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Transcription factor FOXA2-centered transcriptional regulation network in non-small cell lung cancer

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ABSTRACT

Lung cancer is the leading cause of cancer-mediated death. Although various therapeutic approaches are used for lung cancer treatment, these mainly target the tumor suppressor p53 transcription factor, which is involved in apoptosis and cell cycle arrest. However, p53-targeted therapies have limited application in lung cancer, since p53 is found to be mutated in more than half of lung cancers. In this study, we propose tumor suppressor FOXA2 as an alternative target protein for therapies against lung cancer and reveal a possible FOXA2-centered transcriptional regulation network by identifying new target genes and binding partners of FOXA2 by using various screening techniques. The genes encoding Glu/Asp-rich carboxy-terminal domain 2 (CITED2), nuclear receptor subfamily 0, group B, member 2 (NR0B2), cell adhesion molecule 1 (CADM1) and BCL2-associated X protein (BAX) were identified as putative target genes of FOXA2. Additionally, the proteins including highly similar to heat shock protein HSP 90-beta (HSP90A), heat shock 70 kDa protein 1A variant (HSPA1A), histone deacetylase 1 (HDAC1) and HDAC3 were identified as novel interacting partners of FOXA2. Moreover, we showed that FOXA2-dependent promoter activation of BAX and p21 genes is significantly reduced via physical interactions between the identified binding partners and FOXA2. These results provide opportunities to understand the FOXA2-centered transcriptional regulation network and novel therapeutic targets to modulate this network in p53-deficient lung cancer.

1. Introduction

Lung cancer is known as the leading cause of cancer-mediated death, and ~80% of lung cancers is identified as non-small cell lung cancer (NSCLC) histologically [1]. Since NSCLC has high proliferative and metastatic activities, its prognosis is very poor. Although many therapeutic approaches for NSCLC including radiation and chemical reagents target tumor suppressor p53 to activate a p53-centered transcriptional regulation network [2], there is a growing need to identify another tumor suppressor protein as an alternative therapeutic target due to the existence of p53 mutations in more than 50% of lung cancers [3,4]. Therefore, the investigation of other tumor suppressor proteins and their associated transcriptional regulation networks, including their target genes and interaction partners, may allow the design of therapies to target lung cancers regardless of their p53 status.

Forkhead box transcription factor A2 (FOXA2) is a member of the forkhead box gene superfamily [5]. It has crucial roles not only in embryonic development, including the formation of node, nervous system, and endoderm-derived structures [6,7], but also in postnatal development via regulation of normal bile acid and lipid homeostasis [8,9]. In lung tissue, FOXA2 is highly expressed in type II pneumocytes and the bronchioloalveolar portal and modulates the transcription of lung-specific genes including Titf1, Sftpb, and Scgblal, which have important roles in lung homeostasis and morphogenesis [10–13]. In contrast to normal lung tissue, it has been reported that FOXA2 expression is downregulated in a large proportion of lung cancer cell lines [14,15], implying that FOXA2 is a candidate suppressor of lung cancer. Indeed, the NCI-H358 NSCLC cell line shows limited proliferation rates and increased apoptotic cell death when FOXA2 expression is introduced [15]. Moreover, FOXA2-dependent down-regulation of SLUG transcription leads to suppression of tumor metastasis via inhibition of invasion and the epithelial-to-mesenchymal transition in human lung cancer cells [16]. However, it has not been fully elucidated which genes are targeted by FOXA2 or how and when FOXA2 interacts with binding

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proteins to regulate gene transcription in the lung cancer environment.

In this study, we identified several FOXA2 target genes with potential tumor suppressive functions and FOXA2 binding proteins using DNA-protein interaction- and protein–protein interaction-based analyses. Using binding proteins and target gene promoters, we revealed that FOXA2 transcriptional activation is specifically regulated in cells of the NSCLC cell line H1299. This study suggests that the FOXA2-centered transcriptional regulation network includes targets that may be considered for the design of novel therapeutic approaches for p53-deficient lung cancers.

