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N-*myc* Downstream-regulated Gene 2, a Novel Estrogen-targeted Gene, Is Involved in the Regulation of Na^+/K^+-ATPase*[S]

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Na^+/K^+-ATPase, a plasma membrane protein abundantly expressed in epithelial tissues, has been identified and linked to numerous biological events, including ion transport and reabsorption. In Na^+/K^+-ATPase, the β-subunit plays a fundamental role in the structural integrity and functional maturation of holoenzyme. Estrogens are important circulating hormones that can regulate Na^+/K^+-ATPase abundance and activity; however, the specific molecules participating in this process are largely unknown. Here, we characterize that N-*myc* downstream-regulated gene 2 (*NDRG2*) is an estrogen up-regulated gene. 17β-Estradiol binds with estrogen receptor β but not estrogen receptor α to up-regulate *NDRG2* expression via transcriptional activation. We also find that NDRG2 interacts with the β1-subunit of Na^+/K^+-ATPase and stabilizes the β1-subunit by inhibiting its ubiquitination and degradation. NDRG2-induced prolongation of the β1-subunit protein half-life is accompanied by a similar increase in Na^+/K^+-ATPase-mediated Na^+ transport and Na^+ current in epithelial cells. In addition, *NDRG2* silencing largely attenuates the accumulation of β1-subunit regulated by 17β-estradiol. Our results demonstrate that estrogen/NDRG2/Na^+/K^+-ATPase β1 pathway is important in promoting Na^+/K^+-ATPase activity and suggest this novel pathway might have substantial roles in ion transport, fluid balance, and homeostasis.

Na^+/K^+-ATPase, a plasma membrane ion pump, has numerous physiological functions. Of note, it is consisted of three subunits (1), α, β, and γ, and the holoenzyme activity required by the participation of the three subunits. α is the catalytic subunit of the enzyme that utilizes ATP hydrolysis to pump K^+ into the cell in exchange for Na^+, which is essential for maintaining normal resting membrane potentials and facilitating the exchange of other materials needed for cellular homeostasis and activity (2–4). β-Subunit is responsible for the formation and integrity of the holoenzyme. In vertebrate cells, β-subunit may stabilize the correct folding of the α-subunit to facilitate its delivery to the plasma membrane (5–7). In addition, evidence showed that β-subunit is related to the cell motility and invasion (8, 9). At present, four α-isoforms known as α1, α2, α3, α4 as well as three different β-polypeptides termed as β1, β2, and β3 (3) have been identified. Among these isoforms, $\alpha1\beta1$ distributes in nearly every tissue whereas other isoforms exhibit a tissue-specific pattern of expression. γ-Subunit, a small hydrophobic polypeptide that has only one isoform, is involved in the modulation of Na^+/K^+-ATPase function (10, 11). The ionic homeostasis maintained by the Na^+/K^+-ATPase is also critical for cell survival, differentiation, and cell apoptosis (12, 13).

As an important molecule in charge of so many biological events, Na^+/K^+-ATPase is regulated by a number of hormones, including aldosterone, thyroid hormone, glucocorticoid, catecholamines, insulin, carbachol, and androgen. These circulating hormones can exert either short term or long term regulation on Na^+/K^+-ATPase via changing the gene expression level, reversible phosphorylation of its subunits, or modulating its trafficking (14, 15). Recently, accumulated evidence indicates that estrogen has a great effect on the activity and expression of Na^+/K^+-ATPase. It has been demonstrated that 17β-estradiol (E_2)[4] can enhance the activity of Na^+/K^+-ATPase in H9C2 cardiac myocytes and rat hearts (16, 17). Meanwhile, ovariectomized rats showed a decreased Na^+/K^+-ATPase activity (18). In erythrocytes in women, the activity of Na^+/K^+-ATPase was increased when estrogen levels reached a peak during the menstrual cycle (19, 20). Subsequent studies showed that estrogen also associates with Na^+/K^+-ATPase-induced Na^+ transport in other cells such as alveolar epithelial cells and osteoblasts (21, 22). Together, these studies suggest that estro-

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[4] The abbreviations used are: E_2, 17β-estradiol; ENaC, epithelial sodium channel; ER, estrogen receptor; ERE, estrogen response element; NDRG, N-*myc* downstream-regulated gene; PPT, 4,4′,4″-(4-propyl-[1*H*]-pyrazole-1,3,5-triyl)trisphenol); DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile); HSG, human salivary gland.

gen may influence Na^+/K^+-ATPase activation as well as ion transport. The regulation of estrogen on Na^+/K^+-ATPase is likely to be responsible for the cardioprotective effects, fluid balance and homeostasis. However, the precise molecular mechanisms by which estrogen alter pump activity and expression are still poorly understood.

N-*myc* downstream-regulated gene 2 (*NDRG2*) is a member of the *NDRG* family and was first cloned in our laboratory from a normal human brain cDNA library (23). *NDRG2* shares roughly 60% residue identity with the other members *NDRG1*, *NDRG3*, and *NDRG4* (24). The currently available bioinformatic analysis does not indicate any known motif or domain in NDRG2 (25). NDRG2 is involved in cell growth, differentiation, stress, and hormonal responses (26). Previous studies have shown that the expression of *NDRG2* is regulated by many hormones, including dexamethasone, insulin, androgens, and aldosterone (27–29). Analysis of the promoter region flanking 5′ of the *NDRG2* gene revealed a putative estrogen-response element (ERE) in the region (5′-nnAGTCAnnnTGACCnn-3′) (supplemental Fig. S1), which suggests that estrogen, may also play a regulative role in *NDRG2* expression.

To gain further insights into the function of NDRG2, we screened for NDRG2-binding partners by using a yeast two-hybridization assay and identified Na^+/K^+-ATPase β1 as a protein that interacts with NDRG2. Our previous studies have shown that as a cytoplasmic protein NDRG2 is expressed in many human normal tissues and is expressed highly in the salivary glands, brain, heart, skeletal muscle, and kidney where Na^+/K^+-ATPases were enriched (30, 31). Importantly, the interaction of NDRG2 with Na^+/K^+-ATPase β1 also occurred in human HEK293 cells as determined by using a mammalian two-hybrid system (supplemental Fig. S2*B*).

Based on these previous studies, we wondered about the following: first, whether estrogen exerts its regulation on *NDRG2*; second, what would be the significance of the interaction between NDRG2 and Na^+/K^+-ATPase β1; and third, whether there are any possibilities that NDRG2 is involved in the estrogen-mediated regulation of Na^+/K^+-ATPase. In this study, we tested the hypothesis that a possible estrogen/NDRG2/Na^+/K^+-ATPase β1 pathway participates in the regulation of Na^+/K^+-ATPase.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

All cells were cultured in a humidified incubator under 5% CO_2 in a Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. DNA and RNA transfection of cells was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. All cell-based assays were performed 48 h after transfection.

Construction of Plasmids

The plasmid (pGL3) for the luciferase reporter of the human *NDRG2* promoter has been described previously (32). The mutants of the human *NDRG2* promoter were generated by PCR using the cloned promoter as template. The plasmids for the mammalian expression of ERα and ERβ were generated by PCR amplification of the ER gene from pRST7-ERα and PSG5-ERβ (generously provided by Drs. Donald P. McDonnell (33) and Jan-Åke Gustafsson (34)), and the PCR products were subcloned into pcDNA3.1, respectively. The authenticity of newly constructed plasmids was confirmed by sequencing.

Real Time PCR

Total RNA was extracted from individual samples using TRIzol (Invitrogen) and reversely transcribed into cDNA using the RevertAid™ First-Strand cDNA synthesis kit (Fermentas, Lithuania), according to the manufacturer's instruction. The levels of mRNA transcripts were determined by quantitative real time PCR using the cDNA as the template, specific primers, and the standard SYBR® Green RT-PCR kit (Takara, Japan), according to the manufacturer's instructions. The PCRs (25 μl/tube, in triplicate) were performed first at 95 °C for 10 s for denaturation and subjected to 35 cycles of 95 °C for 5 s and 60 °C for 34 s using the specific primers (supplemental Table S1). The relative levels of target gene mRNA transcripts were normalized to the control β-actin.

