MicroRNA-mediated Regulation of Ubc9 Expression in Cancer Cells

Fangting Wu,1 Shuomin Zhu,1 Yanna Ding,1 William T. Beck,2 and Yin-Yuan Mo1

Abstract

Purpose: As an E2-conjugating enzyme for sumoylation, Ubc9 plays a critical role in sumoylation-mediated cellular pathways, ultimately impacting cell growth and cancer development. The aim of this study was to investigate the regulation of Ubc9 in cancer cells.

Experimental Design: Immunohistochemistry and Western blot were used to determine Ubc9 expression in paraffin-embedded tumor tissue and frozen specimens of the matched tumors from the same patient, respectively. To establish the causal relationship between miR-30e and Ubc9 expression, we overexpressed miR-30e and then determined the resultant effects on Ubc9 expression. To determine whether miR-30e directly targets Ubc9, we did luciferase assays using luciferase reporters carrying the 3′-untranslated region (3′-UTR) of the Ubc9 gene.

Results: We found that Ubc9 is up-regulated in breast, head and neck, and lung cancer specimens. In addition, an examination of eight pairs of matched breast tumor specimens by Western blot analysis revealed that, on average, the level of Ubc9 is 5.7-fold higher in tumor than in the matched normal breast tissue. Of interest, we present evidence that Ubc9 is subjected to post-transcriptional regulation by microRNA, and the miR-30 family, such as miR-30e, negatively regulates Ubc9 expression. In contrast to Ubc9, miR-30e is underexpressed in tumors. Moreover, ectopic expression of miR-30e suppresses cell growth, which can be partially reversed by Ubc9. Finally, using luciferase-Ubc9-3′-UTR reporters, we show that Ubc9 is a direct target for miR-30e by interactions with the putative miR-30e binding sites.

Conclusion: These results provide new insight into regulation of Ubc9 in cancer cells.

Posttranslational modifications play an important role in protein function through the regulation of their activity, turnover and localization and/or interactions. One such modification involves the covalent attachment of the small ubiquitin-related polypeptide SUMO (small ubiquitin-like modifier) to different cellular protein substrates (1, 2). Although SUMO conjugation or sumoylation is similar to ubiquitination in structure, conjugation process, and attachment to target proteins, the biological consequences of these two pathways can be quite distinct. Unlike ubiquitination that normally targets proteins for degradation through proteasome pathways, sumoylation has been implicated in the regulation of protein stability, protein-protein interactions, transcriptional activity, and subcellular localization (3).

Ubc9 is an E2-conjugating enzyme essential for sumoylation, and it transfers the activated SUMO to protein substrates (4, 5).

In particular, Ubc9 has been shown to play a key role in nuclear trafficking (6, 7), transcriptional regulation (8–11), and protein stability (12–15) through regulation of the sumoylation machinery. In addition, recent evidence indicates that Ubc9 is a multifunctional protein that can exert its functions independent of sumoylation (16–18). In the past years, we have learned that many important proteins, including tumor suppressors and oncoproteins, as well as the cell cycle and proliferation-related proteins, are targets for sumoylation or interact with Ubc9; their expression or their activity is regulated by Ubc9 (9, 19). Thus, alterations of Ubc9 could ultimately have an impact on cell growth and cancer development. Indeed, our previous studies indicate that Ubc9 plays a role in tumorigenesis and drug responsiveness (20, 21).

Ubc9 is a single-copy gene and is ubiquitously expressed in all human organs and tissues. However, levels of Ubc9 vary in different organs or tissues (22). In tumors Ubc9 is frequently up-regulated. We and several other groups have reported up-regulation of Ubc9 in various tumors. For example, Ubc9 is up-regulated in lung adenocarcinoma, as detected by microarray analysis (23). By semiquantitative reverse transcription-PCR (RT-PCR) analysis we detected overexpression of Ubc9 in ovarian carcinoma compared with the matched normal ovarian epithelium (20). Moreover, Ubc9 is the most highly expressed protein in protein extracts from melanoma-infiltrated lymph nodes identified by antibody array technology (24). However, little is known about the molecular mechanism of Ubc9 up-regulation in cancer. In this study, we examine Ubc9 regulation and present evidence that Ubc9 expression is subjected to microRNA regulation at the posttranscriptional level, where miR-30e negatively regulates Ubc9 expression by translation...
Suppression of Ubc9 by miR-30