2. Materials and methods

2.1. Cell culture and transfection

Human non-small cell lung cancer cell line H1299 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (50 units/ml; Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. Transient transfection with different plasmid DNA was performed by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.2. Chromatin immunoprecipitation (ChIP)

A ChIP assay was performed following the protocol provided by Millipore (Temecula, CA). Briefly, the TSA-treated H1299 cells were cross-linked with 1% paraformaldehyde (#15710, Electron Microscopy Sciences, Hatfield, PA) in PBS for 10 min at 37 °C. The cells were then washed with ice-cold PBS and resuspended in 200 μl of SDS-sample buffer containing protease inhibitor mixture. The suspension was sonicated three times for 10 s with a 1-min cooling period on ice, after which it was precleared with 20 μl of protein A/G-agarose beads blocked with sonicated salmon sperm DNA for 30 min at 4 °C. The beads were then removed, after which the chromatin solution of each experimental group was immunoprecipitated overnight with anti-FOXA2 at 4 °C followed by incubation with 50 μl of protein A-agarose beads (Millipore) for an additional hour at 4 °C. The immune complexes were eluted with 100 μl of elution buffer (1% SDS and 0.1 M $NaHCO_3$), and formaldehyde cross-links were reversed by heating at 65 °C for 4 h. Proteinase K (P2308, Sigma) was added to the reaction mixtures and incubated at 45 °C for 1 h. DNA of the immunoprecipitates and control input DNA were purified using the PCR purification kit (Qiagen, Valencia, CA) and then analyzed by quantitative PCR using promoter-specific primers (Supplementary Materials).

2.3. Oncomine database analysis

The use of database is described previously [17]. Data sets with differential expression of possible FOXA2 target genes between normal and lung cancer tissues were selected.

2.4. MALDI-TOF/MS analysis

Proteins separated by one-dimensional gel electrophoresis were in-gel digested. Coomassie Blue-stained sample bands were excised and gel particles were washed with 30% MeOH at room temperature with occasional mixing for 5 min. Then, supernatant methanol was discarded and 100 μl of 100% acetonitrile was treated. After gel drying using speed vacuum centrifuge, the gels were rehydrated in 50 mM ammonium bicarbonate buffer containing 0.1 μg/μl trypsin (SIGMA, sequencing grade). Peptide samples were lyophilized and reconstituted with 0.5% trifluoroacetic acid solution, and the resulting droplets were air-dried. Mass spectra of trypsin-digested proteins were obtained for the mass range of 500–2000 Da using a Voyager-DE™ STR Biospectrometry Workstation mass spectrometer (Applied Biosystems Inc.). Mass spectra were calibrated using known masses of internal standards. Proteins were identified by the sets of proteolytic peptide masses using the Peptide Fingerprint option in Protein Prospector (ver 5.2.2; University of California, San Francisco) (http://prospector2.ucsf.edu/prospector/mshome.htm). The accuracy of MH + mass determination was 0.04% and the possible modification of cysteine residues by acrylamide and methionine oxidation was taken into consideration.

2.5. Immunoprecipitation and Western blotting

Transfected H1299 cells were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet P-40, and 1 mM PMSF. The cell suspensions were incubated on ice for 20 min and centrifuged at 12 000 rpm at 4 °C for 20 min. The supernatants were precleaned with 20 μl of protein A/G agarose bead (50% slurry) and then incubated at 4 °C overnight with 40 μl of fresh protein A/G bead in the presence of GFP or FLAG antibodies. The beads were washed 3 times in PBS, resuspended in SDS sample buffer, and boiled for 10 min. The protein samples were electrophoresed on a 10% or 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman, PROTRAN). The membrane was blocked with 5% skim milk in a solution of 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, and 0.1% Tween 20 and incubated with appropriate dilutions of the primary antibody at room temperature for 3 h. Samples were analyzed by Western blotting using the appropriate antibodies to detect interacting proteins. Monoclonal antibodies against green fluorescence protein (GFP-1814 460) and FLAG-M2 (F3165) antibodies were purchased from Roche Diagnostics and Sigma, respectively.

2.6. Luciferase assay

H1299 cells were cultured in 60 mm dishes and were transfected using Lipofectamine 2000, with the luciferase reporter constructs (0.1 μg), pCMV-β-galactosidase and GFP-FOXA2, or with expression plasmids for FOXA2 binding proteins. The cells were lysed in reporter lysis buffer 48 h after transfection (Promega). Cell lysates were then analyzed with the luciferase reporter assay system, using a glomax luminometer (Promega). Luciferase activities were normalized on the basis of the β-galactosidase activity of the cotransfected vector. All transfection experiments were repeated independently at least three times.

3. Results

3.1. Identification of novel target genes of FOXA2

To identify novel target genes of FOXA2, we collected genes that were differentially expressed following the overexpression of FOXA2 from previously reported microarray data [18] (Fig. 1A). Among these candidates, we identified genes that have FOXA2 binding sequences within their promoter regions (between 0 and −1 kb) by using the MOTIF search program (TRANSFAC database, http://motif.genome.jp); this was followed by an analysis of these identified FOXA2 binding sites by scanning with a FOXA2 position weight matrix (PWM) (Fig. 1B). Finally, we selected genes that were down-regulated in lung cancer by using the Oncomine database (http://www.oncomine.org). This search showed that the expression of CITED2 was decreased in various lung cancer tissue

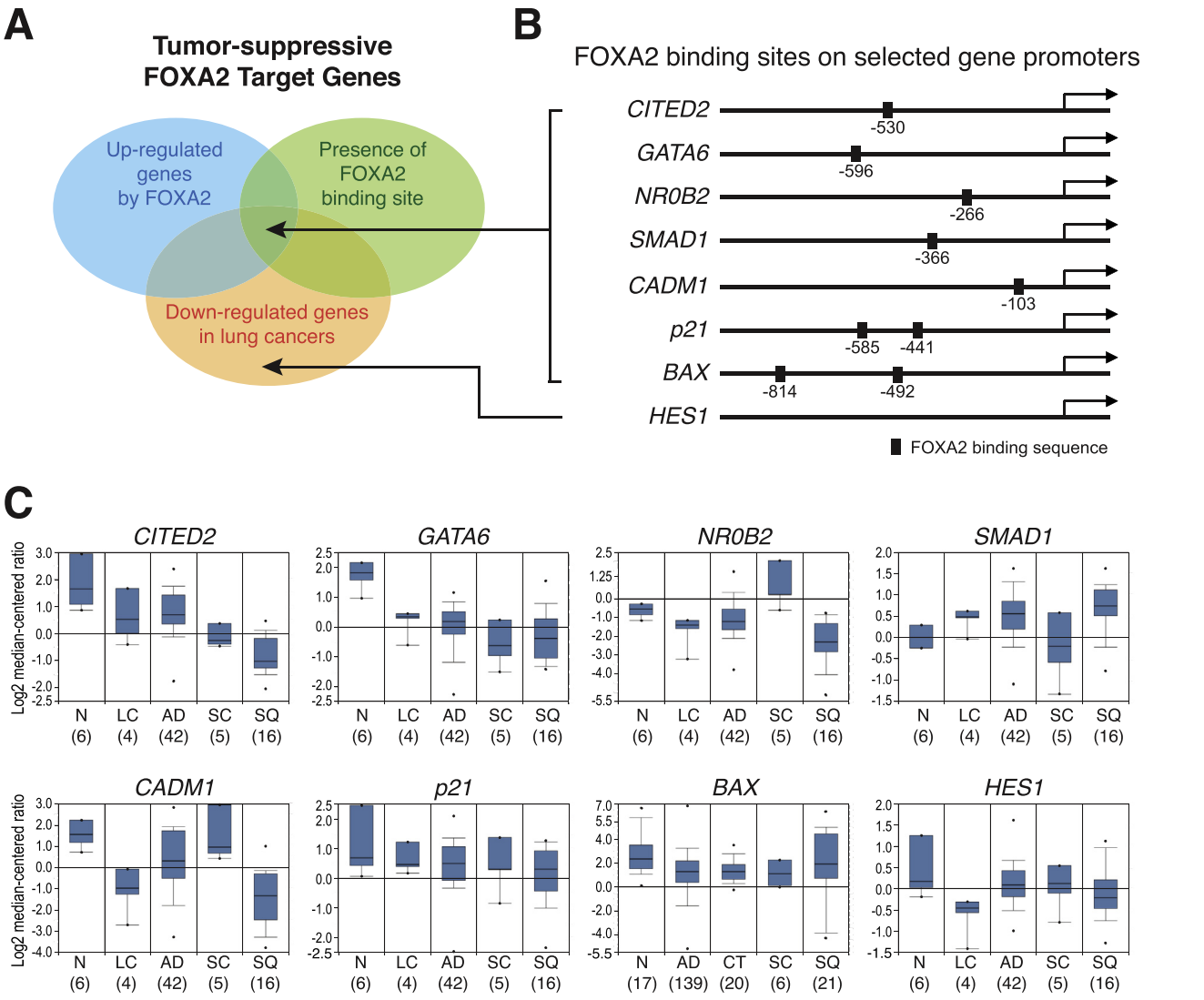

Fig. 1. Identification of putative target genes of FOXA2. (A) Schematic diagram of selection procedure of FOXA2 response genes. Using opened microarray data set, transcriptionally increased target genes mediated by FOXA2 expression were collected. And then, promoter sequences of these genes were analyzed for searching FOXA2 binding elements using bioinformatic programs. Finally, down-regulated genes in lung cancer were selected. (B) Possible FOXA2 binding sites are shown with their distance from the transcription start site (TSS; arrow, +1). (C) Expression levels of selected genes in human normal and lung cancer tissues using Oncomine database. N, normal lung tissues; LC, lung carcinoma; AD, lung adenocarcinoma; SC, small cell lung carcinoma; SQ, squamous cell lung carcinoma; CT, lung carcinoid tumor.

samples, especially those from small cell lung carcinoma and squamous cell lung carcinoma, when compared with matched normal lung tissues (Fig. 1C). Moreover, GATA6 expression was markedly reduced not only in the above cancer tissues but also in lung carcinoma and lung adenocarcinoma. Other genes were also down-regulated in various lung cancers in a cell-type-dependent