The Iroquois Homeobox Gene 5 Is Regulated by 1,25-Dihydroxyvitamin D3 in Human Prostate Cancer and Regulates Apoptosis and the Cell Cycle in LNCaP Prostate Cancer Cells

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Abstract

1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃], the most active metabolite of vitamin D, has significant antitumor activity in a broad range of preclinical models of cancer. In this study, we show that the Iroquois homeobox gene 5 (Irx5) is down-regulated by 1,25(OH)₂D₃ in human prostate cancer samples from patients randomly assigned to receive weekly high-dose 1,25(OH)₂D₃ or placebo before radical prostatectomy. Down-regulation of Irx5 by 1,25(OH)₂D₃ was also shown in the human androgen-sensitive prostate cancer cell line LNCaP and in estrogen-sensitive MCF-7 breast cancer cells. Knockdown of Irx5 by RNA interference showed a significant reduction in LNCaP cell viability, which was accompanied by an increase in p21 protein expression, G₂-M arrest, and an increase in apoptosis. The induced apoptosis was partially mediated by p53, and p53 protein expression was increased as a result of Irx5 knockdown. Cell survival was similarly reduced by Irx5 knockdown in the colon cancer cell line HCT116 and in MCF-7 breast cancer cells, each being derived from clinical tumor types that seem to be inhibited by 1,25(OH)₂D₃. Overexpression of Irx5 led to a reduction of p21 and p53 expression. This is the first report that Irx5 is regulated by 1,25(OH)₂D₃ in humans and the first report to show that Irx5 is involved in the regulation of both the cell cycle and apoptosis in human prostate cancer cells. Irx5 may be a promising new therapeutic target in cancer treatment.

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) promises to be effective in the treatment and prevention of cancer. Numerous in vitro and in vivo studies have shown the ability of biologically active analogues of vitamin D to potently inhibit the proliferation of many different cell types, including carcinomas of the prostate, breast, colon, skin, brain, and myeloid leukemia cells (1, 2). 1,25(OH)₂D₃ has also been shown to induce apoptosis in several tumor models (3–8) and to inhibit angiogenesis and tumor invasion (9–14). However, the molecular factors that mediate these processes have yet to be clearly defined.

Members of the Iroquois gene family play multiple roles in patterning and regionalization of embryonic tissues during development and are highly conserved from Drosophila to mammals (15–21). The Iroquois homeobox (Irx) genes were first identified in Drosophila because of their role in the control of proneural gene expression (16, 22). In Drosophila, the Iroquois gene cluster consists of three Irx genes, whereas in mammals, six genes exist in two clusters. The mammalian IrxA cluster (Irx1, Irx5, and Irx4) is located on chromosome 8, whereas the IrxB cluster (Irx3, Irx2, and Irx6) is found on chromosome 16. Iroquois protein products bind DNA, at least in part through their homeodomain motif, a highly conserved 63–amino acid sequence, and seem to act as both transcriptional activators and repressors (23–25). Outside the homeodomain, Irx family members share little sequence similarity, but all Irx transcription factors have a conserved motif called the Iro box.

Homeobox genes are developmental regulators that are essential for growth and differentiation, including early embryonic patterning, cell-type specification, and organogenesis. Increasing evidence indicate that normal development and tumorigenesis are intimately related, and the anomalous expression of many homeobox genes has been detected in a number of cancers, including prostate (26–29). Evidence that homeobox genes are involved in the regulation of proliferation through their interaction with several cell cycle regulators, including p21, p27, and cyclin D1, is accumulating (30). Recent data also suggest that homeobox genes play a role in regulating apoptosis, angiogenesis, and/or metastasis; however, very little is known about the specific signaling pathways involved (31, 32). A number of homeobox genes have been shown to be regulated by vitamin D (33).
frozen tumor samples were obtained from a subset of patients for the purposes of biomarkers using immunohistochemistry in fixed specimens, snap-frozen tissue was the study of prostate cancer tissue for a series of biomarkers using immunohistochemistry in fixed specimens, snap-frozen tissue was used for the study of prostate cancer tissue for a series of biomarkers.

Materials and Methods

Chemicals. 1,25(OH)2D3 was a kind gift from Dr. Milan Uskokovic, Hoffmann-La Roche. A 50 μg/mL stock solution dissolved in absolute ethanol was stored at -80°C and protected from light.

