Gene silencing in alveolar type II cells using cell-specific promoter in vitro and in vivo

Deming Gou, Telugu Narasaraju, Narendranath Reddy Chintagari, Nili Jin, Pengcheng Wang and Lin Liu*

Department of Physiological Sciences, Oklahoma State University, 264 McElroy Hall, Stillwater OK 74078, USA

Received May 12, 2004; Revised July 27, 2004; Accepted August 20, 2004

ABSTRACT
RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing process. Although it is widely used in the loss-of-function studies, none of the current RNAi technologies can achieve cell-specific gene silencing. The lack of cell specificity limits its usage in vivo. Here, we report a cell-specific RNAi system using an alveolar epithelial type II cell-specific promoter—the surfactant protein C (SP-C) promoter. We show that the SP-C-driven small hairpin RNAs specifically depress the expression of the exogenous reporter (enhanced green fluorescent protein) and endogenous genes (lamin A/C and annexin A2) in alveolar type II cells, but not other lung cells, using cell and organ culture in vitro as well as in vivo. The present study provides an efficient strategy in silencing a gene in one type of cell without interfering with other cell systems, and may have a significant impact on RNAi therapy.

INTRODUCTION
RNA interference (RNAi) is a post-transcriptional process triggered by the introduction of double-stranded RNA, which leads to gene silencing in a sequence-specific manner (1). Specific gene silencing may be achieved in a variety of cell systems using chemically synthesized or in vitro-transcribed small interfering RNA (siRNA) (2) as well as PCR or DNA-vector-based short hairpin RNA (shRNA) (3–6). A few promoters have been reported to drive shRNA expression in cells, including RNA polymerase-III-based promoters, U6 and H1, and the RNA polymerase II promoter, CMV. However, the use of these promoters to drive shRNA expression in animals would silence a gene in all types of cells and thus produce undesirable effects in non-target cells. Cell-specific targeting of siRNA is an important issue to consider in RNAi therapeutics (7). To silence a gene in a particular type of cell without affecting its expression in another type of surrounding cells, a cell-specific promoter has to be used. Till date, there are no successful reports in the use of a cell-specific promoter to drive shRNA expression in cells. Most recently, the use of tissue-specific recombination to produce tissue-specific knock-down has been reported (8).

The lung is one of the major targets for gene therapy. The alveolar epithelium is composed of morphologically- and functionally distinct type I and type II cells. A number of genes that are expressed in a highly cell-selective manner in the respiratory epithelium have been isolated and characterized. These include the genes encoding the surfactant proteins (SP)-A, B and C, and the Clara cell secretory protein. Among them, SP-C is exclusively expressed in alveolar type II cells of the distal airways. The human SP-C promoter has been successfully used to express transgenes in a cell-specific manner (9).

Here, we sought to develop a strategy for specifically silencing a gene in alveolar epithelial type II cells of rat lungs, allowing us to directly elucidate its function in type II cells and to develop more refined disease models. We constructed adenoviral vectors containing various shRNA under the control of SP-C promoter. Using these vectors, we demonstrated a specific silencing of target genes in type II cells, but not in other lung cells both in vitro and in vivo.

MATERIALS AND METHODS
Design and construction of shRNA and viral vectors
A DNA fragment containing 3.7 kb of human SP-C promoter, rtTA coding sequence and 0.45 kb SV40 poly(A) was PCR-amplified with Pfu DNA polymerase (Stratagene, La Jolla, CA) and the primer pair, 5'-CACCTCGAGATCGATGAAGAC-3' and 5'-TTCGAACCG-GTAATTCCAGCTCGGTACCGG-3' from pSP-C-rtTA vector (9) (a kind of gift from Dr Jeffrey Whitsett, Children’s Hospital Medical Center, Cincinnati, OH). The pENTR/SP-C-rtTA vector was obtained by directionally cloning the purified PCR product into a pENTR/D-Topo vector (Invitrogen, Carlsbad, CA).Later, the pENTR/SP-C-rtTA vector was digested by Sall and EcoRI restriction enzymes to remove the rtTA fragment between SP-C promoter and poly(A) sequences. The annealed shRNAs with Sall–EcoRI overhangs were then cloned into the SP-C vector through Sall–EcoRI sites to obtain a new vector, pENTR/SP-C-shRNA-pA with a poly(A) terminal sequence. We replaced the poly(A) sequence with a minimal poly(A)
ACTTCAAGAGAGTAGTTGTACTCCAGCTTGTGTTG-3

