hCLCA2 Is a p53-Inducible Inhibitor of Breast Cancer Cell Proliferation

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Abstract

***hCLCA2* is frequently down-regulated in breast cancer and is a candidate tumor suppressor gene. We show here that the *hCLCA2* gene is strongly induced by p53 in response to DNA damage. Adenoviral expression of p53 induces *hCLCA2* in a variety of breast cell lines. Further, we find that p53 binds to consensus elements in the *hCLCA2* promoter and mutation of these sites abolishes p53-responsiveness and induction by DNA damage. Adenoviral transduction of *hCLCA2* into immortalized cells induces p53, CDK inhibitors p21 and p27, and cell cycle arrest by 24 hours, and caspase induction and apoptosis by 40 hours postinfection. Transduction of the malignant tumor cell line BT549 on the other hand does not induce p53, p21, or p27 but instead induces apoptosis directly and more rapidly. Knockout and knockdown studies indicate that growth inhibition and apoptosis are signaled via multiple pathways. Conversely, suppression of *hCLCA2* by RNA interference enhances proliferation of MCF10A and reduces sensitivity to doxorubicin. Gene expression profiles indicate that *hCLCA2* levels are strongly predictive of tumor cell sensitivity to doxorubicin and other chemotherapeutics. Because certain Cl^- channels are proposed to promote apoptosis by reducing intracellular pH, we tested whether, and established that, hCLCA2 enhances Cl^- current in breast cancer cells and reduces pH to ~6.7. These results reveal hCLCA2 as a novel p53-inducible growth inhibitor, explain how its down-regulation confers a survival advantage to tumor cells, and suggest both prognostic and therapeutic applications.** [Cancer Res 2009; 69(16):6624–32]

Introduction

The evolution of tumor cell from primary cell requires the disabling of a succession of safeguards that enforce the differentiated state (1). One of the first obstacles to be overcome by a hyperplastic cell is the activation of the DNA damage checkpoint in response to telomere erosion (2). Activating this checkpoint in mammary epithelial cells culminates in permanent cell cycle arrest or death (3). DNA damage signaling also figures in arrest at later stages of tumor progression, for example in response to chromosome breaks and chemotherapy with agents such as doxorubicin (4).

DNA damage signals converge on p53, which then activates a battery of genes involved in DNA repair, cell cycle arrest, apoptosis, and other antineoplastic functions (5, 6). Loss of p53 is one of the most common mutations in cancer, allowing the cell to escape this safeguard mechanism (7). Consequently, p53 mutation is one of the most powerful prognostic markers in breast cancer (8). The restoration of p53 itself being technically formidable, there is great interest in identifying p53 target genes for therapeutic potential (9, 10).

The Chloride Channel Accessory (CLCA) protein family has four members in humans and at least seven in mouse, with various members expressed throughout epithelia, endothelia, and other cell types (11). The CLCA archetype is an ~900 amino acid, Type I transmembrane protein that does not closely resemble any other protein type (12). Ectopic expression of CLCA proteins has consistently been shown to produce a novel Cl^- current that can be activated by calcium and blocked by typical Cl^- channel inhibitors; this led to their designation as chloride channels (13). However, we recently showed that hCLCA2 has only a single, COOH-terminal transmembrane segment followed by a 16 amino acid cytoplasmic tail, a structure incompatible with function as a channel (14). Similar conclusions have been reached for other CLCA family members, suggesting that they regulate Cl^- current rather than forming an ion-conductive pore (15–17).

Ion channels influence cell fate decisions by a number of avenues, e.g., by regulating cell volume, intracellular pH (pHi), concentration of signaling molecules such as calcium, and activity of protein kinases and phosphatases (18). Certain ion channels have been proposed to be either oncogenic or tumor suppressive (18–21). Recent observations suggest that the *CLCA* family may harbor tumor-suppressor genes. We reported previously that several mouse *CLCA* genes are transcriptionally down-regulated in mammary tumor cell lines and that ectopic reexpression of these genes inhibits tumor cell growth and survival *in vitro* (22, 23). Down-regulation of *hCLCA2* itself was observed by *in situ* hybridization and reverse transcription-PCR of both human breast cancer specimens and breast cancer cell lines (24, 25). Methylation of the *hCLCA2* promoter region seems at least partly responsible for down-regulation; in addition, missense mutations were detected in some tumor specimens (25). A breast cancer cell line stably transfected with *hCLCA2* cDNA produced smaller tumors in nude mice than vector-transfected controls; however, no effect on *in vitro* proliferation was observed (24).

Certain members of the CLCA family are stress-inducible genes. For example, we recently determined that two mouse paralogs of *hCLCA2* are up-regulated during mammary involution and can be induced *in vitro* by stresses such as cell detachment (22, 23). To gain insight into human breast cancer, we asked whether human *hCLCA2* was also stress-inducible. We show here that *hCLCA2* is potently induced by several DNA damaging agents and that the primary arbiter of stress-response, p53, binds to the *hCLCA2*

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promoter and strongly activates its expression. We find that ectopic expression of hCLCA2 promotes arrest and apoptosis, whereas knockdown has opposite effects, indicating that hCLCA2 is a novel effector of the p53 growth-inhibitory stress response.

Materials and Methods

Cell lines and cell culture. The human immortalized mammary epithelial cell line MCF-10A and transformed derivative MCF10CA1d (referred to herein as CA1d) were obtained from the Barbara Ann Karmanos Cancer Institute and were grown in CM (1:1 DME/F-12), 5% fetal bovine serum (FBS) plus hEGF (1 ng/mL; Invitrogen), insulin (10 μg/mL; Sigma), and hydrocortisone (0.5 μg/mL; Sigma) for all experiments. The human cancer cell lines MCF-7, BT-549, HCT116, and low passage HEK293 were grown in NM (DME/10% FBS). The HCT116 cell lines bearing homozygous deletions of p53, p21, or Bax were a generous gift from B. Vogelstein (Johns Hopkins University, Baltimore, MD).

Quantification of mRNA in mammary cell lines. RNA was extracted and reverse transcribed as described (23). Quantitative PCR was performed on an ABI 7500 instrument using the SybrgreenER premix (Invitrogen). Primers for hCLCA2 were 5′-cagctagtctttggattccaggaa-3′ and 5′-tcagggcttgcagagaatgat-3′. Reactions were performed in triplicate and expression was normalized to β-actin. Bax primers were 5′-tccggggagcagcccagaggc-3′ and 5′-agacacgtaaggaaaacgcatta-3′ and p21 primers were 5′-cacacacagaatctgactccc-3′ and 5′-ctcacatcctccttcttcag-3′.

