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Master of Science

Simultaneous visualization of mRNA and protein in living cells using a CRISPR-Cas9 system

CRISPR-Cas9을 이용한 살아있는 세포에서의 mRNA와 단백질의 동시 시각화 기법

The Graduate School

of the University of Ulsan

Department of Medicine

Jinwook Yang

Simultaneous visualization of mRNA and protein in living cells using a CRISPR-Cas9 system

Supervisor: Yongsub Kim

A Dissertation

Submitted to
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for the Degree of

Master of Science

by

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December 2018

Simultaneous visualization of mRNA and protein in living cells using a CRISPR-Cas9 system

This certifies that the dissertation

of Yang, Jinwook is approved.

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December 2018

Abstract

mRNA and proteins play critical roles as spatiotemporal steps maintaining cell homeostasis and regulating biosynthesis. Proteins are important as catalytic materials for various chemical reactions in cells. Thus, detecting mRNA and protein is necessary to understand their regulation. However, there is currently no technology to simultaneously image mRNA and protein in living cells. In this study, a heat shock transcription factor (HSF) 1 donor DNA knock-in cell line was developed using CRISPR-Cas9 to image HSF1 mRNA and HSF1 protein at the same time. A HSF1 knock-in cell line was prepared by inserting donor DNA by homologous recombination around the HSF1 stop codon. Specifically, HSF1 donor DNA contained homologous arms of appropriate length on both sides. EGFP was used for protein tagging, and a puromycin resistance cassette was the selection marker. I also applied the MS2 RNA-labeling system in donor DNA. Live imaging of mRNA dynamics at a single RNA molecule resolution was achieved in live cells using this binary system to express mRNA of the target tagged with 12 copies of RNA hairpin MS2-binding site and fluorescent MS2 bacteriophage coated protein (MCP). In addition, I designed single-guide RNA with low off-target and high cleavage efficiency. A stable HSF1 cell line was constructed using CRISPR-Cas9, and the HSF1 protein was visualized through an enhanced green fluorescent protein signal. Furthermore HSF1 mRNA was visualized in the cytoplasm by exogenously expressing fluorescent MCPs. This system provides a new technique for visualizing specific mRNA and protein to study dynamic changes in endogenous mRNAs and proteins in living cells.

Keywords**:** CRISPR-Cas9, Homologous recombination, Knock-in, mRNA, MS2

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Introduction

All genetic information encoded in DNA functions as genetic material after it is an RNA transcript. mRNA transcribed from DNA moves to functional locations in the cell and plays a variety of roles in accordance with the function of each. mRNA is translated into a protein to demonstrate the function of a gene or to regulate the amount of gene expression. However, sometimes mRNA can be difficult to transcribe, modify, or degrade in a tissue environment [1]. Thus, errors related to expression, movement, function, and degradation of mRNA may result in tumors and neurodegenerative diseases [2]. Proteins are representative molecules comprising the body of an organism that play a very important role in the growth and maintenance of cells. Proteins also participate as catalysts in various biochemical reactions. Additionally, proteins have complex structures to function in various locations. An abnormally structured protein can cause disease. Thus, mRNA and protein are involved in cell homeostasis and regulating biosynthetic processes. Techniques for studying the dynamics of mRNA and proteins have been available for a long time. The length and sequence of mRNA, measurements of the amount and structure of mRNA, and how to locate the proteins that interact with mRNA have been realized [3-6]. In addition, many studies have visualized mRNA and proteins in live cells by fluorescence to observe the dynamics of proteins and understand their regulation and interaction in a variety of situations.

Detecting localized mRNA in live cells is difficult due to the unstable properties of mRNA and the regulatory action compared to DNA. RNA is single stranded and does not have a complementary sequence, so there is no repair system. Nevertheless, methods to study RNA have expanded greatly. A typical method, fluorescence *in situ* hybridization (FISH), is an invaluable method for detecting target RNA by injecting an oligonucleotide probe conjugated to a fluorophore to directly label mRNA. The oligonucleotide probe is complementarily annealed to the RNA of interest. RNA for

FISH must be fixed in its native subcellular location through chemical cross-linking [7]. Therefore, FISH is not used in living cells. Molecular beacons have developed as a derivative of FISH to reduce background signals. Molecular beacons contain a DNA probe connected to a fluorophore at one end and a quencher at the opposite end. The probe is folded into a hairpin structure when unbound to the target mRNA. Fluorescence is suppressed because the fluorophore and quencher are attached. Once the target RNA is recognized, the probe stretches out and the quencher is separated from the fluorophore enabling fluorescence [8]. The signal to noise ratio of a molecular beacon is greater than that of a standard FISH probe because the fluorescence signal of the reporter probe is quenched when unconjugated. However, molecular beacons can become mislocated in living cells when delivered by microinjection [9]. Hybridization-based RNA detection is an outstanding tool for use in fixed samples, but the probes are based on modified nucleic acids and are neither cell permeable nor can they be intracellularly synthesized. Furthermore, hybridization must occur in local areas of the target RNA without secondary structure, and hybridization conditions are not commonly optimized for a tissue environment. These methods of RNA visualization measure RNA that has been exogenously overexpressed rather than RNA that has been expressed in actual cells. It is difficult to quantitatively compare the actual expression quantity and make experimental error due to overexpression [10].

RNA aptamers are another nucleotide-based probe but they work on a different principle from hybridization probes. Aptamers are single-stranded oligonucleotides capable of binding specific target molecules grounded on their structure that can be identified by *in vitro* selection [11-12] One of the aptamers, spinach, emits fluorescence through binding of a small molecule fluorophore with its special internal structure [13-15]. Alternatively, RNA-binding proteins that bind to RNA aptamers can be used. Bacteriophage-derived RNA-binding proteins have been used to visualize RNA in live cells. This feature is part of the MS2-MS2 coated protein (MS2-MCP) system. MCP recognizes a specific RNA sequence consisting of a hairpin that targets RNA extended by repeating the MS2-binding sites (MBSs) [16]. An MS2-MCP

system has been used to image mRNA in living mice and to visualize the expression of β-actin mRNA in mouse primary fibroblasts [17]. Although this study is capable of resolving and tracking β-actin mRNA molecules, it is time-consuming to produce transgenic mice. In addition, the size of the MBS should be considered and the aptamer adjacent to the target mRNA may interfere with the function of mRNA and localization. Moreover, the amount of MS2 aptamer used must be sufficient to combine the target mRNA without increasing the background signal.

Techniques to visualize mRNA using the bacterial clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein 9 (Cas9) system have been developed. This system has achieved great success in introducing site-specific DNA double-strand breaks (DSBs) with high accuracy and efficiency. The system is a proven versatile tool for genomic engineering and gene regulation in a broad range of prokaryotic and eukaryotic cell types [18-20]. Specifically, CRISPR/Cas9 generates DSBs precisely in DNA at the desired location in living cells. Single guide RNA (sgRNA) complexing with the Cas9 nuclease recognizes various 20 nucleotide target sequences neighboring a protospacer adjacent motif (PAM) and generates a DSB in the target DNA sequence [21]. Hereafter, a DSB triggers distinct DNA repair via homology directed repair (HDR) or non-homologous end-joining (NHEJ) pathways. The HDR pathway involves exchanging DNA strands to repair the DNA correctly based on the existing homologous DNA sequence. In contrast, the NHEJ pathway repairs the DSB through a homology-independent process that often creates random mutations, such as small insertions or deletions (indels). Thus, CRISPR/Cas9-mediated DNA cleavage following the NHEJ pathway has been used to produce loss-of-function in protein-coding genes [22].

A method for detecting mRNA by modifying the CRISPR/Cas9 system has been developed. This recently reported method exploits catalytically inactive dCas9[Cas9(D10A, H840A)] which inhibits nuclease function and delivers it to a specific position on the genome by sgRNA and dCas9, which have the green fluorescent protein (GFP) attached. The dCas9-EGFP/sgRNA complex and a DNA

nucleotide called PAMmer, which hybridizes to the target mRNA, are used to visualize the target mRNA by fluorescence [23]. This approach has the advantage of minimizing functional side effects in the cell and does not cause any variation in the RNA itself. However, If it is for stable detection of mRNA, this method requires a continuous supply of PAMmer from the outside to accurately detect mRNA in the cytoplasm, which is difficult to achieve in cells with low delivery efficiency of DNA [24].