previously (12). A mixed lung cell preparation was obtained

Cell culture and adenoviral infection

Alveolar type II cells were isolated from rat lungs as described previously (12). A mixed lung cell preparation was obtained by using elastase digestion (4.5 U/ml) as described previously (13). The resulting cell mixture consisted of alveolar type I cells, type II cells, Clara cells, ciliated airway epithelial cells, fibroblasts, macrophages and lymphocytes. To preserve the type II cell phenotype, type II cells and mixed lung cells were cultured on an air–liquid culture system as described previously (14). The cells (3 \times 10^6) were plated on a 30 mm filter insert (Millipore, Bedford, MA) coated with rat-tail collagen and Matrigel (4:1, v/v, Collaborative Biomedical Products, Bedford, MA). One milliliter of DMEM containing 5% rat serum, 10 ng/ml keratinocyte growth factor and 10 nM dexamethasone were added to each side of the insert. The plates were placed on a rocking rotator inside an incubator with 5% CO2. After a 20 h culture, 0.3 ml of the same medium containing adenoviruses (400 multiplicities of infection or MOI) was added to the apical surface, and 1.5 ml of the medium was placed on the outside of the insert. A 1:8 ratio was used when Ad/CMV-EGFP and Ad/SP-C-shRNA adenovirus were added simultaneously. The medium was changed on alternate days. On the sixth day, the type II cells were directly examined for the EGFP fluorescence, and the mixed cells collected by disrupting the collagen gel with a pipette and filtering through a 160 μm pore-size filter. Cells were cytospinned to coverslips for immunocytochemistry.

**In vitro lung organ culture**

The lungs were excised from 3-day-old rat pups and cut transversely into 5 mm slices using a sterile blade. The lung slices were cultured on a 30 mm filter insert. Serum-free, hormone-free BGGb medium (0.3 ml) containing 0.2 mg/ml ascorbic acid, 0.5 U/ml penicillin, and 0.5 g/ml streptomycin was added on the inside of the insert and 1.5 ml outside of insert (15). In this design, the lung slices were situated just above the median and remained suspended with medium by capillary action through the membrane. The adenovirus (6 \times 10^9 particles) was added on the inside of the insert at the time of plating. After culturing for 6 days, the organ culture was fixed with 4% (v/v) formaldehyde, embedded in paraffin, sectioned and immunostained with anti-annexin II and anti-SP-C antibodies.

**Adenoviral delivery into the rat lungs**

Adult male Sprague–Dawley rats (200–250 g) were used for *in vivo* studies. Oklahoma State University Animal Use and Care Committee approved the animal procedures. Endotracheal intubation and administration of the virus was carried out as described previously (16). In brief, the animals were anesthetized with an intraperitoneal injection of Ketamine and Xylazine. The epiglottis and trachea of the animal were visualized using a modified intubation wedge. The animals were then orally intubated using a sterile18-guage intravenous catheter. Immediately prior to the administration of the adenovirus, the animals were forced to exhale by circular compression of the thoracic cavity and then 200–400 μl of the adenovirus (5 \times 10^{11} particles) in phosphate-buffered saline containing 50% Survanta (Abbot Laboratories, Columbus, OH), 1 mg/ml of proteamine sulfate and 250 μg/ml hydrocortisone (17,18) was administered. The virus was incubated for 10 min with proteamine sulfate before being administrated into the animal. Animals were sacrificed on the fifth day. The lungs were instilled with 4% formaldehyde (w/v) in phosphate-buffered saline (pH 7.4), embedded in paraffin, and processed for immunostaining.

**Northern blotting**

Total RNA was isolated with TRI reagents (Molecular Research Center, Inc., Cincinnati, OH) from the cultured type II cells. RNA (20 μg/lane) was electrophoretically separated on a 15% polyacrylamine–7 M urea gel and transferred by electoblotting onto to Hybond N+ membrane (Amersham
The sense oligonucleotides (100 pmol) of shEGFP or shCon were end-labeled with polynucleotide kinase and [³²P]ATP (150 µCi), purified through a G-25 MicroSpin Column (Amersham Pharmacia Biotech), heated for 5 min to 65°C, and then used for hybridization at 37°C overnight. The membrane was washed two times at 5 min intervals at room temperature in 2× SSC plus 0.1% SDS, three times for 10 min in 0.1× SSC plus 0.1% SDS and exposed on BioMax MS films (Kodak).

