Intracellular antigen labeling and manipulation by antibodies have been long-thought goals in the field of cell research and therapy. However, a central limitation for this application is that antibodies are not able to penetrate into the cytosol of living cells. Taking advantages of small sizes and unique structures of the single-domain antibodies, here, we presented a novel approach to rapidly deliver the nanobody/variable domain of heavy chain of heavy-chain antibody (V_{H}H) into living cells via introducing its coding mRNA, which was generated by in vitro transcription. We demonstrated that actin-green fluorescent proteins (GFP) and Golgi-GFP can be recognized by the anti-GFP nanobody/V_{H}H, vimentin can be recognized by the anti-vimentin nanobody/V_{H}H, and histone deacetylase 6 (HDAC6) can be recognized by the anti-HDAC6 nanobody/V_{H}H, respectively. We found that the anti-GFP nanobody expressed via in vitro-transcribed (IVT) mRNA can be detected in 3 h and degraded in 48 h after transfection, whereas the nanobody expressed via plasmid DNA, was not detected until 24 h after transfection. As a result, it is effective in delivering the nanobody through expressing the nanobody/V_{H}H in living cells from its coding mRNA.

INTRODUCTION

Over the past decades, tremendous progress has been made in modifying conventional antibodies to variable forms of antibodies in different structures and sizes, and now antibodies are widely used from basic lab research to medical therapies. However, a major limitation is that conventional antibodies are not able to penetrate into living cells, since the high molecular weight of antibodies prevents them from crossing the cell membranes to access intracellular targets. Up to now, there are four major approaches to deliver antibodies into living cells, including intrabodies, protein transfection (profection), peptides as protein transduction domains (transmabs/transbodied), and fusion to targeting proteins. Antibodies delivered using the above approaches usually bear a cytosol-penetrating ability.

Developing antibodies in a smaller size is a fundamental problem in this field. The antigen-binding fragment (Fab) of a heavy-chain antibody, which is devoid of light chains and recognizes antigens via their variable domain from Camelidae sp. (referred to as variable domain of heavy chain of heavy-chain antibody (V_{H}H) or nanobody), represents the smallest intact antigen-binding fragment. Nano-bodies/V_{H}Hs are small (13–14 kDa) antibody fragments that consist of just one variable heavy-chain domain of a heavy-chain antibody. To date, the most advanced method in delivering antibodies to the endoplasmic reticula (ERs) is transfection with a plasmid-containing coding sequence of nanobodies/V_{H}Hs. However, as recombinant plasmid transfection is associated with multiple serious safety issues, this approach is still limited to research purposes.

In vitro-transcribed (IVT) mRNA is an ideal carrier to deliver genetic information into living cells. Over the last years, many interesting techniques of IVT mRNA were proposed and shown to be useful for many applications. So the development of IVT mRNA-based therapeutics has gained broad attention. One of the main uses of IVT mRNA is to deliver protein into living cells, but recently, nanobodies/V_{H}Hs have been expanded toward mining from traditional antibodies.

Since the foundations of nanobodies/V_{H}Hs transfected into living cells, as well as the burst of IVT mRNA, the problem of nanobodies/V_{H}Hs expressed with high efficiency and safety has been in a scope of interest of numerous researchers from this field. Here, we focus only on transferring the nanobody/V_{H}H targeting cell skeletons, organelle, or other cytoplasmic molecules via expressing its IVT mRNA. We chose an anti-green fluorescent protein (GFP) nanobody/V_{H}H sequence (CAN 375), anti-vimentin nanobody/V_{H}H sequence (CAN 2391), as well as anti-histone deacetylase 6 (HDAC6) nanobody/V_{H}H sequence (unpublished) expressed via IVT mRNAs and studied their distribution in cells. Also, we investigated how the stability of IVT mRNA is regulated. Moreover, we compared the relative fluorescence intensity of the anti-GFP nanobody/V_{H}H expressed via IVT mRNA and via plasmid, including production and degradation. Our study suggests a potential strategy to expand the applications of the nanobody/V_{H}H via expressing its IVT mRNA in living cells.
RESULTS