Antibody-based probes called antibody fragments (Fab) and single-chain variable fragments (ScFv) are useful to image protein dynamics. These modified antibody-based probes have been used to image protein subpopulations, including nascent polypeptides and post-translationally modified proteins in live cells. Fabs are a heterodimer chain of about 50 kDa, made up of a digested antibody, which leaves only the epitope-binding sites [25-27]. Fabs are conjugated with synthetic dyes for live cell imaging. scFv is a plasmid similar in shape to Fab but it has a linker between the heavy and light chains and is expressed along with the fluorescent protein. The advantage of using Fabs and scFvs for live-cell imaging is that they can pass through the nuclear pores and bind directly to target proteins inside the nucleus. Fab and scFv also prevent aggregation and interference due to their monovalency (bind to one target) [28, 29]. However, these proteins generate background signals from the free-diffusing probes that are not associated with the target. Other methods of labeling endogenous proteins include self-complementing split fluorescent proteins (FPs), which split super-folder GFP (sfGFP) into two fragments, a FP-11 tag and a FP 1-10 tag that can be used to image target proteins using the recovery of GFP by binding of the fragments through protein-protein interactions [30]. This method has been applied to localize proteins [31,32] and to aggregate, quantify, identify cell contacts and protein scaffolding [33-35]. If a relatively small FP11 tag is inserted into the endogenous genomic loci in which the target protein expressed using CRISPR/Cas9, the target protein can be visualized by injecting only FP1-10 which solves the background signal problem. However, knocking-in tandem FP11 tags is necessary to increase endogenous expression for visualization. In that case, cloning is

required because long donor ssDNAs with longer homology arms are required.

In this study, a new platform was developed that avoids the limitations of conventional RNA visualization methods, but also includes possible methods for protein imaging. Cas9, sgRNA, and donor DNA can be knocked in as an expression cassette with homologous arms of appropriate length into the target gene where DSBs occur [36]. An expression cassette containing EGFP, a puromycin resistance gene, and 12 copies of MS2 RNA aptamers to apply the MS2-MCP system was knocked into HSF1 exon 13 in Hela cells using CRISPR/Cas9 targeted genome editing via the HDR pathway. Inserting a gene at the HSF1 stop codon allows stable gene expression thorough an endogenous promoter. EGFP can be identified in live cells by establishing a stable cell line and selecting a single cell where HSF1 mRNA is translated into the protein. However, mRNA transcribed from HSF1 DNA is visualized by applying the MS2-MCP system via strong binding affinity [37]. HSF1 mRNA fluorescence was compared with the MS2-MCP system using a MCP-free fluorescent construct. Finally, a fluorescent dot of HSF1 mRNA was observed that was dependent on the presence of fluorescent MCP in the cytoplasm of a live cell. Background signals were detected frequently during this conventional method of RNA detection, but because fluorescent MCPs contain a nuclear localization sequence (NLS), the exogenous fluorescent MCPs used for this technique lower the background signal in the cytoplasm. Thus, only the mRNA seen in the cytoplasm was imaged. As a result, endogenous mRNA can be visualized in the actual live cell. In this study, approach was established to enable live-cell imaging of a single endogenous mRNA and protein at the same time. This method demonstrates how single gene dynamics in native cells can be used to unearth details of the molecular mechanisms of different cell types and offer potential applications imaging endogenous genes and proteins in their native tissue environment.

2. Materials and methods

2.1 HSF1 donor DNA constructs

The donor plasmids were cloned by adding 12xMS2 aptamer to pUC19-$(GGGGS)_2$-EGFP-P2A-Puromycin DNA. First, the product was amplified (Phusion DNA polymerase, Thermo Scientific, Waltham, MA, USA) using the HSF1_MS2_F/HSF1_MS2_R primers with the pSL-MS2-12x plasmid (#27119; Addgene, Cambridge, UK) as the template (Table1). After splicing with HindIII, the pUC19-$(GGGGS)_2$-EGFP-P2A-Puromycin vector with calfintestine phosphatase (CIP) was cloned using the Gibson assembly method. Second, the left homologous arm (LHA) was amplified using the HSF1_left_arm_F/HSF1_left_arm_R primers and the previously completed pUC19-(GGGGS)2-EGFP-P2A-Puromycin-HSF1 as the template. These were cloned into pUC19-$(GGGGS)_2$-EGFP-P2A-Puromycin-12xMS2,cut with EcoRI, and treated with CIP. Third, the right homologous arm (RHA) was amplified with the HSF1_Right_arm_F/HSF1_Right_arm_R primers using pUC19-$(GGGGS)_2$-EGFP-P2A-Puromycin-HSF1 as the template. Finally,the polymerase chain reaction (PCR) product was cloned into the pUC19-leftarm-$(GGGGS)_2$-EGFP-P2A-Puromycin-12xMS2, cut with Hind III, and treated with CIP.

2.2 Cell culture

The HeLa cervical cancer cell line was grown in DMEM. The HeLa cell lines were cultured at 37°C under 5% CO_2 in Dulbecco's Modified Eagle Medium (WELGENE Gyeongsang buk-do, Gyeongsan-si, South Korea) medium containing 10% fetal bovine serum (WELGENE).

2.3 sgRNA design

To design the sgRNA that recognizes the target sites, the HSF1 exon 13 sequence flanking the stop codon was imported into sgRNA design tools (Table 2). Each crRNA sequence was designed as a 20-bp target sequence, and three sgRNA candidates with the lowest off-target efficiency were selected. The sgRNA expression plasmid with a U6 promoter was digested with the BbsI enzyme (New England Biolabs, Ipswich, MA, USA) and the HSF1-sgRNA#1-3 plasmid was constructed by inserting a pair of annealed oligos (Table 1).

2.4 T7E1 assay

sgRNA expression vector #1–3 (500 ng) was transfected into 8 × 10^4HeLa cells/ml seeded in a 24-well plate using Lipofectamine®2000 DNA transfection reagent (1mg/mL) (Invitrogen, Carlsbad, CA, USA). Genomic DNA was prepared after 48h of transfection. Using the targeting primer (Table1), the position of the target was amplified by first PCR and second PCR using gDNA as the template. The second PCR product was denatured at 95°C for 18 min and re-annealed at 95°C/3 min, 95°C C/0.01 min, −1°C/s (40 cycles), 55°C/0.02 min, −1°C/s (40 cycles), 16°C. The T7E1 assay was performed using 10 μL of the hybridized second PCR product, 2.0 10× NE Buffer, and 0.5 μL T7 endonuclease I (T7E1), incubated at 37°C for 20 min, and confirmed by agarose gel electrophoresis.

2.5 Fluorescent MCP constructs

The lenti MS2-p65-HSF1-Hygro plasmid was cut with BamHI-HF and EcoRI at 37°C for 2h to prepare the vector. The insert was amplified from GFP (mCherry)_BamHI_F/BSD_EcoRI_primers in pUC19-mCherry (Table 1). mCherry-P2A-blasticidin was the product, which was cloned into the vector. Thus, the lenti-MS2-mCherry plasmid was constructed. However, cloning was performed again for mammalian expression because it was a lentivirus vector. The p3s plasmid as a vector and the MS2-NLS-mCherry and NLS-mCherry as PCR inserts were

inserted under the CMV promoter. We prepared p3s-MS2-NLS-mCherry and p3s-NLS-mCherry constructs. p3s-MS2-NLS-mCherry was used to detect RNA in cytoplasm. The DNA sequence encoding the MS2-coated protein was fused with SV40 NLS and the mCherry sequence. The other sequence only had the MS2 binding site. We cloned these fusion proteins into a p3s vector containing a CMV promoter using standard ligation-independent cloning.

