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Development of cytotoxicity-sensitive human cells using overexpression of long non-coding RNAs

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Biosensors using live cells are analytical devices that have the advantage of being highly sensitive for their targets. Although attention has primarily focused on reporter gene assays using functional promoters, cell viability assays are still efficient. We focus on long non-coding RNAs (lncRNAs) that are involved in the molecular mechanisms associated with responses to cellular stresses as a new biological material. Here we have developed human live cells transfected with lncRNAs that can be used as an intelligent sensor of cytotoxicity for a broad range of environmental stresses. We identified three lncRNAs (GAS5, IDI2-AS1, and SNHG15) that responded to cycloheximide in HEK293 cells. Overexpression of these lncRNAs sensitized human cells to cell death in response to various stresses (cycloheximide, ultraviolet irradiation, mercury II chloride, or hydrogen peroxide). In particular, dual lncRNA (GAS5 plus IDI2-AS1, or GAS5 plus SNHG15) overexpression sensitized cells to cell death by more cellular stresses. We propose a method for highly sensitive biosensors using overexpression of lncRNAs that can potentially measure the cytotoxicity signals of various environmental stresses.

[**Key words:** Non-coding RNA; Biosensor; Human cells; Cellular stress; Cytotoxicity]

Whole cell biosensors have been the focus of increasing interest worldwide as methods for detecting and quantifying environmental toxicity, including biochemical oxygen demand (BOD), heavy metals, antibiotics, pesticides and herbicides (1). In particular, biosensors using live cells can potentially report functional information of the effects of certain stimuli on various signaling cascades. Thus, these biosensors have great applicability in the fields of toxicology and/or environmental assessment (2).

One method to obtain functional information from intact live cells is to monitor the expression levels of specific genes by reporter assays using functional promoters. Heat shock protein (HSP) genes are upregulated by a wide range of cellular stresses via the binding of heat shock factor to the heat shock element in the HSP promoter region (3,4). Live cells transfected with a reporter vector containing a functional HSP promoter can be used as an intelligent sensor for cytotoxicity (5,6). However, this method is limited to HSP responsive stresses. A novel method that has more broad applications is required.

Long non-coding RNA (lncRNAs) are non-protein-coding transcripts longer than 200 nt that are involved in various biological functions (7–9). The majority of lncRNAs are transcribed by RNA polymerase II (Pol II) with 5′ caps and polyadenylation (10). Increasing evidence has demonstrated lncRNA roles as signals, decoys, guides, and scaffolds in diverse biological processes (11). Several human lncRNAs show alterations in expression levels in response to stress. The psoriasis susceptibility-related RNA gene induced by stress (PRINS) is increased by many stressors such as ultraviolet (UV)-B irradiation, virus transfection, and translational inhibition (12). Our previous studies showed that MAGI2 antisense RNA 3 (MAGI2-AS3) and LOC730101 were increased by genotoxic agents, such as mitomycin C or doxorubicin (13). MIR22 host gene (MIR22HG), GABPB1 antisense RNA 1 (GABPB1-AS1), FLJ33630, long intergenic non-protein coding RNA 152 (LINC00152), IDI2 antisense RNA 1 (IDI2-AS1), and small nucleolar RNA host gene 15 (SNHG15) are upregulated by several chemical stresses, such as cisplatin, cycloheximide, or mercury (II) chloride (14). Moreover, depletion of the promoter of CDKN1A antisense DNA damage-activated RNA (PANDA) markedly sensitized human fibroblasts to apoptosis by doxorubicin. PANDA interacts with the transcription factor NF-YA to limit the expression of pro-apoptotic genes and enables cell cycle arrest (15,16).

The aim of this study was to construct highly sensitive cytotoxicity sensor cells for a broad range of cellular stresses. We hypothesized that certain lncRNAs sensitize human cells to cell death in response to several environmental stresses and sought to identify lncRNAs that respond to model environmental stresses.

MATERIALS AND METHODS

Cell culture Human embryonic kidney 293 (HEK293) cells are provided by Japan Health Sciences Foundation. HEK293 were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in a humidified incubator with 5% CO_2.