Patients. The eligibility criteria and procedures of our randomized trial of high-dose 1,25(OH)2D3 or placebo before prostatectomy have been previously described (34). Briefly, 44 patients were randomly assigned to receive 1,25(OH)2D3 (0.5 μg/kg) or identical placebo weekly for 4 wk before prostatectomy. Prostatectomy was carried out 4 ± 1 d after the last dose of 1,25(OH)2D3. Whereas the primary goal of the study was the interrogation of prostate cancer tissue for a series of biomarkers using immunohistochemistry in fixed specimens, snap-frozen tumor samples were obtained from a subset of patients for the purposes of exploratory studies of the effect of high-dose 1,25(OH)2D3 on gene expression at the RNA level. After surgical removal, prostate tissue was fixed in Cryomatrix (Thermo Shandon), snap frozen in a solution of liquid pentane and dry ice, and stored at -80°C.

Cell lines. LNCaP and MCF-7 cells were obtained from American Tissue Culture Collection. Cells were subcultured every 48 to 72 h in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Cells were incubated at 37°C with standard 5% CO2 content in a humidified incubator. HCT 116 cells were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University). HCT 116 cells were cultured in McCoy's 5A medium (Invitrogen) with 10% fetal bovine serum. The LNCaP p53 dominant-negative pC53Tetp248 cells (248 mutant) and the control pRC-CMV were generously provided by Dr. Mark Garzotto (Portland VA Medical Center). The pC53Tetp248 plasmid contains a mutant form of p53, which has a Trp substitution at the 248 position that allows it to inactivate wild-type p53. LNCaP cells stably transfected with the pC53Tetp248 plasmid (LNCaP/p53DN) markedly overexpress dominant-negative p53 by immunoblot analysis compared with control (pRC-CMV)–transfected cells. Results from a transient transfection of LNCaP/p53DN cells with a p21 promoter-luciferase reporter plasmid showed that overexpression of the 248-p53 mutant inactivates the function of wild-type p53 in LNCaP cells (data not shown).

RNA extraction from human tumors. Snap-frozen human prostatectomy specimens containing ≥90% cancer (determined by histology analysis by a clinical pathologist) were pulverized by mortar and pestle to inactivate wild-type p53. LNCaP cells stably transfected with the pC53Tetp248 plasmid (LNCaP/p53DN) were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University). HCT 116 cells were cultured in McCoy's 5A medium (Invitrogen) with 10% fetal bovine serum. The LNCaP p53 dominant-negative pC53Tetp248 cells (248 mutant) and the control pRC-CMV were generously provided by Dr. Mark Garzotto (Portland VA Medical Center). The pC53Tetp248 plasmid contains a mutant form of p53, which has a Trp substitution at the 248 position that allows it to inactivate wild-type p53. LNCaP cells stably transfected with the pC53Tetp248 plasmid (LNCaP/p53DN) markedly overexpress dominant-negative p53 by immunoblot analysis compared with control (pRC-CMV)–transfected cells. Results from a transient transfection of LNCaP/p53DN cells with a p21 promoter-luciferase reporter plasmid showed that overexpression of the 248-p53 mutant inactivates the function of wild-type p53 in LNCaP cells (data not shown).

RNA extraction from cell lines. LNCaP cells grown in 60-mm plates were washed with PBS and subsequently lysed in RSBI lysis buffer [10 mmol/L Tris-Cl (pH 7.4), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.65% NP40]. The lysis was centrifuged (2,500 × g) at 4°C for 10 min, and RNA was extracted in 0.8 mL phenol and 0.6 mL chloroform. RNA was precipitated in 100% ethanol at 80°C for at least 24 h and reconstituted in nuclease-free water. Resuspended RNA was further purified using the RNeasy cleanup kit, and DNase digestion was carried out on the column (Qiagen, Inc.) according to the manufacturer's instructions.

Real-time reverse transcription–PCR. Purified RNA (1 μg) was reverse-transcribed by Superscript II enzyme (Invitrogen) and random hexamers. cDNA was cleaned-up using the Minelute PCR kit (Qiagen). cDNA (100-400 ng) was combined with TaqMan PCR Master Mix (Applied Biosystems) and run on an ABI PRISM 7000 Sequence Detection System using Irx5 and 18S RNA (endogenous control) specific primers and MGB probes (Applied Biosystems) as previously described (35). A standard curve was run to determine efficiency of primer/probe binding. All reactions were carried out in triplicate. RNA expression was quantified by the comparative ΔΔCt method (Applied Biosystem's User Bulletin 2).