Luciferase Assays

Cells (2×10^4 cells/well) were cultured in 10% FBS DMEM in 96-well plates overnight and transfected with one of the reporter vectors (0.1 μg) containing WT, truncated mutants, or point mutants (M1, M2, or M3) of *NDRG2* promoter along with 5 ng of pHRL using Lipofectamine 2000 (Invitrogen) for 48 h and then treated with 10 nM E_2, PPT, DPN, or ethanol for 12 h. The luciferase activities of individual samples were determined using the Dual-Luciferase reporter assay system.

Chromatin Immunoprecipitation

HSG and HeLa cells (2×10^6 cells/dish) were transfected with 10 μg of WT ERβ or control vector for 48 h in 10-cm culture dishes, respectively, and treated with 10 nM E_2 for 12 h. The cells were harvested, cross-linked with 1% formaldehyde, and then lysed, as described previously (35). The chromatin DNA was sonicated to yield an average size of 300–500 bp and incubated with rabbit anti-ERβ polyclonal antibodies (Santa Cruz Biotechnology) or rabbit IgG (negative control) in a ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 2 mM EDTA, 167 mM NaCl, 16.7 mM Tris-HCl, pH 8.1, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A) at 4 °C overnight with gentle rotation. The immunocomplex was precipitated by the salmon sperm DNA/protein A-agarose 50% beads. After washing, the precipitated DNA was amplified by PCR using the primers flanking the ERE of the *NDRG2* promoter (−1378/−1240) or the control region (−75/+50). The plasmid containing the *NDRG2* promoter and the original portions of lysates containing the sonicated input chromatin were used as positive controls. The PCR products were resolved by agarose gel electrophoresis, and their identities were determined by DNA sequencing.

Electrophoretic Mobility Shift Assay

HSG cells were transfected with human WT ERβ or control vector and treated with 10 nM E_2 for 12 h. EMSA was performed using the EMSA kit, according to the manufacturer's protocol

(Pierce). Endogenous or exogenous ERβ protein was prepared using the NE-PER nuclear and cytoplasmic extraction reagents. Briefly, the candidate WT ERE in the *NDRG2* promoter and the corresponding complementary sequence were synthesized and labeled with, or without, biotin at the 5′ terminus. A mutant ERE sequence (supplemental Table S1) in the *NDRG2* promoter was synthesized and used as a control. The biotinylated sequence was used as the hot probe, and the unbiotinylated sequence and the mutant were used as the cold competitors. HSG cells (1×10^7) were incubated with 200 μl of cytoplasmic extraction reagent I containing protease inhibitors on ice for 10 min and treated with 11 μl of cytoplasmic extraction reagent II for 1 min, followed by centrifuging at 12,000 × *g* for 10 min. The cell pellets were resuspended in 50 μl of nuclear extraction reagent containing protease inhibitors and rotated at 4 °C for 1 h, followed by centrifuging at 12,000 × *g* for 15 min for the recovery of nuclear proteins in the supernatants. The collected nuclear proteins (3 μg) from individual samples were incubated with 10 fmol of the biotinylated hot probe (WT *NDRG2* ERE) in the presence or absence of 1 μg of anti-ERβ or control rabbit IgG at 4 °C for 1 h. Subsequently, the DNA-protein complexes were separated on a 6% nondenaturing polyacrylamide gel in 2.5% glycerol, 0.5×TBE and transferred to nylon membranes. After UV-cross-linking and blocking, the membranes were probed with peroxidase-conjugated streptavidin and visualized using the enhanced chemiluminescence (Pierce) and exposing to x-ray film. In addition, the nuclear proteins were preincubated with 50–200-fold excess of the cold probes (WT or its mutant) for 1 h at room temperature and then interacted with the hot probe for competition.

Immunoprecipitation

Transfected or untransfected cells were incubated with 1 ml of lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% Lubrol, and 5 mM EDTA and protease inhibitors for 30 min at 4 °C. The insoluble fraction was eliminated through centrifugation at 10,000 × *g* for 30 min at 4 °C. After the centrifugation, the lysates were incubated with the antibody of interest, and protein A or G was conjugated to Sepharose (Pierce) for 8 h at 4 °C. To quantify the total amount of protein loaded, 20 μl of the lysates was saved. Beads were washed four times with lysis buffer. Proteins were eluted in SDS-PAGE sample buffer and separated by SDS-PAGE and analyzed by immunoblotting. Blots were then probed with peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies and visualized by enhanced chemiluminescence reagent (Pierce).

Immunofluorescent Assay

HSG cells were fixed in a freshly prepared solution of 4% paraformaldehyde, rinsed, and permeabilized with 0.1% Triton X-100 in PBS. Permeabilized cells were then incubated with horse serum in PBS to block nonspecific binding. After washing with PBS, cells were incubated overnight at 4 °C with mouse anti-NDRG2 antibody (diluted 1:150), rabbit anti-Na^+/K^+-ATPase β1 (diluted 1:150) and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody (diluted 1:400; Sigma) or Cy3-conjugated anti-rabbit antibody (diluted 1:400; Sigma). The isotype mouse and rabbit IgGs were used as negative controls. Dual-color detection was performed by a laser confocal microscope after treatment with 4′,6-diamidino-2-phenylindole (DAPI) to label nuclear DNA.

Transepithelial Electrical Resistance

HSG cells (5×10^5 cells/well) were cultured in 10% FBS DMEM on polyester filters of 6-well Transwell-coll culture chambers (Costar, Cambridge, MA), and the resistance of the HSG cell monolayer was measured using a Millicell-ERS volt-ohmmeter (Millipore Corp., Billerica, MA), according to the manufacturers' instruction. Individual values were normalized by subtracting the background resistance of a filter and medium alone and recorded as ohms/cm^2.

Ionic Transport Measurements

HSG cells (5×10^5 cells/well) were cultured in 10% FBS DMEM on the collagen I-coated filters in the apical chamber of 6-well Transwell-col culture chambers. When confluent, the cells were exposed to 1.6 and 2.5 ml of fresh minimal medium in the apical and basolateral chambers for down-regulating or up-regulating the expression of *NDRG2*. The medium in both the apical and basolateral chambers was harvested separately. The ionic concentrations in the medium were determined with a Hitachi 7600 chemical auto-analyzer (Hitachi, Co., Ltd., Tokyo, Japan).

Whole-cell Patch Clamp Recordings

Na^+-K^+-ATPase—HSG cells were voltage clamped using the whole-cell patch technique. Patch electrodes were made from borosilicate glass capillaries (Clark Electromedical Instruments, Reading, UK) and were fire-polished (DMG Universal Puller, Zeitz-Instrumente VentreibsGmbH, Germany). The electrodes had a resistance of 3–5 megohms when filled with the standard pipette solution. Current signals were recorded using an Axopatch 1-C single electrode voltage clamp amplifier (Axon Instruments) controlled by a microcomputer running pClamp software (version 6, Axon Instruments). A gigaohm seal was rapidly established between the electrode and the cell surface, and the resistance was monitored by a repetitive +5-mV pulse (five is duration) from a holding potential of 0 mV. Access resistance on gaining access to the inside of the cell was <6 megohms. The pipette and extracellular solutions were designed to inhibit all voltage-gated channels and the Na^+/Ca^{2+} exchanger. The standard pipette solution was made of deionized water and contained the following (Sigma) in mM: NaCl 15, $MgCl_2$ 1, CsCl 8, HEPES 10, EGTA 5, MgATP 5, creatine phosphate 5, $CsCH_3O_3S$ 90, $NaCH_3O_3S$ 35, pH 7.2. The standard extracellular solution contained the following (in mM): NaCl 140, KCl 5, $MgCl_2$ 1, $NiCl_2$ 2, $BaCl_2$ 1, glucose 10, HEPES 10, pH 7.4, or without KCl and correction for osmolarity for the potassium-free extracellular solution. Under these conditions, the Na^+/K^+-ATPase current can be defined as that inhibited by the removal of extracellular potassium.