Translational Relevance

As an essential E2-conjugating enzyme for sumoylation, Ubc9 plays a central role in sumoylation-mediated cellular pathways. Available evidence suggests that Ubc9 is a tumor-promoting factor. Little is known, however, about the regulation of Ubc9. In this study, we first show that Ubc9 is overexpressed in several types of cancers, highlighting its clinical significance. We then investigate the underlying mechanism of Ubc9 up-regulation and show that miRNAs such as miR-30e are able to specifically silence Ubc9, thus providing new insight into Ubc9 regulation. Therefore, our study suggests that Ubc9 may serve as a potential biomarker for diagnosis or prognosis as well as a therapeutic target for cancer intervention.

Repression. Therefore, these findings might provide a molecular explanation as to why Ubc9 is frequently overexpressed in tumors.

Materials and Methods

Reagents. Primary monoclonal Ubc9 antibody from BD Biosciences or custom-made polyclonal Ubc9 antibody was used in both Western blot and immunofluorescence microscopy. Anti–SUMO-1 antibody for Western blot and secondary antibodies conjugated with Alex 566 used for immunofluorescence staining were obtained from Invitrogen. Secondary antibodies conjugated with IRDye 800CW were purchased from LI-COR Biosciences. PCR primers were purchased from Sigma-Genosys.

Cell culture. All cell lines were purchased from the American Type Culture Collection. Both HeLa and 293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma-Aldrich). All media contained 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were incubated at 37°C and supplemented with 5% CO2 in the humidified chamber.

Transfection. HeLa cells were transfected using DNAfection reagent (Applied Biological Materials) following the manufacturer’s protocol. In brief, cells were seeded at 40% confluence in a 12- or 6-well plate and then transfected with 1 or 3 μg of miRNA expression vectors in serum-free medium the following day when the cells reached about 70% confluence. The serum-free media were replaced by normal growth media after 15 h of transfection. The 293T cells were transfected using the calcium phosphate method, as described previously (25). The transfected cells were grown overnight before they were harvested and lysed for luciferase assay or extraction of protein or RNA.

Plasmids. pCMV-Ubc9 was described previously (26). To construct pre-miRNA expression vectors, we first amplified ∼0.5 kb DNA fragment covering a pre-miRNA, using genomic DNA from a healthy blood donor as a template. PCR reactions were done using the high-fidelity Phusion enzyme (New England Biolabs) and the corresponding specific primers:

miR-200c-5.1 (sense) 5’- AAATCGGTGTGGTCCGGGC.  
miR-200c-Not1-3.1 (antisense) 5’-GGGTGCAAGCTGGATCG.  

The amplified fragment was first cloned into a PCR cloning vector and subsequently cloned a pmCV vector or lentiviral vector (pCDH-CMV-MCS-EF1-copGFP from System Biosciences) at the EcoRI and Not1 sites. Expression of the mature microRNAs was verified by TaqMan real-time PCR kit (Applied Biosystems) or QuantmiR kit (System Biosciences).

The luciferase-untranslated region (UTR) reporter plasmid (pluc-Ubc9-3’-UTR) was constructed by introducing the Ubc9 3’-UTR carrying putative microRNA binding sites into pGL3 control vector (Promega). Thus, we amplified the Ubc9 3’-UTR sequence from MCF10a cDNA by PCR using the following primers:

Ubc9-UTR-5.1 (sense), 5’-GCGGCCGCACCGCACCCATCCGCAATCTCAAG.  
Ubc9-UTR-Not1-3.1 (antisense) 5’-GCGGCCGCACCGCACCCATCCGCAATCG.  

For the construction of deletion mutant pluc-Ubc9-3’-UTR clones, we used primers Ubc9-UTR-5.2 (see below) and Ubc9-UTR-Not1-3.1, resulting in pluc-Ubc9-3’-UTR-d1 where the first putative miR-30e binding site was deleted. We then used primers Ubc9-UTR-5.1 and Ubc9-UTR-Not1-3.6 (see below) to generate pluc-Ubc9-3’-UTR-d2 where the second putative miR-30e binding site was eliminated. Finally, to delete both sites, we used primers Ubc9-UTR-5.2 and Ubc9-UTR-Not1-3.6 to generate pluc-Ubc9-3’-UTR-d1-d2.

