Journal of Bioscience and Bioengineering
VOL. 119 No. 5, 604–608, 2015

www.elsevier.com/locate/jbiosc

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

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Received 6 August 2014; accepted 17 October 2014
Available online 14 November 2014

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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http://dx.doi.org/10.1016/j.jbiosc.2014.10.012

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

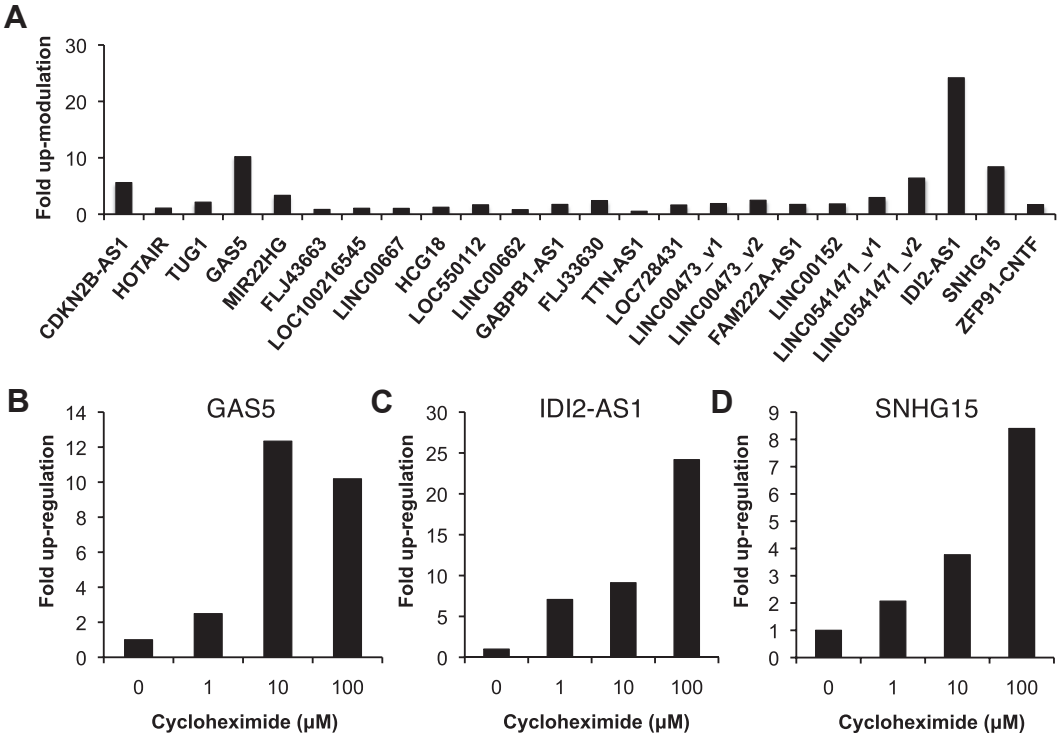

FIG. 1. Alterations in lncRNA expression levels in HEK293 cells by the chemical stress. (A) HEK293 cells were treated with 100 μM cycloheximide for 24 h. Expression levels of the indicated RNA were determined by RT-qPCR. Quantitative values in response to vehicle alone were set to 1. GAPDH mRNA levels 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.

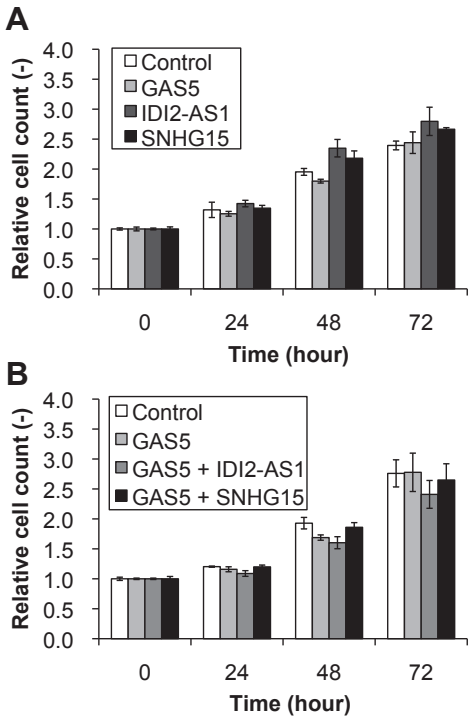

FIG. 2. Overexpression of the indicated lncRNAs does not change the cell proliferation rate. HEK293 cells were treated with one plasmid vector (A) or two plasmid vectors (B) as indicated. The numbers of viable cells were counted using the Cell Counting Kit-8. Relative cell count values at 0 h were set to 1 in each sample. Values represent mean ± SD obtained from four independent experiments.

RT-qPCR. GAS5, IDI2-AS1, and SNHG15 expressions were increased by approximately 60-, 40,000-, and 50-fold in each lncRNA-transfected cell, respectively, compared with cells transfected with mock vector. The rates of cell proliferation of single or co-transfected cells were not significantly different from control cells, indicating that these lncRNAs had no impact on cell proliferation (Fig. 2).

Single overexpression of lncRNAs enhances stimulation of cell death by environmental stresses We next examined whether the overexpression of lncRNAs sensitized human cells to cell death by environmental stress. HEK293 cells were transfected with a single plasmid vector expressing GAS5, IDI2-AS1, or SNHG15, and then subjected to various cellular stresses 24 h later. Cells were exposed to cycloheximide, UVC irradiation (DNA damage), mercury II chloride (heavy metal), or hydrogen peroxide (oxidative stress) for 24 h of treatment, and cell viability was determined (Fig. 3). The expression of GAS5, IDI2-AS1, and SNHG15 were also assessed using RT-qPCR. Similarly to Fig. 2, GAS5, IDI2-AS1, and SNHG15 expressions were increased by approximately 60-, 40,000-, and 50-fold in each lncRNA-transfected cell, respectively, compared with cells transfected with mock vector. In response to 1 μM cycloheximide treatment, overexpression of GAS5 caused a decrease in cell viability compared with control cells, whereas IDI2-AS1 and SNHG15 had no effect (Fig. 3A). Under treatment with 20,000 μJ/cm^2 UVC irradiation, overexpression of IDI2-AS1 caused a decrease in viability compared with control cells, whereas GAS5 and SNHG15 had no effect (Fig. 3B). Although overexpression of GAS5 increased the rate of apoptosis in previous report (19), the similar condition did not change the sensitivity against UV irradiation in Fig. 3B. This is because HEK293T cells were used in