RT–PCR

After being treated with DNase, 1 µg of total RNA was reverse-transcribed into cDNA using M-MLV reverse transcriptase (200 U) in the presence of 100 ng 18mer oligo(dT) and 5 ng EGFP reverse primer. One microliter of cDNA was used to amplify the EGFP fragment with 1× PCR buffer, 1.5 mM MgCl₂, 200 µM of each dNTP, 200 µM of primers and Taq DNA polymerase. For normalization of RNA loading, the housekeeping gene, β-actin, was also amplified from each sample. The primer sequences are as follows: EGFP, forward 5'-TGCCACCTACGGCAAGCTGA-3' (111–130), reverse 5'-TCGATTTGGGCGCATCTT-3' (499–518); β-actin, forward 5'-GGCATTGTAACCAACTGGGACGATATG-3' (220–246), reverse 5'-TTTATGGTGCCACAGGGTCC-3' (807–828). PCR amplification was performed using the following conditions: 1 cycle of 95°C for 3 min, 25 cycles of 94°C for 30 s, 56°C for 1 min, 72°C for 1 min, followed by a final elongation step of 72°C for 7 min. After amplification, 10 µl aliquots of PCR products from each condition were separated on a 1.5% agarose gel. Signals were quantified by densitometric analysis using the Bio-Rad Quantity One 4.0.3 software.

Western blotting

The cells were lysed at 4°C for 1 h in the lysis buffer [50 mM Tris–HCl (pH 7.4) 250 mM sucrose, 1% Triton X-100, 10 mM EGTA, 2 mM EDTA, 20 µg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride]. Ten to twenty micrograms of the proteins were resolved on 12% SDS–PAGE under reducing conditions and electrophoretically transferred onto a nitrocellulose membrane. The membranes were blocked with Tris-buffered saline plus 0.1% Tween-20 (TTBS) containing 5% non-fat milk for 1 h, incubated with the appropriate primary antibodies (anti-β-actin, 1:4000 dilution; anti-EGFP, 1:1000 dilution) in TTBS containing 1% BSA for 2 h, followed by incubation with secondary antibodies (horse-radish peroxidase-conjugated IgG, 1:5000 dilution) for 1 h. Finally, the proteins were visualized by enhanced chemiluminescence reagents. Signals were quantified by densitometric analysis using Bio-Rad Quantity One 4.0.3 software.

Immunostaining

Immunohistochemistry and immunocytochemistry were performed as described previously (19). The primary and secondary antibodies were polyclonal goat anti-SP-C (1:50), monoclonal anti-lamin A/C (1:50) and polyclonal rabbit anti-annexin A2 (1:50) antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA), and Alexa 488- or Alexa 546-conjugated anti-goat (1:200), Alexa 488-conjugated anti-mouse (1:200), Cy 3-conjugated anti-mouse (1:200) and Alexa 546-conjugated anti-rabbit (1:200) IgG (Molecular Probes, Eugene, OR).