Chromatin immunoprecipitation. For ectopically expressed p53, two 10-cm dishes of confluent CA1D cells were infected with Ad-p53 at an moi of 200 for 24 h. For chromatin immunoprecipitation (ChIP) of endogenous p53, MCF7 cells were treated with 1 μmol/L doxorubicin or DMSO for 24 h. ChIP was performed as described (26). Details are available upon request. The hCLCA2 promoter interval −359 to −157 containing the p53 binding consensus was amplified with the primers 5′-cagtccagatatactgatttc-3′ (forward) and 5′-ctgggacctgcctctacaag-3′ (reverse); the negative control lacking a p53 consensus was the interval −2,925 to −2,553, amplified with 5′-gccaggttgaactacagagtcag-3′ (forward) and 5′-aggctgaccttacagcctga-3′ (reverse). For the p21 promoter, the primers 5′-caccactgagccttcctcac-3′ and 5′-ctgactcccagcacacacactc-3′ were used to amplify the interval −2,478 to −2,029 containing an established p53 binding site (26).

Western blot analysis. Levels of hCLCA2 were measured by immunoprecipitation and Western blotting with affinity-purified antibody TVE20, raised against the peptide TVEPETGDPVTLRLLDDGAG. Cleared lysates were prepared from confluent 10-cm dishes, and immunoprecipitation and western analysis were performed as described (14). Primary antibody was detected with donkey anti-rabbit antibody tagged with IR680, then scanned and quantified on an Odyssey IR detector (Licor). To detect other proteins, cleared lysates or total cell lysates were analyzed, loading 50 μg of protein per lane. Other antibodies were from Cell Signaling except anti-tubulin monoclonal antibody (mAb) 6G7 (W. Halfter via the Developmental Studies Hybridoma Bank, University of Iowa). Size marker, Dual color (Bio-Rad).

Adenoviral methods. hCLCA2-Ad-Easy was created by inserting a *Not I* fragment containing hCLCA2 into pAdTrack. Recombinant adenovirus was then generated according to established protocols, and viral supernatants were titrated on HEK293 cells and on each breast cell line. p53-AdEasy was obtained from Vector Laboratories. Adherent MCF10A, CA1D, and BT549 cells were typically infected overnight at an moi of 20.

Luciferase assays. A BAC clone containing the hCLCA2 promoter region (#RK11-268K17; Oakland Research Institute) was used as template for PCR (Prime-Star; Takara). Error-free pGEM-T clones were inserted into pGL4 luciferase vector and cotransfected with Renilla control phRL-TK into 293T cells at a ratio of 25:1 using Lipofectamine2000 (Invitrogen). Plasmids encoding p53 fused to green fluorescent protein (GFP; pC1-p53-GFP; gift of Y. Mo, Southern Illinois University School of Medicine, Springfield, IL) or empty vector were included. Luciferase assays were performed on cell lysates using the Dual Luciferase kit (Promega) and quantified on a SIRIUS U3.1 luminometer. Data are reported as the mean of two experiments.

Growth curves. Cell proliferation was measured by seeding 12- or 6-well plates with 100,000 cells per well, 3 wells per time point, and counting at daily intervals on a Vi-Cell Analyzer. Adenoviral infection was performed at the time of seeding.

Mitochondrial extraction. Cells were washed with cold PBS and lyzed in mitochondrial isolation buffer (20 mmol/L HEPES/KOH, 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L EGTA/EDTA, 1 mmol/L DTT, 250 mmol/L Sucrose) using a dounce homogenizer followed by passage through a 26 gauge needle. Homogenized samples were centrifuged at 1,000 *g* for 10 min at 4°C. Supernatants were spun at 10,000 *g* for 20 min, and pellets washed with mitochondrial isolation buffer then resuspended in SDS-PAGE sample buffer, heated, and analyzed by SDS-PAGE. Supernatants were again spun at 14,000 *g* for 30 min to obtain the pure cytosolic fraction.

Cell synchronization and flow cytometry. For cell cycle analysis, CA1d cells were infected with adenoviruses at an moi of 10. Twenty-four hours after infection, cells were arrested with hydroxyurea (1 mmol/L for 24 h) followed by wash-out and nocodazole (0.2 μg/mL for 12 h). Cells synchronized in G_2-M were then released into fresh medium. Cell samples were fixed, treated with RNase A, stained with 50 μg/mL propidium iodide, and analyzed on a FACSCaliber instrument.

RNA interference. Expression of hCLCA2 was suppressed by infection with lentiviruses expressing shRNAs (GIPZ, Open Biosystems clones VLHS-181798, −183886 and nonsilencing control RHS4346). Packaging was performed by cotransfection into 293T cells with plasmids pCMV-dR8.74 and pCMV-VSV-G, purchased from Addgene. Cells were infected in the presence of 8 μg/mL polybrene and selected with 3 μg/mL puromycin with frequent splitting until all cells were GFP positive.

Measurement of pHi. BT549 cells were infected with Ad-GFP or Ad-hCLCA2 at a multiplicity of infection (m.o.i.) of 20. After 48 h, cells were loaded with SNARF-AM (Invitrogen) and pHi was determined by flow cytometry as described by the manufacturer.[3] Details upon request.

Bioinformatics. Gene expression profiles were studied using Oncomine[4] and Gene Expression Omnibus.[5] The hCLCA2 promoter was analyzed using TFSearch[6] and TESS.[7]

Results

DNA damaging agents induce *hCLCA2*. To determine whether *hCLCA2* was induced in response to DNA damage, breast cell lines were treated with the topoisomerase inhibitor doxorubicin, the DNA polymerase inhibitor aphidicolin, or ionizing radiation (IR). All cause DNA damage, leading to either senescence, mitotic catastrophe, or apoptosis, depending on dosage and genetic background (4, 27, 28). An acute treatment of MCF10A-immortalized cells with doxorubicin increased *hCLCA2* mRNA expression by 40-fold (Fig. 1*A*). Similar treatment of the p53-positive breast cancer cell line MCF7 with either doxorubicin or aphidicolin caused a 25- to 30-fold induction of *hCLCA2* expression. Because MCF-7 cells are resistant to IR (4), we instead irradiated CA1d, a tumorigenic derivative of MCF10A, producing a 30-fold induction of hCLCA2 by 48 hours postirradiation (Fig. 1*A, right*).

A survey of the cDNA microarray databases Oncomine and GEO using the key words "human CLCA2 and doxorubicin" revealed multiple studies that confirm and extend these observations. For example, hCLCA2 is induced by 45-fold in response to doxorubicin treatment of HT1080 fibrosarcoma cells (29) and 14-fold in Ewing's sarcoma A673 (30), placing it among the most highly induced genes in those studies (Supplementary Fig. S1). Thus, several forms of DNA damage induce *hCLCA2* expression, and the phenomenon is not limited to breast.