ENaC—Whole-cell macroscopic current recordings of ENaC reconstituted in HSG cells were made under voltage clamp conditions using standard methods. Current through ENaC was the inward amiloride-sensitive Na^+ current with a bath solution of (in mM) NaCl 124, KCl 2.5, $CaCl_2$ 2, $MgSO_4$ 1, $NaHCO_3$

25, NaH_2PO_4 1, glucose 10, pH 7.2, and the pipette solution of (in mM) CsCl 140, NaCl 5, $MgCl_2$ 2, EGTA 1, HEPES 10, MgATP 2, Na_3GTP 0.1, pH 7.4. All currents were filtered at 1 kHz. The cells were clamped to a −60 mV holding potential voltage ramp (500 ms) step protocols with potentials ranging from −100 mV to 40 mV in 20-mV increments to elicit current. Series resistances, on average 3–5 megohms, were also compensated.

Plasma Membrane Na^+/K^+-ATPase Activity Assays—HSG cells (5×10^5cells/well) were infected with NDRG2 or control LacZ adenovirus (5 and 20 multiplicity of infection) or transfected with the *NDRG2*-specific siRNA or control siRNA (100 and 300 pmol, Invitrogen). The cells were harvested, and their Na^+/K^+-ATPase activities were measured essentially as described (36). Briefly, the cell suspension (50 μl) was first cooled on ice, and one portion of it was transferred to the Na^+/K^+-ATPase assay medium. The reactions were shocked at −20 °C and then at 37 °C for 15 min, and the contained proteins were precipitated by trichloroacetic acid/charcoal, followed by centrifugation. The ^{32}P liberated in the supernatant was counted. The Na^+/K^+-ATPase activity was calculated as the difference between testing samples (total ATPase activity) and samples assayed in a medium devoid of Na^+ and K^+ and in the presence of 2 mM ouabain (ouabain-insensitive ATPase activity). Protein determination was performed according to the BCA protein assay kit (Pierce).

Cellular Fractionation

Cells were washed twice with ice-cold phosphate buffered saline, scraped, and homogenized in 1 ml of lysis buffer (in 250 mM sucrose, 10 mM HEPES, pH 7.4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 100 μM Na_3VO_4, 100 μM PMSF, and complete protease inhibitor (Roche Applied Science)). After two centrifugations at 1,000 × *g* for 10 min at 4 °C, the anucleated supernatant were sonicated (power of 3 watts, using a Microson 2425 from Misonix, Inc.) for 10 s on ice and centrifuged at 28,000 × *g* for 15 min at 4 °C. The membrane and cytosolic pellets were resuspended in 150 μl of lysis buffer, and protein concentration was measured using the BCA method.

Statistical Analyses

All values were expressed as mean ± S.D. The difference among the different groups was analyzed by analysis of variance and post hoc analysis, and the difference between two groups was analyzed by Student's *t* test using the GraphPad Prism software version 4.0 (GraphPad software, San Diego). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Estrogen Up-regulates Expression of NDRG2—Given that the promoter region of *NDRG2* had a putative estrogen-response element, we tested the hypothesis that estrogen may directly modulate the *NDRG2* expression. We found that treatment with E_2 significantly increased the levels of *NDRG2* mRNA transcripts in a dose- and time-dependent manner in HeLa cells (Fig. 1, *A* and *B*). A similar pattern of NDRG2 protein was detected following treatment with different doses of E_2 for varying periods (Fig. 1, *C* and *D*).

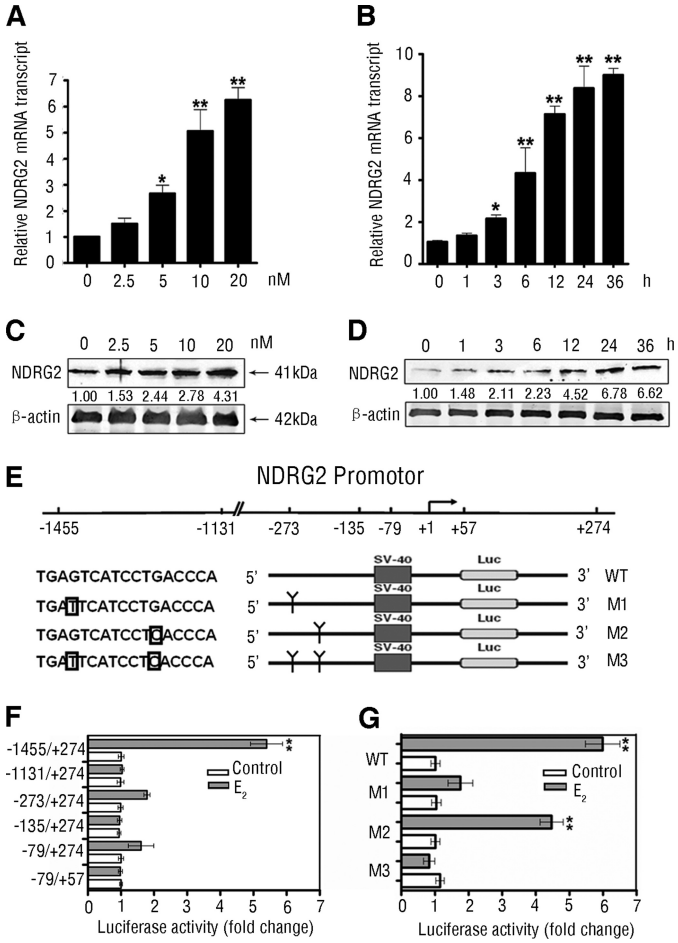

FIGURE 1. **Estrogen up-regulates expression of NDRG2 by binding to the putative ERE in *NDRG2* promoter.** *A* and *C,* dose-dependent up-regulation of NDRG2 expression. HeLa cells were treated with 0–20 nM E_2 for 24 h, and the NDRG2 expression was determined by real time PCR (*A*) and immunoblotting (*C*). *B* and *D,* time-dependent up-regulation of NDRG2 expression. HeLa cells were treated with 10 nM E_2 for 0–36 h and the NDRG2 expression was determined by real time PCR (*B*) and immunoblotting (*D*). Data are expressed as means ± S.D. of the relative levels of *NDRG2* mRNA transcripts to control β-actin (*A* and *B*) and representative images (*C* and *D*) of each group of cells from three separate experiments. *E,* scheme of *NDRG2* promoter region and the deletion and point mutants. The *framed letter* indicates the point mutation. *F* and *G,* HeLa cells were transfected with different deletion and point mutants of the *NDRG2* promoter for 48 h and treated with E_2, or control ethanol for 12 h, and the relative promoter reporter luciferase activity (firefly luciferase/*Renilla* luciferase) of individual groups of cells was analyzed. Luciferase activity experiments were performed in triplicate. Data are expressed as the means ± S.D. of relative changes of individual groups to the control. *, $p <$ 0.05; **, $p < 0.01$ *versus* control.

To explore the mechanism by which E_2 up-regulated *NDRG2* expression, we cloned the promoter region flanking 5′ of the *NDRG2* gene (−1455/+274) (32), and we generated a series of the truncated promoters for testing their responses to E_2 stimulation by luciferase reporter assays. We found that E_2, but not control ethanol, dramatically increased the luciferase activity in HeLa cells that had been transfected with the wild type (WT) *NDRG2* promoter but not in the cells transfected with any of the truncated promoters (Fig. 1*F*). These data suggest E_2 response region located within −1455 to −1131 bp of the *NDRG2* promoter. Accordingly, we generated three mutants of the *NDRG2* promoter by point mutation of one or two nucleotides in the putative ERE in HeLa cells (Fig. 1*E*). We found that