RESULTS AND DISCUSSION

To determine whether the SP-C promoter can be used to produce siRNA and silence gene expression in a cell-specific fashion, we constructed an adenoviral vector, in which shRNA in the form of a small hairpin structure, was placed under the control of the 3.7 kb human SP-C promoter followed by a mA (Figure 1A). The minimal poly(A) has been used successfully for the CMV-driven shRNA (3). We chose the adenoviral vector because of its high transfection efficiency in primary culture cells and animals. We first tested whether SP-C-driven shRNA depressed the expression of the exogenous reporter gene, EGFP, in primary culture of alveolar type II cells. It is well-known that type II cells lose their phenotype and SP-C expression, and trans-differentiate into type-I-like cells when cultured on plastic dishes. We, therefore, used an air–liquid culture system that mimics the in vivo conditions expected in the lung. This system has been reported to maintain the type II cell phenotype including the expression of SP-C (14). The exogenous EGFP expression in type II cells was obtained with a CMV-EGFP adenoviral vector, containing EGFP under the control of the CMV promoter. The infection of Ad/CMV-EGFP adenovirus alone resulted in a high expression of EGFP in isolated type II cells by a direct visualization under a fluorescence microscope (Figure 1B a). EGFP expression was markedly reduced when type II cells were co-infected with Ad/CMV-EGFP and SP-C-driven shRNA targeted to EGFP (Ad/SPC-shEGFP) adenoviruses [Figure 1B (c)]. Inhibition was sequence-specific because the co-infection of Ad/CMV-EGFP and a control virus Ad/SP-C-shCon expressing unrelated shRNA failed to reduce the EGFP expression [Figure 1B (b)]. The quantitation by RT–PCR and western blotting indicated that SP-C-driven shEGFP decreased the mRNA and protein levels of EGFP by 74 and 81%, respectively, but did not alter β-actin expression (Figure 1D and E). Another vector, Ad/SP-C-shEGFP-pA, containing 0.45 kb SV40 poly(A) instead of the 66 bp mA, did not show a significant inhibition of EGFP expression under the same conditions (data not shown), consistent with a previous report on the CMV promoter (3). To examine whether SP-C-driven shRNA is specific to type II cells, we repeated the experiment above with a number of cell lines that do not express SP-C. No reductions of EGFP mRNA and protein levels were observed in a rat lung epithelial cell line, L2 cells infected with Ad/SP-C-shEGFP as determined by RT–PCR, western blotting, and a fluorescence microscopy (Figure 1C–E). Similar results were obtained with two mouse fibroblast cell lines, NIH 3T3 and L929 (data not shown).

Northern blotting analysis showed that Ad/SPC-shEGFP- or Ad/SPC-shCon-infected type II cells expressed a ~57 bp RNA specific to shEGFP or shCon sequences (Figure 2), consistent with the predicted size of the transcripted shRNA, further indicating that the SP-C promoter can be used to express shRNAs and silence gene expression specifically in type II cells.
Figure 1. SP-C-driven shRNA silences exogenous EGFP expression in type II cells, but not in L2 cells. (A) A schematic illustration of the human SP-C promoter-driven shRNA adenoviral vector (Ad/SP-C-shRNA). The shRNA contains a sense strand (S), a 9 nt loop (L) and an antisense strand (AS), followed by TT and a 66 bp of mA. (B and C): Effect of siRNA expressed from SP-C promoter constructs on EGFP expression on primary culture of alveolar type II cells (B) and L2 cells (C). The cells were infected with Ad/CMV-EGFP (a and d), Ad/CMV-EGFP and the siRNA control, Ad/SP-C-shCon (b and e), and Ad/CMV-EGFP and Ad/SP-C-shEGFP (c and f). After 5 days, the cells were examined with a fluorescence microscope (a–c), and the nuclei stained with DAPI to visualize the cells in the fields (d–f). Scale bar, 20 μm. mRNA (D) and protein (E) levels of EGFP in type II and L2 cells as determined by RT–PCR and western blotting. β-actin was used as a loading control. Upper panels show a representative gel or blot. Lane 1, Ad/CMV-EGFP; lane 2, Ad/CMV-EGFP and Ad/SP-C-shCon; lane 3, Ad/CMV-EGFP and Ad/SP-C-shEGFP. Lower panels, mRNA and protein levels were quantitated after being normalized with β-actin signals. The results are expressed as a percentage of control (Ad/CMV-EGFP). Data shown are means ± SE (n = 3). The asterisk denotes a P of <0.01 versus Ad/CMV-EGFP group.
We next tested whether the shRNAs controlled by SP-C promoter work on endogenous genes. We selected two endogenous genes with diverse functions and different cellular locations, lamin A/C and annexin A2. Lamin A/C is a nuclear membrane protein involved in the organization of nuclear architecture (20). Annexin A2 is a cytosolic Ca\(^{2+}\)-dependent phospholipid-binding protein and plays an important role in the membrane fusion during the exocytosis of lamellar bodies from alveolar epithelial type II cells (21). The siRNA sequence, targeted to the coding region of 607 to 625 of rat lamin A/C gene, was chosen based on the previous reports on human lamin A/C gene (2,4). In this region, there is one base difference between rat and human sequences, which we switched to the rat sequence (C\(^{611}\)→T\(^{611}\)). We infected a mixed lung cell culture with Ad/SP-C-shLamin or Ad/SP-C-shCon. After a 4-day culture, the cells were double-labeled with anti-lamin A/C and anti-SP-C antibodies to determine the protein expression level of lamin A/C and to identify alveolar type II cells, respectively. As shown in Figure 3B (a–d), lamin A/C was expressed at a similar level in all the different cells when they were infected by Ad/SP-C-shCon adenovirus. However, the expression of lamin A/C was specifically reduced in type II cells [arrows, Figure 3B (e–h)], but not in non-type-II cells (asterisks) by Ad/SP-C-shLamin adenovirus. Quantified data by counting type II cells (red) with or without a reduced lamin A/C expression (green) revealed that the silence of lamin A/C occurred in ~44% of type II cells.