Illustration and Characterization of the Anti-GFP Nanobody/VH\textsubscript{H} Expressed via the IVT mRNA Concept

Most nanobodies/VH\textsubscript{H}s can be functionally expressed as intrabodies via plasmids transfected into a eukaryotic cell. This makes nanobodies/VH\textsubscript{H}s ideal tools to recognize structural or dynamic features observed by biochemical measurements in living cells. Previous reports have indicated that the epitope-recognizing fragment of heavy-chain antibodies from Camelidae sp. with fluorescent proteins to generate fluorescent, antigen-binding nanobodies/VH\textsubscript{H}s (chromobodies)\textsuperscript{12} can be expressed in living cells via plasmid transfection. Here, we constructed GFP-binding nanobody/VH\textsubscript{H} expressed in the form of RNA instead of DNA, termed as nanobody-mCherry expressed via IVT mRNA. We quickly produced anti-reverse cap analog (ARCA)-capped and poly(A)-tailed mRNA with untranslated regions (UTRs) and chemically modified nucleotides \textit{in vitro}. The mRNA synthesized with the kit can be used for transfection (Figures 1A and 1B).

We then investigated the ability of the anti-GFP nanobody-mCherry expressed via IVT mRNA in accessing and binding its epitopes in living cells. To test the expression of the anti-GFP nanobody/VH\textsubscript{H} in living cells, we fused it to mCherry to generate a “visible” anti-GFP antibody. Actin is a typical epitope in the cytoplasm, which is incorporated into growing actin filaments and can be visualized directly. Thus, we cotransfected HeLa cells with the filamentous actin (F-actin)-GFP expression construct and the nanobody-mCherry expressed via IVT mRNA. A representative confocal image of a double-transfected cell showed green and red fluorescence at the cytoskeleton (Figure 1C), which is indicative of correct incorporation of F-actin-GFP into the actin filaments and efficient recognition by the nanobody-mCherry expressed via IVT mRNA. The green field represented GFP, which was coexpressed with F-actin, and the red field represented mCherry, which was coexpressed with the nanobody/VH\textsubscript{H}. Double-immunofluorescent development revealed colocalization of F-actin and the nanobody/VH\textsubscript{H} in HeLa cells.

Nanobodies/VH\textsubscript{H}s Expressed via IVT mRNAs Recognizing Specific Cytoplasmic Antigens in Living Cells

We cotransfected the F-actin-GFP expression construct with the anti-GFP nanobody-mCherry expressed via IVT mRNA into A549 cells. We also cotransfected the Golgi-GFP expression construct with the nanobody-mCherry expressed via IVT mRNA into HeLa cells and analyzed them by live cell microscopy (Figures 2A and 2B). As a result, the nanobody-mCherry expressed via IVT mRNA was
successfully generated, which can bind F-actin and Golgi in A549 and HeLa cells, respectively.

Vimentin is the major intermediate filament of mesenchymal cells and is mainly involved in tissue integrity and cytoarchitecture. Vimentin nanobodies in A549 and HeLa, HEK293T, and Chinese hamster ovary (CHO)-K1 cells were transfected with the IVT mRNA coding anti-vimentin nanobody-mCherry and analyzed by live cell microscopy (Figure 2C). As a result, the anti-vimentin nanobody-mCherry expressed via IVT mRNA was successfully generated and can be distributed in cytoplasm mainly, not shown in the cell nucleus.