2.6 HSF1 stable cell line

HeLa cells were plated in a six-well plate (1×10^6cells/well) and co-transfected with the sgRNA expression plasmid (1μg), HSF1 donor plasmid (5 μg), Cas9 plasmid (1 μg), and 100 μL of SE solution using the Lonza 4D-Nucleofector. After 7days, the cells were detached with 0.05% trypsin-EDTA (1×) (Gibco, Grand Island, NY, USA) and genomic DNA-prep was performed to confirm the indel efficiency using T7E1, and the remaining amount was transferred to a six-well plate. After puromycin (0.1 μg/mL) selection (14 days), the cells were replated in a 96-well plate (1 cell/well). Junction PCR was performed using HSF1left_arm_F/LHA_junction_1st_R, target_specific_PCR_F/R (Table 1) and the positive band was selected. HSF1 cell lines 10 and 12 were finally selected with a high concentration of gel extracted after PCR. Sequencing of each PCR product demonstrated that the HSF1 donor DNA was inserted endogenously at the target site.

2.7 Transfection and preparation of the imaging sample

To determine whether this technique allows for RNA imaging, Cells were plated onto six-well plates (4×10^5)and p3s-MS2-NLS-mCherry(2.5μg) was transfected into the HSF1 donor cell line using Lipofectamine®2000 DNA transfection reagent (1mg/mL) (Invitrogen). p3s-NLS-mCherry(2.5μg) was transfected to compared with the positive control. The cells were transferred to a confocal dish by 1/10. 24h after transfection, and confocal microscopy was used to image the RNA 48 hours later.

3. Results

3.1 Design of HSF1 donor DNA

I first designed the donor DNA template containing EGFP and RNA aptamers to insert in HSF1exon 13. The RNA aptamers of this donor DNA consisted of 12 repeated MS2 stem-loops. Donor DNA structures generally include the LHA (508 bp), a GS linker (GGGGSGGGS) sequence, an EGFP coding sequence, the puromycin selection sequence ($Puro^R$), the 12xMS2 aptamer sequence, and the RHA (820bp) (Figure1). The donor DNA template was inserted at the stop codon of the HSF1 sequence in the genomic DNA. EGFP fluoresces in th is template when expressed as a protein. We cloned this construct into pUC19 vector using Gibson assembly cloning. Only the HSF1 donor-inserted cell expressed the puromycin resistance gene by the endogenous cell promoter to proceed with the selection process. Transfection of the fusion protein with MS2 coated protein, NLS, and mCherry can be used to visualize HSF1 RNA by binding to the 12xMS2 aptamer in HSF1 RNA.

3.2 Selection of sgRNA to insert HSF1 donor DNA into the stop codon of HSF1 exon 13

Three sgRNAs with the lowest potential off-target effect were selected to introduce the HSF1 donor construct. This sgRNA target nucleotide sequence was located near the stop codon of HSF1 exon 13 (Figure 2). sgRNA oligos were annealed and inserted into the sgRNA expression plasmid expressing HSF1-sgRNA#1. HeLa cells were transfected with the Cas9 expression plasmid and the sgRNA plasmid, and then HeLa gDNA was extracted and confirmed by T7 endonuclease I (T7EI) assay to determine sgRNA production efficiency. The analysis showed that the efficiency of inducing mutations in target loci was similar (Figure 3A, B). Increasing the amount of donor DNA increases HDR efficiency when nucleofection of HSF1-sgRNAs, Cas9,

and donor DNA is performed. Deep sequencing was used to analyze indel efficiency of the two vectors when expressing sgRNA and Cas9 respectively and px330 vector in which sgRNA and cas9 are expressed in one vector. As a result, the highest indel efficiency was confirmed by using two vectors and sgRNA#1 (Figure 4).

3.3 Establishment of the HSF1 cell line

HeLa cells were selected because they are a human cell line with high transfection efficiency. The HeLa cells were transduced with Cas9, HSF1-sgRNA#1, and HSF1 donor DNA template to sort out the puromycin-resistant cells (Figures 5, 6). The cells were expanded for 18 days in media containing puromycin to obtain a single clone, and then separated on a 96-well plate. Then, 14 growing candidates were selected from single cells.

Fourteen monoclonal cells were grown to confirm correct targeting of the gene from sequencing. The PCR products of the four positive bands were obtained among the junction PCRs of the 14 clones. Each of the four clones were transferred to six-well plates for expansion. To confirm the sequence of homologous recombination in the HSF1 gene, four PCR-processed target cells were used for junction PCR (Figure 7A). When the HSF1 donor template was inserted into the genomic DNA by homologous recombination, the sequences between the LHA and the GS linkers and the sequences between the 12xMS2 aptamer and the RHA were identified. Here, the nested PCR primer and the targeted PCR primers were used. It was confirmed by sequencing that the endogenous donor template was inserted using primers that read in the forward direction (Figure 7B).

3.4 Deep sequencing analysis of the HSF1 cell line

The DSBs that occur when nucleofection of CRISPR/Cas9, HSF1_sgRNA#1 are moved into a target sequence are restored by homologous recombination if HSF1 donor DNA is present. At this time, we checked whether the HSF1 gene that has

DSBs was homozygously repaired or heterozygously repaired. If one DNA strand is heterozygously repaired with a donor template, an indel occurs in the remaining DNA strand and the cleavage efficiency of Cas9 can be confirmed. The first PCR was performed with nested PCR primers. Then, the second PCR was performed using a designed deep sequencing primer using the first PCR product. As a result, it was confirmed that homologous recombination occurred in only one strand, and deep sequencing was performed. The deep sequencing showed that the cleavage efficiency of Cas9 was close to 90%. The mutation sequence also confirmed that one deletion pattern prevailed. This finding confirms that the established HSF1 cell line was obtained from a single clone (Figure 8).

3.5 HSF1 RNA detection by confocal microscopy

I identified the ability of the HSF1 donor cell line containing the 12xMS2 RNA aptamer to recognize RNA in human cells by evaluating the degree of red fluorescent protein fluorescence seen outside the nucleus (Figure 9). Specifically, we tested whether the mCherry-tagged MS2 expression vector containing a NLS can be seen in the cytoplasm and recognized HSF1 RNA (Figure 10). This method visualized the HSF1 RNA in the cytoplasm compared to the MS2-mCherry construct without NLS.

4. Discussion

I have described a new approach to accurately image endogenous RNA in the cytoplasm using CRISPR/Cas9-mediated genome editing via HDR. Inserting the donor DNA by Cas9 and sgRNA was essential as a prerequisite in this study. Effective DSBs by Cas9 in living cells while avoiding the off-target effect of the target gene relies on careful design of the sgRNA. First, 10 sgRNAs with a low off-target effect were screened by targeting HSF1 exon 13 using an *in silico* program. Second, three sgRNAs that precisely targeted the stop codon position of HSF1 exon 13 were selected. Hereafter, it was necessary to control the amount of donor DNA to increase the nucleofection efficiency of donor DNA by HDR. As the amount of donor DNA was increased by using the px330 vector expressing Cas9 and sgRNA simultaneously, the indel efficiency of the one vector system and the conventional two vector system was compared through deep sequencing. In subsequent experiments, the two-vector system was used to achieve Cas9 indel efficiency.