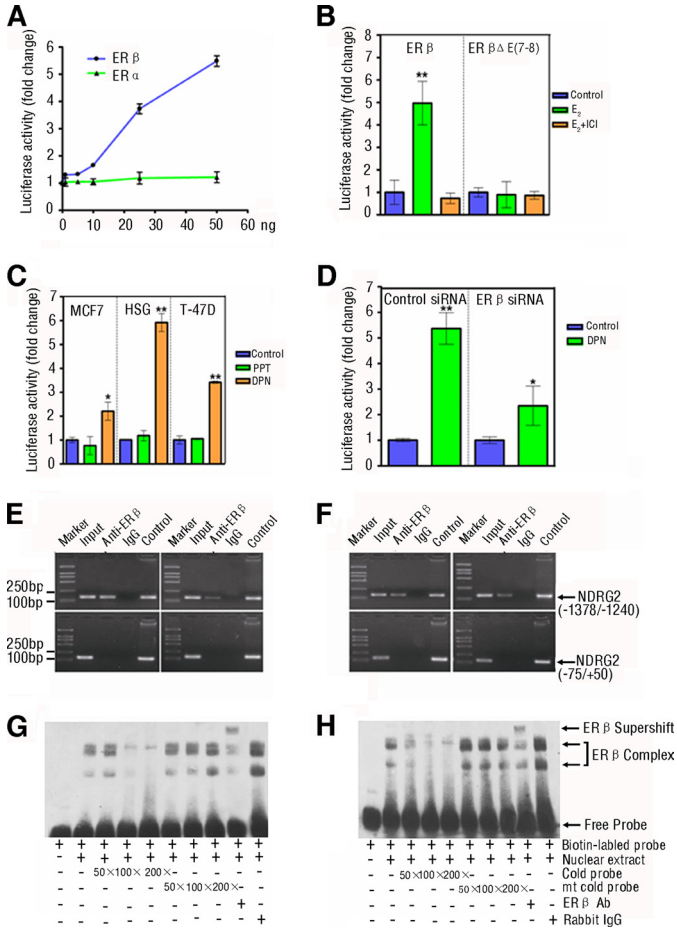

FIGURE 2. **ERβ mediates the estrogen-induced *NDRG2* transcriptional activation by binding to the ERE of *NDRG2* promoter *in vivo* and *in vitro*.** *A,* ERβ, but not ERα, mediates the estrogen-induced *NDRG2* transcriptional activation. HEK293 cells were co-transfected with *NDRG2* promoter and increasing amounts of the expression vectors for human WT ERα or ERβ for 48 h, and then the cells were treated with 10 nM E_2 for 12 h. The luciferase activity was measured. *B,* mutation in the ligand binding domain of ERβ loses the function. HEK293 cells were co-transfected with *NDRG2* promoter and WT ERβ or the mutant with a deletion in ligand binding domain (ERβΔE(7–8)) for 48 h and treated with 10 nM E_2 for 12 h in the presence or absence of 10 nM ICI 182,780 (ER antagonist). The luciferase activity was measured. *C,* DPN, but not PPT, enhances the *NDRG2* transcriptional activation. MCF7, HSG, and T-47D cells that express endogenous ERα and ERβ were transfected with the *NDRG2* promoter for 48 h and treated with ERα- or ERβ-selective agonist, 10 nM PPT or 10 nM DPN, or control ethanol for 12 h. The luciferase activity of individual groups of cells was measured. *D,* silence of *ERβ* expression reduces their responses to DPN. HSG cells were co-transfected with the *NDRG2* promoter and 100 pmol of *ERβ*-specific siRNA or control siRNA for 48 h and treated with DPN for 12 h. The luciferase activity of individual groups of cells was measured. *B–D,* above experiments were performed in triplicate. Data are expressed as the means ± S.D. of relative changes of individual groups of cells to the control cells. *, $p < 0.05$; **, $p < 0.01$ *versus* control. *E* and *F,* binding of exogenous (*left panel*) or endogenous (*right panel*) ERβ to the ERE in the *NDRG2* promoter *in vivo* was measured by ChIP assay. HSG (*E*) and HeLa (*F*) cells were transfected with expression vector for human ERβ or control vector for 48 h and treated with 10 nM E_2 for 12 h. Subsequently, cell lysates were immunoprecipitated with anti-ERβ-specific antibody and analyzed by PCR amplification using the primers flanking the ERE in the *NDRG2* promoter (−1378/−1240) or the control region (−75/+50), as indicated in supplemental Table S1. *NDRG2* promoter plasmid and input chromatin (the remaining portion of cell lysates) were used as positive controls. ERβ antibody isotype rabbit IgG was used as negative control. *G* and *H,* binding of exogenous (*G*) or endogenous ERβ (*H*) to the ERE in *NDRG2* promoter *in vitro* was measured by EMSA. HSG cells were transfected with the expression vector for human ERβ or control vector for 48 h and treated with 10 nM E_2 for 12 h. Their nuclei were extracted and incubated with biotinylated WT probe for *NDRG2*-ERE in the presence or absence of increasing amounts of competitive unlabeled cold probes for *NDRG2*-ERE (WT or mutant) or the ERβ-specific antibody, respectively. The DNA-protein complexes were separated on a 6% TBE gel, transferred to nylon membranes, and visualized using peroxidase-conjugated streptavidin and substrate. Data shown are representative images from two repeated experiments.

M1 and M3, not M2, significantly attenuated the luciferase activity induced by E_2 (Fig. 1*G*). These data strongly suggest that the putative ERE in the *NDRG2* promoter is critical for the responsiveness to E_2.

ERβ Participates in the Transcriptional Regulation of Estrogen on NDRG2 Promoter—Estrogens usually bind to their nuclear receptors, ERα or ERβ, which act as transcription factors, regulating the expression of downstream genes (37), and the ERα and ERβ are encoded by distinct genes and expressed in many tissues with different patterns (38). To determine which type of the ER is responsible for transcriptional regulation on *NDRG2*, we analyzed the expression of ERα and ERβ in human salivary tissues and HSG (human submandibular epithelial duct) cells where high levels of *NDRG2* mRNA transcripts were detected (30). Both ERα and ERβ appeared to express predominantly in the salivary duct cells and HSG cells, which were co-localized with NDRG2, but the intensity of anti-ERβ staining was much stronger than that of anti-ERα (supplemental Fig. S3). To further illustrate the role of ERα or ERβ in the estrogen-mediated up-regulation of *NDRG2* expression, HEK293 cells were transfected with the *NDRG2* promoter-controlled luciferase reporter and the plasmid of ERα or ERβ and treated with different doses of E_2. High levels of luciferase activity were detected in the cells transfected with ERβ, but not ERα, and the E_2-induced activation of the *NDRG2* promoter appeared to be dose-dependent (Fig. 2*A*). This activity was not mediated by the ERβ mutant and was completely abrogated by treatment with ER antagonist ICI 182,780 (Fig. 2*B*). Next, we examined whether the endogenous ERβ was a necessary component for transactivation of *NDRG2* promoter. We transfected MCF7 (human breast cancer), HSG, and T-47D (human breast cancer) cells that express both endogenous ERα and ERβ with the *NDRG2* reporter and treated with the ERα- or ERβ-specific agonist, PPT or DPN, respectively. We found that DPN, the ERβ-specific agonist, activated the *NDRG2* promoter and elevated endogenous NDRG2 protein expression in these cells (Fig. 2*C* and supplemental Fig. S4*A*). Moreover, we examined whether *ERβ* silencing could attenuate the responsiveness of the *NDRG2* promoter to DPN. We found that there were significantly reduced levels of *NDRG2* promoter luciferase activity and NDRG2 protein expression in HSG cells that had been transfected with *ERβ*-specific siRNA, as compared with that in cells transfected with control siRNA (Fig. 2*D* and supplemental Fig. S4*B*). Therefore, ERβ, but not the ERα, mediated the regulation of estrogen on the activation of the *NDRG2* promoter.