Stress treatments HEK293 cells were treated with cycloheximide (final concentrations of 1, 10, or 100 μM; Biovision), mercury (II) chloride (10, 100, or

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1000 μg/ml; Wako), hydrogen peroxide (20, 40, 60, or 80 μM; Wako, Osaka, Japan), or UV 254 nm (20,000, 40,000, 60,000, or 80,000 μJ/cm^2). Cycloheximide and mercury II chloride were diluted in dimethyl sulfoxide (DMSO). Hydrogen peroxide was diluted in ultrapure water. After UV exposure, the irradiated medium was replaced by fresh medium.

Construction of expression vectors GAS5 (1–631), IDI2-AS1 (1–1088), and SNHG15 (1–808) expression vectors were constructed by subcloning the full-length GAS5, IDI2-AS1, or SNHG15 sequences lacking a poly A tail (based on the GAS5 sequence NR_002578 in NCBI; IDI2-AS1 sequence NR_024628 in NCBI; and SNHG15 sequence NR_003697 in NCBI). The GAS5, IDI2-AS1, or SNHG15 cDNAs were amplified from total RNA purified from HeLa Tet-off cells, and then cloned into pcDNA3.1 (+) (Life Technologies, Carlsbad, CA, USA). Additional information on vector construction can be provided upon request.

Overexpression of lncRNAs and cell count Seed the 10^6 cells in 60 mm dishes using DMEM supplemented with 10% FBS. Expression vectors (1 μg/ml) were transfected into cells using Lipofectamine 2000 (Life Technologies), according to the manufacturer's instructions. Cells were treated with chemical stresses 24 h after transfection. The number of viable cells was counted 24 h after treatment using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. Cells were also harvested 48 h after transfection, and total RNAs were isolated using RNAiso Plus (Takara, Shiga, Japan), according to the manufacturer's instructions.

Reverse transcription-quantitative real-time polymerase chain reaction Total RNA was extracted from cells with RNAiso Plus (Takara) according to the manufacturer's instructions. The isolated RNA was reverse transcribed into cDNA using PrimeScript RT Master Mix (Perfect Real Time; Takara). The resulting cDNA was amplified using the primer sets listed in Supplemental Table S1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were used for normalization. Relative RNA quantities were the treated values normalized to untreated values. Thunderbird SYBR qPCR mix (Toyobo, Osaka, Japan) was used according to the manufacturer's instructions. Reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) analysis was performed using a MyiQ2 (Bio-Rad, Hercules, CA, USA).

RESULTS AND DISCUSSION

Screening of lncRNAs in chemical stress response We first selected 24 lncRNAs that are short-lived ($t_{1/2} < 4$ h) in HeLa Tet-off cells (17), longer than 200 nt, and fulfilled the established criterion for lncRNA classification (14,17). To investigate potential responses of the 24 lncRNAs to cellular stress, we examined alterations in their expression levels following treatment of HEK293 cells with a model chemical stress, cycloheximide, which functions as a translation inhibitor. After treatment with 100 μM cycloheximide, we found significant increases in the expression levels of GAS5, IDI2-AS1, and SNHG15 (Fig. 1A). We selected the GAS5, IDI2-AS1, and SNHG15 for checking the dose responses because these expression levels are higher than other lncRNAs including CDKN2B-AS1 or LINC0541471_v2 in Fig. 1A. We also examined alterations in lncRNA expression levels following treatment with cycloheximide at various doses. As expected, GAS5, IDI2-AS1, and SNHG15 levels increased with increasing concentrations of cycloheximide (Fig. 1B–D). These data indicate that these lncRNAs respond to cell stress by cycloheximide in a dose-dependent manner.