[3] http://probes.invitrogen.com/media/pis/mp01270.pdf
[4] http://www.oncomine.org
[5] http://ncbi.nlm.nih.gov/sites/entrez
[6] http://www.cbrc.jp/research/db/TFSEARCH.html
[7] http://www.cbil.upenn.edu/tess/

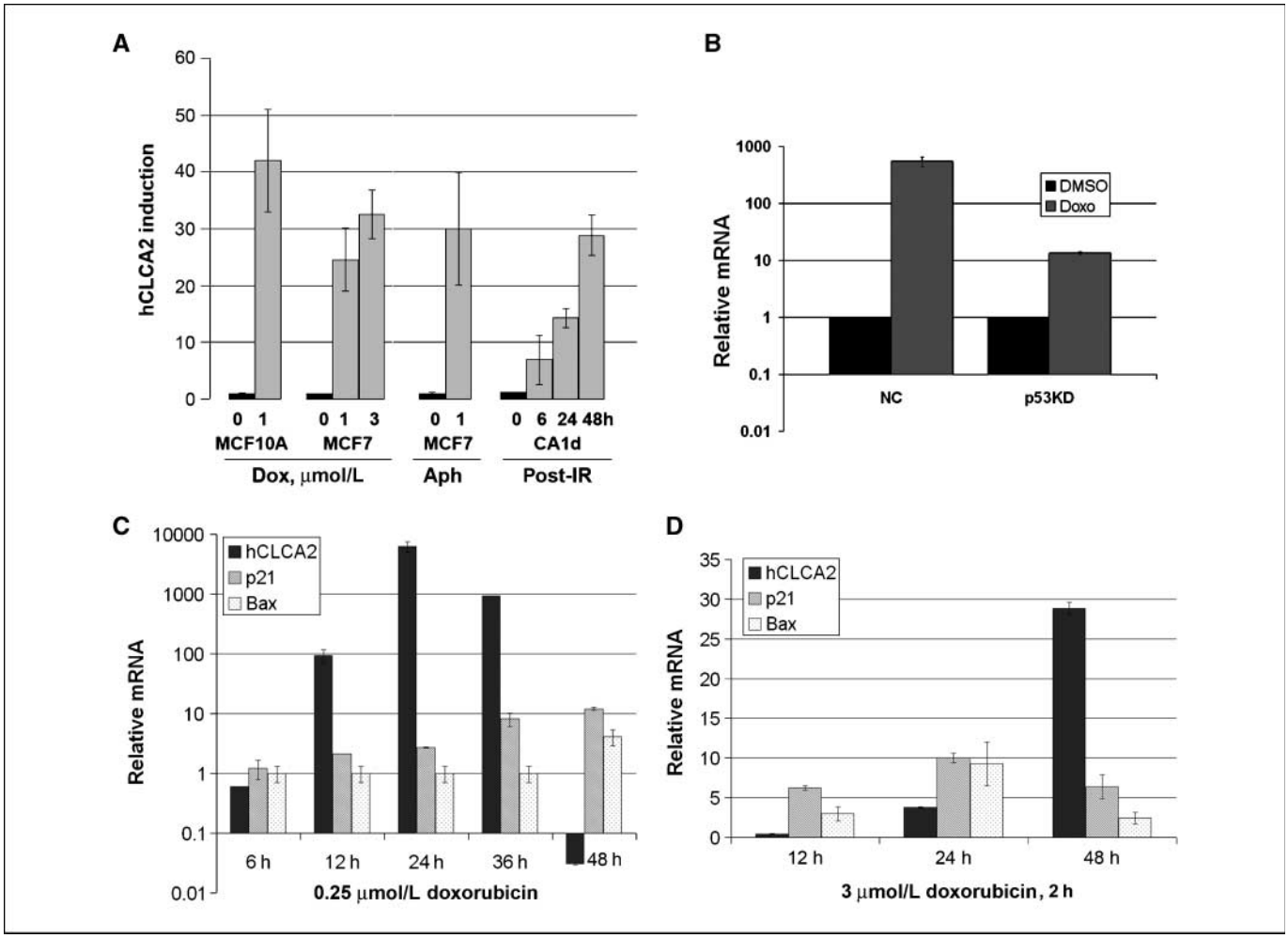

Figure 1. Induction of hCLCA2 by chemotherapeutic agents in breast cancer cell lines. *A,* diverse forms of DNA damage induce hCLCA2. Cells were treated with doxorubicin or 1 μg/mL aphidicolin and induction of hCLCA2 was measured by RT-qPCR. For IR, CA1d cells were treated with 8 Gy of IR and allowed to recover for the indicated times, followed by RT-qPCR. Expression is relative to untreated cells (0 h). *B,* inhibition of induction by p53 knockdown. MCF7 cells transduced with p53shRNA or nonsilencing control were treated with 0.25 μmol/L doxorubicin for 24 h, followed by RT-qPCR. *C* and *D,* kinetics and scale of induction vary with dosage. MCF7 cells were treated with different doses of drug, and kinetics of induction were compared for hCLCA2, p21, and Bax by RT-qPCR. For drug treatments, expression is relative to that of the vehicle control, DMSO.

Because of the well-established role of p53 in DNA damage response, we knocked down expression of p53 in MCF7 cells and measured induction of *hCLCA2* in response to doxorubicin. Induction was reduced by 90% in the knockdown cells, implying that *hCLCA2* is a p53 target gene (Fig. 1*B*).

The kinetics of induction of p53 target genes can provide insight into their function. Genes responsible for arrest and repair tend to be induced earlier than those involved in apoptosis. This kinetic difference is mediated by auxiliary transcription factors and the severity of DNA damage (31). We measured the kinetics of *hCLCA2* induction relative to *p21* and Bax and found that the profile depended on the strength of the insult. At a low dose of doxorubicin, hCLCA2 induction peaked at 24 hours, whereas *p21* and Bax peaked at 48 hours, and hCLCA2 was no longer expressed (Fig. 1*C*). However, at a higher dose, the kinetics were reversed, with hCLCA2 peaking at 48 hours and *p21* and Bax peaking at 24 hours (Fig. 1*D*). The scale of induction also differed. At the low dose, hCLCA2 induction was >1,000-fold, compared with about 30-fold at the higher dose. These results suggest that hCLCA2 may be involved in cell cycle arrest in response to moderate stress but apoptosis or senescence under extreme stress.

p53 binds to the hCLCA2 promoter and activates it. The p53 consensus binding site was recently defined (Fig. 2*A, top*; refs. 9, 10). We found two matches for this sequence within 250 bp of the hCLCA2 initiation codon (Fig. 2*A, bottom*). Comparison with the promoter regions of *hCLCA2* orthologs from mouse, rat, and dog revealed that these elements are evolutionarily conserved (32). To establish that p53 bound to at least one of these elements *in vivo*, we performed ChIP analysis. We found that a p53-specific antibody could immunoprecipitate the *hCLCA2* promoter segment containing these consensus sites from cells transduced with p53-Ad (Supplementary Fig. S2) or from cells treated with doxorubicin to induce endogenous p53 (Fig. 2*B*). A distal promoter segment that lacked such sites was not bound (Fig. 2*B*). In addition, a high-affinity site from the *p21* promoter was also precipitated by the p53 antibody, whereas an antibody against a cell surface protein, CD34, did not precipitate any of the fragments. Identical results were obtained in two cell lines. Thus, both ectopic and endogenous p53 bind to the *hCLCA2* promoter.