Ubc9-UTR-5.2 (sense), 5’-ACATTTTGACAAATCTCAAG.  
Ubc9-UTR-Not1-3.6 (antisense), 5’-GCGGCCGCACCAACCAAGGAAGC-CCATATGAC.  

The PCR product was also first cloned into a PCR cloning vector and then subcloned into a modified pGL3 control vector where an EcoRI site and a Not1 site were introduced into the Xba1 site so that an insert could be unidirectionally cloned downstream of the luciferase gene. All the amplified products were verified by DNA sequencing before cloning into the final destination vector.

Luciferase assay. Luciferase assays were carried out in 293T cells to determine the effect of microRNAs on the activity of Luc-Ubc9-3’-UTR and the deletion mutant constructs. First, cells were transfected with appropriate plasmids in 12-well plates. Then, the cells were harvested and lysed for luciferase assay 24 h after transfection. Luciferase activity was determined by using a luciferase assay kit (Promega) according to the manufacturer’s protocol. β-Galactosidase was used for normalization.

PCR/RT-PCR and real-time RT-PCR. PCR was done to amplify pre-microRNA sequences or the Ubc9 3’-UTR sequence according to the standard three-step procedure. Annealing temperature varied depending on the primers used. For RT-PCR, we isolated total RNA using Trizol reagent (Invitrogen) per the manufacturer’s protocol and used 1 μg RNA to synthesize cDNA by SuperScriptase III (Invitrogen) with random primers. Finally, the resultant cDNA was used in regular PCR or real-time PCR reactions. To detect Ubc9 mRNA levels, we used the SYBR Green method with primers Ubc9-5.10 and Ubc9-3.10.

Ubc9-5.10 (sense) 5’-CAGGAGAGGAGAAAAGCATGAG.  
Ubc9-3.10 (antisense) 5’-TGGGTTTAAATGTGTGAG.  

To detect mature microRNA expression, we also used Trizol reagent to isolate total RNA, which was then amplified by QuantmiR method (System Biosciences) or TaqMan stem-loop RT-PCR method (27, 28) using specific primer sets and TaqMan probe from Applied Biosystems. Real-time PCR reactions were done in ABI 7500 HT thermal cycler...
according to the manufacturer’s protocol. Average levels of U6, 5s RNA, and β-actin were used as an internal control. The fold-change between vector control and pre-microRNA expression vector was calculated with the 2-ΔΔCt method (27, 28).

Cell growth assay. Cell growth assays were carried out by MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as described previously (29). In brief, cells were seeded in 96-well plates and incubated for various days before adding MTT. Absorbance at 570 nm was measured in the multiwell plate reader (Thermo Scientific). The relative values were calculated by expressing the first day data as 1.

Western blot. Cells were harvested and protein was extracted 2 d after transfection as previously described (30). Protein concentration was determined by protein assay kit (Bio-Rad) and samples were separated in 12% SDS polyacrylamide gels. Signals were revealed by a secondary antibody labeled with IRDye 800CW and the signal intensity was determined by Odyssey Infrared Imaging System (LI-COR Biosciences).

Immunofluorescence microscopy. To detect miR-30e-mediated suppression of Ubc9 by immunofluorescence staining, HeLa cells were first transfected with vector control or miR-30e expression vector and then transferred to coated coverslips in a 12-well plate. After overnight growth, the cells were fixed with 3% paraformaldehyde (Sigma-Aldrich) and permeabilized by 80% cold methanol, followed by washing with PBS. Coverslips were then incubated with 3% bovine serum albumin in PBS for 10 min at room temperature. Primary antibodies against Ubc9 in PBS plus 0.1% Tween 20 were then added and incubated for 1 h at room temperature. After three washes with PBS, the cells were incubated with a fluorescence-conjugated secondary antibody in the

---

Fig. 1. Expression of Ubc9 in the matched tumor specimens. A, paraffin-embedded specimens were stained by immunohistochemistry using anti-Ubc9 antibody as described in Materials and Methods. Shown here are representatives of three cases for each type. Note strong Ubc9 signals in tumors compared with the matched normal tissues. B, representative gels for Ubc9 levels in freshly frozen samples of matched breast tumor tissue, as detected by Western blot. Also shown are Ubc9 levels in tumor (T) versus normal tissue (N) after normalization with β-actin. C, relative expression levels of Ubc9 between tumors and matched normal breast tissues (n = 8) derived from means of two experiments. The Ubc9 levels were first normalized with β-actin and were then compared with each other; the relative value of normal tissues was set at 1.