GAS5 (~650 nt in humans) was originally isolated in a screen for potential tumor suppressor genes expressed at high levels during growth arrest (18). Overexpression of GAS5 causes both an increase in apoptosis induced by UV and cisplatin and a reduction in the rate of progression through the cell cycle (19). A recent study showed that the RNA degradation pathway can regulate the function of GAS5 in human cells (20). IDI2-AS1 (~1100 nt in humans) and SNHG15 (~810 nt in humans) were upregulated by cisplatin and cycloheximide in HeLa Tet-off cells (14), but the biological significance of these lncRNAs is largely unknown. Thus, we focused our analyses on GAS5, IDI2-AS1, and SNHG15.

Overexpression of lncRNAs does not impact cell proliferation rate We next examined whether the overexpression of the lncRNAs affected the cell proliferation rate. HEK293 cells were transfected with either a single plasmid vector encoding GAS5, IDI2-AS1, or SNHG15, or co-transfected with vectors encoding GAS5 plus IDI2-AS1 or GAS5 plus SNHG15. Viable cell numbers were determined in a time-course analysis after transfection, and expressions of GAS5, IDI2-AS1, and SNHG15 were assessed using

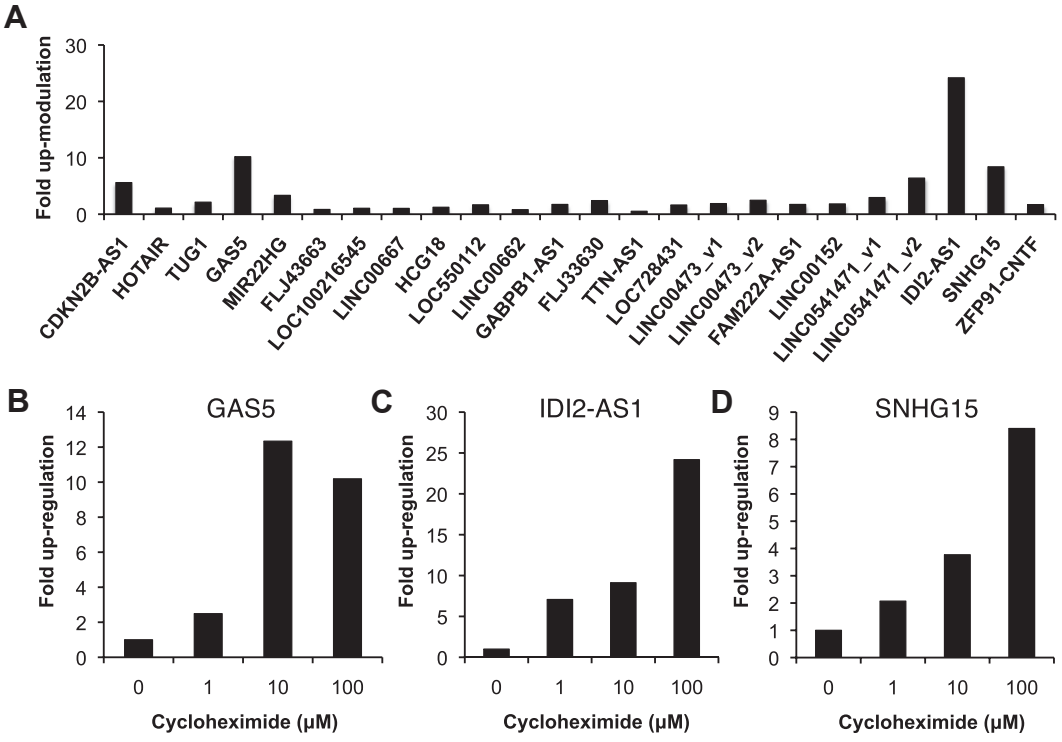

FIG. 1. Alterations in lncRNA expression levels in HEK293 cells by the chemical stress. (A) HEK293 cells were treated with 100 μM cycloheximide for 24 h. Expression levels of the indicated RNA were determined by RT-qPCR. Quantitative values in response to vehicle alone were set to 1. GAPDH mRNA levels were used for normalization. (B–D) HEK293 cells were treated with cycloheximide at various doses for 24 h. The expression levels of GAS5 (B), IDI2-AS1 (C), and SNHG15 (D) were determined by RT-qPCR.