To establish whether p53 binding is functionally relevant, we tested segments of the hCLCA2 promoter for the ability to drive a luciferase reporter gene in response to cotransfection of p53 into

293T cells (Fig. 2*C*). This analysis revealed that only segments containing one of the two p53 consensus sites could drive such expression. A segment containing both consensus sites (−274) produced a 200-fold response to p53, whereas loss of one site (−236) halved the activity. Mutation of the innermost site by deletion or C/G to A substitutions in the consensus abolished p53-responsive expression (−184M). Thus, p53 binding to these elements activates *hCLCA2* gene expression. The reporter constructs were similarly p53-responsive in MCF7 cells except that the basal level of *hCLCA2* expression was much higher than in 293T, suggesting that basal expression depends on breast-specific transcription factors absent from 293T (data not shown).

To confirm that p53 plays a role in the induction of *hCLCA2* by DNA damage that was observed in p53$^+$ MCF7 cells, these cells were transfected with the p53-responsive or p53-nonresponsive promoter constructs and treated with aphidicolin (Fig. 2*D*). Only the construct bearing an intact p53-binding site showed induction, consistent with a major role for p53 in DNA damage responsiveness of *hCLCA2*.

p53 transduction induces hCLCA2. To further confirm that p53 drives expression of hCLCA2 from the natural promoter, we infected several breast cell lines with an adenovirus encoding p53, Ad-p53 (10). A prior survey of breast cancer cell lines established that MCF10A and BT549 were especially responsive to adenoviral expression of p53 (33). Accordingly, we found that transcription of hCLCA2 was induced greater than a 100-fold by p53 in MCF10A cells (Fig. 3*A*). Expression of hCLCA2 protein was induced 7.4-fold in parallel with a 4.3-fold induction of p53 (Fig. 3*B*). In the metastatic p53-deficient cell line BT549, expression of p53 peaked around 40 hours postinfection, and hCLCA2 protein was again induced in parallel (Fig. 3*C*). Interestingly, although the cell cycle arrest effector p21 had peaked by 20 hours and then subsided, hCLCA2 expression was robust at both times, consistent with roles in both cell cycle arrest and apoptosis.

hCLCA2 inhibits growth of breast cancer cell lines. To determine whether hCLCA2 reexpression could inhibit proliferation of a malignant tumor cell line, BT549 cells were transduced with adenoviruses expressing hCLCA2 or a tumoricidal control, p53 (Fig. 4*A–C*). This approach avoided artifacts associated with antibiotic selection, allowing short-term effects to be assessed more clearly, and enabled reexpression in transfection-refractory cell lines. In addition, the adenoviral backbone expressed GFP, allowing the virus to be easily titered and the extent of infection assessed. Infection with hCLCA2-Ad resulted in a steady increase in