---
For nuclear staining, the cells were subsequently stained in 0.5 μg/mL Hoechst dye (Sigma-Aldrich) for 5 min before examinations under a fluorescence microscope.

Immunohistochemistry. Paraffin-embedded tissue was pretreated at 65°C for 2 h, followed by deparaffinization using standard procedures. Antigen retrieval was carried out in antigen retrieval solution (10 mmol/L Tris, 1 mmol/L EDTA, pH 9.0) before applying the primary Ubc9 antibody. Thereafter, slides were incubated for 2 h at room temperature followed by extensive washes with PBS plus 0.1% Tween 20 and further incubated for 1 h at room temperature with the secondary antibody conjugated with horseradish peroxidase. Horseradish peroxidase activity was detected using Histostain Plus kit (Invitrogen) according to the manufacturer’s instruction. Finally, sections were counterstained with hematoxylin and mounted.

Patient specimens. Matched breast, head and neck, and lung tumor specimens were obtained from the Cooperative Human Tissue Network Midwestern Division (Columbus, OH) or the SIU SimmonsCooper Cancer Institute Tissue Bank. The use of these specimens in this study was approved by the Institutional Review Board of Southern Illinois University School of Medicine. Where it was necessary, total protein was isolated in protein extraction buffer using a tissue homogenizer as described previously (30) and protein concentration was determined by protein assays kit (Bio-Rad).

Statistical analysis. Statistical analysis of data was done using the Student’s t test. Differences with P < 0.05 are considered significant.

Results

Ubc9 is up-regulated in tumor specimens. We have previously shown that overexpression of Ubc9 enhances tumor growth in the xenograft mouse model (20). To determine the clinical relevance of this finding, we examined the expression levels of Ubc9 in the matched patient specimens including breast, head and neck, and lung by immunohistochemistry. From four cases for each of the three types of cancer, we found that the Ubc9 level was higher in tumor than in the matched normal tissues. Shown in Fig. 1A are representative fields for each of three cases where the tumor specimens revealed intensive Ubc9 staining, concentrated in the nucleus. However, the matched normal tissues displayed very weak staining, suggesting that Ubc9 is overexpressed in tumors.

To better quantitate the Ubc9 expression in tumor specimens, we examined eight pairs of frozen samples from the matched breast tumors by Western blot analysis. We found that Ubc9 was up-regulated in all eight cases (Fig. 1B). On average, breast tumors expressed 5.7-fold higher than the matched normal tissues (Fig. 1C), which is consistent with the immunohistochemistry data from paraffin-embedded samples (Fig. 1A).

Table 1. Putative microRNAs targeting Ubc9

<table>
<thead>
<tr>
<th>Name</th>
<th>BS</th>
<th>Predicted by</th>
</tr>
</thead>
<tbody>
<tr>
<td>mir-30e</td>
<td>2</td>
<td>T, M, P, R</td>
</tr>
<tr>
<td>mir-30c</td>
<td>2</td>
<td>T, M, P, R</td>
</tr>
<tr>
<td>mir-30a</td>
<td>1</td>
<td>T, M, P, R</td>
</tr>
<tr>
<td>mir-30b</td>
<td>1</td>
<td>T, M, P, R</td>
</tr>
<tr>
<td>mir-30d</td>
<td>1</td>
<td>T, M, P, R</td>
</tr>
<tr>
<td>mir-188</td>
<td>1</td>
<td>T, M, P, R</td>
</tr>
<tr>
<td>mir-200c</td>
<td>1</td>
<td>T, M, P, R</td>
</tr>
<tr>
<td>mir-195</td>
<td>1</td>
<td>M, P, R</td>
</tr>
<tr>
<td>mir-548a</td>
<td>1</td>
<td>M, R</td>
</tr>
<tr>
<td>mir-450b</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-361</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-10b</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-376c</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-200b</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-877</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-802</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-49</td>
<td>1</td>
<td>M</td>
</tr>
<tr>
<td>mir-652</td>
<td>1</td>
<td>M</td>
</tr>
</tbody>
</table>

Abbreviations: BS, binding site; T, Targetscan4; M, miRBase Target5; P, PicTar; R, miRanda.