ERs can act as transcription factors, activating the downstream genes. To explore whether ERβ could directly bind to the ERE of *NDRG2* promoter, HSG and HeLa cells were transfected with ERβ plasmid, and the potential complex of the ERβ/*NDRG2* promoter was characterized by chromatin immunoprecipitation (ChIP) using anti-ERβ and amplifying the ERE-containing fragment of the *NDRG2* promoter (−1378/−1240 bp). Interestingly, the ERE-containing fragment of *NDRG2* pro-

moter (−1378/−1240 bp), but not the control sequence (*NDRG2* promoter, −75/+50 bp), was precipitated by anti-ERβ in both types of cells with or without ERβ transfection (Fig. 2, *E* and *F*), indicating that inducible and endogenous ERβ bound to this region of *NDRG2* promoter. In addition, the direct binding of ERβ to the ERE region of *NDRG2* promoter was further demonstrated by electrophoretic mobility shift assay (EMSA). Incubation of the biotinylated WT ERE-*NDRG2* DNA with the nuclear extracts from the ERβ-transfected (Fig. 2*G*) or nontransfected HSG cells (Fig. 2*H*) changed the mobilization behaviors of the probe, which was further shifted by the addition of anti-ERβ, indicating the formation of ERβ-DNA complex. The binding of ERβ to the ERE region of *NDRG2* promoter was competitively inhibited by preincubation of nuclear proteins with an excessive amount of WT cold probe but not with the same amount of mutant cold probe. Together, the data clearly demonstrate that estrogen up-regulates the expression of *NDRG2* through direct binding ERβ to the ERE region (position of −1455 to −1131 bp) of *NDRG2* promoter.

NDRG2 Interacts with the β1-subunit of Na^+/K^+-ATPase—Data obtained from yeast and mammalian two-hybrid assays suggest that NDRG2 might interact with Na^+/K^+-ATPase β1 (supplemental Fig. S2). To further confirm that NDRG2 interacts with β1-subunit of Na^+/K^+-ATPase, co-immunoprecipitation was performed using lysates prepared from HEK293 cells that were transfected with pCMV-HA-NDRG2 and pCMV-Myc-Na^+/K^+-ATPase β1. A 41-kDa protein, which corresponded to NDRG2, was precipitated by anti-Myc mAb and probed by anti-HA antibody, whereas a 38-kDa protein corresponding to Na^+/K^+-ATPase β1 was precipitated by the anti-HA antibody and probed by anti-Myc mAb (Fig. 3*A*). We also demonstrated that endogenous Na^+/K^+-ATPase β1 co-immunoprecipitated with endogenous NDRG2 in HSG cells, and vice versa (supplemental Fig. S5). Collectively, these experiments revealed that NDRG2 bound to Na^+/K^+-ATPase β1.

The interaction between NDRG2 and Na^+/K^+-ATPase β1 prompted us to examine their subcellular distribution. In accordance with previous studies, NDRG2 was predominantly localized in the cytoplasm (31), whereas Na^+/K^+-ATPase β1 was in plasma membrane and perinuclear region of the cytoplasm (7). Indeed, we found that a portion of endogenous NDRG2 and endogenous Na^+/K^+-ATPase β1 were co-located in the peri-nuclear cytoplasm region in HSG cells (Fig. 3*B*).

NDRG2 Modulates the Ionic Transportation via Regulating the Na^+/K^+-ATPase Activity—Na^+/K^+-ATPase is responsible for maintaining membrane potential and ionic gradients by transporting sodium and potassium ions against their concentration gradients (3). To investigate the potential impact of NDRG2 on Na^+/K^+-ATPase, we detected the transepithelial ions transport by establishing a polarized monolayer of salivary duct cells cultured on Transwell filters (39), as shown in Fig. 4*A*. HSG monolayer cells on the filters of the apical chamber exhibited a high trans-epithelial electrical resistance (3.71 ± 0.12 kilo-ohms/cm^2), which meant that our model can mimic the *in vivo* environment. In salivary glands, Na^+/K^+-ATPase localizes mainly at the basolateral plasma membrane of the striated duct and excretory ducts (40). Under basal conditions, cells generated basolateral to apical Na^+ and Cl^- gradients, indicating a Na^+/Cl^- reabsorption from the apical to the basolateral side. A significant decrease in Na^+ reabsorption and comparable decrease in Cl^- reabsorption were induced in *NDRG2*-silenced HSG cells; however, it did not alter K^+ and Ca^{2+} gradients (Fig. 4*B*). Consistent with above result, induction of *NDRG2* overexpression by infecting HSG cells with NDRG2 adenovirus significantly elevated the Na^+ and Cl^- gradients but did not affect the K^+ and Ca^{2+} gradients in HSG cells (supplemental Fig. S6*C*). We also checked the effect of NDRG2 on Na^+-activated currents in HSG cells using whole-cell patch clamp. *NDRG2* silencing significantly reduced Na^+/K^+-ATPase-mediated Na^+ current (Fig. 4*C*). Consistent a with previous study in oocytes (41), *NDRG2* silencing could also reduce ENaC-mediated Na^+ current.

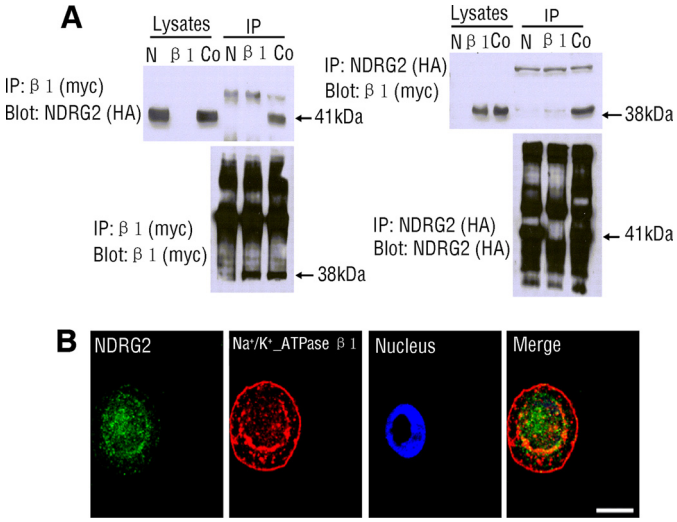

FIGURE 3. **NDRG2 interacts with Na^+/K^+-ATPase β1.** *A,* HA-tagged full-length NDRG2 and/or Myc-tagged Na^+/K^+-ATPase β1-subunit were/was transfected into HEK293 cells. β1-subunit can be detected in an NDRG2 immunoprecipitate (*IP*), and conversely, NDRG2 can be detected in a β1-subunit immunoprecipitate, when the β1-subunit and NDRG2 are transfected together. Vector transfection alone showed no bands in either direction. Immunoprecipitation antibodies were either anti-Myc (β1-subunit) or anti-HA (NDRG2), with detection antibodies either anti-Myc or anti-HA. The location of various proteins is indicated with an *arrowhead*. *N,* pCMV-HA-NDRG2 transfection alone; *β1,* pCMV-Myc-Na^+/K^+-ATPase β1 transfection alone; *Co,* co-transfection with both plasmids; *Blot,* immunoblotting; *IP,* immunoprecipitation. *B,* co-localization of NDRG2 and Na^+/K^+-ATPase β1 in HSG cells. The distribution of endogenous NDRG2 and endogenous Na^+/K^+-ATPase β1 was characterized by immunofluorescent assays using FITC and Cy3 antibodies. Data shown are representative images from three independent experiments. *Scale bars,* 10 μm.

Given that NDRG2 interacted with Na^+/K^+-ATPase β1, and it affected the Na^+ transport and Na^+ current, we next measured the Na^+/K^+-ATPase activity, the motor of sodium reabsorption. As shown in Fig. 5*A*, *NDRG2* silencing reduced Na^+/K^+-ATPase activity, and accordingly, *NDRG2* overexpression increased Na^+/K^+-ATPase activity in HSG cells. Taken together, these results suggest that NDRG2 is a positive regulator of Na^+/K^+-ATPase activity.