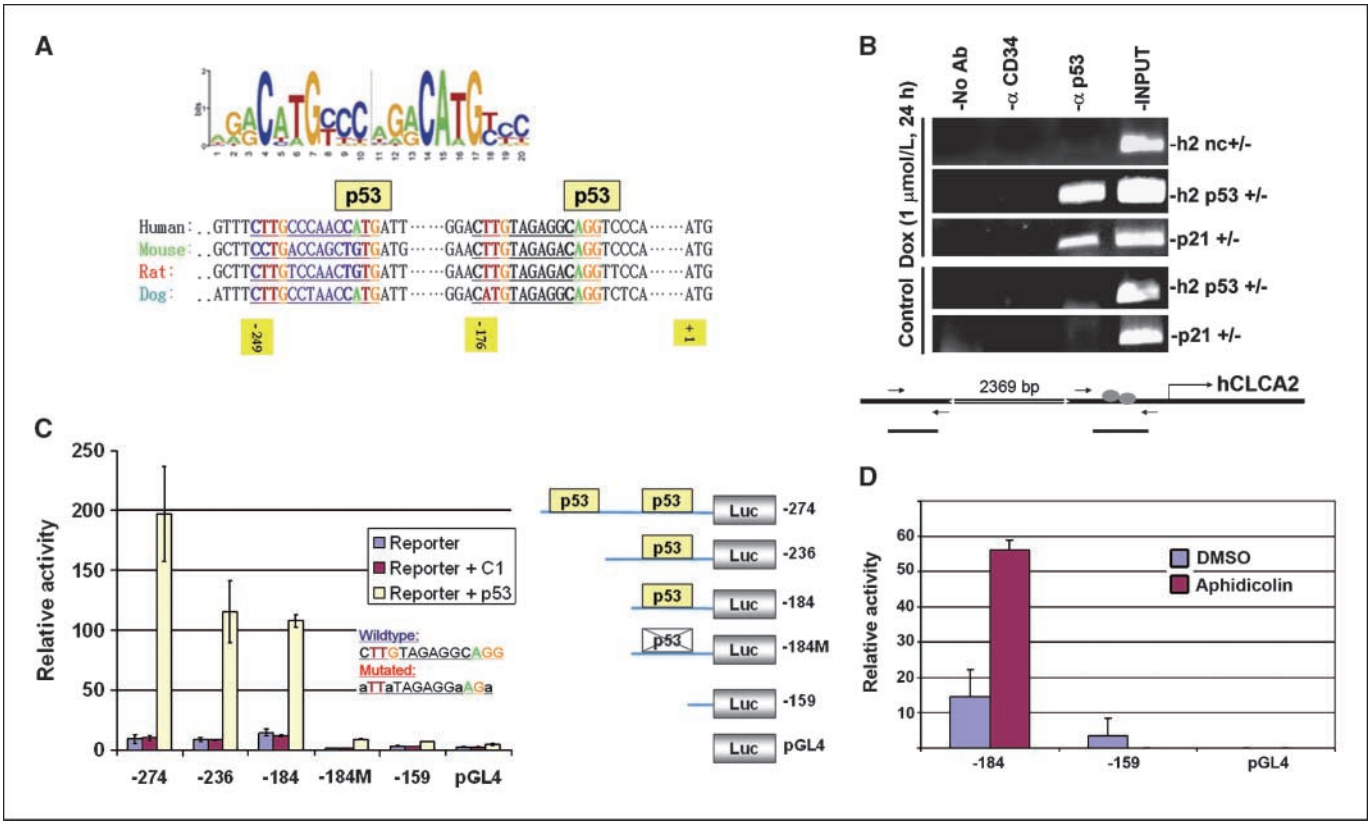

Figure 2. p53 binds to a consensus element in the *hCLCA2* promoter. *A, top,* p53 binding consensus, reprinted from Wei and colleagues (9) with permission from Elsevier. *Bottom,* matches for the consensus in the *hCLCA2* promoter are conserved in orthologs from mouse, rat, and dog. Positions within the human promoter relative to the translation initiation codon are indicated. *B,* ChIP analysis with anti-p53 antibody 1C12 (Cell Signaling) showing p53 binding to the consensus elements in the *hCLCA2* promoter (h2 p53) and to an established binding site from the *p21* promoter (p21) but not to an adjacent region in the *hCLCA2* promoter (h2 nc). An anti-CD34 mAb (Pharmingen) served as an additional negative control. *INPUT*, 10% of the lysate was subjected to PCR directly. Relative positions of the PCR products from the *hCLCA2* promoter are depicted in the schematic. *Dox,* doxorubicin. *C,* demonstration that the putative p53 binding sites from the *hCLCA2* promoter are responsible for transcriptional activation by p53. *Right,* PCR products containing these sites or not were fused to a firefly luciferase reporter gene in pGL4. The constructs were transfected into 293T cells alone or along with a plasmid encoding p53 or no insert (C1). A plasmid expressing Renilla luciferase was cotransfected and readings were normalized to this control. Activities are expressed relative to the pGL4 vector control. Promoter segments include the interval from the indicated upstream nucleotide to the A of the translation initiation codon (+1). *Inset,* mutations introduced into the p53 binding site in the −184M construct are shown. *Luc,* luciferase. *D,* p53 role in induction by DNA damage. MCF7 cells were transfected for 8 h with constructs bearing or lacking the p53 binding site, treated with aphidicolin for 2 h, and lysed 24 h later. *Columns*, mean; *bars,* SE.

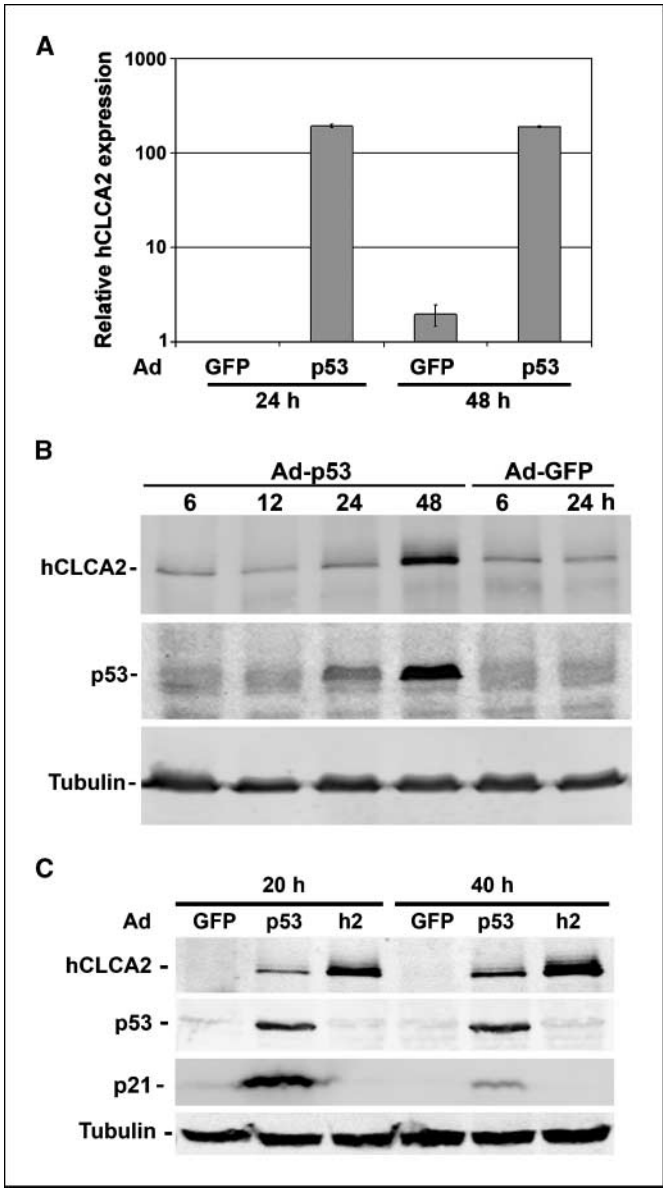

Figure 3. Induction of hCLCA2 by adenoviral transduction of p53. *A,* RT-qPCR shows 200-fold induction of hCLCA2 by 24 h postinfection. Subconfluent MCF10A cells were infected with adenoviral supernatants at an m.o.i. of 30, and RNA was extracted and analyzed. *B,* induction of hCLCA2 protein in parallel with p53. CA1d cells were infected with adenoviral supernatants and lysed at indicated intervals. Lysates were immunoblotted directly for p53 and tubulin or subjected to immunoprecipitation for hCLCA2 followed by immunoblotting. *C,* timing of hCLCA2 induction relative to p21. BT549 cells were infected and immunoblotted 20 or 40 h later. Ad-GFP indicates Ad-Easy vector sans insert; *p53,* p53-Ad-Easy; *h2,* hCLCA2-Ad-Easy.

hCLCA2 protein expression to about thrice the level induced by p53 at 40 hours postinfection (Fig. 3*C*). Similar to p53-Ad but less potent, hCLCA2-Ad arrested proliferation of BT549 (Fig. 4*A*). Quantification of apoptosis by flow cytometry of propidium iodide–stained cells revealed a 4-fold increase in the sub-G_1 fraction (Fig. 4*B*). In a separate experiment, the frequencies of cell detachment and apoptotic nuclei were increased by 2.7- and 5-fold, respectively (data not shown). Notably, hCLCA2-Ad did not induce expression of either p53 or the CDK inhibitors p21 (Fig. 3*C*) or p27 (data not shown) in BT549. On the contrary, we observed induction of initiator caspases 8, 9, and 12, as well as executioner caspase 3 (Fig. 4*C*). Because caspase 8 was strongly induced at 15 hours, well before 9 or 12, hCLCA2 seems to signal apoptosis via the extrinsic pathway. Cross-talk between pathways then activates intrinsic pathways, manifested by the late induction of caspase 9 and translocation of Bax at 30 hours (Fig. 4*C*).

To determine the effect of hCLCA2 transduction on cells that normally express it, we infected MCF10A at the same m.o.i. as BT549. Growth curves showed a strong growth-inhibitory effect, arresting proliferation by 48 hours postinfection and causing ~40% cell detachment and death by 72 hours (Fig. 5*A*). Detached cells showed blebbing typical of apoptosis. Biochemical analysis of apoptotic signaling 40 hours postinfection revealed activation of extrinsic, intrinsic, and endoplasmic reticulum initiator pathways as well as executioner caspase 3 in cells infected with either p53-Ad or hCLCA2-Ad (Fig. 5*B*); notably, hCLCA2-Ad induced apoptosis about 1 day later than p53-Ad at similar m.o.i. Cleavage of PARP and cell death in response to p53 and hCLCA2 were also observed in MCF-7 and nonmammary cell lines as well (data not shown). The induction of apoptosis in MCF10A indicates that there is a threshold beyond which more hCLCA2 expression leads to cell death.