RNA is important as a protein as well as a functioning genetic material in cells. Protein is mainly a functional substance, but RNA also plays an important role in the maintenance of homeostasis by mediating between DNA and protein and controlling expression in the cell [38]. In recent years, long non-coding RNAs (lncRNAs) or microRNAs (miRNAs), which have been regarded as waste *in vivo*, were found to play an important role regulating cell homeostasis, and their mutations have been reported to lead to disease [39-41]. If the RNA is distributed in an atypical location or if it sends a false signal to the stimulus, regulation of the cell becomes dysfunctional, leading to diseases, such as cancer and aging. Thus, visualization techniques for RNA have become increasingly important to determine the function of RNA, Including RNA that has not yet been characterized [42]. Previous approaches to visualize RNA not only measured the state of RNA in fixing the cell but also to visualize RNA dependent on overexpression of exogenously injected genes in live cells [43, 44]. Thus, methods based on FISH result in many experimental errors due to overexpression. To overcome this problem, some methods

can be used to directly visualize endogenous RNA in the cell using CRISPR/Cas9. dCas9 (catalytically dead Cas9) with fluorescence can analyze transformation, migration pathways, and degradation processes from the transcriptional stage of RNA in living cells [23, 24, 45]. In addition, a visualization technique for diverse intracellular proteins has developed rapidly along with the discovery and application of fluorescent proteins, such as GFP [46]. Although antibody-based probes, such as Fab, scFv and sfGFP, were developed to study protein dynamics in live cells, these have limitations. In the present study, a fluorescent protein was directly tagged to a target gene of interest and an RNA aptamer was inserted using CRISPR/Cas9. Ultimately, this method was able to co-visualize mRNA and proteins different from the conventional RNA visualization method. Furthermore, adding an inducer that activates the HSF1 gene may result in a further increase in expression of HSF1 mRNA and protein. In the case of non-coding RNA, such as RNA with a low expression rate, increasing the number of aptamers may amplify fluorescence intensity. Since the HSF1 protein is a heat shock protein, the formation of stress granules different from the distribution of existing EGFP in live cells is expected to be more apparent when heat is provided. In addition, using an activator that promotes HSF1 DNA transcription is useful to measure changes in mRNA. So, the developments from this study could be applied to provide a new means to image RNA in live cells as well as other RNA that regulates a variety of RNA-processing steps in biotechnology and disease research for treatment. Subcellular localization of mRNA and protein is crucial in many situations. For example, asymmetric localized mRNA is translated into a *bicoid* protein according to localized translation when the *Drosophila* oocyte is developing, which determines the anterior-posterior polarity of the developing larva [47, 48]. Localization of mRNA is also particularly important in nerve cells, as localized mRNA leads to translation at the synapse and activity-dependent changes [49]. Finally, the HSF1 stable cell line provides a powerful instrument for biotechnology. Because a stable cell line with donor DNA inserted is able to produce the target RNA appended to MBS, so additional study of the dynamics of endogenous RNA will be possible. This technology has been linked

to research on protein function, leading to causes of diseases and the development of therapeutic agents.

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Table 1. DNA primers used for donor cell establishment

Name	Sequence
Construction of HSF1 donor DNA	
12xMS2 aptamer	
HSF1_MS2_F	**GCAAGCCCGGTGCCTGAACCCGGGCCCTATATAT**
HSF1_MS2_R	**ATGATTACGCCAAGCTTCTCTGTACATGTCCGCG**
Left homology arm	
HSF1_Left_arm_F	**AAACGACGGCCAGTTTCTGACTTCCCTCCCTCCT**
HSF1_Left_arm_R	**GCCACCGCCACCGGAGACAGTGGGGTCCTTG**
Right homology arm	
HSF1_Right_arm_F	**GGACATGTACAGAGAGGCCCCGGAGGAG**
HSF1_Right_arm_R	**ACCATGATTACGCCGCAAAGCCTGCTCCACT**
Construction of MS2 fusion mCherry DNA	
mcherry_BamHI_F	**CCGGGATCCATGGTGAGCAAGGGCGA**
mcherry_EcoRI_R	**CCGATGAATTCTTAGCCCTCCCACACATAAC**
Nested PCR primers for T7E1	
HSF1_1st_F	**GGATTCAGGTGAGCCAAGTC**
HSF1_1st_R	**AACAACCTGCAGGGTCAGTC**
HSF1_1st_R	**GAAGCAGCTGGTGCACTACA**
HSF1_1st_R	**TTGTCCTACTGGGGCTATGG**
Junction PCR primers	
HSF1_new_left_arm_F	**AAACGACGGCCAGTTTCTGACTTCCCTCCCTCCT**
LHA_junction_1st_R	**GCCGGTGGTGCAGATGAACT**
Target_specific_PCR_F	**GGTTGTTAACGTTAGCCGGC**
Target_specific_PCR_R	**GAGATGGAGGCATCTCCTTG**
Deep sequencing primers	
HSF1_Deep_F	**ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTG GGAGAGGGCTCCTAC**
HSF1_Deep_R	**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCC ACCAGTGCCCAAGACC**
CCR5_1st_F	**CTCCATGGTGCTATAGAGCA**
CCR5_2nd_F	**GAGCCAAGCTCTCCATCTAGT**
CCR5_R	**GCCCTGTCAAGAGTTGACAC**
Index primers	
501	**AATGATACGGGGACCACCGAGATCTACA**
502	**AATGATACGGGCGACCACCGAGATCTACA**
503	**AATGATACGGCGACCACCGAGATCTACA**
504	**AATGATACGGCGACCACCGAGATCTACA**
505	**AATGATACGGCGACCACCGAGATCTACA**
701	**CAAGCAGAAGACGGCATACGAGATCGA**

Table 2. Sequence of sgRNA oligos

sgRNA oligo sequence	
sgRNA#1_F	**CACCGCGGGGCCTCTAGGAGACAGT**
sgRNA#1_R	**AAACACTGTCTCCTAGAGGCCCCGC**
sgRNA#2_F	**CACCGCCGGGGCCTCTAGGAGACAG**
sgRNA#2_R	**AAACCTGTCTCCTAGAGGCCCCGGC**
sgRNA#3_F	**CACCGAGGACCCCACTGTCTCCTAG**
sgRNA#3_R	**AAACCTAGGAGACAGTGGGGTCCTC**

Table 3. Available online resources for CRISPR/Cas9 system

Name	Remarks	Reference
Primer 3 Input	Oligo design tools	http://bioinfo.ut.ee/primer3-0.4.0
sgRNA design tools	guide RNA design tools	http://rgenome.net/cas-designer

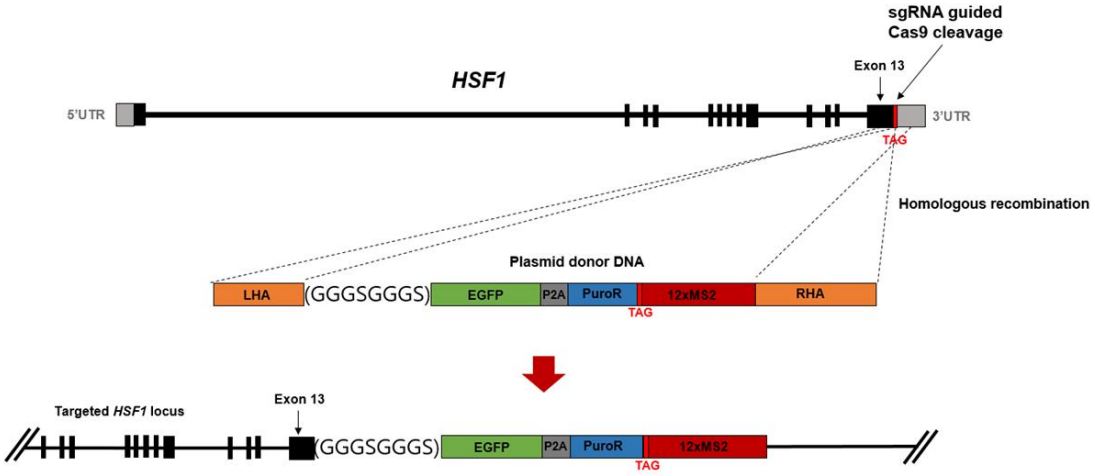

Figure 1. Schematic of heat shock transcription factor (HSF)1 donor DNA

A schematic diagram for inserting the HSF1 donor gene at the site of the HSF1 stop codon using Cas9-induced homologous recombination (HR). Donor DNA is inserted into the target site using Cas9 and sgRNA. Enhanced green fluorescent protein (EGFP) and puromycin ($Puro^R$) were inserted by HR and the left/right homology arm (LHA/RHA).