Suppression of Ubc9 by miR-30. To better understand the up-regulation of Ubc9 in tumors, we first examined the potential transcriptional regulation. Therefore, we cloned the putative Ubc9 promoter into a luciferase reporter plasmid and then introduced into several cell lines that expressed different levels of Ubc9. However, no significant difference in luciferase activity was seen, suggesting that transcriptional regulation may not be
important for the observed difference of Ubc9 expression. Furthermore, we found that epigenetic factors such as methylation and acetylation did not seem to play a significant role in Ubc9 expression because the demethylation agents such as 5-Aza-deoxycytidine or histone deacetylase inhibitors such as trichostatin A had only a marginal effect on Ubc9 expression (not shown).

Therefore, we investigated the posttranscriptional regulation of Ubc9. Newly discovered small noncoding RNAs, microRNAs, have been shown to silence protein-coding genes in a variety of organisms including mammals by translation repression or mRNA degradation (31–33). MicroRNAs are believed to target mRNAs by partial sequence homology to the 3′-UTR of the target gene. Thus, we searched for potential microRNAs that might play a role in the regulation of Ubc9 using several commonly cited microRNA target prediction programs such as TargetScan4 (34), miRBase Target5, PicTar (35), and miRanda (36).

These four prediction programs all identified seven putative microRNAs (miR-30a to miR-30e, miR-188, and miR-200c; Table 1). In addition, some other microRNAs were identified by either two or three of these programs.

We focused on the miR-30 family and other two microRNAs, miR-188 and miR-200c. Among the miR-30 family, whereas both miR-30e and miR-30c target Ubc9 at two potential binding sites (Table 1), the rest of the family have only one site. Thus, we chose miR-30e and miR-30c to represent the miR-30 family. We first confirmed that ectopic expression generated mature microRNAs by real-time RT-PCR (Fig. 2A) and then determined the effect of each microRNA on Ubc9 expression.

Western blot analysis revealed that both miR-30e and miR-30c suppressed Ubc9 expression at the protein level (Fig. 2B). In contrast, we detected no significant effect on Ubc9 expression for miR-188 and miR-200c (Fig. 2B), highlighting the specificity of this suppression although both miR-188 and miR-200c are also predicted to target Ubc9. To determine whether miR-30e and miR-30c affect the Ubc9 mRNA level, we carried out real-time RT-PCR analysis for the same cells transfected with miR-30e and miR-30c, and found that these two microRNAs had no effect on the Ubc9 mRNA level (Fig. 2C), suggesting that they regulate Ubc9 expression mainly through translation repression.

To further confirm the suppressive effect of miR-30e on Ubc9 expression, we introduced the miR-30e expression vector into HeLa cells and then immunostained with Ubc9-specific antibody. As shown in Fig. 3, ectopic expression of miR-30e remarkably suppressed Ubc9 expression because the red signal was clearly reduced (upper panels). In contrast, the vector control (pCDH) had no effect on Ubc9 (Fig. 3, bottom panels), further supporting the notion that Ubc9 is a target for miR-30e.

**Effect of miR-30e on cell growth.** Given that Ubc9 is an E2 enzyme for sumoylation, suppression of Ubc9 by miR-30e would inhibit sumoylation. To test this hypothesis, we determined the effect of miR-30e on the overall levels of protein sumoylation using SUMO-1 antibody. As expected, miR-30e reduced total protein sumoylation (Fig. 4A) as compared with vector control. In particular, we found that miR-30e suppressed the level of sumoylated RanGAP1 (Fig. 4A) because RanGAP1 is a major SUMO substrate (37). In agreement with this, the free SUMO-1 level was higher in miR-30e–transfected cells than in vector control (Fig. 4A), presumably because reduction of overall sumoylation leads to the accumulation of the free SUMO-1.