HDACs are enzymes that catalyze the removal of acetyl groups from the lysine residues located on histone and nonhistone proteins. HDAC6 is a cytoplasmic enzyme that uniquely features two catalytic domains. HDAC6 is a component of aggresome, which could be induced by the proteasome inhibitor MG132. A549 and HEK293T cells were transfected with the IVT mRNA coding anti-HDAC6 nanobody-mCherry and analyzed by live cell microscopy (Figure 2D). As a result, the anti-HDAC nanobody-mCherry expressed via IVT mRNA was successfully generated, can recognize HDAC6 within the inclusion body in cytoplasm in A549 and HEK293T cells after MG132 treatment. Confocal midsections of living cells are shown. Scale bars, 10 μm.

Thus, the nanobodies/VHHs expressed via IVT mRNAs were strongly expressed and can recognize antigens of cell skeletons, organelle, and cytosol in different kinds of living cells. The distribution of nanobodies presented structures in shapes of the cell skeleton or the Golgi.

The Anti-GFP Nanobody-mCherry Expressed via IVT mRNA Produced and Degraded Earlier Than the pmCherry-N1/Nanobody

To accelerate the production and increase the transfection efficiency of nanobody-mCherry, HeLa cells were transfected with the same concentration of the IVT mRNA and pmCherry-N1/anti-GFP nanobody. Western blot and microscopy analyses of transfected cells showed that the anti-GFP nanobody-mCherry expressed via IVT mRNA can be expressed at 3 h after transfection, whereas the nanobody-mCherry expressed via the plasmid was not expressed until 24 h after transfection (Figures 3A and 3B).

Furthermore, the existing of DNA in cell lines may expose a risk of genome integration and cause multiple serious safety issues. The nanobody-mCherry expressed via IVT mRNA peaked at 24 h after transfection and then degraded quickly. At 72 h after transfection, the nanobody-mCherry expressed via IVT mRNA
degraded mostly. Compared to IVT mRNA, the nanobody-mCherry expressed via the plasmid progressively produced from 24 h to 72 h and did not disappear at 72 h after transfection. The relative fluorescence intensity of the nanobody was shown in Figure 3C.

In A549 cells, the same results of Figure 3 were shown (Figure S1). A549 cells were also transfected with the same concentration of IVT mRNA and the plasmid. Western blot and microscopy analyses of transfected cells showed that the nanobody-mCherry expressed via IVT mRNA can be expressed at 3 h, peaked at 24 h, then degraded at 48 h, and disappeared mostly at 72 h after transfection. At the same time, the nanobody-mCherry expressed via the plasmid progressively produced from 24 h to 72 h and did not disappear at 72 h after transfection (Figures S1A and S1B). The relative fluorescence intensity of the nanobody was shown in Figure S1C. The relative fluorescence intensity of cells expressing the nanobody-mCherry was fitted to the phenomenon of Figure 3, in which the nanobody-mCherry expressed via IVT mRNA produced and degraded earlier than the plasmid.

Optimization IVT mRNA Construction for the Best Protein Production

To be efficiently translated, most eukaryotic mRNAs require a 7-methyl guanosine (m7G) cap structure at the 5’ end and a poly(A) tail length of 300 nt at the 3’ end,20 as well as UTRs.21 We also allow for partial incorporation of 5-methyl-cytosine-5’-triphosphate (5mCTP), pseudo-UTP, and other modified nucleotides into mRNA, which abrogate mRNA interaction with Toll-like receptor (TLR)3, TLR7, TLR8, and retinoid-inducible gene I (RIG-I), resulting in low immunogenicity and higher stability in mice.22

In order to optimize the highest production and determine the mRNA function in the anti-GFP nanobody-mCherry translation, we produced four different mRNA mutations, such as no incorporation of 5mCTP and pseudo-UTP into mRNA, no UTRs (including 5’ and 3’ UTR) in T7 RNA polymerase templates, the poly(A) tail length of 150 nt at the 3’ end, and no m7G cap structure at the 5’ end of mRNA. They were termed as mRNA with nucleoside triphosphates (NTPs), mRNA with 5mCTP and pseudo-UTP without UTRs, mRNA with 5mCTP and pseudo-UTP with half poly(A) tail, and...
mRNA with 5mCTP and pseudo-UTP without 5'-cap, respectively (Figure 4A). The RNA length of mRNAs after tailing for 15 min (called half poly(A) tail) and 30 min (called mRNA) on an Agilent 2100 bioanalyzer was also shown (Figure S2).