sgRNA target site(5′ to 3′)	Mismatches		
	0	1	2
CTTGACACCCCCACCCCCGCAGG	1	0	4
CACCAGCTGCTTCCCTGCGGGGG	1	0	0
GCACCAGCTGCTTCCCTGCGGGG	1	0	2
CCGCTGTTCCTGCTGGACCCCGG	1	0	1
GTCAAGGCGGTGGGGGGTTGGGG	1	0	2
TCAAGGCGGTGGGGGGTTGGGGG	1	0	5
AGGACCCCACTGTCTCCTAGAGG	1	0	3
CCGGGGCCTCTAGGAGACAGTGG	1	1	1
CGGGGCCTCTAGGAGACAGTGGG	1	0	0
CTGTCTCCTAGAGGCCCCGGAGG	1	0	7

Figure 2. Design of a single guide RNA that induces cleavage near the heat shock transcription factor (HSF)1 target sequence

sgRNAs were designed to create double strand breaks (DSBs) around the stop codon of the HSF1 exon 13 sequence using sgRNA design tools (http://www.rgenome.net/cas-designer). The top 10 sgRNA candidates with the lowest off-target effect were selected first, followed by three sgRNAs showing cleavage around the stop codon of HSF1 exon 13, with relatively few mismatches. To confirm the cleavage efficiency of Cas9 and sgRNA, nested PCR primers used for T7E1 assay were designed using Primers 3 Input (http://bioinfo.ut.ee/primer3-0.4.0).

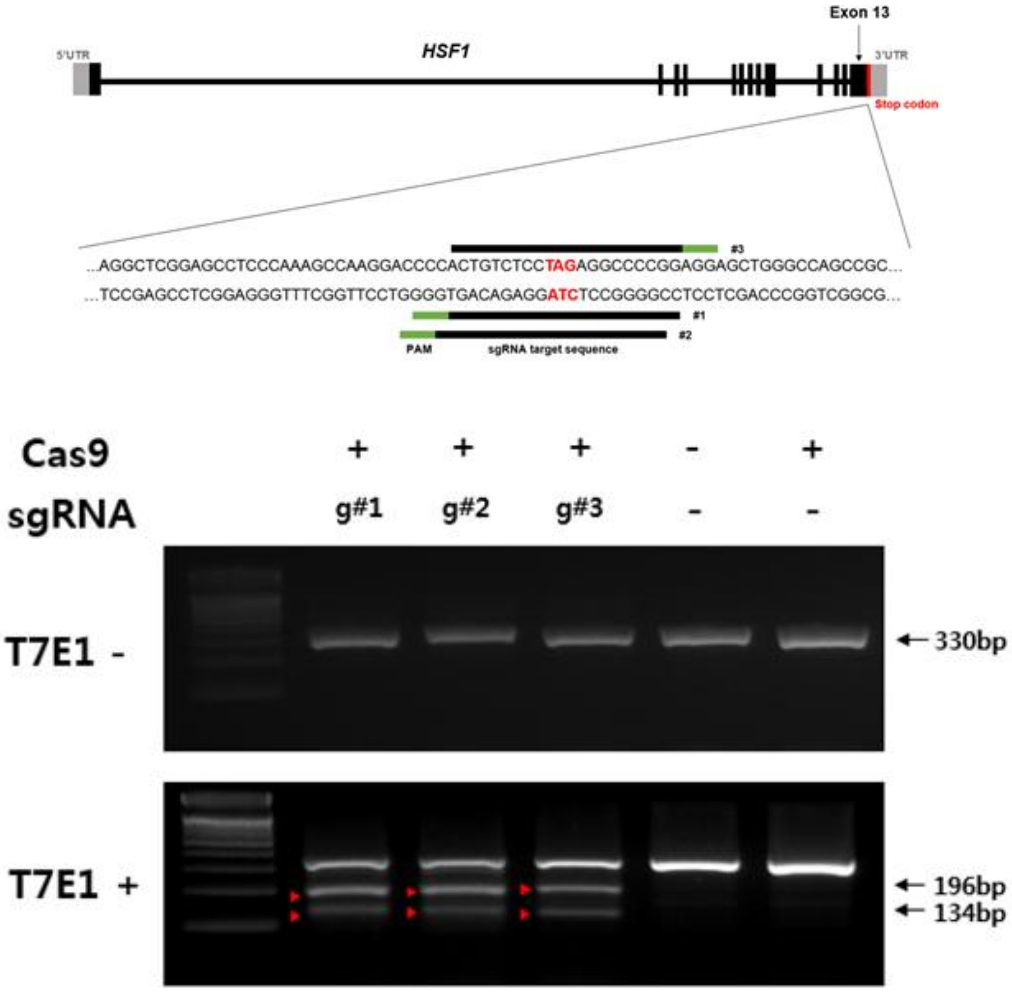

Figure 3. Identification of sgRNA position and indel efficiency of the heat shock transcription factor (HSF)1 locus

(A) The sgRNA target position of the HSF1 exon is indicated by the red solid line. The target sequence of sgRNA #1 and PAM are shown as green and blue solid lines, respectively. (B) sgRNA targeting efficiency of the HSF1 exon was confirmed in HeLa cells using the T7E1 assay. All sgRNA expression plasmids were transfected into HeLa cells with Cas9. Using gDNA as the template, the region containing the target site was amplified by the first PCR using a primary F/R primer. The first PCR product was amplified using the second F/R primer, and the second PCR (330 bp) was performed. The T7E1 assay was then performed. Red arrows indicate cleavage fragments.

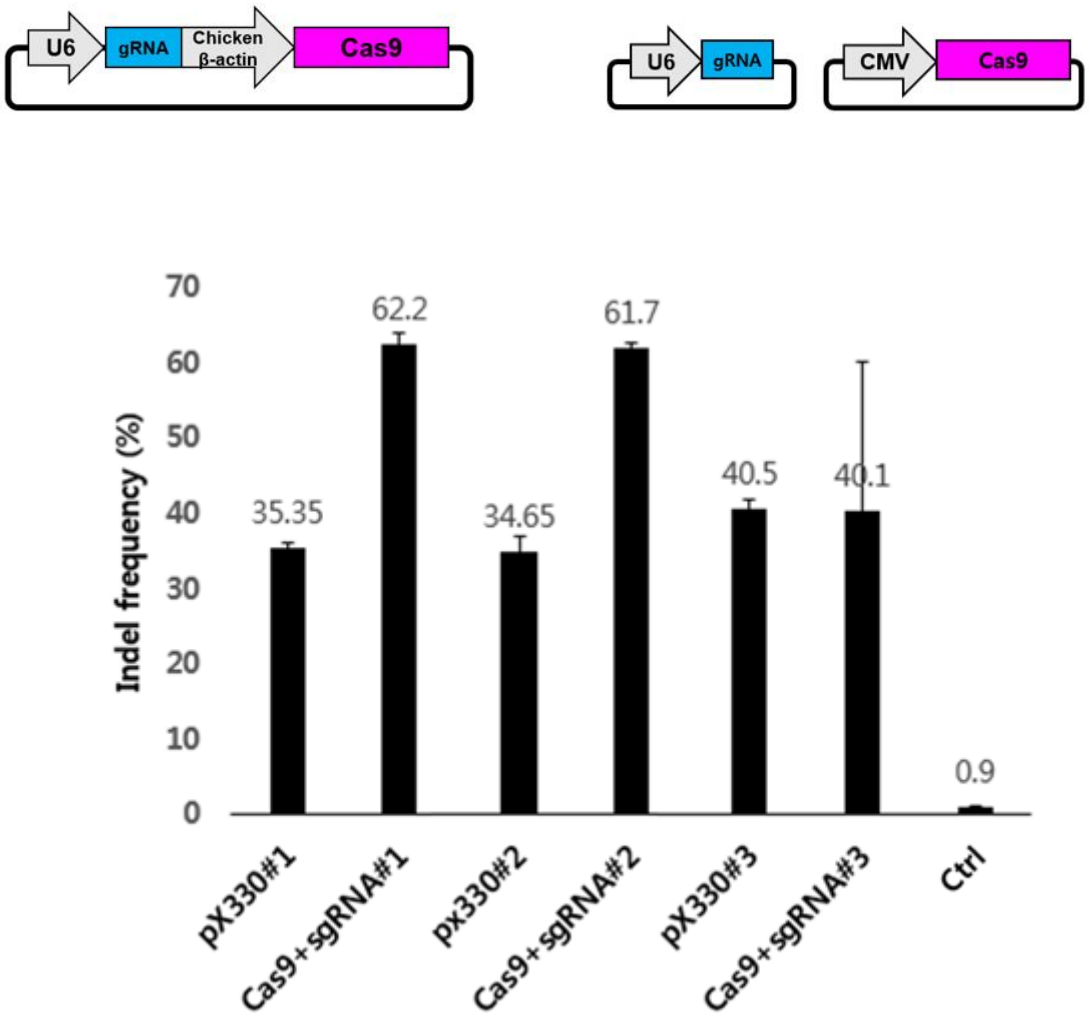

Figure 4. One vector system and two vector systems are compared to confirm cleavage efficiency