NDRG2 Inhibits the Ubiquitination and Degradation of β1-subunit—To investigate the mechanism of increased Na^+/K^+-ATPase activity regulated by NDRG2, we examined whether NDRG2 regulates the abundance of Na^+/K^+-ATPase β1. Notably, NDRG2 did not change the levels of Na^+/K^+-

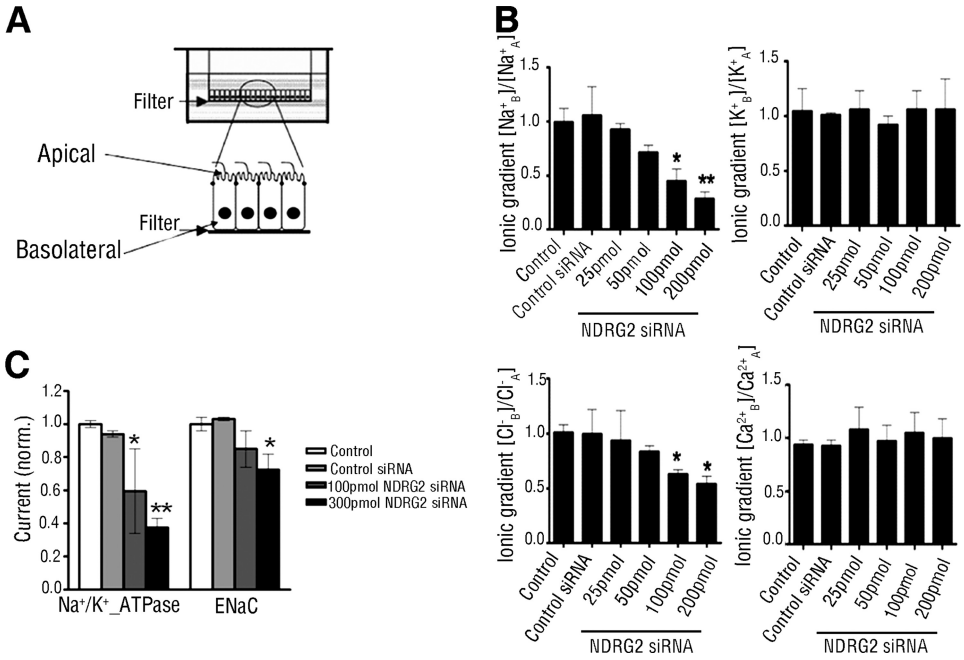

FIGURE 4. **NDRG2 stimulates ionic transport and Na^+/K^+-ATPase-mediated Na^+ current.** *A,* schematic diagram of HSG cells grown on a filter of the top well. HSG cells were cultured on collagen I-coated Transwell Col filters for monolayer. *B,* HSG cells on the filter were transfected with different doses of *NDRG2*-specific or control siRNA for 48 h. The concentrations of Na^+, K^+, Cl^-, and Ca^{2+} in the supernatants from the apical and basolateral compartments were measured, and the ratio of individual electrolyte concentrations in the basolateral to that in the apical well was calculated for the ionic gradients. The value of control cells was designated as 1. Data are expressed as means ± S.D. of the relative values of individual electrolytes in individual groups of cells from three independent determinations. *A,* apical; *B,* basolateral. *C,* HSG cells were transfected with different doses of *NDRG2*-specific siRNA or control siRNA for 48 h. Ouabain-sensitive current (Na^+/K^+-ATPase-mediated Na^+ current) and amiloride-sensitive current (ENaC-mediated Na^+ current) were measured under whole-cell patch clamp conditions. Na^+/K^+-ATPase-mediated Na^+ current was the outward current at a cell potential of 0 mV, which was blocked by 1 mM ouabain. ENaC-mediated Na^+ current was the inward current at a cell potential of −60 mV, which was blocked by 10 μM amiloride. Data are expressed as means ± S.D. of the relative values of each group of cells from 12 independent experiments, and the value of control cells was designed as 1. *, $p < 0.05$; **, $p < 0.01$ *versus* control.

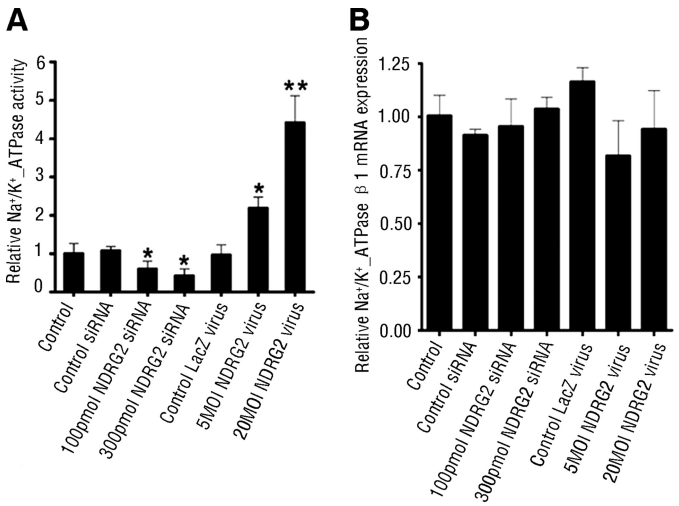

FIGURE 5. **NDRG2 promotes Na^+/K^+-ATPase activity but not affects Na^+/K^+-ATPase β1 mRNA transcripts.** *A,* HSG cells were infected with NDRG2 adenovirus or transfected with *NDRG2*-specific siRNA for 48 h. The Na^+/K^+-ATPase activity in different groups of cells was determined. Results are expressed as means ± S.D. of the relative values of each group to control from three independent determinations, and the value of control cells was designated as 1. *B,* HSG cells were infected with NDRG2 adenovirus or transfected with *NDRG2*-specific siRNA for 48 h. The levels of Na^+/K^+-ATPase β1 mRNA transcripts were determined by real time PCR. Data are expressed as the mean ± S.D. of the relative levels of Na^+/K^+-ATPase β1 mRNA transcripts to control β-actin of individual groups of cells from three independent experiments and the value of control cells was designated as 1. *, $p < 0.05$; **, $p < 0.01$ *versus* control.

ATPase β1 mRNA transcripts in HSG cells (Fig. 5*B*), and because of that, the amount of Na^+/K^+-ATPase at the plasma membrane could be modified by changes in the rate of synthesis or degradation (14, 42). We hypothesized that NDRG2 may exert a regulation on the degradation of Na^+/K^+-ATPase β1. To test this hypothesis, HSG cells that had been infected with NDRG2 adenovirus were then treated with emetine, a protein synthesis inhibitor. We detected significantly high levels of Na^+/K^+-ATPase β1 protein in *NDRG2* overexpression cells 60 min post-emetine, as compared with that in controls (Fig. 6*A*). The amounts of Na^+/K^+-ATPase β1 in the membrane and cytosolic fractions were substantially increased in *NDRG2*-overexpressed HSG cells (Fig. 6*B*). Thus, NDRG2 could inhibit the natural degradation and prolong the half-life of Na^+/K^+-ATPase β1.

To investigate the mechanism that NDRG2 inhibits Na^+/K^+-ATPase β1 protein degradation, we first detected the protein degradation pathway of the β1-subunit. MG132, a proteasome inhibitor, led to greater accumulations of Na^+/K^+-ATPase β1 in HSG cells (Fig. 6*C*), indicating that the β1-subunit degradation was proteasome-mediated, consistent with a previous study that ubiquitylation, as well as endocytosis, was involved in the fast degradation of the β1-subunit (42). Furthermore, ectopic *NDRG2* expression led to a decrease in Na^+/K^+-ATPase β1 ubiquitination in HSG cells (Fig. 6*D*). These results indicated that overexpressed NDRG2 bound to Na^+/K^+-ATPase β1 and inhibited the proteasome-mediated Na^+/K^+-ATPase β1 ubiquitination and degradation, leading to