In order to determine the proper amount of mRNA transfection, we compare different concentrations of the nanobody-mCherry expressed via mRNA fused with a red fluorescent protein (mCherry) from 0 to 2 μg per 10⁶ cells. As the relative fluorescence intensity of the nanobody was shown in Figure S3B, production of the anti-GFP nanobody-mCherry expressed via IVT mRNA in 1.5 μg and 2 μg per 10⁶ cells has reached the highest and is enough for transfection.

At 24 h after the same concentration of mRNAs and plasmid, the western blot and microscopy analyses of the nanobody-mCherry production showed significant results in HeLa cells (Figures 4B and 4C), whereas Figure 4D displays the relative fluorescence intensity of the top ten cells producing the nanobody-mCherry expressed via mRNA without UTRs and with half poly(A) tail decreased almost 20% and 40% and expressed via mRNA without 5'-cap was none. The relative fluorescence intensity of cells producing the nanobody-mCherry expressed via plasmid was done as a control. It showed that the 5'-cap structure is crucial to protein production, and the poly(A) tail length of 300 nt, UTRs, and incorporation of modified nucleotides enhanced protein producing.

So far, we have constructed the most stable, efficient, and productive nanobody-mCherry expressed via IVT mRNA, such as anti-GFP, anti-vimentin, and anti-HDAC6 nanobodies. All of them can be rapidly, intracellularly transfected into different living cells to specifically target and trace cell skeletons, organelle, and cytoplasmic molecules.

**DISCUSSION**

The dramatic shift in the types of modified antibodies has been exposed in reaching clinical trial studies. There is a main focus on new advances in targeting antibodies to the central nervous system and intracellular compartments. However, their target repertoire is limited to few numbers of tumor-specific or associated cell-surface antigens. Intracellular molecules represent almost one-half of the human proteome and provide a broadly potential therapeutic target.²³ So antibodies have been developed into binding externalized antigens and have also been engineered to enter into cells or expressed intracellularly with the aim for combining intracellular antigens.
Intracellular antibodies are these antibodies which produce and bind antigens within the same cell. This is a different delivery strategy from antibodies produced extracellularly and engineered to penetrate cell membranes for accessing their intracellular targets. There are some strategies for targeting intracellular tumor antigens with antibody therapy. Antibodies can be fused to cell-penetrating peptides, which allow internalization of the antibody. However, the approach achieving direct cytosolic protein delivery via modification with a cyclic peptide requires more manipulation. The antibody depicted on the diagram could represent a full-length immunoglobulin G (IgG), a Fab fragment, single-chain variable fragment (scFv), or a single domain antibody. As mentioned before, the nanobody/V_{13}H has an advantage in this field, representing the smallest intact antigen-binding fragment with an increased stability and solubility compared with those of conventional monoclonal antibodies. Caplacizumab, anti- von Willebrand factor (vWF) nanobody/V_{13}H to treat acquired thrombotic thrombocytopenic purpura (aTTP), has the potential to become an important new component in the standard of care. KN035 is an anti-programmed death ligand 1 (PD-L1) nanobody/V_{13}H that can strongly induce T cell responses and inhibit tumor growth. Here, we present a possibility of rapidly generating nanobodies/V_{13}Hs in living cells to target intracellular antigens. The anti-GFP nanobody/V_{13}H expressed via IVT mRNA can recognize and trace F-actin-GFP and Golgi-GFP in skeleton and organelle compartments after transfection. We showed that nanobodies/V_{13}Hs expressed via IVT mRNA are efficiently and specifically target antigens. The anti-GFP nanobody-mCherry can be found as early as within 3 h after transfection with IVT mRNA, which was not found until 24 h after transfection with the pmCherry-N1/anti-GFP nanobody. Furthermore, the nanobody/V_{13}H expressed via IVT mRNA degraded as early as 48 h after transfection, which would not happen to the plasmid. Last, but not least, due to the inclusion of chemically modified nucleotides, UTRs, the poly(A) tail length of 300 nt, and the 5'-cap structure, it suffices to increase the stability of IVT mRNA and the production of the nanobody-mCherry. This research focused on the development of nanobodies/V_{13}Hs delivered into living cells with IVT mRNAs for the first time. It is an essential step for the use of nanobodies/V_{13}Hs in clinical study. Ultimately, the nanobodies/V_{13}Hs expressed via IVT mRNAs are tools toward potentially any antigenic cellular structure, being available to make possible functional studies.