Cleavage efficiency was checked by deep sequencing when the Cas9 encoding plasmid (500 ng) and sgRNA (500 ng) were used respectively and px330 (1 μg) co-expressing Cas9 and sgRNA were used.

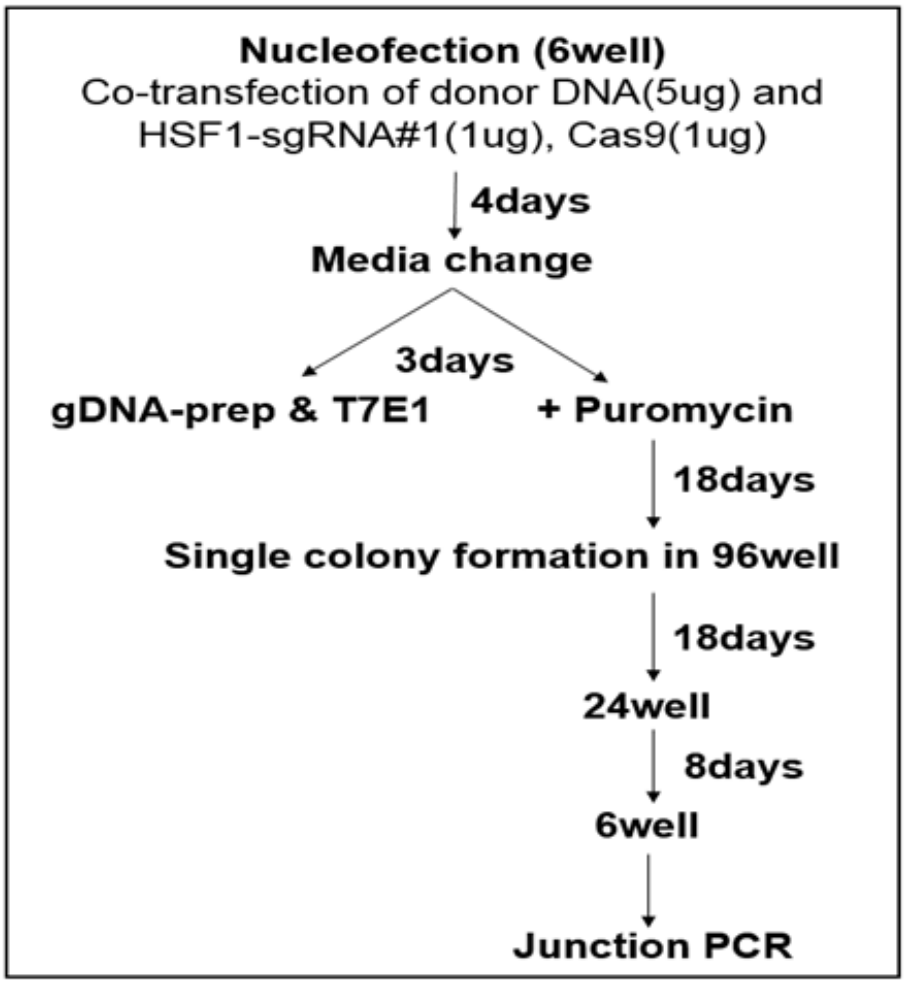

Figure 5. Scheme for making the heat shock transcription factor (HSF)1 stable cell line

HSF1-sgRNA#1 and donor DNA were transfected into HeLa cells, and the media were changed 4 days later. After 3 days, the cells were split, and the T7E1 assay was performed to confirm operability of Cas9. Some cells were seeded in six-well plates and selected for 15 days with puromycin (1 μg) After that, seeding was performed by diluting 1 cell/well in a 96-well plate. Target PCR was performed with a single clone grown for 14 days.

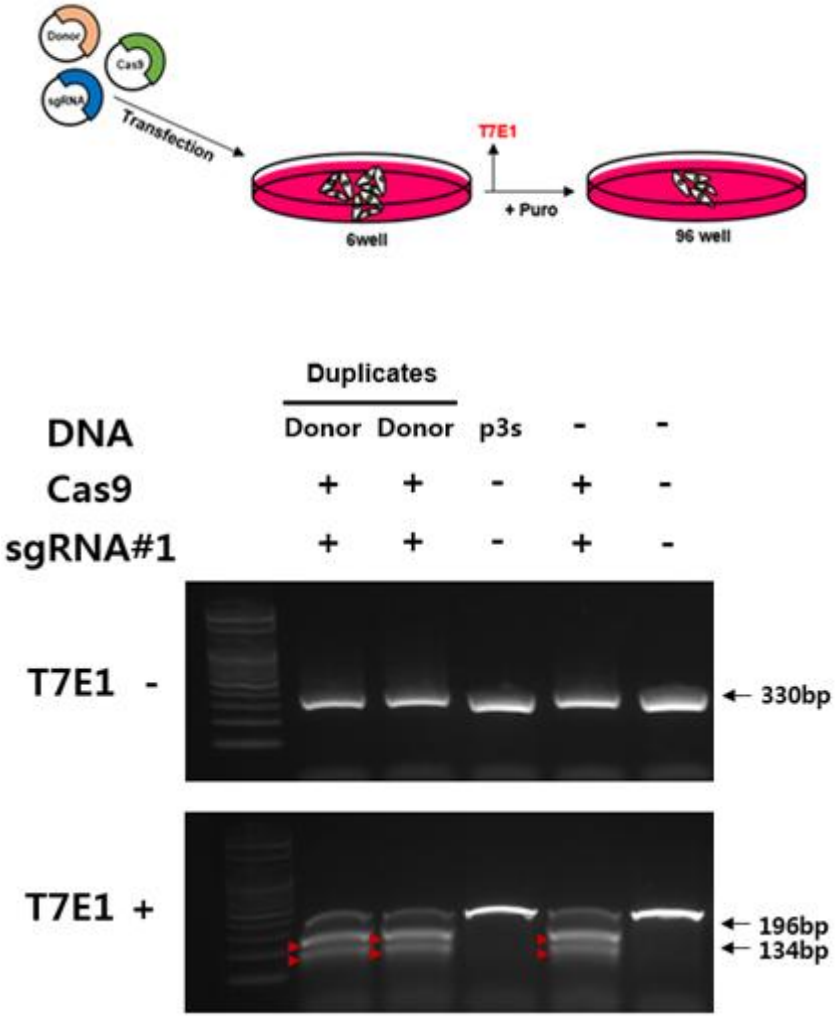

Figure 6. Identification of sgRNA targeting efficiency after nucleofection of heat shock transcription factor (HSF)1 donor DNA

(A) A schematic diagram for co-transfection of HSF1 donor DNA with Cas9 and sgRNA to obtain a single cell. (B) HSF1 donor DNA, Cas9 plasmid, and HSF1-sgRNA#1 were co-transfected into Hela cells. After 7 days, gDNA was obtained, and the T7E1 assay was performed. Duplicates: Co-transfection of HSF1 donor DNA and Cas9 plasmid, HSF1-sgRNA#1, Ctrl: Co-transfection of p3s and HSF1 donor DNA, D3: Ctrl donor DNA and Cas9 plasmid, HSF1-sgRNA#1. Red arrow indicates the expected fragment.