Cell proliferation. LNCaP cells (100,000 per well in six-well plates) were treated with indicated concentrations of 1,25(OH)2D3 24 h after plating. Cells were harvested by trypsinization 96 h after treatment and resuspended in media containing 0.4% trypan blue (Invitrogen). Live cells were counted under a microscope using a hemocytometer (Hauser Scientific).

Small interference RNA. Small interference (siRNA) molecules aligned with Irx5 mRNA were synthesized by Dharmaco as desalted duplexes with the following sense sequences: si1 5′-GCC UCA GCG ACU AGU UAU UdT dt-3′, si2 5′-GAG AGA GAC GAG AGA AdT dt-3′, si3 5′-CAU CGU CGG ACA AGG UCA AdT dt-3′. Cells were plated in RPMI 1640 containing 10% fetal bovine serum and transfected 36 h later with 133 μmol/L siRNA using Oligofectamine reagent (Invitrogen) in serum-free media according to the manufacturer's protocol. 10% Fetal bovine serum was added to transfected cells 4 h after transfection. Effects of Irx5 knockdown were normalized to CY3-labeled luciferase GL2 duplex (control; Dharmaco) with target sequence 5′-CGT ACC GGG AAT ACT TCG A′-3′. Western blot analysis. LNCaP cells were washed twice in PBS and lysed in radioimmunoprecipitation assay buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.01% phenylmethylsulfonyl fluoride, 1× complete protease inhibitor cocktail; Roche). The lysate was incubated on ice for 1 h. Growth media containing dead cells was aspirated and centrifuged for 5 min in chilled tabletop centrifuge at 4,000 × g. Pelleted cells were washed with PBS and combined with radioimmunoprecipitation assay buffer lysate. Cellular debris was pelleted in chilled microcentrifuge at 10,000 × g for 30 min, and the supernatant was isolated. Protein concentrations were measured using Bradford reagent (Bio-Rad). Protein samples were resolved on a 10% NuPAGE precast gel (Invitrogen), transferred to a polyvinylidene difluoride membrane (Amersham), and probed with polyclonal antibody anti–poly (ADP-ribose) polymerase (PARP) β85 fragment (Promega) or polyclonal anti-p21 antibody (Santa Cruz). Loading control was accessed using monoclonal anti–β-tubulin antibody (Sigma), polyclonal anti-actin antibody, or anti–glyceraldehyde-3-phosphate dehydrogenase (Santa Cruz). A horseradish peroxidase–conjugated secondary antibody (Santa Cruz) was used to visualize proteins with standard luminal reagents (Perkin-Elmer Life Sciences, Inc.).

Hoechst staining. Trypsinized and floating cells were washed in PBS, pelleted, and resuspended in 3% paraformaldehyde/PBS solution (Sigma). To visualize apoptotic cells, cells were subsequently stained with Hoechst 33258 pentahydrate (Molecular Probes) and counted under a microscope with UV light source (excitation, 350-365 nmol/L). An average of 300 to 500 cells was counted per sample. Apoptotic cells with at least three nuclear fragments were scored and represented as a total of counted cells.

Overexpression. 1 × 105 per well LNCaP cells were plated into six-well plates. At ~60% confluence (36 h), cells were transfected with either 1 μg of the PCM6v/XL-5 (Origene) parent vector or the same vector containing the entire Irx5 open reading frame using Lipofectamine (Invitrogen) according to the manufacturer's instructions.

Cell cycle analysis. Approximately 2 × 106 LNCaP cells were harvested 48 and 72 h after treatment. Cells were washed in PBS, fixed in 70% ethanol for 1 h at 4°C, and stored for up to 2 wk at -20°C. At that time, cells were washed in PBS, stained with 1 ml of propidium iodide staining solution [3.8 mmol/L sodium citrate (Fisher Scientific) and 50 μg/ml propidium iodide (Sigma in PBS)], then combined with RNase A (Worthington Biochemicals), and added to a final

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concentration of 0.5 μg/mL. Samples were stored at 4°C overnight and analyzed by flow cytometry on a FACSCalibur (Becton Dickson).