MATERIALS AND METHODS

Expression Plasmids and PCR Templates

Lifeact is a 17-amino acid peptide for staining F-actin structures in eukaryotic cells. The DNA sequence of the peptide fused to the GFP was synthesized by GENEWIZ, and the construct was called F-actin-GFP. For targeting human Golgi-resident enzyme N-acetyl-galactosaminyltransferase 2, a molecular probe called CellLight Golgi-GFP, BacMam 2.0, was purchased from Invitrogen. GFP-binding, vimentin-binding, and HDAC6-binding nanobody/V_{13}H DNA sequences were synthesized by GENEWIZ. The sequences of three nanobodies were shown in Figure S4. To test the distribution of three nanobodies/V_{13}Hs in living cells, we fused them to a red fluorescent protein (mCherry) to generate three visible antibodies, and the sequences also used the vector pcDNA3.1 (+). The reconstructed plasmids were amplified by PCR using the following oligonucleotides: 5'-TAA TAC GAC TCA CTA TAG GGG AGA CCC AAG CTG GCT A-3', 5'-AGA ATA GAA TGA TGA CAC CTA CTC-3' to generate PCR templates.
General Synthesis of Nanobodies/Vα4Hs Expressed via IVT mRNAs
The ARCA was used. PCR templates containing a T7 promoter in the correct orientation can be transcribed using the HiScribe T7 ARCA mRNA kit (with tailing; NEB; E2060S). PCR product should be purified and run on an agarose gel to confirm amplicon size prior to its use as a template in the T7 ARCA mRNA transcription reaction. Generally, 0.1–0.5 μg of PCR fragments can be used in a 20-μL in vitro transcription reaction. After the preparation of PCR templates, standard mRNA synthesis with modified nucleotides should be started with thawing the necessary kit components, mixing, and pulse spinning in a microfuge to collect solutions to the bottoms of the tubes. The reaction was assembled at room temperature in the following order: nuclease-free water (to 20 μL), 2 × ARCA/NTP mix (10 μL), 5mCTP (10 mM, 2.5 μL), pseudouridine-UP (10 mM, 2.5 μL), template DNA (1 μg), and T7 RNA polymerase mix (2 μL); mixed thoroughly; and pulse spun in a microfuge. Incubation was at 37°C for 30 min. DNase treatment to remove template DNA included adding 2 μL of DNase I, mixing well, and incubating at 37°C for 15 min. poly(A) tailing starts with setting up the tailing reaction as below. Standard tailing reaction volume was 50 μL: H2O (20 μL), IVT reaction (20 μL), 10 × poly(A) polymerase reaction buffer (5 μL), and poly(A) polymerase. It was mixed thoroughly, pulse spun in a microfuge, and incubated at 37°C for 30 min. Synthesized mRNA can be purified by LiCl precipitation, phenol/chloroform extraction, followed by ethanol precipitation. To the 50-μL tailing reaction, 25 μL LiCl solution was added and mixed well. Incubation at −20°C for 30 min was completed, as was centrifugation at 4°C for 15 min at top speed to pellet the RNA. The supernatant was removed carefully, the pellet was rinsed by adding 500 μL of cold 70% ethanol and centrifuged at 4°C for 10 min, ethanol was removed carefully, the tube was spun briefly to bring down any liquid on the wall, residual liquid was removed carefully using a sharp tip, the pellet was air dried, the mRNA was resuspend in 50 μL of 0.1 mM EDTA or a suitable RNA storage solution, and the RNA was heated at 65°C for 5–10 min to completely dissolve the RNA and mix well.

Immunoblotting Analysis
In total, 100 ng of cell lysate transfected with the same concentration of DNA- or RNA-encoded, GFP-binding nanobody/Vα4H was loaded onto a 12% SDS-PAGE gel. Separated proteins were transferred to a nitrocellulose (NC) membrane. Blots were blocked in Tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) with 5% low-fat milk and incubated with 1.5 μg/mL anti-mCherry antibody and anti-tubulin antibody in TBST with 0.1% low-fat milk at 4°C. The membranes were then washed in TBST and incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG.

Cell Culture and Transfection and Image Analysis
The majority of the molecular biology, biochemistry, and cell biology reagents and chemicals used in this study was purchased from Sigma. Plasmid and RNA transfections were delivered via lipofection. Fetal bovine serum (FBS)-free medium was used during transfection to protect RNA from degradation. In addition, all of the consumables and reagents used in the experiment were RNase free. Cells were cultured on glass slides by using Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, 100 U/mL penicillin-streptomycin, and 100 U/mL L-glutamine at 5% CO2 and 37°C. Slides were rinsed in phosphate-buffered saline (PBS), and cells were fixed in PBS with 4% paraformaldehyde (PFA) for 10 min. VectaShield (Vector Laboratories, Burlingame, CA, USA)-mounted microscopy slides were assessed with an LSM 710 confocal station (Zeiss).

ImageJ software (https://imagej.nih.gov/ij; version 1.51) was used to quantify the fluorescence intensities. We set an arbitrary threshold based on the difference in intensity between the cells and the background regions. The sum of the pixels with intensities above the threshold was recorded by ImageJ. For comparison of fluorescence intensities among different samples, all samples were processed simultaneously and under identical conditions. For each samples, the top 10 highest fluorescence intensities of all cells were collected from 5 separate images. All statistical analyses were performed with a Mann-Whitney U test using GraphPad Prism 7. The methods used for the statistical analyses in all statistical graphs are described in the source data.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2020.01.008.

AUTHOR CONTRIBUTIONS
W.X. and R.H. conceived the idea. X.Z. and C.C. performed the experiments. X.Z., C.C., and W.X. analyzed the data. X.Z. and W.X. discussed and wrote the manuscript. R.H., Z.S., and Z.L. discussed the results and commented on the manuscript. Z.S. and L.Z. provided technical support.

CONFLICTS OF INTEREST
The authors declare no competing interests.

ACKNOWLEDGMENTS
We thank Dr. Chengqi Lin for critical comments and discussion on the manuscript. We also thank other members of the Xie laboratory for assistance and comments on this work. The authors declare that the main data supporting the findings of this study are available within the article and its Supplemental information. Extra data are available from the corresponding author on request. Comprehensive documentation of the nanobody database is an open source in Institute Collection & Analysis of Nanobody (http://ican.ils.seu.edu.cn/). This work was supported by the National Natural Science Foundation of China (NSFC), China (81570743 to R.H. and 81303019 to L.Z.) and an SEU-Alphamab grant (SA2017001 to W.X.).

REFERENCES
1. Strohl, W.R. (2018). Current progress in innovative engineered antibodies. Protein Cell 9, 86–120.
2. Leavy, O. (2010). Therapeutic antibodies: past, present and future. Nat. Rev. Immunol. 10, 297.