To further determine the effect of suppression of Ubc9 by miR-30e on cellular processes, we examined the cell growth for miR-30e–transfected cells because we have previously reported that suppression of Ubc9 causes cell growth inhibition (29). As expected, we found that miR-30e caused growth inhibition in a time-dependent manner. For example, on the first two days,

![Suppression of Ubc9 by miR-30e by immunofluorescence microscopy. HeLa cells were transfected with miR-30e or vector (pCDH). One day later, the transfected cells were harvested and seeded on glass coverslips in a 12-well plate, and grown for an additional day before immunostaining with anti-Ubc9 antibody (red). Note reduction of Ubc9 expression (red signal) in miR-30e–transfected cells (green cells, upper panels). Merge 1, green plus red; Merge 2, green plus blue (nuclear staining).](http://microrna.sanger.ac.uk/targets/v5/)

---

3 http://microrna.sanger.ac.uk/targets/v5/
4 http://microrna.org
there was no significant difference between vector and miR-30e. However, on days 3 and 4, miR-30e inhibited cell growth by almost 30% compared with the vector control (Fig. 4B). Of interest, this growth inhibition was partially reversed by overexpression of Ubc9 (Fig. 4B), suggesting that Ubc9 is an important target for miR-30e. In addition, miR-30e was able to sensitize cells to the anticancer agent topotecan (Fig. 4C), which is consistent with our previous finding that suppression of Ubc9 by Gam1 can increase the sensitivity to this agent (29). These results suggest that as a negative regulator of Ubc9, miR-30e plays a role in cell growth and drug response, in part through suppression of Ubc9 expression.

**Ubc9 is a direct target for miR-30e.** To determine whether miR-30e directly targets Ubc9, we cloned the Ubc9-3′-UTR (Fig. 5A) into pGL3 control vector, resulting in pLuc-Ubc9-3′-UTR (Fig. 5B). After transfection of 293T cells with this reporter construct along with miR-30e or miR-30c, we found that both miR-30e and miR-30c suppressed the luciferase activity by about 50% compared with the vector control (Fig. 5B), suggesting that Ubc9 is a direct target for these two microRNAs. As shown Fig. 5A, there are two potential microRNA binding sites in the 3′-UTR of Ubc9. To determine whether any of these two binding sites is important for microRNA suppression, we deleted the first (pLuc-Ubc9-3′-UTR-d1) or second binding site (pLuc-Ubc9-3′-UTR-d2) or both (pLuc-Ubc9-3′-UTR-d1-d2). As shown in Fig. 5C, deletion of the first binding site impaired the suppression of luciferase activity, but we still detected about 30% suppression; deletion of the second binding site had a similar effect. When both sites were deleted, however, miR-30e–mediated suppression of luciferase activity was abolished. These results suggest that both binding sites are critical for microRNA regulation.

Finally, to investigate the clinical relevance of miR-30e–mediated regulation of Ubc9, we amplified the entire UTR region from eight pairs of tumors by RT-PCR so that we were able to determine whether there is any mutation or deletion in the Ubc9-3′-UTR, which could be responsible for the observed Ubc9 overexpression. DNA sequencing analysis of the PCR products indicated that both normal and tumor cells expressed wild-type sequences in all eight cases. Then we examined the level of miR-30e in these samples. Of interest, we found that the level of miR-30e was lower in tumor than in normal tissue in seven of them (Fig. 5D and E), suggesting that Ubc9 overexpression in tumors could be in part due to down-regulation of miR-30e.

**Discussion**

Despite its ubiquitous expression in normal tissues, we report here that Ubc9 is deregulated in several types of cancers including breast, head and neck, and lung cancer. In particular, in breast cancer the Ubc9 level is >5-fold higher than the matched normal tissues. Little was known, however, about the basis of Ubc9 deregulation in tumors. Although we cannot exclude the possibility of transcriptional regulation of Ubc9, our study indicates that the miR-30 family, particularly miR-30e, plays a regulatory role in Ubc9 expression and Ubc9 is a direct target for miR-30e.