manner. Although HES1 gene has putative FOXA2 binding sequences in its promoter, we used this gene as a negative control because of its low FOXA2 motif-matching scores. Data analysis revealed seven genes as candidate novel FOXA2 targets, including the p21 gene, which we used as the positive control [19] (Fig. 1B). Candidate genes that were expressed at low levels in lung cancer tissues showed expression levels consistent with that of FOXA2, which was found to be down-regulated in human lung cancer tissues and lost in many non-small cell lung cancer (NSCLC) cell lines, suggesting that recovery of FOXA2 expression may normalize the transcriptional regulation of its target genes and recover their expression.

To determine whether FOXA2 regulates the transcription of the selected genes, cells of the NSCLC cell line H1299 were transiently transfected with FLAG-tagged FOXA2 expression plasmids, and the mRNA levels of candidate genes were analyzed using quantitative real-time PCR. As shown in Fig. 2A, overexpression of FOXA2 induced increased expression levels of the genes CBP/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain 2 (CITED2), nuclear receptor subfamily 0, group B, member 2 (NR0B2), cell adhesion molecule 1 (CADM1), and BCL2-associated X protein (BAX). As we previously reported, the transcription of p21 was significantly induced by FOXA2 expression [19]. However, transcription levels of the genes GATA binding protein 6 (GATA6), SMAD family member 1 (SMAD1), and hes family bHLH transcription factor 1 (HES1) were not significantly increased by FOXA2 overexpression. These in vitro results showed that FOXA2 regulates the transcription of some of the putative target genes that we identified in silico.

We recently found that the expression of FOXA2 protein is specifically induced by trichostatin A (TSA) treatment [19]. Therefore, we examined whether FOXA2 regulates the transcription of its candidate target genes via binding to their promoter regions in vitro, using TSA-treated H1299 cells. We performed a ChIP assay