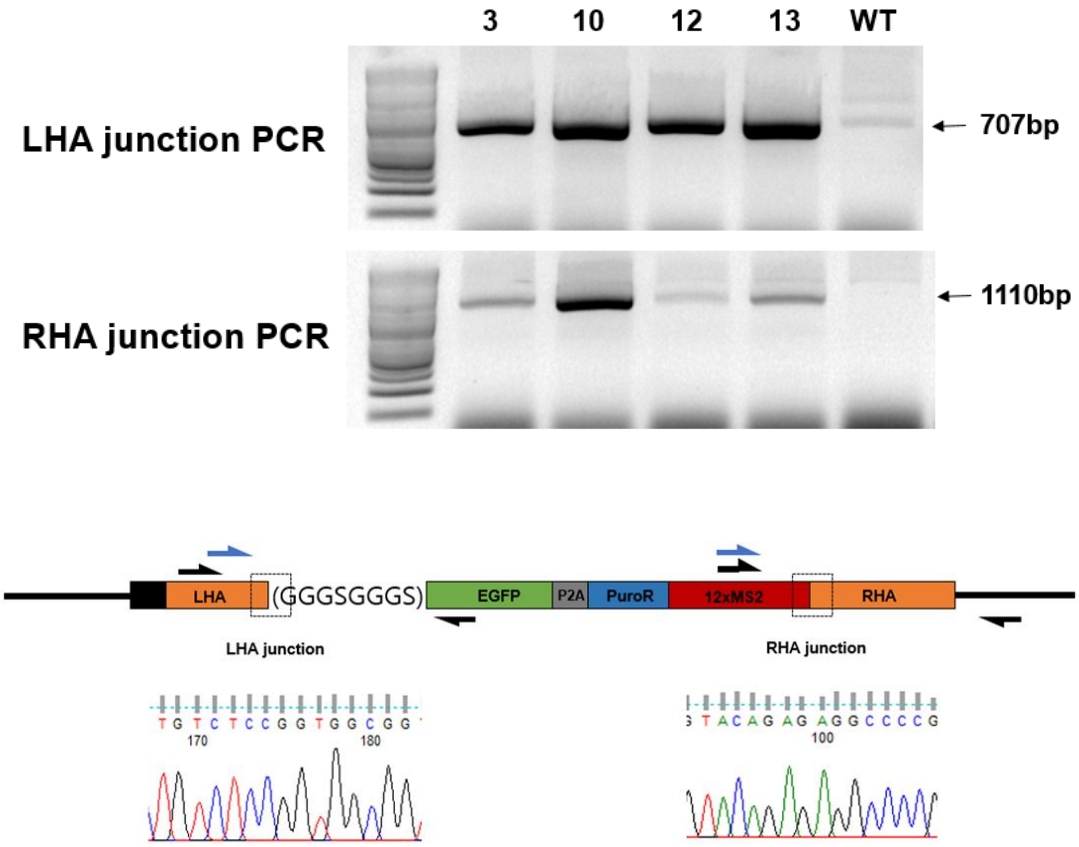

Figure 7. Junction PCR and chromatogram of the heat shock transcription factor (HSF) donor cell line

Single cells were selected after 18 days, moved to a 24-well plate and target PCR was performed to identify a single clone. Junction PCR was performed to confirm the correct insertion of HSF1 donor DNA into HeLa cells (among the 96 clones obtained from each cell). (A) F primer binding to the 12xMS2-aptamer and R primer for target specific PCR were designed to confirm whether the right homologous arm (RHA) was integrated (1,110 bp) on the gDNA. I used a primer that binds to the left homologous arm (LHA) sequence in the HSF1 genomic DNA and a R primer that binds to enhanced green fluorescent protein (EGFP) to identify the inserted sequence between the LHA and the GS linker (707 bp). (B) HSF1 donor cell lines 10 and 12 were subjected to junction PCR. The junction sequence between the LHA and GS linker was confirmed by PCR and sequencing. The junction sequence

between the RHA and the 12xMS2 aptamer was confirmed by PCR and sequencing. Black arrow, primers used in PCR; Blue arrows, primers used for sequencing.

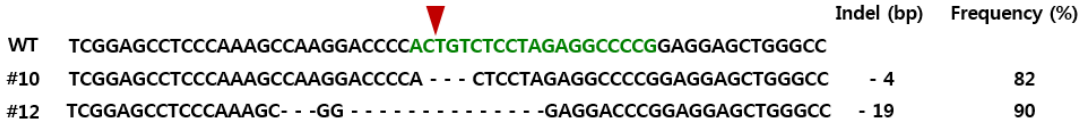

Figure 8. Result of deep sequencing of the heat shock transcription factor (HSF)1 cell line

Scheme for verifying whether the donor template was heterozygous or homozygous in the HSF1 donor cell line. Deep sequencing result of HSF1 donor cell and mutational sequence pattern. The indel efficiency was about 90%. Error-bar does not appear well.

