity 98, member A	-1.825
FKBP14	FK506 binding protein 14, 22 kDa	-1.8255
C14orf153	chromosome 14 open reading frame 153	-1.8413

LRRCC1	leucine rich repeat and coiled-coil domain containing 1	-1.8471
FAM83A	family with sequence similarity 83, member A	-1.8473
CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa	-1.8476
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	-1.8523
ISG20L2	interferon stimulated exonuclease gene 20kDa-like 2	-1.8601
ENC1	ectodermal-neural cortex 1 (with BTB-like domain)	-1.8625
LOC728026	hypothetical LOC728026	-1.8632
LAMC2	laminin, gamma 2	-1.8662
C20orf177	chromosome 20 open reading frame 177	-1.8665
HSPA2	heat shock 70kDa protein 2	-1.8715
DTL	denticleless homolog (Drosophila)	-1.8737
CPA4	carboxypeptidase A4	-1.8751
MCM4	minichromosome maintenance complex component 4	-1.8809
C18orf55	chromosome 18 open reading frame 55	-1.8923
HSPH1	heat shock 105kDa/110kDa protein 1	-1.8927
LOC646993	similar to high mobility group box 3	-1.8959
BARD1	BRCA1 associated RING domain 1	-1.8966
CHORDC1	cysteine and histidine-rich domain (CHORD)-containing 1	-1.897
CCNA2	cyclin A2	-1.9088
KIAA1751	KIAA1751	-1.9093
LOC441087	hypothetical LOC441087	-1.91
DUSP19	dual specificity phosphatase 19	-1.9262
HMGB1L1	high-mobility group box 1-like 1	-1.9432
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	-1.9432

LOC100130178	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae) pseudogene	-1.9434
LOC728809	hypothetical LOC728809	-1.9515
CDCA7	cell division cycle associated 7	-1.9604
LOC100128288	hypothetical protein LOC100128288	-1.9798
LSM3	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae)	-1.9866
LOC644422	splicing factor, arginine/serine-rich 6 pseudogene	-1.9881
LOC100129362	hypothetical LOC100129362	-1.9973
H2AFX	H2A histone family, member X	-2.0056
ITPK1AS	ITPK1 antisense RNA (non-protein coding)	-2.0167
KIFC1	kinesin family member C1	-2.0186
NFIC	nuclear factor I/C (CCAAT-binding transcription factor)	-2.0187
EPN3	epsin 3	-2.0246
ERAP2	endoplasmic reticulum aminopeptidase 2	-2.0366
CREB1	cAMP responsive element binding protein 1	-2.0403
GRIPAP1	GRIP1 associated protein 1	-2.0465
LOC646817	SET nuclear oncogene pseudogene	-2.0473
GNL3L	guanine nucleotide binding protein-like 3 (nucleolar)-like	-2.064
EID2B	EP300 interacting inhibitor of differentiation 2B	-2.0694
FAM83D	family with sequence similarity 83, member D	-2.0722
HSPA1B	heat shock 70kDa protein 1B	-2.097
CDKN2AIPNL	CDKN2A interacting protein N-terminal like	-2.1033
NBPF8	neuroblastoma breakpoint family, member 8	-2.1192
MCM3	minichromosome maintenance complex component 3	-2.1218
C3orf34	chromosome 3 open reading frame 34	-2.1364

ARID4B	AT rich interactive domain 4B (RBP1-like)	-2.1485
DLGAP5	discs, large (Drosophila) homolog-associated protein 5	-2.1515
ASPM	asp (abnormal spindle) homolog, microcephaly associated (Drosophila)	-2.1642
NOP56	NOP56 ribonucleoprotein homolog (yeast)	-2.1671
MCM6	minichromosome maintenance complex component 6	-2.1822
GMNN	geminin, DNA replication inhibitor	-2.2147
FBXO5	F-box protein 5	-2.2841
BMP4	bone morphogenetic protein 4	-2.2873
FEN1	flap structure-specific endonuclease 1	-2.5511
MCM10	minichromosome maintenance complex component 10	-2.5936
HSPA8	heat shock 70kDa protein 8	-2.73
RN28S1	RNA, 28S ribosomal 1	-3.0581
HNRNPL	heterogeneous nuclear ribonucleoprotein L	-3.0607