This presented a conundrum, however, because confluent monolayers of MCF10A express hCLCA2 without displaying apoptosis, suggesting that its function in normal cells is to effect cell cycle arrest rather than apoptosis. To address this possibility, we infected MCF10A and analyzed cells 24 hours postinfection. Consistent with the preceding results, no cell detachment or apoptosis was observed at this time. Instead, immunoblots revealed induction of p53 and the CDKIs p21 and p27 (Fig. 5*C*). Fluorometry showed that, after normalization to actin, p53 was induced 2.4-fold; p21, 7.2-fold; and p27, 3.5 fold. In contrast, no induction or cleavage of caspase 3 was detected at this time (Fig. 5*C*). These results suggest that hCLCA2 causes cell cycle arrest in normal cells by activating p53 and CDKIs.

To determine whether hCLCA2 expression caused cell cycle arrest, we took advantage of CA1D cells, which we found exhibited an inhibition of proliferation but little apoptosis during the first 72 hours postinfection (data not shown). We synchronized cells by the double block technique with hydroxyurea followed by washout and nocodazole treatment (Fig. 5*D*). Upon release, most of the hCLCA2-transduced population remained in G_1 phase, whereas Ad-GFP–infected cells resumed cycling.

To determine the genetic requirements for growth inhibition and apoptosis by hCLCA2, we obtained an isogenic set of HCT116 colon carcinoma cell lines bearing homozygous deletions of p53, p21, or Bax and infected them with hCLCA2-Ad or control. None of these deletions reduced growth-inhibition or cell death in response to hCLCA2 (Supplementary Fig. S3). However, inhibition of p53 by pifithrin in the p21 knockout line did reduce cell death compared with p21 knockout alone (Supplementary Fig. S3*B*). Knockdown of p27 also failed to reduce cell death (data not shown). These results indicate that hCLCA2 signals arrest and death via multiple pathways.

Knockdown of hCLCA2 enhances growth rate and resistance to doxorubicin. If hCLCA2 is a growth-inhibitor, then suppressing its expression in normal cells might enhance their growth rate or render them resistant to DNA damaging agents. To attenuate its expression in MCF10A, we used a lentiviral system encoding shRNAs embedded in a microRNA and bearing GFP and puromycin-resistance markers. This hybrid system has been reported to enhance knockdown efficiency and reduce off-target effects.

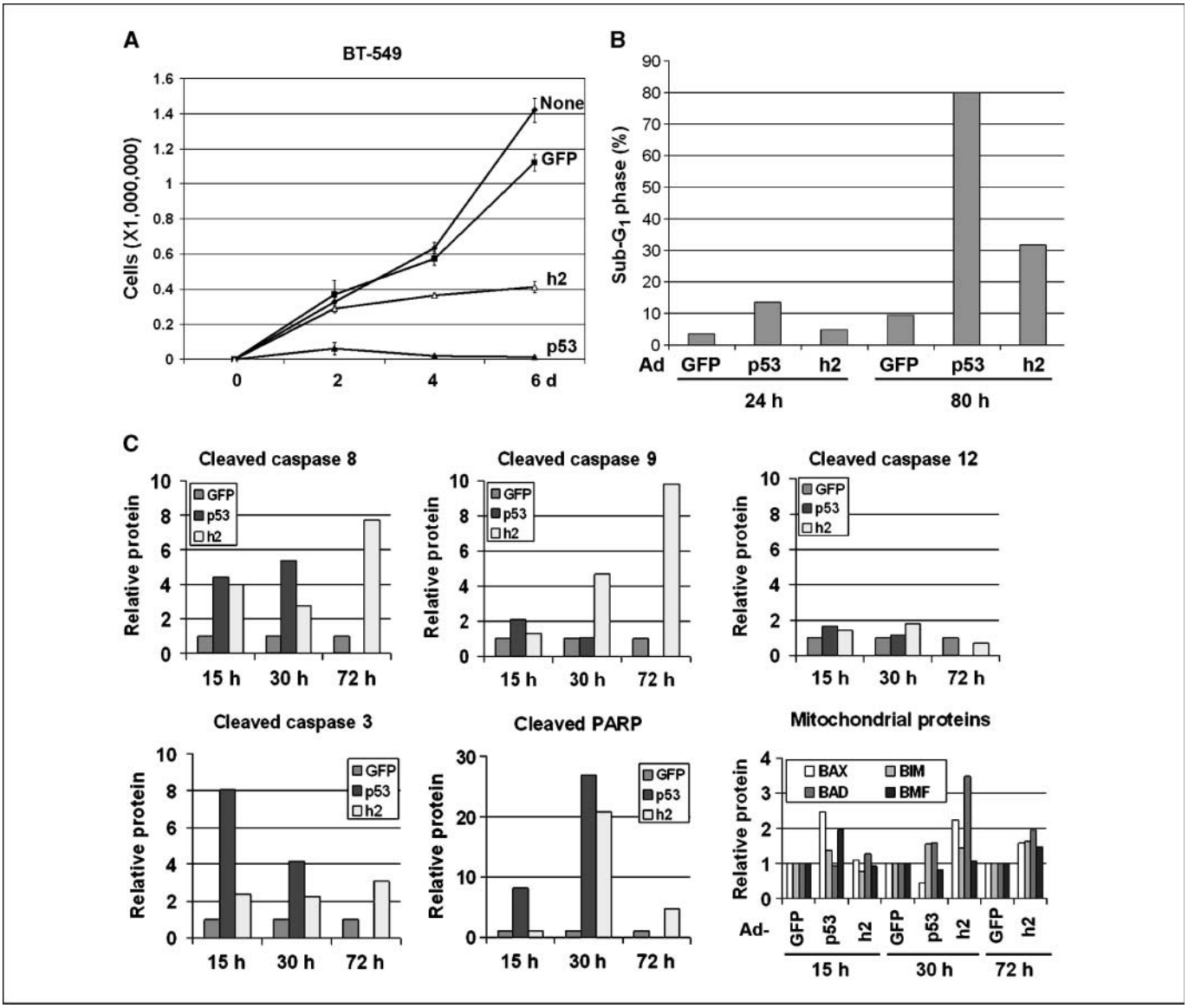

Figure 4. Restoration of hCLCA2 expression in breast cancer cells is growth inhibitory. *A* to *C,* BT549 breast cancer cells were infected with adenoviruses bearing a GFP marker, p53, or hCLCA2 (*h2*) at an m.o.i. of 10. *A,* growth curves measured on a Vi-Cell instrument. *B,* flow cytometry of propidium iodide–stained cells 48 h after infection showed a steep increase in the sub-G_1 DNA peak. *C,* lysates were analyzed by immunoblotting for apoptotic markers. Signals were quantified by fluorometry (Licor) and normalized to actin. Units are relative to Ad-GFP control. *Bottom right,* mitochondrial fractions were analyzed for translocation of Bax, Bad, Bim, and Bmf.

Transduced populations were selected and knockdown was tested by immunoblot. Although H2sh2 suppressed expression by ~90%, H2sh1 suppressed expression by only 50% compared with the nonsilencing control insert, NC (Fig. 6*A, inset*). Nevertheless, both knockdown populations had significantly faster growth than the control over a 4-day time course (Fig. 6*A*).