Statistical analysis. Means were compared using Student’s t test or ANOVA when more than two samples were analyzed simultaneously.

Results

1,25(OH)2D3 down-regulates Irx5 in human prostate cancer tissue and in the androgen-sensitive prostate cancer cell line LNCaP. To explore the effects of 1,25(OH)2D3 on human prostate cancer, a randomized, placebo-controlled trial of high-dose 1,25(OH)2D3 before prostatectomy was carried out in patients with localized prostate cancer (34). After RNA extraction, the Affymetrix U133A chip was used to compare gene expression profiles in 10 prostate cancer specimens (samples confirmed to consist predominantly of adenocarcinoma by frozen section examined by a pathologist) collected after 4 weeks of high-dose 1,25(OH)2D3 or placebo therapy. The results showed that the homeobox gene Irx5 is down-regulated in 1,25(OH)2D3-treated tumors relative to controls (raw P = 0.0000008, FDR = 33%; data not shown).

Subsequently, the effect of 1,25(OH)2D3 therapy on down-regulation of Irx5 expression was confirmed in the same human specimens using real-time reverse transcription–PCR (RT-PCR). As shown in Fig. 1A, Irx5 mRNA levels, measured by real-time RT-PCR, are reduced 3-fold in 1,25(OH)2D3-treated patients compared with placebo-treated controls.

To further validate this target and develop a model system for laboratory investigation, the effect of 1,25(OH)2D3 therapy on Irx5 expression was examined in the LNCaP prostate cell line using real-time RT-PCR. A persistent 60% to 70% (P < 0.001, ANOVA) reduction in Irx5 expression from 48 to 120 hours was observed with a single treatment of 1,25(OH)2D3 (10⁻⁸ mol/L; Fig. 1B). The presence of Irx5 mRNA was also examined in androgen-independent PC-3 cancer cells and was found to be significantly lower in these cells (~30-fold lower than in LNCaP cells), as measured by real-time RT-PCR (data not shown).

Similar to the effects on LNCaP cells, Irx5 mRNA expression is significantly down-regulated by 1,25(OH)2D3 in the hormone-dependent breast cancer cell line, MCF-7 (Fig. 1C). This experiment provides evidence that the effect of 1,25(OH)2D3...
on Irx5 is not restricted to prostate cancer and may play a central role in other hormonally regulated tumors. 1,25(OH)2D3 and its analogues have been shown to have significant antiproliferative effects on prostate cancer cells. The effect of 1,25(OH)2D3 on LNCaP cell growth was examined over a range of concentrations to compare the effects of 1,25(OH)2D3 with those resulting from targeting Irx5 directly. A 1,25(OH)2D3 dose-response curve shows that 10 nmol/L 1,25(OH)2D3 reduces LNCaP cell numbers by ~50% at 96 hours (IC50 ≈ 10 nmol/L; Fig. 1D).

**Down-regulation of Irx5 by RNA interference reduces LNCaP cell numbers.** To examine more closely the function of Irx5 in prostate cancer cells, LNCaP cells were transfected with two different 21-nucleotide Irx5-specific siRNAs (si1 and si2) or control siRNA to knockdown Irx5 expression, and viable cells were counted at 96 hours. Irx5 si1 targets the open reading frame of the Irx5 gene, and si2 targets the 3′ untranslated region. Transient transfection of siRNA oligos in LNCaP cells consistently exhibited ~60% transfection efficiency (data not shown). Treatment with si2 reduced Irx5 gene expression by ~3-fold at 24 hours as determined by real-time RT-PCR (data not shown) and, as shown in Fig. 2A, was associated with a 50% reduction in LNCaP cell numbers (P < 0.001, t test) at 96 hours. A time course of the effect of si2 on LNCaP cell survival shows that cell numbers were reduced by ~60% at 120 hours compared with control siRNA-transfected cells (Fig. 2B).

All siRNA oligonucleotides were searched against the human genome, and no identical sequences were found. However, considering the possibility that Irx5 RNA interference oligonucleotides may interact with a similar mRNA sequence in another gene, a third Irx5 siRNA (si3) targeting the open reading frame of Irx5 was tested to ensure that the effects observed were specific to Irx5. Figure 2C shows that this siRNA construct (si3) also significantly reduced LNCaP cell numbers at 96 hours compared with controls. When compared with the results with 1,25(OH)2D3, