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Gene expression and cell growth are modified by silencing SUMO2 and SUMO3 expression

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ABSTRACT

Small ubiquitin-like modifier (SUMO) is a group of proteins binding to lysine residues of target proteins and thereby modifying their stability, activity and subcellular localization. Here we report that blocking SUMO2 and SUMO3 conjugation by silencing their expression markedly modifies gene expression. A microRNA-based RNAi system was used to specifically silence SUMO2 and SUMO3 expression simultaneously and stably transfected neuroblastoma B35 cells expressing dual SUMO2/3 microRNA were created. In cells stably expressing SUMO2/3 microRNA, mRNA levels of 105 and 58 known genes were significantly up- and down-regulated, respectively. About 20% of differentially regulated genes were associated with pathways involved in cell growth and differentiation. Cell division was significantly suppressed in SUMO2/3 miRNA expressing cells. Elucidating what effect the silencing of SUMO2/3 expression has on gene expression will help to identify the impact of SUMO2/3 conjugation on the various cellular pathways.

Introduction

Small ubiquitin-like modifier (SUMO) conjugation is a post-translational protein modification that was identified 12 years ago [1]. Four SUMO paralogues have been identified, denoted SUMO1–4. Of these, SUMO2 and SUMO3 are highly homologous and are, therefore, referred to as SUMO2/3. SUMO is conjugated to the ε amino group of lysine residues of target proteins thereby modifying their activity, stability and cellular localization. The SUMO conjugation pathway is massively activated in cells exposed to various stress conditions of high clinical relevance, including oxidative stress, thermal stress (hypo- and hyper-thermia), and transient cerebral ischemia [2–8]. It is, therefore, of key interest to us to understand the possible significance of the activation of the SUMO conjugation pathway in determining the fate of cells exposed to stressful conditions.

Proteomic analyses have revealed that many of the SUMO conjugation target proteins are transcription factors or other nuclear proteins modulating gene expression [9]. Any marked change in levels of SUMO conjugated proteins can, therefore, be expected to have a major impact on gene expression. To date no information has been published on the effect of SUMO conjugation on global gene expression. One strategy towards elucidating how gene expression is modified by SUMO conjugation is to silence SUMO expression and perform genomic analyses. We decided to focus on SUMO2/3 conjugation, because this pathway is dramatically activated both in-vitro and in-vivo in cells exposed to various forms of cellular stress conditions. We have created stably transfected cell lines expressing designed microRNA (miRNA) that effectively silence SUMO2/3 expression, and have analyzed the effects of silencing SUMO2/3 expression on the cellular transcription pattern and cell proliferation.

Materials and methods

Experiments were performed on B35 cells, a neuroblastoma cell line [10]; by courtesy of Dr. P.F. Maness, University of North Carolina, Chapel Hill, USA. To produce miRNA-based RNAi vectors, the pcDNA6.2-GW/EmGFP-miR (Invitrogen) containing enhanced green fluorescent protein (EGFP) and a blasticidin resistance cassette was used as vector backbone. We screened for effective target sequences in transient transfection experiments. The miRNA expression vectors miR-SUMO2 and miR-SUMO3 containing the target sequences GTTTGTCAATGAGGCAGATCA or AATCGAA TCTGCCTCATTGAC demonstrated best efficiency in silencing HA-SUMO2 or HA-SUMO3 expression, respectively. To generate the dual vector miR-SUMO2/3 expressing both target sequences in one primary transcript, the pre-miRNA SUMO2 was inserted into the vector miR-SUMO3. The vector pcDNA6.2-GW/EmGFP-miR-neg

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(miR-Neg) containing a pre-miRNA sequence not related to any mammalian gene served as a negative control (Invitrogen). All constructs were verified by DNA sequencing. Subcellular localization of SUMO2/3 conjugated proteins and the effect of silencing SUMO2/3 expression were evaluated by confocal microscopy. The Affymetrix Rat Genome 230 2.0 Array was used for analysis of miRNA expression-induced changes in gene expression in the stable cell lines. Array data were verified by quantitative PCR. To evaluate the effect of silencing SUMO2 and SUMO3 expression on cell proliferation, we quantified the change in the number of viable cells/per well over a period of 72 h. An extended Materials and methods section can be found at the Biochemical Biophysical Research Communication website.

Results

First, we screened various SUMO2 and SUMO3 pre-miRNA sequences to identify the most effective one in silencing SUMO2 and SUMO3 expression, respectively. B35 cells were transiently co-transfected with miR-SUMO2 or miR-SUMO3 constructs and plasmids expressing HA-tagged SUMO2 or SUMO3, respectively.

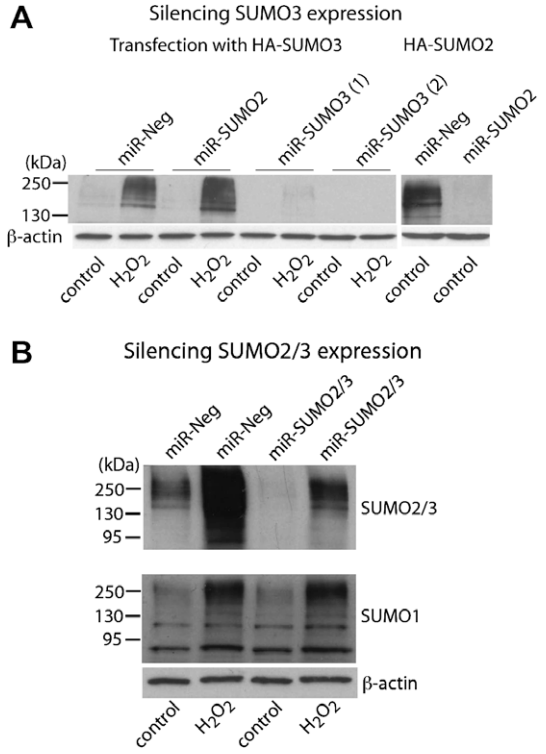

Fig. 1. Establishing a miRNA-based approach to the silencing of SUMO2/3 expression. (A) Screening for target sequences to silence SUMO expression. B35 cells were transiently co-transfected with a construct expressing HA-tagged SUMO3 and constructs expressing miR-Neg, miR-SUMO2 or miR-SUMO3, respectively, or a construct expressing HA-tagged SUMO2 and miR-Neg or miR-SUMO2 (right two lanes). Four days after transfection, cells were left intact (control) or exposed to 50 μM H_2O_2 for 10 min to activate SUMO conjugation (H_2O_2). Levels of SUMO2 or SUMO3 conjugated proteins were evaluated by Western blot analysis using a HA-tag antibody. Expression of miR-SUMOs effectively silenced co-expressed SUMO2 or SUMO3 expression. (B) Establishing cells stably expressing SUMO2/3 miRNA. A dual vector miR-SUMO2/3 expressing both SUMO2 and SUMO3 target sequences was generated, and cells were stably transfected using blasticidin for selection. Cells stably transfected with the miR-Neg vector served as controls. Cells were left intact or exposed to 50 μM H_2O_2 for 10 min to activate SUMO conjugation. Levels of free SUMO2/3 and SUMO2/3 conjugated proteins were evaluated by Western blot analysis using a SUMO2/3-specific antibody (by courtesy of Dr. John M. Hallenbeck, Stroke Branch, NINDS/NIH/Bethesda, dilution 1:1000). Expression of miR-SUMO2/3 effectively silenced endogenous SUMO2/3 expression.

An example of this miRNA sequence screening is illustrated in Fig. 1A. Cells were transfected with HA-SUMO3 and constructs expressing miR-Neg, miRNA related to SUMO2 (miR-SUMO2) and two miRNA sequences related to SUMO3 (miR-SUMO3). Peroxide exposure to activate SUMO conjugation induced the expected marked increase in levels of high molecular weight HA-SUMO3 conjugated proteins. This pattern was not changed by expression of miR-SUMO2, whereas miR-SUMO3 expression markedly reduced levels of high molecular weight HA-SUMO3 conjugated proteins. The effect of miR-SUMO2 expression on silencing HA-SUMO2 expression is illustrated in the last two lanes of Fig. 1A.

Next, we designed constructs for expression of both SUMO2 and SUMO3 miRNA within one transcript. An example is given in Fig. 1B. Cells were stably transfected with constructs expressing miR-Neg or dual vectors containing different combinations of target sequences for silencing SUMO2/3 expression (miR-SUMO2/3). Cells were left intact (control) or exposed to H_2O_2 to activate SUMO2/3 conjugation (Fig. 1B). Expression of miR-SUMO2/3 dramatically reduced levels of SUMO2/3 conjugated proteins, but did not induce any changes in levels of SUMO1 conjugated proteins (Fig. 1B). The subcellular distribution of SUMO2/3 conjugated proteins and the effect of silencing SUMO2/3 expression were verified by confocal microscopy. In control cells expressing miR-Neg, strong SUMO2/3 immunoreactivity was detectable concentrated in the cell nuclei. In cells expressing miR-SUMO2/3, SUMO2/3 immunoreactivity was almost below detection level (Supplementary material, Fig. S1).

To evaluate the effect of silencing SUMO2/3 expression on gene transcription, we used the Affymetrix GeneChip Rat Genome 230 2.0 Arrays ($n = 3$/group). Using a threshold of 2-fold up- or down-regulation and a significance level of $p < 0.05$, we identified 261 probe sets, with 105 known genes where expression was significantly up-regulated and 58 known genes with down-regulated expression (for a complete list see Supplementary material, Table. S1). Expression of SUMO2 and SUMO3 were down-regulated −2.2- and −4.4-fold, respectively, in SUMO2/3 miRNA expressing cells, while SUMO1 mRNA levels were not changed (100.8 ± 2.3% compared to control cells expressing miR-Neg), indicating the high selectivity of the miRNA sequence in silencing SUMO2/3 expression. To validate microarray data, we ran quantitative PCR (qPCR) for eight selected genes. qPCR analysis provided results similar to microarray analysis data for all eight genes investigated (Fig. 2).

Using the Ingenuity Pathways Analysis software to identify pathways associated with genes where expression was modified by silencing SUMO2/3 expression, we found genes associated with cell growth and differentiation to be particularly affected by miR-SUMO2/3 expression (Fig. 3). Expression of 18 genes associated with cell growth and differentiation was significantly activated (2.0- to 6.4-fold), expression of 14 genes was significantly down-regulated (−2.0- to −4.4-fold). To elucidate the net effect that silencing of SUMO2/3 expression has on cell growth and proliferation, we compared the growth rate of cells expressing miR-Neg to that of cells expressing miR-SUMO2/3. The growth rate of cells in which SUMO2/3 expression was silenced was significantly reduced compared to miR-Neg expressing cells, to 74% and 64% of control after 48 and 72 h, respectively (Fig. 4).

Discussion

One strategy towards identifying genes where the expression is modified by SUMO conjugation is to elucidate the effects of silencing of SUMO expression on global gene expression. Whereas conjugated forms of mouse SUMO2 and SUMO3 share 96.7% amino acid identity, they have only 69.3% nucleotide sequence identity. The miRNA approach used in the study enabled us to create a dual miRNA silencing vector miR-SUMO2/3 expressing both designed

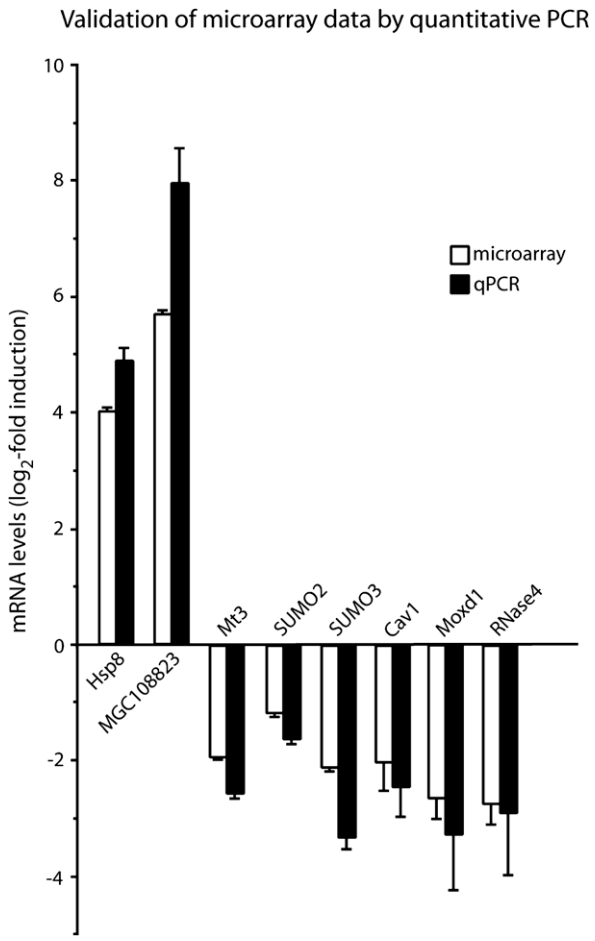

Fig. 2. Validation of microarray data by quantitative PCR (qPCR). Using the samples processed for microarray analyses, total RNA was reverse transcribed into cDNA which was used as template for qPCR. Data are presented as $\log_2$ (fold increase/decrease) (mean ± SD, $n = 3$). Full gene names are provided in Table S1 (Supplementary material).

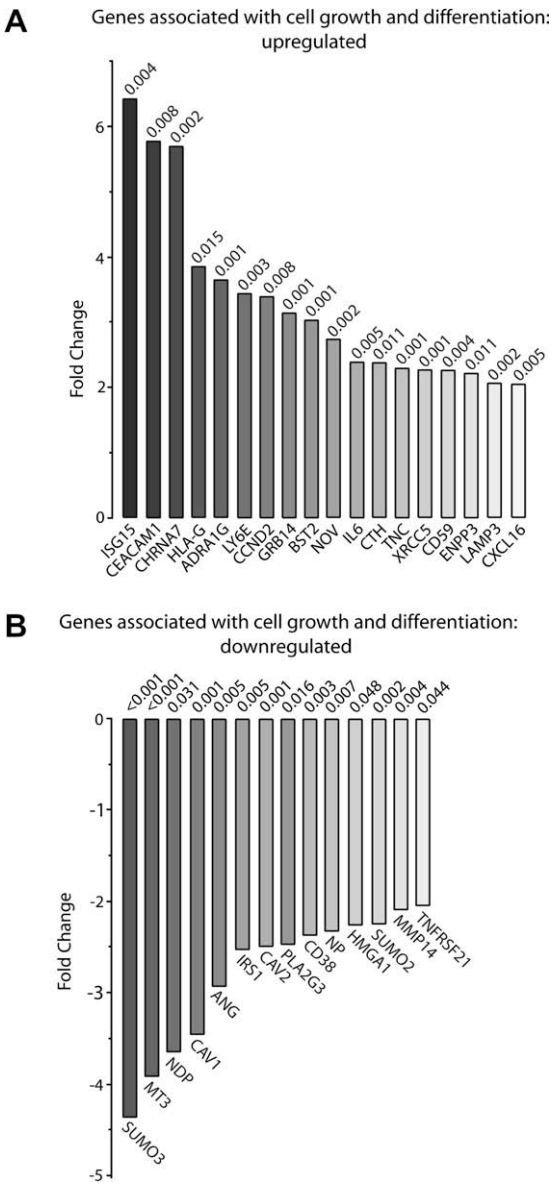

Fig. 3. Effect of miR-SUMO2/3 on expression of genes associated with cell growth and differentiation. The Ingenuity Pathway Analysis software was used to analyze microarray data. About 20% of the genes where expression was significantly modified in cells expressing miR-SUMO2/3 compared to miR-Neg expressing cells were found to be associated with cell growth and differentiation pathways. (A) Genes where expression was significantly up-regulated more than 2-fold. (B) Genes where expression was significantly down-regulated more than 2-fold. Data are presented as mean values ($n = 3$). Gene names and p values are displayed below and above bars, respectively. Full gene names are provided in Table S1 (Supplementary material).

miR-SUMO2 and miR-SUMO3. Using this construct, we have successfully established a stable cell line in which expression of both SUMO2 and SUMO3 was significantly silenced. By exploiting this unique cell line, we evaluated the effects of silencing SUMO2/3 expression on global gene expression and identified 105 and 58 known genes where expression was significantly up- or down-regulated in SUMO2/3 miRNA expressing cells respectively. At present, it remains to be established whether the changes in gene expression observed in SUMO2/3 miRNA expressing cells resulted directly from de-sumoylation of transcription factors where the activity is modified by SUMO conjugation or from an indirect effect on the functional state of cells. For example, if SUMO2/3 conjugation plays a key role in cellular function, silencing SUMO2/3 expression could impair cellular function and thus activate a stress response to restore function. We here provide evidence that cellular function is affected by silencing SUMO2 and SUMO3 expression. Cell growth was significantly reduced in SUMO2/3 expressing cells (Fig. 4). Furthermore, pathway analysis revealed that many of the genes differentially expressed in SUMO2/3 miRNA expressing cells are associated with cell growth and differentiation (Fig. 3). Several of the up-regulated genes have been associated with suppression of cell growth, including CEA-related cell adhesion molecule 1 (Ceacam1), lymphocyte antigen 6 (Ly6E), Cyclin D2 (Ccnd2), growth factor receptor bound protein 14 (Grb14) and interleukin-6 (IL6) (Supplementary material, Refs. [2–6]). We, therefore, expect miR-SUMO2/3 expression-induced activation of the expression of these genes to play a role in the observed suppression of cell growth (Fig. 4). On the other hand, several of the down-regulated genes have been found to activate cell growth, including methallothionein-3 (Mt3), Caveolin-1 (Cav1), the insulin receptor substrate-1 (Irs1), and the membrane type-1 matrix metalloproteinase (Mmp14) (Supplementary material, Refs. [7–13]), which could also contribute to the slowing down of cell growth in miR-SUMO2/3 expressing cells. Since we did not observed any change in levels of SUMO1 conjugated proteins in cells expressing miR-SUMO2/3 that induced a massive silencing of SUMO2/3 expression, it is evident that the observed changes in gene expression and cell growth were indeed caused by depletion of SUMO2/3 conjugated proteins. It has been shown using SUMO1 knockout animals that SUMO1

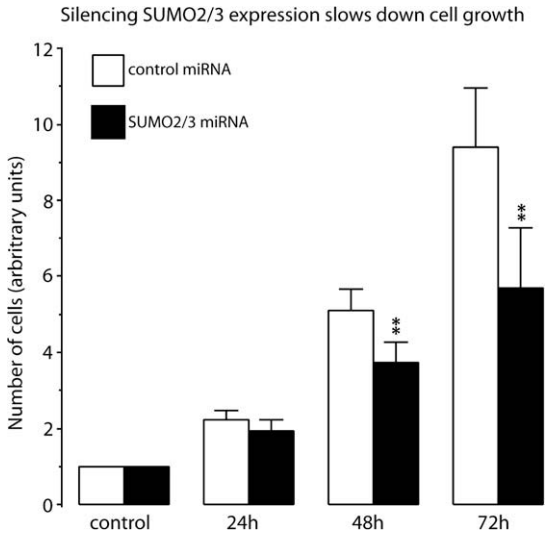

Fig. 4. Silencing of SUMO2/3 expression results in slowing down of cell growth. Stably transfected cells expressing miR-Neg or miR-SUMO2/3 were plated at a density of 7×10^3 cells/well in 96-well plates. On the day of plating (control) and 24, 48, and 72 h after plating changes in the number of cells were evaluated as described in Materials and methods. Cell growth was significantly suppressed in cells expressing miR-SUMO2/3. Data are presented as means ± SD with four independent experiments and 3 samples each/experiment (Student's *t*-test; $^{**}P < 0.01$).

function is dispensable in normal mouse development, and that fibroblasts derived from these animals grow and differentiate as wild-type fibroblasts [11]. It is, therefore, unlikely that silencing SUMO1 expression in cultured cells will influence cell growth as does silencing SUMO2/3 expression.

To date, little information has been published regarding the impact of the individual SUMO paralogues SUMO1, SUMO2 and SUMO3 on cellular functions. It is well established that completely blocking SUMO conjugation by expressing dominant negative Ubc9, the only identified SUMO conjugating enzyme, is lethal for animals and potentially toxic for cells [11]. On the other hand, SUMO1$^{-/-}$ mice of both sexes have been found to be viable and fertile, and it has been concluded that in SUMO1$^{-/-}$ mice most, if not all, SUMO1 functions are compensated for by SUMO2 and SUMO3 [12]. Little attention has been given to elucidating the role of SUMO2/3 conjugation in cellular function. SUMO2/3 conjugation plays a key role in mitosis where it binds to the microtubule motor protein CENP-E essential for kinetochore localization [13], and regulates topoisomerase II [14]. Furthermore, the p66 subunit of DNA polymerase delta has been found to be modified by SUMO3 but not SUMO2 [15]. Taken together, these observations suggest that blocking SUMO2/3 conjugation by silencing its expression will impair cell growth as illustrated in the present study. Moreover, the stably transfected cells expressing SUMO2/3 miRNA that have been developed in this study could be a useful tool for elucidating the role of SUMO2/3 conjugation in normal functioning of the cell and in pathological states associated with a marked increase in levels of SUMO2/3 conjugated proteins.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.013.

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