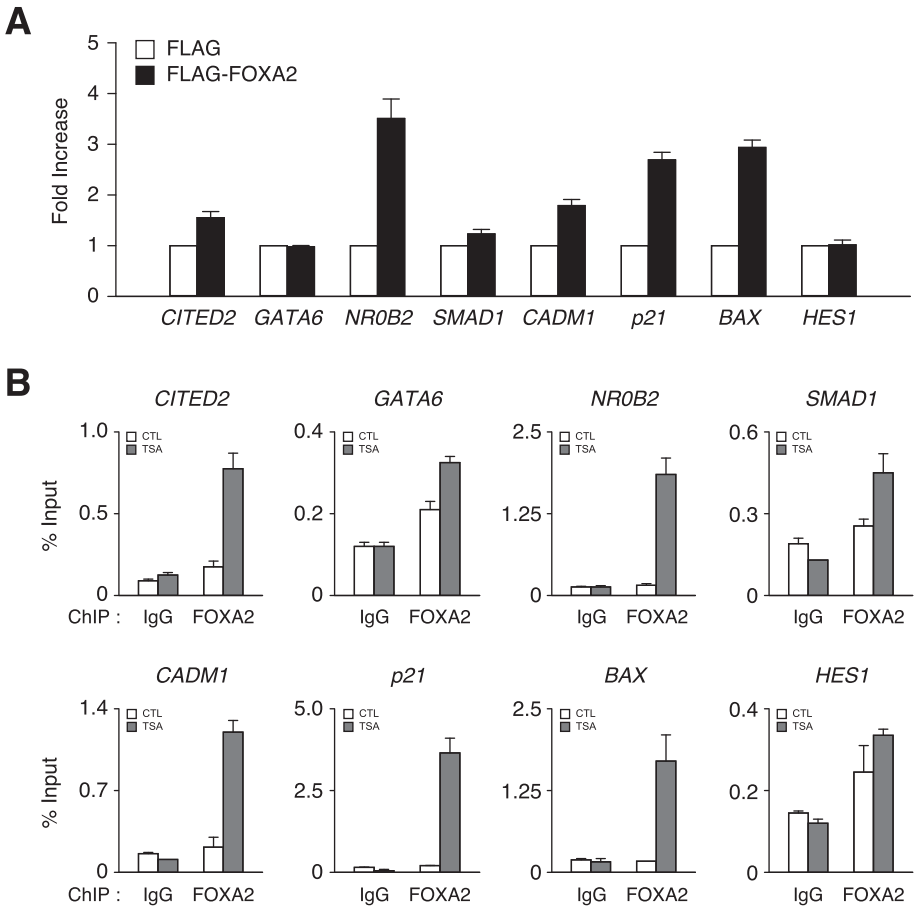

Fig. 2. Transcriptional regulation of FOXA2 for putative target genes. (A) H1299 cells were transfected with FLAG-control vector or FLAG-tagged FOXA2 expression constructs. After 24 h, cells were collected for isolation of total RNA and analyzed mRNA expression of selected genes using reverse transcription and qRT-PCR. Transcription levels of selected genes were normalized by 18SrRNA gene. All data are representative of three independent experiments. (B) Recruitment of FOXA2 to possible target gene promoters in response to TSA treatment. H1299 cells were incubated with TSA for 24 h, and FOXA2 occupancy at the new target gene promoter was analyzed with ChIP assays using anti-FOXA2 antibodies. The precipitated chromatin fragments were analyzed using primers specific to the selected gene promoter as described in Material and methods. All data are the mean ± SEM of three independent experiments performed in triplicate.

with an anti-FOXA2 antibody in H1299 cells that were treated with 1 μM TSA. As shown in Fig. 2B, there was no significant co-precipitation of the selected gene promoters with FOXA2 in control cells; levels were similar to those of IgG-mediated precipitation, which was expected because endogenous FOXA2 is barely expressed in H1299 cells [16,19]. In contrast, the induced FOXA2 protein that was expressed in response to TSA was significantly recruited to the regions of the CITED2, NR0B2, CADM1, and BAX promoters containing FOXA2 binding sequences, whereas FOXA2 was not recruited to its binding sites within the GATA6, SMAD1, and HES1 promoters. Taken together, these results establish the novel target genes of FOXA2.

3.2. Identification of novel binding proteins of FOXA2

To identify interacting proteins of FOXA2, we transfected an expression vector encoding the open reading frame of full-length FOXA2 tagged with FLAG into H1299 cells. Cell lysates were passed through an anti-FLAG M2 affinity column for binding of FLAG-FOXA2 interacting proteins. Affinity purified FLAG-FOXA2 protein and co-purified proteins were subsequently eluted and then run on a 10% SDS-PAGE gel and stained with Coomassie blue (Fig. 3A). Individual bands that were observed only in samples from the FLAG-FOXA2 transfected group were excised. Their identification was attempted by MALDI-TOF/MS (electrospray tandem mass spectrometry) analysis of peptides obtained after trypsin digestion. Among these bands, four bands were unequivocally identified, including that of myosin 9 (NP_002464), which is highly similar to heat shock protein HSP 90-beta (HSP90A) (BAG57821), GRP78 precursor (AAA52614), and heat shock 70 kDa protein 1A variant (HSPA1A) (BAD93055). In addition to identifying these interacting candidates by IP/MALDI-TOF/MS, we further tested for protein interactions between FOXA2 and HDAC family proteins. To confirm the interactions between FOXA2 and its candidate interacting proteins, we selected four candidates including HSP90A, HSPA1A, HDAC1, and HDAC3, and then exogenously expressed each of them together with FOXA2 in HepG2 cells. The whole-cell lysates were immunoprecipitated with anti-FLAG or anti-GFP antibodies to precipitate candidate proteins, and then we performed an immunoblot analysis to identify co-precipitated FOXA2 protein. As shown in Fig. 3B, FOXA2 protein was clearly co-precipitated with the selected candidate proteins in each transfected cell line (lane 2), but no signal was observed in cells transfected with control empty vector (lane 1). These results suggest that the transcriptional activity of FOXA2 could be affected by its physical interactions with its identified binding partners.