Dissection of lung cells from rat lung tissues and the cell isolation procedure may alter cell function and activate gene expression. We, therefore, used an in vitro model of neonatal rat lung organ culture to further test the specific silencing of the endogenous gene, annexin A2. This organ culture system maintains the cellular architecture and thus intracellular contacts and communications (15). We have previously screened six in vitro-transcribed siRNA sequences targeted to different regions of the rat annexin A2 gene and found that the siRNA sequence targeted to 129–147 nt was the most effective in silencing annexin A2 protein expression in type II cells (22). This annexin A2 siRNA sequence was used in the present study to construct an adenoviral vector, Ad/SP-C-shAII. Double-labeling with anti-annexin A2 and SP-C antibodies was used to determine annexin A2 protein expression level (red) and to identify type II cells (green), respectively. The infection of the neonatal lung organ culture with Ad/SP-C-shAII generated green-positive (type II cells) and red-negative (annexin A2 expression level) cells [Figure 4A (e–h)], suggesting the silencing of annexin A2 in type II cells. In the siRNA control-treated group, we observed green- and red-positive cells, indicating a high expression of annexin A2 in type II cells [Figure 4A (a–d)]. A clear difference can be seen in the merged images, in which the yellow spots in the siRNA control group and the green spots in the annexin A2 siRNA group represent the expression and silencing of annexin A2 in type II cells, respectively. The cell counting revealed that the cells showing both SP-C and annexin A2 staining were reduced by 48% when compared to the controls (Figure 4B). In both groups, some of the cells showed green-negative and red-positive cells, indicating no silencing of annexin A2 in the non-type-II cells. The results suggest that the observed RNAi effect on endogenous annexin A2 gene in lung organ culture is cell- and sequence-specific.

![Diagram](https://example.com/diagram.png)
Figure 3. Specific silencing of EGFP and lamin A/C by SP-C-driven-shRNA in a lung cell mixture. (A) EGFP, the mixed lung cells were infected with Ad/CMV-EGFP plus Ad/SP-C-shCon or Ad/SP-C-shEGFP adenoviruses for 5 days. (a and e) EGFP fluorescence; (b and f) immunostaining with anti-SP-C antibodies to identify type II cells. (B) lamin A/C, the mixed lung cells were infected with Ad/SP-C-shCon or Ad/SP-C-shLamin adenovirus for 5 days. The cells were double-labeled with anti-lamin A/C (a and e) to monitor the lamin A/C expression and anti-SP-C (b and f) antibodies to identify type II cells. Arrows, type II cells; asterisks, non-type-II cells. Scale bar, 20 μm. (C) Quantitation of EGFP (top) or lamin A/C (bottom) silencing in Ad/SP-C-shEGFP- or Ad/SP-C-shLamin-treated type II cells: Cell counting was performed based on the number of type II cells (red) with or without EGFP or lamin A/C expression (green). Eighty to hundred type II cells were counted for each slide. The results were expressed as a percentage of control (Ad/CMV-EGFP/SP-C-shCon or Ad/SP-C-shCon). Data are means ± SE (n = 3). The asterisk denotes a P of <0.01 versus control group.
Finally, to examine whether SP-C-driven shRNA works in vivo, we directly delivered the adenoviral vector, Ad/SP-C-shAll, into rat lungs and determined annexin A2 expression in type II cells. The virus was delivered to rat lungs intrabronchially. The surfactant and protamine were included to enhance the efficiency of adenovirus-mediated siRNA expression, and corticosteroids were used to inhibit inflammation and minimize virus-related toxicity (17;18). Rats were sacrificed and the lungs infused with 4% (w/v) formaldehyde 5 days after delivery. The tissue section was double-stained with anti-annexin A2 and anti-SP-C antibodies. In the Ad/SP-C-shAll-treated group, we found that annexin A2 was silenced in type II cells (green-positive and red-negative cells) [Figure 5 (e–h)]. However, the expression of annexin A2 in non-type-II cells was evident (green-negative and red-positive cells). In contrast, both green- and red-positive cells (annexin A2 expressed in type II) and green-negative and red-positive cells (annexin A2 expressed in non-type-II) were seen in the control group [Figure 5 (a–d)]. The number of type II cells showing silencing of annexin A2 was ~15%. Although the current results showed a low silencing efficiency, our studies are promising considering the low infection efficiency in the in vivo studies. We estimated 20–30% infection efficiency in our studies by delivering Ad/CMV-EGFP virus, digesting lungs and counting EGFP-positive lungs. This experiment demonstrates that the SP-C promoter drives siRNA expression and silences gene expression in a cellspecific manner in animals in vivo.