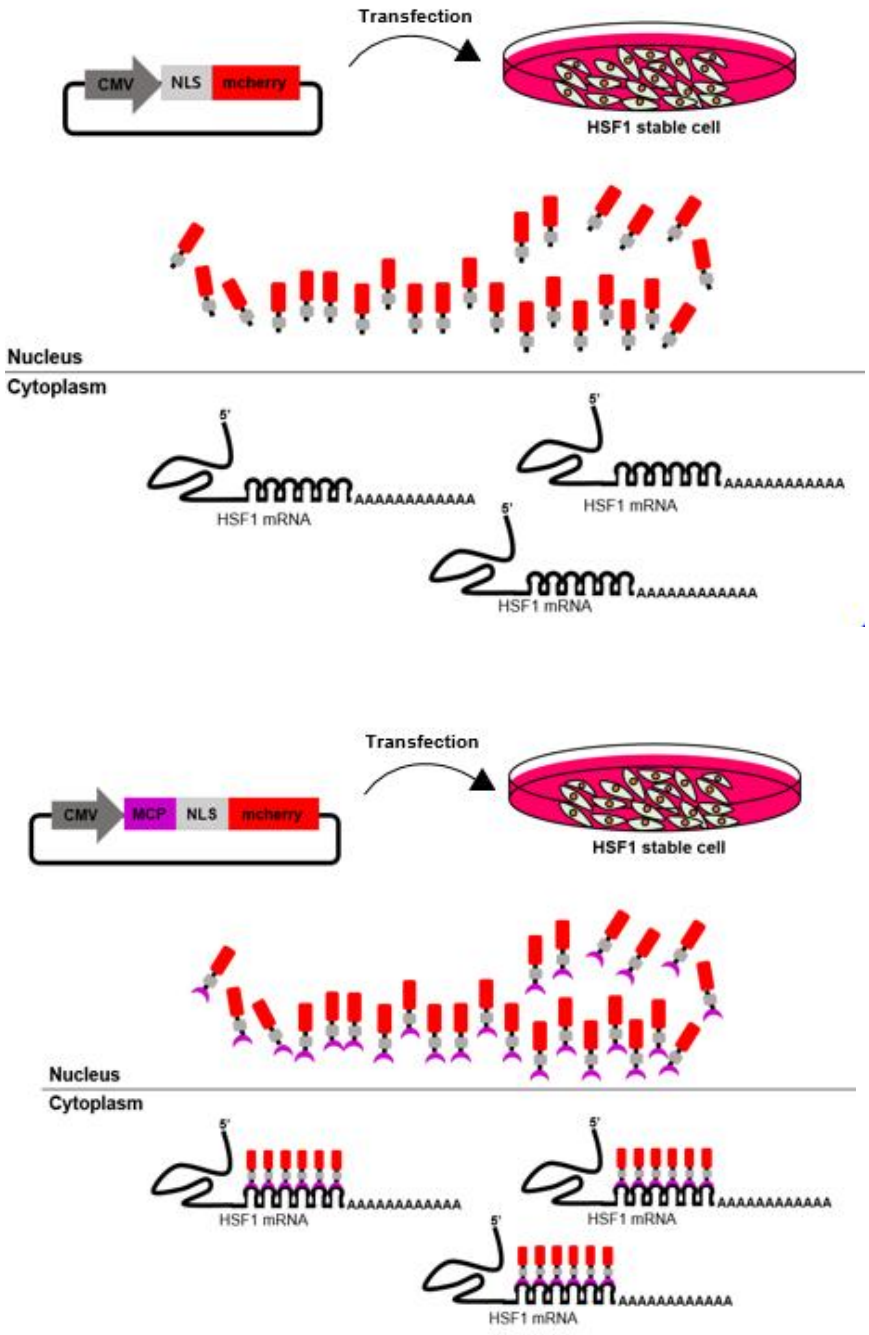

Figure 9. Scheme for visualizing heat shock transcription factor (HSF)1 RNA

(A) MCP-free fluorescent molecules do not bind to HSF1 mRNA and migrate to the nucleus. (B) When fluorescent MCP is expressed in HSF1 cell line, it binds to MBS of HSF1 mRNA.

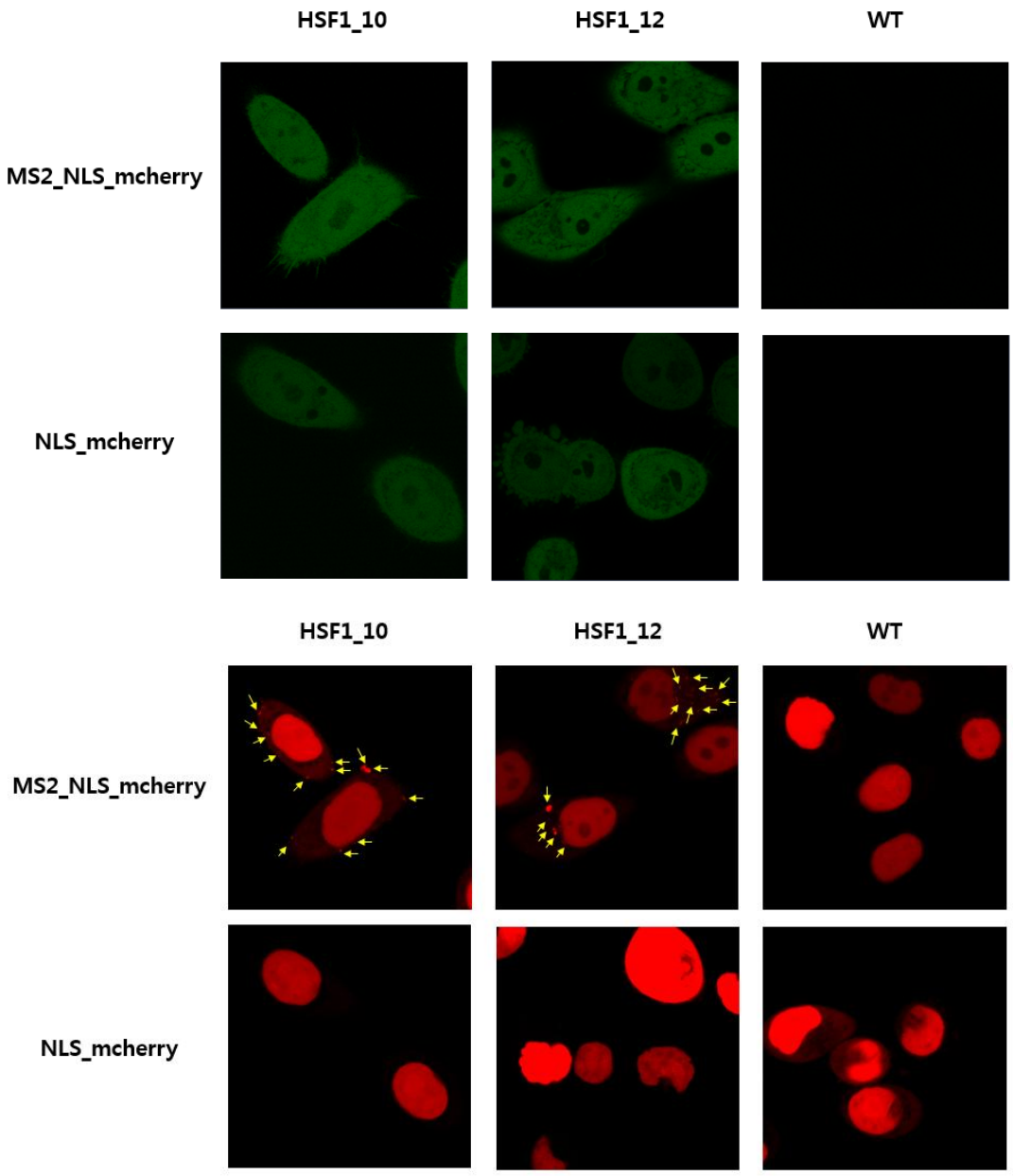

Figure 10. Identification of heat shock transcription factor (HSF)1 RNA by confocal microscopy

p3s-MS2-NLS-mCherry and p3s-NLS-mCherry were transfected into the HSF1 donor cell lines 10 and 12, and confocal microscopic images were taken after 48 h. RNA dots were identified in cytoplasm only when p3s-MS2-NLS-mCherry was transfected into the HSF1 donor cell line.

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국문요약(Summary in Korean)

mRNA와 단백질은 세포의 항상성을 유지하고 생합성을 조절하는 시 공간적인 단계로서 중요한 역할을 한다. 또한 단백질은 세포에서의 다양한 화학 반응을 위한 촉매 물질로서 중요한 인자이다. 따라서 mRNA와 단백질을 탐지하는 것은 그것들의 조절과정을 이해하는 데 필수적이다. 그러나 현재 살아있는 세포에서 mRNA와 단백질을 동시에 시각화하는 기술은 존재하지 않는다. 이 연구에서는 HSF1 mRNA와 HSF1 단백질을 동시에 시각화하기 위해 CRISPR-Cas9를 사용하여 열충격 조절 인자 (HSF) 1 공여자 DNA knock-in 세포주를 개발했다. HSF1 knock-in 세포주는 HSF1 정지 코돈 주위에 상 동성 재조합에 의해 공여체 DNA를 삽입함으로써 제조되었다. 구체적으로, HSF1 공여자 DNA는 양측에 적당한 길이의 상 동성 서열을 포함하며, HSF1 단백질 표지를 위해 EGFP 서열이 사용되었다. 또한 knock-in 세포를 선택적으로 얻어내기 위해 퓨로마이신 내성 서열을 삽입하였고 RNA 시각화를 위한 12카피의 MS2 압타머를 적용하였다. 단일 RNA 분자 수준에서 mRNA 시각화는 RNA 압타머인 MS2 결합 부위와 형광성 MS2 박테리오파지 코팅 단백질 (MCP)의 상호작용으로 표적화 된 HSF1 mRNA를 살아있는 세포에서 시각화 할 수 있도록 한다. 이 연구에서는 CRISPR-Cas9과 함께 사용할 off-target이 낮고 절단 효율이 높은 단일 가이드 RNA를 사용하여, HSF1 공여자 DNA가 상동재조합으로 삽입된 HSF1 안정화 세포주를 개발하였다. 이 세포주는 실제적으로 공초점 현미경을 통하여 HSF1 단백질이 EGFP를 통해 시각화 할 수 있음을 확인 하였고 HSF1 mRNA는 외인성으로 발현시킨 형광성 MCP를 통해 세포질에서 시각화 할 수 있었다. 이 시스템은 살아있는 세포에서 내인성 mRNA와 단백질의 동적 변화를 연구하기 위해 특정 mRNA와 단백질을 시각화하는 새로운 기술을 제공한다.

중심단어: CRISPR-Cas9, 상동 재조합, Knock-in, mRNA, MS2