3.3. Protein binding-dependent FOXA2 transcriptional activity and the FOXA2-centered transcriptional regulation network

Protein interaction results raised the possibility that different combinations of protein–protein interactions modulate the

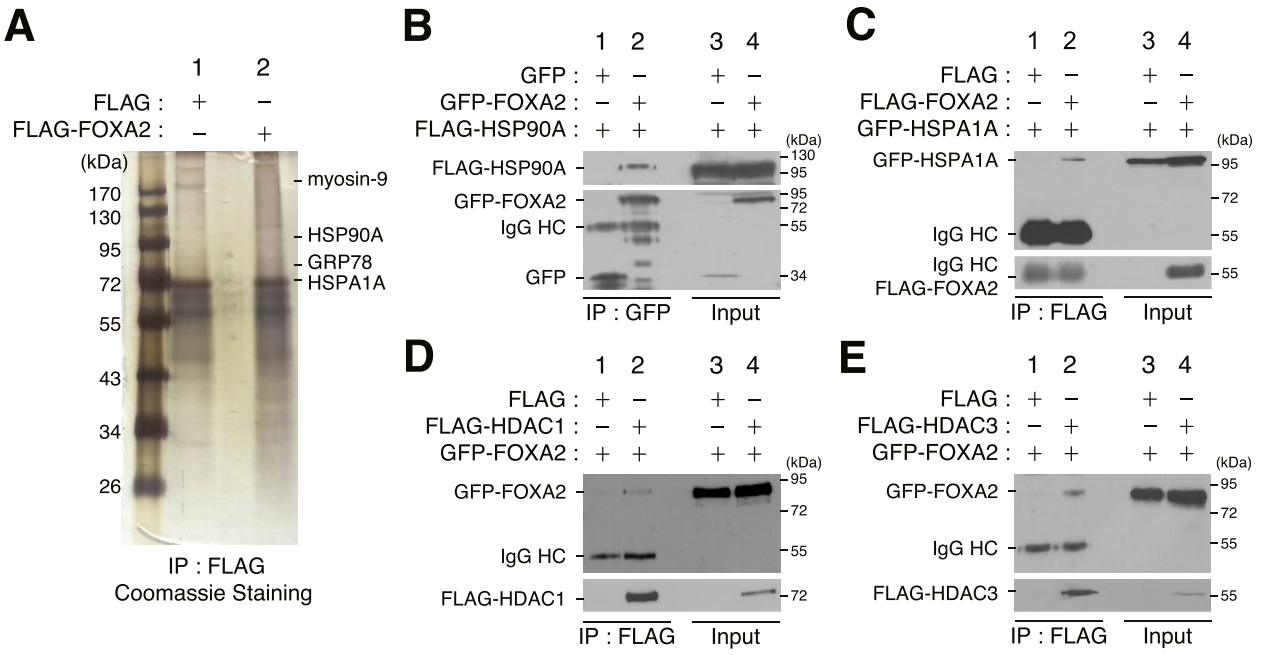

Fig. 3. Identification of novel FOXA2 binding proteins. (A) H1299 cells were transfected with FLAG-empty vector or FLAG-tagged FOXA2 expression constructs. After 24 h, cell lysates were loaded to anti-FLAG M2 affinity column for binding. After stringent bead washing, bound proteins were eluted and boiled for SDS-PAGE gel running. Coomassie blue-stained protein bands that appeared only in FLAG-FOXA2 were subjected to MALDI-TOF/MS analysis. (B–E) Interactions between FOXA2 and novel binding proteins of FOXA2. H1299 cells were transfected with plasmids expressing FOXA2 with (B) HSP90A, (C) HSPA1A, (D) HDAC1, or (E) HDAC3, respectively. Total cell lysates were prepared and immunoprecipitated with anti-FLAG or anti-GFP antibodies. Co-precipitated proteins were detected by Western blot analysis using antibodies as indicated in figure.

