Regulation of Transforming Growth Factor-β-dependent Cyclooxygenase-2 Expression in Fibroblasts*†

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Abnormal transforming growth factor-β (TGF-β) signaling is a critical contributor to the pathogenesis of various human diseases ranging from tissue fibrosis to tumor formation. Excessive TGF-β signaling stimulates fibrotic responses. Recent research has focused in the main on the antiproliferative effects of TGF-β in fibroblasts, and it is presently understood that TGF-β-stimulated cyclooxygenase-2 (COX-2) induction in fibroblasts is essential for antifibroproliferative effects of TGF-β. Both TGF-β and COX-2 have been implicated in tumor growth, invasion, and metastasis, and therefore tumor-associated fibroblasts are a recent topic of interest. Here we report the identification of positive and negative regulatory factors of COX-2 expression induced by TGF-β as determined using proteomic approaches. We show that TGF-β coordinately up-regulates three factors, heterogeneous nuclear ribonucleoprotein A/B (HNRPAB), nucleotide diphosphate kinase A (NDPK A), and nucleotide diphosphate kinase A (NDPK B). Functional pathway analysis showed that HNRPAB augments mRNA and protein levels of COX-2 and subsequent prostaglandin E2 (PGE2) production by suppressing degradation of COX-2 mRNA. In contrast, NDPK A and NDPK B attenuated mRNA and protein levels of COX-2 by affecting TGF-β-Smad2/3/4 signaling at the receptor level. Collectively, we report on a new regulatory pathway of TGF-β in controlling expression of COX-2 in fibroblasts, which advances our understanding of pathophysiological mechanisms of TGF-β.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates a multitude of physiological and pathophysiological processes. TGF-β controls various cell activities ranging from cell differentiation, proliferation, and migration to extracellular matrix production (1–5). Therefore, abnormal regulation of TGF-β-dependent signaling pathways often leads to diseases, such as fibrosis-related diseases (6, 7), cancer (8–11), cardiovascular disease (12, 13), and autoimmune diseases (7). Among the wide range of biological functions regulated by TGF-β, its effect on fibrosis remains one of the most extensively studied research areas because excessive TGF-β signaling is a critical contributor to the pathogenesis of various fibrotic diseases (2, 7).

TGF-β directly promotes expression of type I collagen, a major component of the extracellular matrix, during fibrosis. TGF-β further induces connective tissue growth factor expression in fibroblasts, which synergizes with TGF-β to induce fibrosis. Epithelial to mesenchymal transition by TGF-β is another contributor to the fibrotic response. However, in addition to its well-established profibrotic effects, several recent reports have demonstrated a key role of TGF-β as an antifibrotic regulator through regulation of cyclooxygenase-2 (COX-2).

Cyclooxygenase is a rate-limiting enzyme in the synthesis of prostaglandins, which are autocrine mediators of multiple cellular processes. Although cyclooxygenase-1 (COX-1) is constitutively expressed in most tissues and involved mainly in homeostasis, COX-2 is usually absent under basal conditions and induced by stimuli, such as growth factors and cytokines. Many lines of evidence have shown that the COX-2-prostanoid pathway is involved in many physiological and pathogenetic pathways, including those that regulate fibrosis, cancer, inflammation, angiogenesis, hemodynamics, and renal function (14–17). Although COX-2 and its major product, prostaglandin E2 (PGE2), are generally considered potent proinflammatory mediators, they also possess antifibrotic effects (14). Increased secretion of PGE2 in response to TGF-β has been documented in fibroblasts and shown to be responsible for its antiproliferative effects. Suppression of COX-2 up-regulation and subsequent PGE2 production, which acts in an autocrine fashion to inhibit proliferation and production of collagen, results in loss of the antiproliferative effects of TGF-β (18). Further, COX-2-deficient mice show enhanced susceptibility to pulmonary fibrosis (19) and cardiac fibrosis (20). Thus, TGF-β-stimulated COX-2 induction in fibroblasts is thought to be essential for the antifibroproliferative effects of TGF-β.

Moreover, recent mounting evidence has confirmed that tumor-associated stromal fibroblasts play critical roles in tumor development and progression (9, 10, 21). Stromal fibro-
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Blasts have been shown to be the predominant source of COX-2 in colon adenomas (22), and increased expression of COX-2 has been demonstrated in not only cancer cells per se but also the surrounding fibroblasts in invasive carcinomas (23). Considering that up-regulation of COX-2 and the subsequent prostaglandin cascade play crucial roles in tumorigenesis, tumor invasion, and metastasis (15), TGF-β-induced COX-2 expression in fibroblasts is envisioned to be a critical contributory factor in the tumor microenvironment. However, the involved molecular mechanisms still remain a subject of intense investigation.

Thus, how TGF-β-mediated COX-2 induction is controlled, especially in fibroblasts, is an important question to be addressed for elucidating the biology of these diseases. Here we show a new regulatory pathway of TGF-β dependent COX-2 expression as centered on coordinated regulation of three newly identified factors using a differential proteomic study. Our findings advance our understanding of the underlying mechanisms of TGF-β pathophysiology in various diseases, including fibrotic and cancer diseases.

EXPERIMENTAL PROCEDURES

Cell Culture—Murine 10T1/2 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum with 100 µg/ml streptomycin and 100 units/ml penicillin G. After 24 h of growth arrest with serum-free medium, subconfluent 10T1/2 cells were stimulated with recombinant human TGF-β1 (R&D Systems) (1 ng/ml) or lipo polysaccharide (LPS) (Sigma) (1 µg/ml) and harvested at the indicated times.

Liquid Chromatography—Cell pellets before and 12 h after TGF-β stimulation were suspended in lysis buffer containing 6 M urea, 2 M thiourea, 10% glycerol, 50 mM Tris-HCl, 2% n-octyl glucoside, 5 mM Tris(2-carboxyethyl)phosphine hydrochloride, and 1 mM protease inhibitor (Sigma) and then centrifuged for 1 h at 18 °C. The supernatants were recovered and stored at −80 °C for further use. Cell extracts were analyzed using a two-dimensional liquid chromatography system (ProteomeLab PF2D, Beckman Coulter), according to the manufacturer's instructions and as previously described (24). Briefly, as the first dimension, chromatofocusing was performed on a high performance chromatofocusing column with a linear pH gradient generated with two buffers, the start buffer (pH 8.5 ± 0.1) and the eluent buffer (pH 4.0 ± 0.1). Fractions were collected, and then residual proteins were washed with 1 mM NaCl. The collected fractions were automatically and sequentially applied to a high performance reversed phase column as the second dimension separation, which consists of a linear gradient of 0–100% B against A for 30 min, where A is 0.1% trifluoroacetic acid in water and B is 0.08% trifluoroacetic acid in acetonitrile. Collected fractions were stored at −20 °C for further analysis.

Matrix-assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) Mass Spectrometry—Fractions were reduced to a volume of 16 µl using a vacuum concentrator, and 2 µl of 1 M NH4HCO3, 1 µl of 10 mM dithiothreitol, and 50–150 ng of modified porcine trypsin (Promega) were added. After overnight incubation at 37 °C, 1 µl of 1% trifluoroacetic acid was added to stop digestion. Digests were desalted with C18 ZipTips (Millipore) and then eluted with 70% acetonitrile and 0.1% 20 °C for further analysis.

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trifluoroacetic acid. The eluted peptide samples were mixed with a saturated matrix of α-cyanohydroxycinnamic acid, spotted onto a MALDI plate, and analyzed with a MALDI-TOF mass spectrometer (Voyager-DE STR; Applied Biosystems) in reflectron mode. All data sets were analyzed using the mass spectrometer software (Data Explorer, version 4.0, Applied Biosystems). Each spectrum was base line-corrected with noise reduction and smoothing before it was deisotoped, leaving only the monoisotopic masses. The spectra were internally calibrated before re-analysis of the entire mass list of each sample was entered into the Mascot public search engine (available at the manufacturer’s instructions). The sequences of gene-specific primer sets were as follows: HRN-PAB, 5′-GGGAGGTCTAAACCTGGAAG-3′ and 5′-GGGC- AACCCTGATTTCAC-3′; HRN-PAB (for discrimination of two isoforms), 5′-GGGAGGTCTAAACCTGGAAG-3′ and 5′- ATTCTGTAGCCACACCAGTC-3′; NDPK A, 5′-GGACCT- TCTCAAGGAGCACTAC-3′ and 5′-ACCACAAGCTGATC- TCTTCT-3′; NDPK B, 5′-TCTGAAACACCCTGGAAGC- AGC-3′ and 5′-TAGTGATCGTCTTCTCAGG-3′; COX-2, 5′-ATTCTTCTGCAAGCAGACTTC-3′ and 5′-CCTGAGTG- TCTTGTACTGTG-3′. Error bars indicate S.E.

**Western Blot Analysis**—Cells extracts 24 h after TGF-β stimulation were subjected to SDS-PAGE analysis and immobiloblotted with anti-COX-2 rabbit polyclonal antibody (Cayman Chemical) and anti-COX-1 mouse monoclonal antibody (Cayman Chemical). For analysis of Smad proteins, anti-phospho-Smad2/3 rabbit polyclonal antibody (Cell Signaling) and anti-COX-2 rabbit polyclonal antibody (Cayman Chemical) were used.

**Immunocytochemistry**—Cells plated on glass coverslips were analyzed 24 h after TGF-β stimulation. Cells were washed in phosphate-buffered saline, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 10 min, and then blocked with 1% bovine serum albumin for 30 min. Subsequently, cells were incubated with the same primary antibodies as those for Western blot analysis, exposed to Alexa Fluor 488-conjugated goat anti-rabbit or anti-mouse IgG antibody (Invitrogen), and counterstained with propidium iodide. Fluorescent images were collected with a confocal laser microscope (MRC 1024; Bio-Rad). COX-2, COX-1, or Smad2/3 was visualized with green fluorescence, and nuclei were visualized with red fluorescence.

**Adenoviral Infection**—Recombinant adenoviruses harboring FLAG-tagged Ndpk a, Ndpk b, Hnrpab p37, and Hnrpab p42 were prepared with the AdEasy system using homologous recombination in bacteria as previously described (27). Forty-

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**TABLE 1**

| Band | Protein name | Swiss-Prot accession no. | Mascot score | No. of peptides | Percentage of coverage | Increase* |
|------|--------------|--------------------------|-------------|-----------------|-----------------------|----------|
| A    | Peroxiredoxin-2 | Q61171 | 71 | 5 | 24 | 9.2 |
| B    | T-complex protein 1 subunit β | P08301 | 74 | 6 | 19 | 1.9 |
| C    | Nucleoside diphosphate kinase A | Q01768 | 110 | 6 | 51 | 2.0 |
| D    | Nucleoside diphosphate kinase B | Q01768 | 82 | 5 | 45 | 2.0 |
| E    | Ornithine aminotransferase, mitochondrial precursor | Q88H04 | 61 | 5 | 9 | 4.2 |
| F    | Stress-70 protein, mitochondrial precursor | P29758 | 76 | 8 | 22 | 1.4 |
| G    | Actin, cytoplasmic 1 | Q6ZWM3 | 67 | 5 | 20 | 2.1 |
| H    | Actin, cytoplasmic 2 | P63260 | 67 | 5 | 20 | 2.1 |
| I    | Heat shock cognate 71-kDa protein | P63308 | 122 | 9 | 24 | 2.7 |
| J    | ATP synthase subunit β, mitochondrial precursor | Q3U774 | 186 | 14 | 38 | 3.5 |
| L    | Vimentin | P20152 | 211 | 14 | 36 | 1.8 |
| L'   | Vimentin | P20152 | 170 | 13 | 39 | 1.8 |
| M    | Heterogeneous nuclear ribonucleoprotein A/B | Q93202 | 105 | 7 | 26 | 3.0 |
| N    | 78-kDa glucose-regulated protein precursor | P20029 | 92 | 9 | 18 | 3.8 |

* Fold increase was estimated by calculating change in peak areas. +, proteins whose peaks could not be detected in the control cell lysate.
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A

B

C

**FIGURE 2. Effect of TGF-β stimulation on expression levels of HNRPA, NDPK A, NDPK B, and COX-2.** After 24 h of growth arrest with serum-free medium, cells were stimulated with recombinant human TGF-β1 (1 ng/ml) and then harvested. A, induction of NDPK B, HNRPA, their putative downstream gene, COX-2, and a related gene, NDPK A, by TGF-β as shown by RT-PCR studies. Further, the lowest left panel shows that both isoforms of HNRPA were up-regulated after TGF-β stimulation. Real-time PCR assay results are shown in the graph on the right. In the upper graph, the solid line and circles indicate NDPK A, the dotted line and squares show NDPK B, and the dotted line and circles show HNRPA. B, Western blot analysis confirming the increased protein level of COX-2 24 h after TGF-β stimulation. Note that the expression level of COX-1, which is known to be a relatively constitutive protein, was not affected by TGF-β as confirmed in the lower panel. C, immunofluorescence staining of TGF-β-stimulated cells (a and c) and control cells (b and d) for COX-2 (a and b) and COX-1 (c and d), indicating the induction of COX-2 protein 24 h after TGF-β stimulation, in contrast to the constitutive expression of COX-1, as shown by green fluorescence. Red fluorescence represents nuclei stained with propidium iodide. The subcellular localization pattern of COX-2 is consistent with previous reports showing expression in the nuclear envelope and endoplasmic reticulum.

eight hours after adenoviral infection at 9 MOI, cells were harvested for RT-PCR or Western blot analysis. For determination of mRNA stability, 48 h after adenoviral infection at 3 MOI, transcription was stopped by the addition of 100 ng/ml leupeptin, and 1 μg/ml pepstatin, followed by using an enzyme-linked immunosorbent assay kit following the manufacturer’s protocol (Cayman Chemical). The background concentration of PGE_2 in medium containing 10% fetal bovine serum was negligible (i.e. below the sensitivity of the assay). The enzyme-linked immunosorbent assay was performed in duplicate, and results are shown as means and S.E. Shown are representative data from one of three independent experiments.

**RNAi Transfection**—Silencer pre-designed small interfering RNA targeted to NDPK A, NDPK B, and HNRPA and Silencer Negative Control small interfering RNA was purchased from Ambion. Cells were harvested for RT-PCR or Western blot analysis at 24 or 48 h after transfection of double-stranded small interfering RNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Chromatin Immunoprecipitation Assay**—Approximately 1 × 10^6 cells were fixed by adding formaldehyde directly to media at 37 °C for 10 min to a final concentration of 1%, washed twice with ice-cold phosphate-buffered saline, and then harvested at 0, 40, 80, and 120 min following DRB treatment. For assays of NDPKs, cells were growth-arrested with serum-free medium for 24 h at 24 h after infection at 30 MOI and then stimulated with TGF-β (1 ng/ml).

**Measurement of PGE_2 Synthesis**—Subconfluent cells maintained in Dulbecco’s modified Eagle’s medium (Sigma) with 10% fetal bovine serum were infected at 10 MOI with control empty adenovirus or adenovirus harboring HNRPA. Culture medium was collected at 48 h after adenoviral infection. After the addition of indomethacin, a prostaglandin synthesis inhibitor, at a final concentration of 10 μg/ml, the medium was centrifuged and stored at −80 °C for further analysis. The concentration of PGE_2 in cell culture supernatant was estimated by using an enzyme-linked immunosorbent assay kit following the manufacturer’s protocol (Cayman Chemical). The background concentration of PGE_2 in medium containing 10% fetal bovine serum was negligible (i.e. below the sensitivity of the assay). The enzyme-linked immunosorbent assay was performed in duplicate, and results are shown as means and S.E. Shown are representative data from one of three independent experiments.

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FIGURE 3. Effect of HNRPAB on expression levels of COX-2. Cells were harvested at 48 h after adenoviral infection. A, augmented mRNA expression levels of COX-2 by adenoviral overexpression of HNRPAB as shown by RT-PCR. The right panel shows quantification by real-time PCR assay. B, Western blot analysis confirming up-regulated protein level of COX-2 in the right graph shows the result of a real-time PCR assay. Overexpression of HNRPAB (gray bars for HNRPAB p37 and black bars for HNRPAB p42) resulted in synergistic increase in mRNA levels of COX-2, as compared with infection of control empty adenovirus (white bars). C, induction of COX-2 by HNRPAB as demonstrated by immunocytochemistry. D, increased secretion of PGE2 in culture medium by HNRPAB p37 and HNRPAB p42. E and F show the result of a real-time PCR assay. Overexpression of HNRPAB (gray bars for HNRPAB p37 and black bars for HNRPAB p42) resulted in synergistic increase in mRNA levels of COX-2, as compared with infection of control empty adenovirus (white bars).
sponding data. As shown in this panel, comparison before and after TGF-β stimulation revealed dozens of protein peaks, whose intensity increased or decreased after stimulation. These peaks were identified with MALDI-TOF mass spectrometry, based on the second dimensional elution profiles, whose representative data are presented in Fig. 1B. The identified proteins are listed in Table 1 along with the corresponding band label in Fig. 1, protein name, accession number in the Swiss-Prot data base, Mascot score, and percentage of coverage.

**Up-regulation of NDPK B, HNRPAB, and Their Related Factors as Confirmed by RT-PCR Analysis and Increased COX-2 by Western Blot and Immunocytochemistry**—Heterogeneous nuclear ribonucleoprotein A/B (HNRPAB) and nucleotide diphosphate kinase B (NDPK B) were among the proteins identified as being up-regulated by TGF-β treatment. HNRPAB has been previously reported to bind to the COX-2 3′-untranslated region and stabilize an exogenous COX-2 3′-untranslated region mRNA reporter (28). On the other hand, NDPK B belongs to the NDPK/NM23 gene family, whose member protein NM23-H1, a human orthologue of NDPK A, up-regulates the COX-2 promoter activity in reporter assays (29). Because the effect of these factors on endogenous COX-2 expression level is hitherto unknown, we focused our subsequent studies on understanding the mechanisms underlying the actions of these factors on COX-2 regulation.

First, to validate the proteomic screening analysis, mRNA levels of HNRPAB and NDPK B were analyzed by conventional RT-PCR and real-time PCR assays. As shown in Fig. 2A, the transcription levels of both factors were augmented after TGF-β stimulation in a time-dependent manner. mRNA levels of their related factors, NDPK A and COX-2, were also increased in a similar time-dependent manner. Next, because HNRPAB exists as two isoforms, heterogeneous nuclear ribonucleoprotein A/B (Q99020) and S1 protein C2 (Q20BD0) that differ by a 47-amino acid insertion close to the C terminus (28), we examined which isoform of HNRPAB is induced by TGF-β, using a PCR primer pair that discriminates the two isoforms. As shown in the lowest left panel in Fig. 2A, TGF-β led to the expression of both isoforms of HNRPAB. We refer to the smaller isoform as HNRPAB p37 and to the larger isoform as HNRPAB p42 hereafter.

Further, effects on COX-2 expression were examined by Western blot, which showed a resultant increase in COX-2 protein in contrast to the constitutive expression of COX-1 (Fig. 2B) and immunocytochemistry (Fig. 2C) with further documentation of characteristic prominent staining of the nuclear envelope (30).

**HNRPAB Increases the mRNA and Protein Levels of COX-2 and Leads to the Production of PGE2**—To understand the effects of HNRPAB on COX-2, we overexpressed HNRPAB by adenoviral infection. As indicated in Fig. 3A, both isoforms of HNRPAB augmented the mRNA level of COX-2. HNRPAB p42 was more potent in inducing transcription levels of COX-2 than HNRPAB p37. These effects were accompanied by similar increases in the expression level of COX-2 protein as shown by Western blot analysis (Fig. 3B) and immunofluorescence (Fig. 3C). Also, the secretion of PGE2, a down-
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Stream product of COX-2 metabolism, in cell culture supernatant was measured by enzyme immunoassay. Although the prominent induction of COX-2 protein by HNRNPB p42 did not lead to proportional accumulation of PGE₂, the significant induction of PGE₂ in culture medium of cells overexpressing HNRNPB was seen (Fig. 3D). Thus, overexpression of HNRNPB augmented COX-2 transcript, protein, and enzymatic activity.

In addition, we examined the effect of HNRNPB on TGF-β-induced COX-2 mRNA and protein. As shown in Fig. 3, E and F, TGF-β and HNRNPB showed a synergistic effect on COX-2 expression.

**Adenoviral Transfer of HNRNPB Stabilizes COX-2 mRNA Levels**—HNRNPB has been previously reported to stabilize an exogenous COX-2 3′-untranslated region mRNA reporter in HeLa cells (28). However, it is unknown whether overexpression of HNRNPB can stabilize endogenous mRNA of COX-2. To evaluate this potential post-transcriptional regulation, HNRNPB was overexpressed in cells using adenoviral transfer with further inhibition of transcription by the addition of DRB at 48 h. Quantitative RT-PCR (Fig. 4A) and real-time PCR (Fig. 4B) showed rapid degradation of COX-2 mRNA after DRB treatment in cells infected with control empty adenovirus (lanes 1–4 in Fig. 4A and solid line in Fig. 4B) and prolonged stabilization of COX-2 mRNA in cells overexpressing HNRNPB, especially p42 (lanes 5–12 in Fig. 4A and dotted lines in Fig. 4B). On the other hand, knockdown of HNRNPB using RNAi led to enhanced degradation of COX-2 mRNA (Fig. 4C). In addition, HNRNPB did not show any transactivation effects on a murine Cox-2 promoter in cotransfection reporter assays (data not shown). Collectively, the mechanism of HNRNPB-dependent up-regulation of COX-2 is mainly due to post-transcriptional stabilization of COX-2 mRNA.

**NDPK A and NDPK B Suppress TGF-β-dependent Augmentation of**

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**FIGURE 5.** Effect of NDPK A and NDPK B on the expression level of COX-2. A, suppression of mRNA expression level of COX-2 by adenoviral overexpression of NDPK A or NDPK B as shown by RT-PCR. At 24 h after adenoviral infection, cells were starved for an additional 24 h, treated with TGF-β (1 ng/ml) for 8 h, and then harvested. Under the condition in which TGF-β increased COX-2 mRNA (lane 1 versus lane 4), either NDPK A or NDPK B diminished the stimulatory effect of TGF-β on COX-2 (lane 4 versus lanes 5 and 6). The right graph shows the result of the real-time PCR assay. Overexpression of NDPKs (gray bars for NDPK A and black bars for NDPK B) led to an attenuated increase in mRNA levels of COX-2, compared with infection of control empty adenovirus (white bars). B, Western blot analysis confirming reduced protein levels of TGF-β-induced COX-2 by NDPK A or NDPK B (lane 4 versus lanes 5 and 6) under the condition in which TGF-β increased COX-2 protein (lane 1 versus lane 4). The treatment condition was the same as that of A, but cells were harvested 24 h after TGF-β stimulation. Note that neither TGF-β nor NDPKs affected the expression level of COX-1 as demonstrated by the lower panel. C, attenuation of TGF-β-stimulated COX-2 expression by NDPK A or NDPK B as demonstrated by immunocytochemistry. The treatment condition was the same as that of B. Adenoviral transfer of either NDPK A or NDPK B impaired the expression level of COX-2 protein (b and c) compared with cells infected with control empty adenovirus (a) as shown by green fluorescence. Red fluorescence represents nuclei stained with propidium iodide. D, augmentation of mRNA expression level of COX-2 by RNAi silencing of NDPK A or NDPK B, as indicated by RT-PCR. Cells were harvested 48 h after RNAi transfection. The middle and lower panels on the left demonstrate the specificity of RNAi constructs. The right graph shows the result of a real-time PCR assay. Note that RNAi targeting either NDPK A or NDPK B increased COX-2 mRNA (black bars) under the condition in which each RNAi construct decreased mRNA of each target specifically (gray bars for NDPK A and white bars for NDPK B). E, increased COX-2 protein by RNAi silencing of NDPK A or NDPK B as shown by Western blot analysis. Cells were harvested 48 h after RNAi transfection. The difference of effects between NDPK A and NDPK B might attribute to the different efficiency of RNAi suppression as demonstrated in E.
Next, we assessed whether NDPK A and NDPK B increase COX-2 expression as is consistent with a previous report using reporter assays (29). Unexpectedly, however, ectopic overexpression of these factors diminished TGF-β-dependent induction of COX-2 mRNA (Fig. 5A). Suppressed COX-2 protein levels were also confirmed by Western blot analysis (Fig. 5B) and immunofluorescence (Fig. 5C). RNAi analysis was further used to confirm the effect of NDPK A and NDPK B on COX-2 by depleting these factors. As shown in Fig. 5, D and E, knockdown of either NDPK A or NDPK B led to increased COX-2 expression at both the mRNA and protein levels. Thus, NDPK A and NDPK B repress COX-2.

NDPK A and NDPK B Impair TGF-β-dependent Induction of COX-2 Not at the Transcriptional Level but at the TGF-β Receptor Level—With these apparently conflicting results, we conducted reporter assays similar to that used in a previous report to rule out cell type-dependent effects, which showed that NDPK A and NDPK B can transactivate the Cox-2 promoter (supplemental Fig. S1). It was therefore thought unlikely that NDPK A and NDPK B act on the Cox-2 promoter to impair transcription. We thus speculated that an alternative mechanism might be that NDPK A and NDPK B attenuate COX-2 expression not at the transcription level but at the receptor level, because a recent report has shown that NM23-H1, a human orthologue of NDPK A, interacts with serine-threonine kinase receptor-associated protein, a TGF-β receptor-interacting protein, and negatively regulates a number of TGF-β target genes in other cell lines (31).

TGF-β receptors can activate both Smad-dependent pathways and Smad-independent pathways (32, 33). First, to address whether TGF-β-induced COX-2 expression is mediated by Smad proteins, a chromatin immunoprecipitation assay using anti-Smad2/3 antibody was done. PCR analysis was performed to amplify the region of the Cox-2 promoter that contains possible Smad binding sites (34), including multiple Smad binding elements (AGAC), one AP-1-like site (TGCCTG), and one Sp1 site (GGGCCGG) (35). In vivo binding of Smad2/3 to the Cox-2 promoter was clearly induced in response to TGF-β stimulation (Fig. 6A). In addition, real-time PCR analysis showed that recruitment of Smad2/3 on the promoter is increased by the addition of TGF-β in a time-dependent manner (Fig. 6B). These findings suggest that TGF-β-dependent COX-2 expression is at least in part regulated by Smad2/3/4 pathways.

In the Smad-dependent pathways, the TGF-β receptor phosphorlates Smad2 and Smad3. Phosphorylated Smad2 and
Smad3 then form oligomers with or without Smad4 that translocate to the nucleus, where they regulate the transcription of downstream genes (32–34). Thus, we assessed whether NDPK A and NDPK B can suppress each step of these pathways. First, adenoviral overexpression of these factors decreased TGF-β-mediated phosphorylation of Smad2 as shown by Western blot (Fig. 6C). Next, subsequent nuclear translocation of Smad2/3 was impaired as demonstrated by immunofluorescence (Fig. 6D). Moreover, chromatin immunoprecipitation showed that resultant TGF-β-induced recruitment of Smad2/3 onto the Cox-2 promoter was clearly attenuated by forced expression of NDPK A or NDPK B (Fig. 6E). Collectively, these findings confirmed that NDPK A and NDPK B can suppress TGF-β-stimulated induction of COX-2 by down-regulating the Smad pathways at the receptor level.

**FIGURE 6. Effect of NDPK A and NDPK B on the Smad2/3/4 signaling pathway and resultant Smad2/3 recruitment on the promoter region of Cox-2. A, in vivo recruitment of Smad2/3 on the promoter of Cox-2 upon TGF-β stimulation. After 24 h of growth arrest with serum-free medium, cells were stimulated with TGF-β (1 ng/ml). Chromatin was harvested 8 h after TGF-β treatment and then immunoprecipitated (IP) with anti-Smad2/3 antibody (lanes 2 and 6) or normal mouse IgG as a negative control (lanes 1 and 4). Note that direct recruitment of Smad2/3 to the Cox-2 promoter was only seen with TGF-β treatment (lane 5 versus lane 6). Lanes 1 and 2 are input (1%), confirming that the applied chromatin amounts were the same. B, time course of Smad2/3 binding to the Cox-2 promoter. After 24 h of growth arrest with serum-free medium, cells were stimulated with TGF-β (1 ng/ml) and then harvested at the indicated times. Chromatin precipitated by anti-Smad2/3 antibody (solid line and squares) or normal mouse IgG (dotted line and circles) was quantified by real-time PCR. Data were normalized by input DNA. Error bars, S.E. C, suppression of phosphorylation levels of Smad2 by adenosoral transfer of NDPK A or NDPK B as shown by Western blot analysis. Cells were growth-arrested with serum-free medium for 24 h at 24 h after adenosoral infection, stimulated with TGF-β (1 ng/ml) for 12 h, and then harvested. Under the condition in which TGF-β increased phosphorylated Smad2 (lane 1 versus lane 4), either NDPK A or NDPK B reduced the phosphorylation level of Smad2 (lane 4 versus lanes 5 and 6). D, immunofluorescence staining of Smad2/3, indicating that NDPK A or NDPK B suppressed TGF-β-stimulated nuclear translocation of Smad2/3. Cells were growth-arrested with serum-free medium for 24 h at 24 h after adenosoral infection, stimulated with TGF-β (1 ng/ml) for 8 h, and then harvested. Although TGF-β induced nuclear translocation of Smad2/3 as shown by prominent green fluorescence staining of nuclei of TGF-β-treated cells (a versus b), overexpression of NDPK A or NDPK B attenuated the nuclear translocation of Smad2/3 proteins (c and d), compared with cells infected with control empty adenosoral (b). Red fluorescence represents nuclei stained with propidium iodide. E, chromatin immunoprecipitation assay demonstrating the attenuated recruitment of Smad2/3 on the promoter of Cox-2 by NDPK A and NDPK B. At 24 h after adenosoral infection, cells were starved for an additional 24 h, treated with TGF-β (1 ng/ml) for 8 h, and then harvested. Chromatin was immunoprecipitated with anti-Smad2/3 antibody (lanes 9–12) or normal mouse IgG as a negative control (lanes 5–8). Under the condition in which TGF-β recruited Smad2/3 onto the promoter of Cox-2 (lane 9 versus lane 10), adenosoral forced expression of either NDPK A or NDPK B attenuated the binding of Smad2/3 to the promoter, as compared with infection with empty virus (lane 10 versus lanes 11 and 12). Lanes 1–4 are input (1%), confirming that the applied chromatin amounts were the same. The graph on the right shows results of real-time PCR assay. Data were normalized by input DNA.**