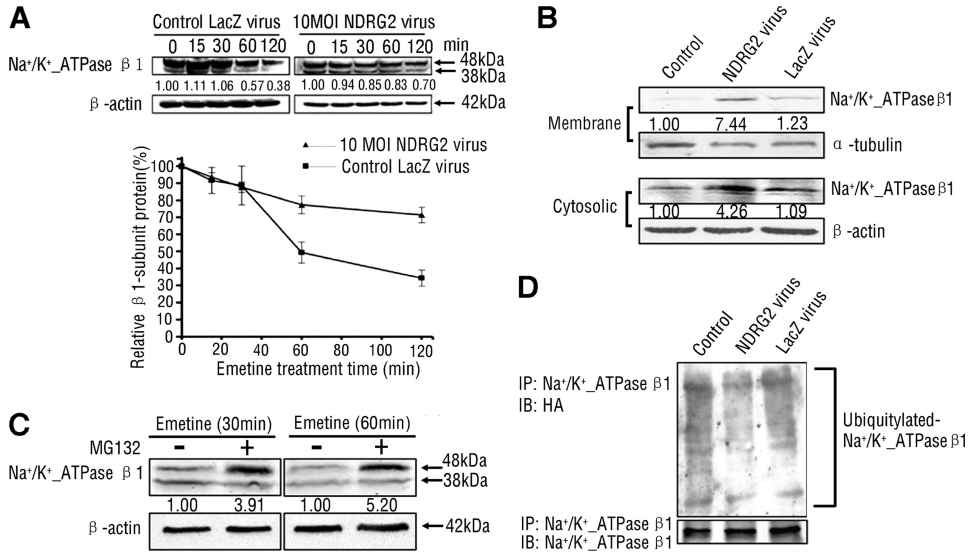

FIGURE 6. **NDRG2 stabilizes Na^+/K^+-ATPase β1 through inhibiting its ubiquitination.** *A, upper panel,* HSG cells were infected with NDRG2 or LacZ adenovirus for 48 h and then treated with 100 μM emetine for the indicated periods. *Bottom panel,* relative levels of β1-subunit to β-actin were quantified by densitometry. Data shown are representative images from three independent experiments. *B,* cell fractions were prepared from HSG cells infected with NDRG2 or LacZ adenovirus for 48 h. Membrane and cytosolic fractions of endogenous β1-subunit were detected. α-Tubulin and β-actin served as loading controls. *C,* HSG cells were treated with DMSO or MG132 for 4 h and emetine for 30 or 60 min; β1-subunit levels were analyzed by immunoblotting. *D,* HSG cells were transfected with HA-ubiquitin for 6 h and then infected with NDRG2 or LacZ adenovirus for 48 h, and cell lysates were collected and subjected to immunoprecipitation and immunoblotting with β1-subunit and HA antibodies. *MOI,* multiplicity of infection; *IP,* immunoprecipitation; *IB,* immunoblotting; *HA,* hemagglutinin.

increased Na^+/K^+-ATPase β1 protein level and Na^+/K^+-ATPase activity.

NDRG2 Is Involved in E_2-mediated Regulation of Na^+/K^+-ATPase β1—Previous studies showed that estrogen up-regulates and activates Na^+/K^+-ATPase (16, 18–20). As our results demonstrated that E_2 can enhance the expression of *NDRG2* and NDRG2 inhibits the degradation of Na^+/K^+-ATPase β1, we postulated that NDRG2 may be involved in the E_2-mediated regulation of Na^+/K^+-ATPase. We found that E_2 can elevate the protein level of Na^+/K^+-ATPase β1. In addition, a significantly lower level of Na^+/K^+-ATPase β1 protein was detected in *NDRG2*-silenced HSG cells compared with control groups with E_2 treatment (Fig. 7*A*). The increased quantity of endogenous Na^+/K^+-ATPase β1 protein in the membrane and cytosolic fractions with E_2 treatment was also attenuated by *NDRG2* silencing (Fig. 7*B*). Next, we found E_2 can inhibit the ubiquitination of Na^+/K^+-ATPase β1, whereas *NDRG2* silencing partly counteracted E_2 mediated above inhibition (Fig. 7*C*). These assays indicated that the regulation of E_2 on Na^+/K^+-ATPase β1 was at least partly mediated by NDRG2.

DISCUSSION

Previous studies have shown that the expression of *NDRG2* is regulated by Myc (32), TNFα, IGF-1 (28), hypoxia (43), DNA damage (44), and many hormones, including dexamethasone, insulin, androgens, and aldosterone (27–29). Nevertheless, regulation of *NDRG2* is still not fully understood and merits further investigation. In our study, we analyzed the promoter region flanking 5′ of *NDRG2* and found a potential ERE. Moreover, we revealed that E_2 up-regulated the expression of *NDRG2* in dose- and time-dependent manner. In addition, we demonstrated that ERβ, but not ERα, bound specifically to the ERE at position of −1455 to −1131 bp of the *NDRG2* promoter and transactivated the *NDRG2* promoter. These data mean that estrogen, as an important circulating hormone, also plays a regulatory role in *NDRG2* expression. *myc* is a downstream target of estrogen and ERs by transcriptional regulation, which has profound mitogenic effects on cancer cells (45). In our previous study, we found Myc represses *NDRG2* gene expression via Miz-1-dependent interaction with *NDRG2* core promoter region (32). In addition, estrogen also regulates the PI3K/Akt pathways (46). Interesting, NDRG2 is one of the phosphorylated substrates of Akt (47). Thus, we infer that estrogen not only regulates *NDRG2* through ERβ-mediated transcriptional regulation but also through many other patterns, such as Myc or Akt-mediated signaling. These complicated yet accurate cross-talk pathways need to be further studied.

NDRG2 seems to be broadly involved in stress responses, cell proliferation, and differentiation (26). To identify NDRG2, interacted proteins may provide important information for understanding its precise molecular and cellular functions. Our previous study characterized a cell cycle-dependent transcription factor MSP58 (58-kDa microspherule protein)/MCRS1 (microspherule protein 1) was a binding partner of NDRG2 (25). NDRG2 could translocate from cytosol to the nucleus to form a complex with MSP58 upon $NiCl_2$ treatment. Data obtained from yeast and mammalian two-hybrid assays suggest that Na^+/K^+-ATPase β1 might also interact with NDRG2 (supplemental Fig. S2). The β-subunit of Na^+/K^+-ATPase serves as a chaperone that promotes proper membrane insertion of the α-subunit. After formation of the holoenzyme, the β-subunit stabilizes the α-subunit in the membrane and modulates cation sensitivity of the pump (48, 49). In this study, we confirmed that NDRG2 directly interacted with Na^+/K^+-ATPase β1 using co-immunoprecipitation. The binding of

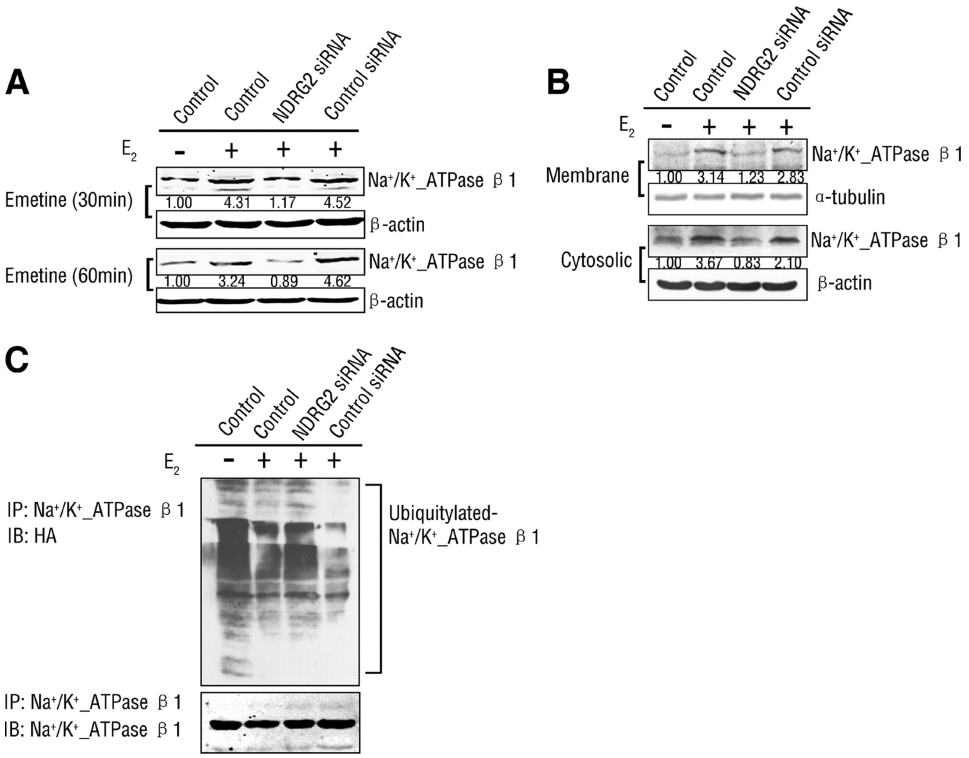

FIGURE 7. **NDRG2 participates in the regulation of estrogen on Na^+/K^+-ATPase β1 protein.** *A–C,* HSG cells were transfected with *NDRG2* siRNA or control siRNA for 48 h and treated with E_2 or ethanol for 12 h. *A,* cell lysates of HSG cells pretreated with 100 μM emetine for 30 or 60 min were analyzed by immunoblotting with β1-subunit antibody. *B,* cell fractions were prepared, and membrane and cytosolic fractions of endogenous β1-subunit were detected. *C,* cells were transfected with HA-ubiquitin plasmid for 6 h, and cell lysates were collected and subjected to immunoprecipitation and immunoblotting with β1-subunit and HA antibodies. *IP,* immunoprecipitation; *IB,* immunoblotting; *HA,* hemagglutinin.

NDRG2 and Na^+/K^+-ATPase β1 prompted us to analyze whether NDRG2 and Na^+/K^+-ATPase β1 localize in the same subcellular compartment. NDRG2 was predominantly localized in the cytoplasm (31), whereas Na^+/K^+-ATPase β1 was in the plasma membrane and perinuclear region of the cytoplasm (7). Our data showed that NDRG2 and Na^+/K^+-ATPase β1 were co-located in the peri-nuclear cytoplasm region.

NDRG2 is expressed in many human tissues, highly in salivary glands, brain, heart, skeletal muscle, and kidney (30), where Na^+/K^+-ATPases are enriched. Based on the salivary duct cell monolayer model, we demonstrated that NDRG2 induces the transport of Na^+ in ductal epithelial cells. Down-regulation of endogenous *NDRG2* by siRNA was associated with a dramatic decrease of Na^+ and Cl^- gradients and the Na^+/K^+-ATPase-mediated Na^+ current. Consistent with the study of Wielputz *et al.* (41) in oocytes and fisher rat thyroid cells, our data showed that *NDRG2* silencing could also reduce ENaC-mediated Na^+ current. It is well known that reabsorption of ion, a vital mechanism for humoral regulation, is an important function in tight epithelium mainly through Na^+/K^+-ATPase, ENaC, and Cl^- channels. Previous studies have indicated that Na^+/K^+-ATPase and ENaC are expressed in tight epithelial cells and are critical components for the control of Na^+ reabsorption (50–52). Our data suggest that NDRG2 may regulate Na^+ reabsorption in epithelial cells by directly interacting with Na^+/K^+-ATPase β1 and affect Na^+/K^+-ATPase holoenzyme, promoting the formation of an electrochemical gradient of Na^+ across the basolateral plasma membrane, leading to low cytosolic concentrations of Na^+, which stimulates the sensors of cytosolic Na^+ and activates ENaC in apical plasma membrane, facilitating the reabsorption of Na^+.

The β-subunit of Na^+/K^+-ATPase facilitates the transport of the α-subunit to plasma membrane, and the α-subunit would execute the catalytic performance (6). We found NDRG2 is a positive regulator of Na^+/K^+-ATPase activity, the motor of sodium reabsorption. To investigate the mechanism of increased Na^+/K^+-ATPase activity regulated by NDRG2, we examined whether NDRG2 regulates the abundance of Na^+/K^+-ATPase β1 and found that Na^+/K^+-ATPase β1 protein, but not RNA, was dramatically affected by NDRG2. Although NDRG2 did not affect the transcript of Na^+/K^+-ATPase β1, it inhibited the ubiquitination and degradation of the β1-subunit. Based on the observation that NDRG2 and Na^+/K^+-ATPase β1 were co-located in the cytoplasm region, we may infer that NDRG2 serves as a chaperone for the β1-subunit that protects it from being degraded during its translocation to the plasma membrane. Therefore, NDRG2 is a physiological regulator of the reabsorption of Na^+ by binding and stabilizing Na^+/K^+-ATPase β1 in salivary duct epithelial cells. Whether this mechanism could apply to brain, heart, skeletal muscle, and kidney where Na^+/K^+-ATPase and NDRG2 were both enriched needs further experiments.

Intricate regulations have developed to adjust the expression and activity of Na^+/K^+-ATPase. Most of the regulators of this pump have been identified to be hormones. Previous studies reported that estrogens can regulate Na^+/K^+-ATPase activity, which is governed by several ways, including an allosteric manner (20) and transcriptional regulation (16). However, the

molecular mechanisms by which estrogen alters Na^+/K^+-ATPase expression and activity have not been fully understood. Here, we demonstrated that human *NDRG2* is up-regulated by E_2 and under the control of E_2-mediated transcriptional regulation. We also found NDRG2 can up-regulate Na^+/K^+-ATPase activity and Na^+/K^+-ATPase-mediated Na^+ transport by binding and stabilizing the Na^+/K^+-ATPase $\beta1$ protein. Based on above data, we postulate that estrogen may affect Na^+/K^+-ATPase via NDRG2-mediated Na^+/K^+-ATPase $\beta1$ regulation. Our results show that E_2 promotes the accumulation of Na^+/K^+-ATPase $\beta1$ protein by inhibiting its ubiquitination and degradation. In addition, knockdown of *NDRG2* could attenuate the effects of E_2 on Na^+/K^+-ATPase $\beta1$, which indicated that the regulation of estrogen on Na^+/K^+-ATPase was at least partly mediated by NDRG2. Interestingly, recent evidence showed that Na^+/K^+-ATPase $\beta1$ also plays an important role in cell-cell tight junctions (9), whereas ERβ-deficient mice failed to maintain the polarized phenotype of epithelial cells (53). This research further implies the possibility of a regulation pathway for E_2/ERβ-induced *NDRG2* expression and enhanced $\beta1$-subunit stability. NDRG2 is able to inhibit proliferation and enhance apoptosis of breast cancer as well as many other malignant tumors (26). In this study, we found that NDRG2 can reduce the natural degradation of Na^+/K^+-ATPase $\beta1$. Given that Na^+/K^+-ATPase $\beta1$ is involved in cell motility and invasion, this may partly explain the reason that *NDRG2* can serve as a tumor suppressor gene candidate in various highly invasive and migratory cancers. However, estrogens exert both genomic and nongenomic effects by complicated signaling pathways. Whether this system is related to breast cancer treatment needs to be directly determined in future studies.

On the basis of our data and previous observations, we propose the following mechanistic model for NDRG2 contributing to estrogen-induced Na^+/K^+-ATPase regulation. The supplemental Fig. S7*A* shows *NDRG2* transcripts at low levels without estrogen treatment or stimulation. Na^+/K^+-ATPase $\beta1$ is ubiquitinated and targeted for rapid destruction by the proteasome in cytosol. Na^+/K^+-ATPase and ENaC mediate Na^+ transport on basolateral and apical sides of plasma membrane, respectively, at low levels in epithelial cells. The supplemental Fig. S7*B* shows in the presence of estrogen that estrogen binds to ERβ, forming a transcription factor, which activates *NDRG2* transcription. NDRG2 protein binds and stabilizes the $\beta1$-subunit by inhibiting the ubiquitin-proteasome pathway-mediated degradation. The holoenzyme of Na^+/K^+-ATPase accumulates and translocates into the basolateral side of cell plasma membrane, where Na^+ is pumped out and an electrochemical gradient of Na^+ is established across the plasma membrane. ENaC localized in the apical membranes senses this Na^+ electrochemical gradient and facilitates the reabsorption of Na^+ in epithelial cells.

Taken together, our data combined with previous studies indicated that estrogen can regulate Na^+/K^+-ATPase via multiple mechanisms. The diversity of the regulatory mechanisms may be required by cells under various physiological and pathological conditions. Therefore, characterization of the novel estrogen/NDRG2/Na^+/K^+-ATPase $\beta1$ regulation pathway broadened the understanding of the regulatory role of estrogen on Na^+/K^+-ATPase, and modulation of this pathway may potentially provide a basis for the intervention of ion transport, fluid balance and internal environment homeostasis.

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