transcriptional activity of FOXA2 as coactivators or corepressors. To test this, H1299 cells were co-transfected with FOXA2 together with the HSP90A, HDAC1, or HDAC3 expression plasmids plus a luciferase reporter gene containing a BAX or p21 promoter region, and a luciferase assay was performed. As shown in Fig. 4A and B, transfection of cells with FOXA2 alone significantly transactivated the BAX and p21 promoters, which is consistent with our previous report (lane 2) [19]. The presence of HSP90A, HDAC1, or HDAC3 without FOXA2 expression did not change the promoter activities of FOXA2 target genes (lane 3). However, the enhancement of luciferase activity by FOXA2 transactivation was repressed by HSP90A, HDAC1, or HDAC3 overexpression (lane 4). These results showed that the novel FOXA2 binding proteins we identified inhibited the transcriptional activities of FOXA2 through their protein–protein interactions and indicated the existence of a FOXA2-centered transcriptional regulation network involving newly identified target genes and binding proteins of FOXA2, which may include targets for novel therapeutic approaches for lung cancer.

4. Discussion

The therapeutic approaches available for p53-mutated or deficient lung cancers are limited. In this study, we found that the tumor suppressor FOXA2 might be effective as an alternative therapeutic target to p53. We also identified novel target genes encoding potential tumor suppressive proteins and novel binding partners of FOXA2 to build a FOXA2-centered transcriptional regulation network. Transcript levels of the CITED2, NR0B2, CADM1, and BAX genes were significantly increased by FOXA2 expression in p53-null H1299 cells (Fig. 2A). Moreover, FOXA2 protein that was induced by TSA treatment was recruited to its consensus binding sequences within the promoter regions of each target gene (Fig. 2B). Furthermore, we showed that the transcriptional activity of FOXA2 was repressed by HSP90A, HDAC1, and HDAC3 by physical protein interactions (Figs. 3 and 4).

Previous reports showed that CITED2 was also down-regulated in primary hepatocellular carcinomas (HCC) compared with their adjacent normal tissues, and ectopic expression of CITED2 in HCC cell lines led to suppression of cell growth, suggesting that CITED2 is a potential tumor suppressor [20]. In addition, LBH589 (Panobinostat, Novartis, Germany), a known HDAC inhibitor, induced CITED2 expression in tumors within CEA424/SV40-T-antigen (CEA/Tag) transgenic mice, leading to an increased efficiency of anthracycline to inhibit tumor growth [21]. NR0B2 expression was also significantly decreased in both human hepatocarcinoma and lung cancer tissues, but not in the normal liver, suggesting that it may act as a tumor suppressor in human liver cancer [22]. Moreover, the tumor suppressor gene CADM1 could hardly be detected in NSCLC [23]. Patients positive for BAX expression had longer median survival rates than did patients negative for BAX expression [24]. Therefore, these reports suggest that the identified target genes of FOXA2 encode potential tumor suppressive proteins.

In contrast to the down-regulation of FOXA2 protein in lung cancer, FOXA2 binding proteins including HSP90A, HDAC1, and HDAC3 are highly expressed in lung cancer. It was reported that HSP90 plays wide-ranging roles in chaperoning proteins associated with tumor cell proliferation [25]. HDACs also induce cell cycle progression and inhibit apoptotic cell death by affecting the transcription of specific target genes via regulating the activity of transcription factors in cancer environments [26]. Therefore, inhibitors or small interfering RNAs (siRNA) for HSP90A and HDACs might have anticancer potential. In our recent report, we revealed that FOXA2 protein is specifically induced by an HDAC inhibitor, TSA [19]. Although there is no possibility of interactions between FOXA2 and its interaction partners in non-treated H1299 cells owing to the negligible presence of endogenous FOXA2 protein in the non-treated cells, these interactions can occur in cells in FOXA2-expressing states following TSA treatment or

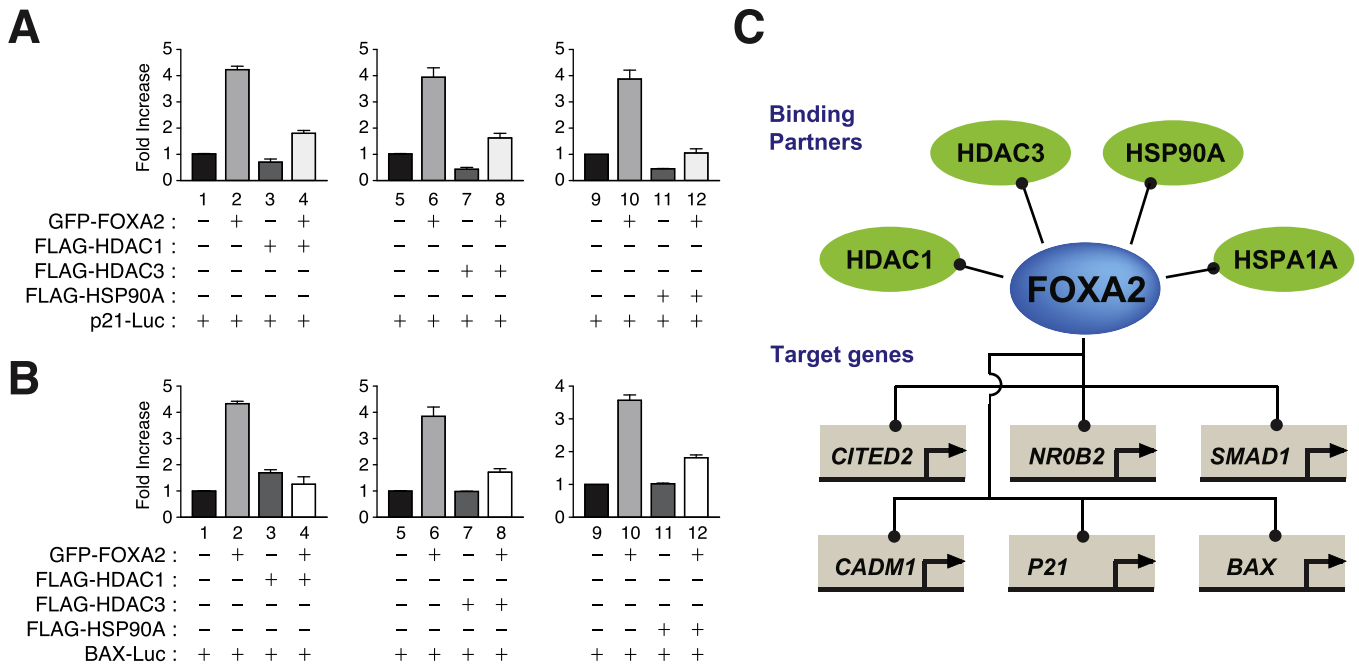

Fig. 4. Construction of FOXA2-centered transcriptional regulation network. (A and B) H1299 cells were transfected with luciferase vector including p21 promoter (A), or BAX promoter (B) together with FOXA2 expression plasmids and constructs for FOXA2 binding protein expression such as HSP90A, HDAC1 and HDAC3. After 48 h, luciferase activity was measured. Data were normalized against β-galactosidase activity and expressed as relative luciferase units compared to the control. (C) FOXA2-centered transcriptional regulatory network model based on combinations between novel FOXA2 interaction proteins and FOXA2 target genes.

overexpression of FOXA2. Under these conditions, we showed that FOXA2 exerts tumor suppressive functions by activating the BAX and p21 promoters, which were significantly repressed by interaction of FOXA2 with its identified binding proteins (Fig. 4). This result suggests that cancer cells operate resistance systems against FOXA2-mediated inhibition of cancer cell growth to enable their continuous proliferation [26,27].

Despite intensive studies on therapeutic approaches for lung cancer, the identity and molecular functions of other tumor suppressor proteins as alternative therapeutic targets to p53 remain to be fully elucidated. Accordingly, it is important to note that our findings contribute to the search for novel therapeutic methods in p53-deficient lung cancer cells. Combining FOXA2 induction with inhibition of its repressor proteins might synergize the effect of treatment and reduce the harmful systemic effects that occur during chemotherapy. Further investigation of therapies targeting the FOXA2-centered transcriptional regulation network is warranted.

Conflict of interest

None.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.042.

Transparency document

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