**DISCUSSION**

A carefully controlled balance of positive and negative signaling pathways regulates normal physiological cellular activities. Their inappropriate alteration often leads to human diseases. Thus, understanding regulatory signaling networks, especially those of growth factors, is a critical step in elucidating mechanisms that regulate development of pathophysiological conditions. Although the complexity of TGF-β signaling is very challenging to analyze, a differential proteomic approach allowed us to discover both positive and negative components of TGF-β-stimulated regulation of COX-2 expression. Although few previous reports have described that TGF-β induces COX-2 in several cell lines (18, 36, 37), surprisingly, almost nothing has been described about underlying molecular mechanisms. This is the first report that shows that HNRPAB is a TGF-β-induced factor that directly increases the expression level of COX-2 protein. Further, our findings on NDPKs are intriguing, considering their possible roles as negative regulatory components of TGF-β-dependent pathways. Moreover, this is the first to report on a direct link between TGF-β-Smad2/3 signaling and COX-2 expression (supplemental Fig. S2).

**Implications in Fibroproliferative Diseases—Fibroproliferative diseases, including cardiac fibrosis, pulmonary fibrosis, systemic sclerosis, liver cirrhosis, progressive kidney disease, and macular degeneration, are one of the leading causes of morbidity and mortality and can affect all tissues and organ systems (6). TGF-β plays a pivotal role in regulating fibrosis and can exert both pro- and antiproliferative effects on fibroblasts. TGF-β induces a biphasic response in lung fibroblasts with stimulation of proliferation at low concentrations and inhibition at high concentrations, the latter of which is accounted for by autocrine synthesis of PGE2 (18). Interestingly, although inappropriate activation of TGF-β signaling leads to excess fibrosis in the pathogenesis of various fibrotic diseases, genetic ablation of Cox-2, one of the downstream targets of TGF-β, also results in excessive fibrosis, as shown in Cox-2 null mice (19, 20). Although antifibrotic therapies are considered to be promising, an incomplete understanding of the molecular machinery of TGF-β-dependent pathways has hindered the development of such therapies. Here we have demonstrated that TGF-β up-regulates HNRPAB. Of note, HNRPAB is a single protein with multiple functions. Although HNRPAB acts as an RNA-binding protein, it was also identified independently as CARG box-binding factor-A on the basis that it binds to single-stranded and double-
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FIGURE 7. Effects of HNRPAB, NDPK A, and NDPK B on the expression level of LPS-induced COX-2. A, effect of LPS stimulation on expression levels of COX-2, NDPK A, NDPK B, and HNRPAB. After 24 h of growth arrest with serum-free medium, cells were stimulated with LPS (1 μg/ml) and then harvested at the indicated times. The right graph shows the results of real-time PCR assay. Although LPS induced transient increase of COX-2 (solid heavy line and large squares), LPS did not show significant effects on the expression levels of NDPK A (solid line and triangles), NDPK B (dotted line and circles), or HNRPAB (dotted line and triangles). B, effect of HNRPAB on COX-2 mRNA induction stimulated by LPS. Cells were growth-arrested with serum-free medium for 24 h at 24 h after adenoviral infection at 10 MOI, stimulated with LPS (1 μg/ml), and then harvested at the indicated times. Under the condition in which LPS increased COX-2 mRNA (lane 1 versus lane 4), HNRPABs augmented the stimulatory effect of LPS on COX-2 at 6 h (lane 7 versus lanes 8 and 9). However, little effect was seen on the peak induction level of COX-2 at 2 h (lane 4 versus lanes 5 and 6). The right graph shows results of real-time PCR assay. Overexpression of HNRPAB (dotted lines and circles for HNRPAB p37 and triangles for HNRPAB p42) up-regulated mRNA levels of COX-2 at 6 h, as compared with infection of control empty adenovirus (solid line and squares). C, effect of NDPKs on LPS-dependent COX-2 mRNA induction. Cells were growth-arrested with serum-free medium for 24 h at 24 h after adenoviral infection at 30 MOI, stimulated with LPS (1 μg/ml), and then harvested at the indicated times. Under the condition in which LPS increased COX-2 mRNA (lane 1 versus lane 4), NDPKs did not show significant inhibitory effect on LPS-stimulated COX-2 expression (lanes 4–9). The right graph shows the results of a real-time PCR assay. Overexpression of NDPKs (dotted lines and circles for NDPK A and triangles for NDPK B) resulted in no decrease or only a slight decrease in mRNA levels of COX-2, as compared with infection of control empty adenovirus (solid line and squares).

stranded DNA in a sequence-specific manner as a transcriptional regulator (38–40). A recent report has demonstrated the key role of this factor in epithelial-mesenchymal transition, a main contributor to tissue fibrosis (40). Thus, HNRPAB is a unique protein that shows bimodal actions; as a profibrotic factor, it is a DNA-binding protein that promotes epithelial-mesenchymal transition, and as an antifibrotic factor, it is a RNA-binding protein that stabilizes mRNA of COX-2. Therapeutic modification of its RNA-binding ability that does not affect its DNA-binding capacity or vice versa may be potentially exploitable for targeted therapeutic intervention against fibroproliferative diseases.

Possible Roles in Carcinogenesis and Metastasis—The significance of TGF-β signaling in carcinogenesis and metastasis has been widely investigated. In general, TGF-β is a tumor suppressor early in carcinogenesis, inhibiting growth and promoting differentiation, and then acts as a tumor promoter later in cancer development to promote growth, survival, invasion, and metastasis (2, 8, 9, 11). COX-2 is also involved in cancer progression. Clinical and experimental data have demonstrated that increased COX-2 expression correlates with poor survival in various cancers, and selective inhibitors of COX-2 inhibit tumor growth (15). Stromal fibroblasts as well as cancer cells per se have been shown to be the source of COX-2 (15, 22, 23). Given that tumor-associated fibroblasts are important contributors to the tumor microenvironment (9, 10, 21), TGF-β-mediated COX-2 expression in fibroblasts is a topic that deserves further attention.

An earlier proteomic report identified HNRPAB as one of eight up-regulated genes in the gene expression signature associated with various tumor metastases (41). Notably, the authors suggested that a considerable proportion of the gene expression signature seems to be derived from stromal components of tumors. Thus, not only TGF-β and COX-2 but also HNRPAB is probably up-regulated in the tumor microenvironment. Although nothing has been shown concerning their relationship in tumor-associated fibroblasts, our findings lead us to propose a straightforward pathway by which TGF-β stimulates HNRPAB, which in turn increases
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COX-2 in tumor-associated fibroblasts, thus resulting in tumor development and progression.

Bifunctional Effects of NDPKs—NDPK A and NDPK B are members of the NDPK/NM23 family, which have multiple activities ranging from DNA-binding in the nuclei to protein-protein interaction in the cytosol. These family proteins have been shown to be involved in many cellular processes, including cell growth, differentiation, metastasis, and development (42–45). Although our findings in the present study are based on experiments using fibroblasts, this novel pathway potentially impacts on the biology of tumor cells. NDPK was first characterized as a metastasis suppressor in melanoma (46). Its high expression was associated with less metastatic potential in various tumors, including melanoma, breast cancer, colon cancer, and liver cancer. In contrast, increased expression in prostate cancer, neuroblastoma, and lymphoma is related to tumor aggressiveness (42, 47). Despite the widely accepted notion of close involvement of NDPKs in tumor progression, no clear consensus has been made regarding its precise roles in tumor biology, or rather, NDPKs are considered to have multifaceted roles in a variety of tumors in a highly context-dependent manner. We note that an earlier study showed that NM23-H1, a human orthologue of NDPK A, up-regulates COX-2 promoter activity in reporter assays (29). This is in agreement with the tumor-progressive property of NDPKs, considering the effect of COX-2 on tumor progression. On the other hand, our finding that NDPKs attenuate COX-2 expression is consistent with classically proposed functions of NDPK as a metastasis suppressor and possible tumor suppressor. The molecular basis of multiple NDPK-dependent signaling pathways is, however, more complex, and further investigation is warranted to better understand the precise underlying mechanisms.

In conclusion, we have shown a new regulatory pathway of TGF-β-dependent COX-2 expression by HNRNPA2B1 and NDPKs, which provides a new perspective in the biology of various diseases, including fibrotic and cancer diseases.

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