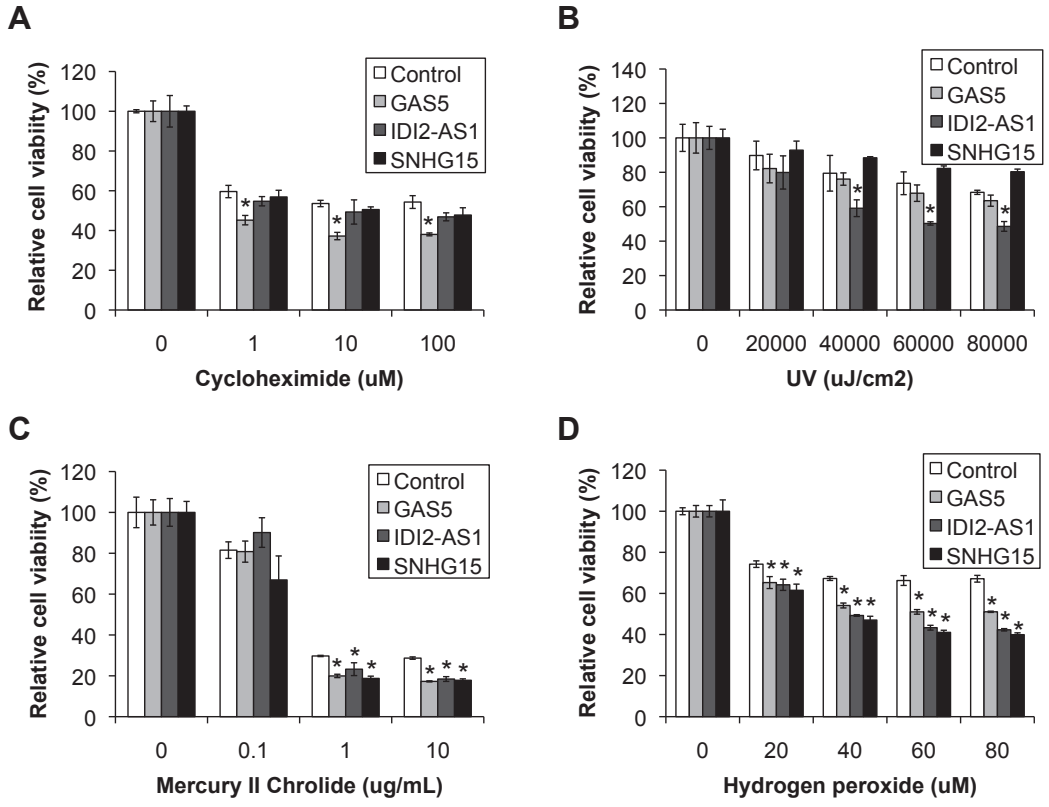

FIG. 3. Single overexpression of the indicated lncRNAs sensitized HEK293 cells to cell death by various chemical stresses. HEK293 cells were treated with one plasmid vector as indicated. Cells were treated with cycloheximide (A), UV (B), mercury (II) chloride (C), or hydrogen peroxide (D) for 24 h. After stress exposure, the number of viable cells was counted using the Cell Counting Kit-8. Relative cell count values at 0 h were set to 100% in each sample. Values represent mean ± SD obtained from four independent experiments. *$P < 0.05$, Student's t-test.

previous report (19). In contrast, HEK293 cells were used in this study. Cell lines are different between previous report and this study. Therefore, we considered that the similar condition did not change the sensitivity against UV irradiation in Fig. 3B. In response to 1 μg/mL mercury II chloride, overexpression of GAS5, IDI2-AS1, and SNHG15 all caused a decrease in cell viability (Fig. 3C). In response to treatment with 20 μM hydrogen peroxide, overexpression of GAS5, IDI2-AS1, and SNHG15 caused a decrease in cell viability (Fig. 3D). These results indicate that each lncRNA shows distinct roles in various cellular stresses and none of these lncRNAs was able to sensitize cells to all stresses. There is increasing evidence of lncRNA involvement in diverse biological processes such as signals, decoys, guides, and scaffolds (11). Although molecular mechanisms of IDI2-AS1 and SNHG15 are not elucidated, over-expression of GAS5 was reported to specifically decrease the expression levels of several apoptosis-inhibitors genes (20). Thus, GAS5 act as a sensitizer of apoptosis. Taken together, we speculate that GAS5, IDI2-AS1, or SNHG15 have some specific molecular mechanisms to show distinct roles in various cellular stresses. Thus, we next examined whether dual overexpression of lncRNAs could increase or broaden sensitivity to these agents.

Dual overexpression of lncRNAs enhances stimulation of cell death by environmental stresses For the dual overexpression experiments, we focused on cycloheximide and UVC as stresses, because only GAS5 or IDI2-AS1 sensitized cells to cell death by cycloheximide or UVC (Fig. 3). HEK293 cells were transfected with GAS5, GAS5 plus IDI2-AS1, or GAS5 plus SNHG15 vectors. After 24 h, cells were exposed to cycloheximide or UVC irradiation and 24 h later, cell viability was determined (Fig. 4). The expression of GAS5, IDI2-AS1, and SNHG15 were also assessed using RT-qPCR. Similarly to Figs. 2 and 3, GAS5, IDI2-AS1, and SNHG15 expressions were increased by approximately 60-, 40,000-, and 50-fold in single or dual lncRNA-transfected cell, respectively, compared with cells transfected with mock vector. Under treatment with 1 μM cycloheximide, overexpression of GAS5, GAS5 plus IDI2-AS1, or GAS5 plus SNHG15 caused a decrease in cell viability (Fig. 4A). In particular, overexpression of GAS5 plus IDI2-AS1 or GAS5 plus SNHG15 significantly sensitized cells to cell death by cycloheximide. At concentrations higher than 10 μM of cycloheximide, the viability of lncRNA-overexpressing cells was similar to that of control cells. Under treatment with 20,000 μJ/cm^2 UVC irradiation, overexpression of GAS5 plus IDI2-AS1 or GAS5 plus SNHG15 decreased cell viability, whereas GAS5 alone had no effect (Fig. 4B). These results indicate that dual lncRNA overexpression is efficient for sensitizing cells to cell death by various stresses.

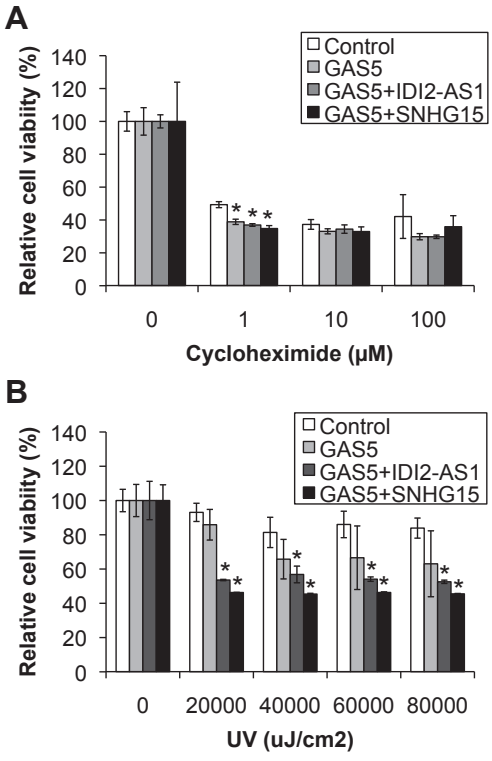

FIG. 4. Dual overexpression of the indicated lncRNAs markedly sensitized HEK293 cells to cell death by various chemical stresses. HEK293 cells were treated with two plasmid vectors as indicated. Cells were treated with cycloheximide (A) or UV (B) for 24 h. After stress exposure, the number of viable cells was counted using the Cell Counting Kit-8. Relative cell count values at 0 h were set to 100% in each sample. Values represent mean ± SD obtained from four independent experiments. *$P < 0.05$, Student's *t*-test.

In this study, we identified three lncRNAs (GAS5, IDI2-AS1, and SNHG15) that respond to several environmental stresses, and found that overexpression of these lncRNAs sensitized human cells to cell death by the stresses. In particular, dual overexpression of lncRNAs (GAS5 plus IDI2-AS1, or GAS5 plus SNHG15) enabled sensitization of cells to more cellular stresses. These sensor cells with overexpressed lncRNAs can potentially report cytotoxicity signals of various environmental stresses. For improvement high resolution of these sensor cells, more screening of other lncRNAs that sensitize the cells to cellular stresses is crucial. Moreover, optimizing of the kind of expression vectors, which enhance the expression level of lncRNAs, is required. More over-expression level of lncRNAs will enable cells to more sensitive to various cellular stresses. Thus, we believe that the highly sensitive sensor cells in this study can feasibly be used to develop biosensors for use in toxicology, environmental assessment and drug screening.

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2014.10.012.

ACKNOWLEDGMENTS

This work was supported by the JSPS Grant-in-Aid for Young Scientists (B) Grant Number 26810100.

References

1. **Pasco, N. F., Weld, R. J., Hay, J. M., and Gooneratne, R.:** Development and applications of whole cell biosensors for ecotoxicity testing, Anal. Bioanal. Chem., **400**, 931–945 (2011).
2. **Keusgen, M.:** Biosensors: new approaches in drug discovery, Naturwissenschaften, **89**, 433–444 (2002).
3. **Wu, C.:** Heat shock transcription factors: structure and regulation, Annu. Rev. Cell Dev. Biol., **11**, 441–469 (1995).
4. **Morimoto, R. I.:** Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators, Genes Dev., **12**, 3788–3796 (1998).
5. **Wada, K., Taniguchi, A., Xu, L., and Okano, T.:** Rapid and highly sensitive detection of cadmium chloride induced cytotoxicity using the HSP70B' promoter in live cells, Biotechnol. Bioeng., **92**, 410–415 (2005).
6. **Wada, K., Taniguchi, A., and Okano, T.:** Highly sensitive detection of cytotoxicity using a modified HSP70B' promoter, Biotechnol. Bioeng., **97**, 871–876 (2007).
7. **Ponting, C. P., Oliver, P. L., and Reik, W.:** Evolution and functions of long noncoding RNAs, Cell, **136**, 629–641 (2009).
8. **Wilusz, J. E., Sunwoo, H., and Spector, D. L.:** Long noncoding RNAs: functional surprises from the RNA world, Genes Dev., **23**, 1494–1504 (2009).
9. **Bergmann, J. H. and Spector, D. L.:** Long non-coding RNAs: modulators of nuclear structure and function, Curr. Opin. Cell Biol., **26C**, 10–18 (2014).
10. **Guttman, M., Amit, I., Garber, M., French, C., Lin, M. F., Feldser, D., Huarte, M., Zuk, O., Carey, B. W., Cassady, J. P., and other 10 authors:** Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals, Nature, **458**, 223–227 (2009).
11. **Mercer, T. R., Dinger, M. E., and Mattick, J. S.:** Long non-coding RNAs: insights into functions, Nat. Rev. Genet., **10**, 155–159 (2009).

12. **Sonkoly, E., Bata-Csorgo, Z., Pivarcsi, A., Polyanka, H., Kenderessy-Szabo, A., Molnar, G., Szentpali, K., Bari, L., Megyeri, K., Mandi, Y., and other 3 authors:** Identification and characterization of a novel, psoriasis susceptibility-related noncoding RNA gene, PRINS, J. Biol. Chem., **280**, 24159–24167 (2005).
13. **Mizutani, R., Wakamatsu, A., Tanaka, N., Yoshida, H., Tochigi, N., Suzuki, Y., Oonishi, T., Tani, H., Tano, K., Ijiri, K., Isogai, T., and Akimitsu, N.:** Identification and characterization of novel genotoxic stress-inducible nuclear long noncoding RNAs in mammalian cells, PLoS One, **7**, e34949 (2012).
14. **Tani, H. and Torimura, M.:** Identification of short-lived long non-coding RNAs as surrogate indicators for chemical stress response, Biochem. Biophys. Res. Commun., **439**, 547–551 (2013).
15. **Hung, T., Wang, Y., Lin, M. F., Koegel, A. K., Kotake, Y., Grant, G. D., Horlings, H. M., Shah, N., Umbricht, C., Wang, P., and other 12 authors:** Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters, Nat. Genet., **43**, 621–629 (2011).
16. **Wang, K. C. and Chang, H. Y.:** Molecular mechanisms of long noncoding RNAs, Mol. Cell, **43**, 904–914 (2011).
17. **Tani, H., Mizutani, R., Salam, K. A., Tano, K., Ijiri, K., Wakamatsu, A., Isogai, T., Suzuki, Y., and Akimitsu, N.:** Genome-wide determination of RNA stability reveals hundreds of short-lived non-coding transcripts in mammals, Genome Res., **22**, 947–956 (2012).
18. **Schneider, C., King, R. M., and Philipson, L.:** Genes specifically expressed at growth arrest of mammalian cells, Cell, **54**, 787–793 (1988).
19. **Mourtada-Maarabouni, M., Pickard, M. R., Hedge, V. L., Farzaneh, F., and Williams, G. T.:** GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer, Oncogene, **28**, 195–208 (2009).
20. **Tani, H., Torimura, M., and Akimitsu, N.:** The RNA degradation pathway regulates the function of GAS5 a non-coding RNA in mammalian cells, PLoS One, **8**, e55684 (2013).