To determine whether knockdown reduced sensitivity to DNA damage, cells were treated with varying doses of doxorubicin (0.5–3 μmol/L) and cell survival was measured. Both the knockdown cell lines were much more resistant to doxorubicin than was the control, especially at the highest dose tested (Fig. 6*B*). The survival advantage was apparent whether drug exposure was acute or continuous and persisted over 20 days of exposure, suggesting that down-regulation of hCLCA2 confers long-term drug-resistance to tumor cells (Fig. 6*C*).

To further establish whether hCLCA2 expression is a determinant of sensitivity to chemotherapy, we searched public databases of gene expression profiles. One large-scale study grouped 30 cancer cell lines according to resistance to the most common chemotherapeutics (34). Using the Oncomine Concepts Map tool to analyze these data, we found that hCLCA2 expression is predictive of sensitivity to doxorubicin, etoposide, mitomycin C, and vinblastine.

hCLCA2 reduces pHi. Several studies link Cl^- channel activation to reduction of pHi and promotion of apoptosis (35–37). Intracellular acidification is known to activate p53, caspases, and a host of other responses (37–40). We tested whether cells transduced with hCLCA2 have lower pHi. We found that hCLCA2 transduction reduced pHi from 7.49 to 6.67, a level shown previously to promote apoptosis (Supplementary Fig. S4; Fig. 5*E*; refs. 35–37). We then confirmed that ectopic hCLCA2 enhanced

transmembrane anion current in breast cancer cells and the current was further enhanced by calcium (Supplementary Fig. S5).

Discussion

Considerable effort has been invested in the identification of new p53-client genes both for insight into the biology of stress response and for their therapeutic potential (9, 10). Many novel tumor suppressor genes have been identified by these studies. We have shown here that human CLCA2 is induced by several forms of DNA damage, that p53 binds directly to its promoter, and that mutation of the p53 binding site abolishes induction by both p53 and DNA damage. We further showed that reexpression of hCLCA2 is growth inhibitory, provoking cell cycle arrest or apoptosis, depending on the cell line and timing. Notably, transduction of immortalized cells initially induced p53, p21, p27, and cell cycle arrest, but sustained expression produced apoptosis (Fig. 5). Yet in BT549 and other malignant cell lines, which are characteristically incapable of G_1 arrest, no induction of p53, p21, or p27 was detected and only apoptosis was observed, with more rapid kinetics than in MCF10A (15 versus 40 hours). Based on these observations, we propose that the function of hCLCA2 in normal cells is to effect cell cycle arrest in response to stress but that in the absence of the capacity to G_1 arrest, as in aggressive tumor cells, sustained expression

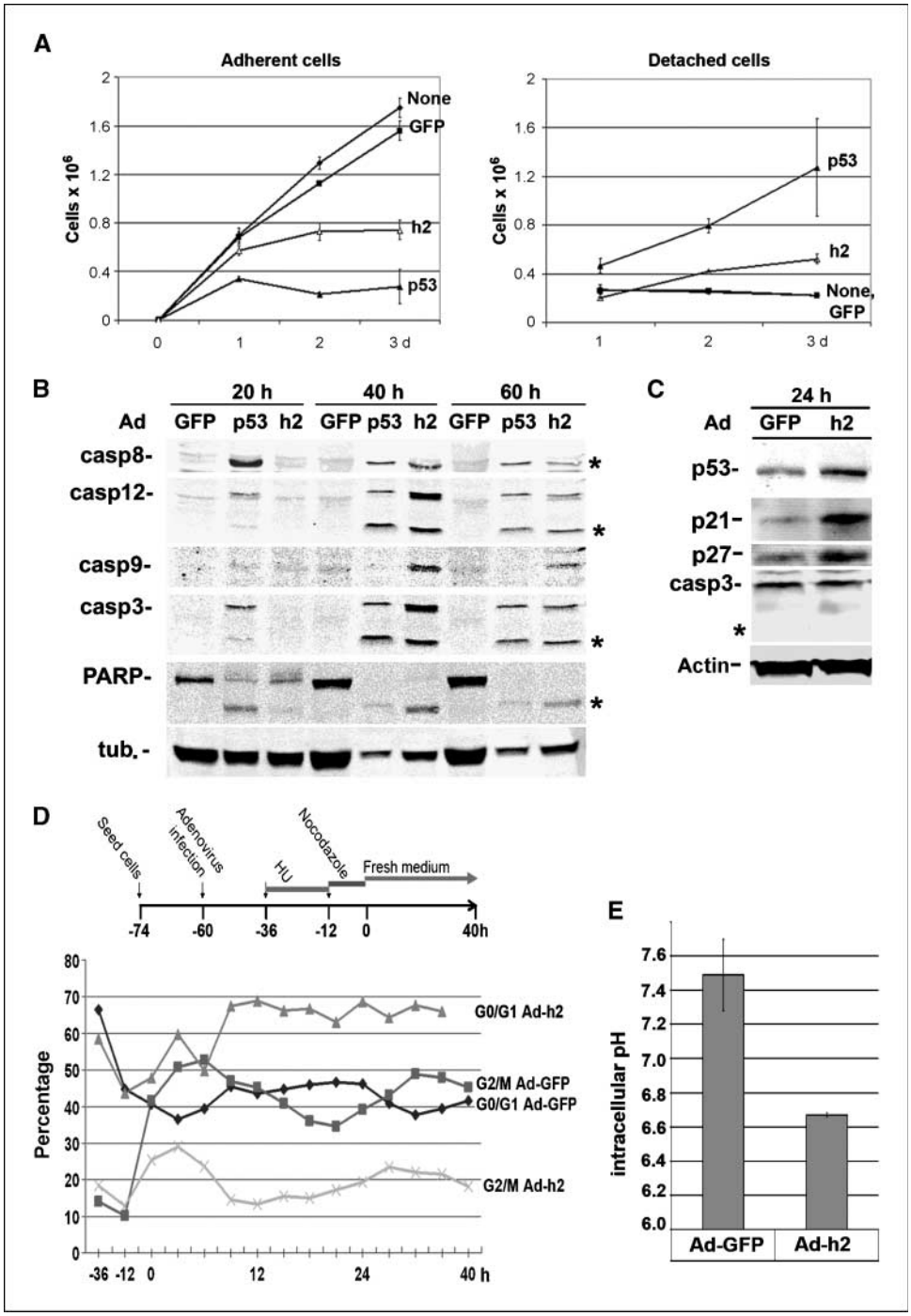

Figure 5. Growth inhibition and apoptosis of MCF10A cells infected with hCLCA2 adenovirus. *A,* cells were infected as in Fig. 4. Adherent (*left*) and detached (*right*) cells were counted daily. *B,* immunoblots of lysates processed at 20 h intervals postinfection. *, proteolytic products diagnostic of apoptosis. *C,* immunoblots of subconfluent MCF10A cells 24 h postinfection show induction of p53, p21, and p27 but not caspase 3. *, absence of caspase 3 proteolytic product. This experiment was repeated four times with similar results. In *B* and *C,* equal amounts of total protein were loaded per lane (50 μg) based on the BCA assay. *D, top,* scheme for cell synchronization by sequential double block and release. *Bottom,* quantification of cells in G_0-G_1 versus G_2-M after release from the double block as measured by flow cytometry. *E,* measurement of pHi in cells transduced with hCLCA2-Ad or control by the SNARF-AM method.

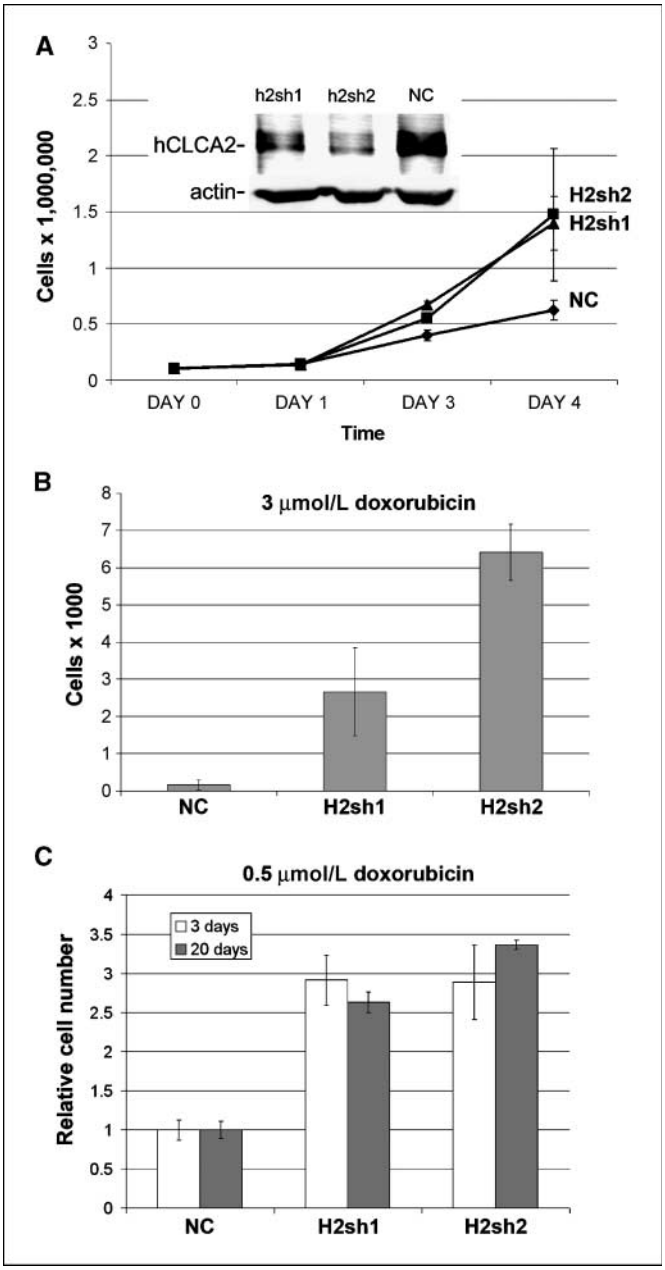

Figure 6. Effect of hCLCA2 attenuation on cell proliferation and survival. Stable knockdown cell lines were generated by infection with lentiviruses expressing shRNAs (H2sh1; H2sh2; or *NC,* negative control), followed by puromycin selection. The extent of knockdown was measured by immunoblot (*A, inset*). To measure proliferation rate, identical numbers of cells were seeded in multiwell plates and counted at one day intervals on a Vi-cell instrument (*A*). *B* and *C,* surviving cell number after treatment with doxorubicin (*B,* 3 μmol/L for 12 h; *C,* 0.5 μmol/L for 3 d) followed by medium change and recovery for 3 or 20 d.

leads instead to apoptosis. These results suggest that transient reestablishment of hCLCA2 expression in a clinical setting might be specifically lethal to tumor cells.

Consistent with this model, we found that attenuation of hCLCA2 expression in immortalized cells confers faster growth and resistance to DNA damage–induced cell death. Together, these results establish hCLCA2 as a multistress responder that may antagonize tumorigenesis at multiple stages. Thus, hCLCA2 satisfies many of the criteria for a tumor suppressor gene. It remains to be determined whether perturbation of its mouse orthologs predisposes to mammary tumorigenesis.

The finding that hCLCA2 is a growth inhibitor leads to the interesting question how immortalized cells are able to tolerate expression of a growth inhibitor. One possibility currently under study is that expression of hCLCA2 is conditional with respect to the cell cycle or other growth parameters as reported for the mouse ortholog mCLCA5 (23).

Growth arrest by hCLCA2 correlates with induction of the growth inhibitors p53, p21, and p27. However, the finding that arrest is not dependent on any of these genes individually suggests that a global change is involved. A number of recent studies link plasma membrane ion channels to progress of the cell cycle and neoplasia (18, 41). For example, Cl^- currents have been observed to vary with the cell cycle, and molecular expression of some Clc channels is cell cycle regulated (18). Moreover, knockdown of Clc-3 induces p21, p27, and cell cycle arrest (42). In breast cancer, the intracellular putative Cl^- channel CLIC4 has been found to be a p53-regulated gene that is down-regulated in tumor cell lines (43). In addition, a subunit of the γ-aminobutyric acid receptor is proposed to be a breast tumor suppressor (44).

Cl^- channels can also potentiate cell death by mediating intracellular acidification (18, 45). For example, transfection of wild-type, but not mutant, CFTR caused a drop in pHi to ~6.7 and potentiated apoptosis in mammary epithelial cells and lung fibroblasts (35–37). Both effects could be prevented by cotransfection with the NHE1 Na+/H+ exchanger (37). Changes in pH have profound effects on cell physiology, influencing the cell cycle, the cytoskeleton, and the activities of regulators such as Bcl-xL, caspases, annexins, and p53 (38, 46–49). Thus, our finding that hCLCA2 acidifies the cytosol could explain its profound inhibitory effects on cell proliferation and survival. We further showed that hCLCA2 stimulates plasma membrane anion current in breast cancer cells. The channel or channels that are regulated by hCLCA2 have not been identified. However, it should be noted that a sister molecule, hCLCA1, is known to interact physically with CFTR (50). Future studies will determine whether hCLCA2 causes intracellular acidification by interacting with this or other channels.

In summary, we have identified a new player in stress response and shown its potency as a growth inhibitor and its relevance to breast cancer progression. These results point to the need for more global studies of hCLCA2 dysregulation in cancer and more directed inquiry into its cellular function(s). Determining the precise mechanism and finding specific agonists or mimics of that action could lead to new therapeutic strategies for cancer treatment, as well as deepen our understanding of epithelial cell biology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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