The most commonly used plasmids for expressing siRNAs in cells contain RNA polymerase-III-based promoters such as U6 and H1 or RNA polymerase II promoters such as CMV (3–6). Although these promoters have a wide applicability in cell systems in vitro, their lack of cell specificity limits their usage in vivo. We have demonstrated that the SP-C-driven shRNA expression effectively and specifically silences exogenous and endogenous gene expression in type II cells using cell and organ culture in vitro as well as in vivo. The current studies, therefore, establish proof-of-principle for using a cell-specific promoter to depress gene expression in a particular type of cell. This provides an efficient strategy in targeting and silencing a specific gene in vivo in one type of cell without interfering with other cell systems. For example, in order to target an oncogene in cancer cells, but not normal cells, a telomerase reverse transcriptase promoter (23) would be used to drive shRNA expression in cancer cells. Considerable interest has developed in the potential for RNAi therapy. As a therapeutic agent, there is a great need for delivering siRNA to and thus silencing a gene in a particular type of target cell. This strategy may have a significant impact on RNAi therapy.

Figure 4. Adenovirus-mediated annexin A2 gene silencing by SP-C-driven shRNA in in vitro lung organ culture. (A) Purified adenoviruses expressing annexin A2 shRNA or the control shRNA under the control of the SP-C promoter (Ad/SP-C-shAll or Ad/SP-C-shCon) were used to infect in vitro lung organ culture. After 5 days, paraffin-embedded sections from cultured lung organs were double-immunostained with anti-annexin A2 (a and e) and anti-SP-C (b and f) antibodies. The merged images and bright fields are shown in (c and g) and (d and h), respectively. The upper right corners show the enlarged images. Scale bar, 20 μm. (B) Quantitation of annexin A2 silencing in type II cells: Cell counting was performed based on the number of type II cells (red) with or without EGFP or lamin A/C expression (green). Eighty to hundred type II cells were counted for each slide. The results were expressed as a percentage of control (Ad/SP-C-shCon). Data shown are means ± SE (n = 3).
The authors thank Dr Jeffrey A. Whitsett of the Children’s Hospital Medical Center, Cincinnati, OH, for kindly providing pSP-C-rtTA vector, Dr Nick Cross for reading the manuscript, Zhixin Wang, Manoj Bhaskaran and Ting-ting Weng for technical help, and Candice Marsh for secretarial assistance. This work was supported by NIH R01 HL-52146, R01 HL-071628 and OCAST HR01-093 (to L.L.).

REFERENCES


Figure 5. Adenovirus-mediated annexin A2 gene silencing by SP-C-driven shRNA in rat lungs. Purified adenoviruses (5 x 10^11 particles) expressing annexin A2 shRNA or the control shRNA under the control of SP-C promoter (Ad/SP-C-shAll or Ad/SP-C-shCon) were directly delivered into rat lungs. After 5 days, paraffin-embedded sections from infused lung were double-immunostained with anti-annexin A2 (a and e) and anti-SP-C (b and f) antibodies. The merged images and bright fields are shown in (c and g) and (d and h), respectively. The upper right corners show the enlarged images. Scale bar, 20 μm.