Up-regulation of Ubc9 expression in tumor specimens, as reported in this study as well as other studies (20, 23, 24), has clinical implications. We have previously shown that although suppression of Ubc9 function by the dominant negative Ubc9 inhibits, the ectopic expression of Ubc9 enhances tumor growth in the animal model (20), suggesting that Ubc9 plays a causal role in tumorigenesis. This is likely due to the fact that Ubc9 is an essential enzyme for sumoylation, and numerous important proteins, such as tumor suppressors or oncoproteins,
are substrates for sumoylation. Thus, deregulation of Ubc9 could lead to alterations of sumoylation pathways, ultimately impacting cell growth and cancer development. In this regard, Ubc9-mediated sumoylation is similar to ubiquitination. It is well known that deregulation of ubiquitination pathways could play a key role in cancer development (38, 39) because the timely and irreversible degradation of critical regulators is essential for normal cellular function and turnover of several regulatory proteins resulting from targeted destruction via ubiquitination. Similarly, Ubc9-mediated sumoylation has been shown to play a role in diverse cellular pathways (3), Therefore, cancer cells may have evolved mechanisms to target the basic functions of these protein modification pathways. One such mechanism could involve microRNA regulation of Ubc9 at the posttranscriptional level, leading to its up-regulation in tumors.

Four lines of evidence support the notion that Ubc9 is a direct target for miR-30e. First, miR-30e specifically suppresses Ubc9 expression, as shown by both Western blot and immunofluorescence staining. Second, ectopic expression of miR-30e inhibits overall protein sumoylation. Third, miR-30e also causes cell growth inhibition that can be attenuated by overexpression of Ubc9. Fourth, analyses of the luciferase reporter carrying the Ubc9 3’-UTR indicate that miR-30e directly interacts with this sequence and the putative miR-30e binding sites are essential for miR-30e regulation.

MicroRNAs are endogenous small noncoding RNAs that are known to posttranscriptionally regulate gene expression (31, 32). Aberrant expression of microRNAs has been reported in many types of tumors because they may function as oncogenes or tumor suppressor genes. Whereas oncogenic microRNAs are often up-regulated (40–42), tumor suppressive microRNAs are often down-regulated in cancer (43–45). Little is known regarding the role of miR-30e. Because ectopic expression of miR-30e causes cell growth inhibition, we suggest that miR-30e is a tumor suppressor gene possibly by suppression of tumor-promoting factors such as Ubc9. Down-regulation of miR-30e in the matched breast tumor specimens supports this notion. Therefore, understanding how miR-30e is expressed in cancer will provide further insight into Ubc9 regulation. As a result, this knowledge will aid in the identification of novel targets in sumoylation-mediated cellular pathways.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

---

**Fig. 5.** Ubc9 is a direct target for miR-30e. The 293T cells were transfected with pLuc-Ubc9-3’-UTR or its deletion constructs. At the same time, the cells were cotransfected with pCDH (V) or miR-30e/pCDH (30e) or miR-30c/pCDH (30c) and then harvested for luciferase assays 24 h later, as detailed in Materials and Methods. A, schematic description of pLuc-Ubc9 3’-UTR with putative binding sites for miR-30e (30e) and miR-30c (30c). B, suppression of pLuc-Ubc9 3’-UTR luciferase activity by miR-30e and miR-30c. C, deletion analysis of the pLuc-Ubc9 3’-UTR. The luciferase activity for each deletion construct was compared between pCDH (100%) and miR-30e. Values are averages of three separate experiments ± SE. D and E, expression expression of miR-30e in matched normal breast tissue (N) and breast tumor (T) as CT value (D) or as relative expression of miR-30e (E) by normalizing normal tissue as 1. Each pair of samples is connected by a line in D.
References


Downloaded from clincancerres.aacrjournals.org on April 14, 2017. © 2009 American Association for Cancer Research.
MicroRNA-mediated Regulation of Ubc9 Expression in Cancer Cells

Fangting Wu, Shuomin Zhu, Yanna Ding, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/15/5/1550

Cited articles
This article cites 45 articles, 26 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/15/5/1550.full.html#ref-list-1

Citing articles
This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/15/5/1550.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.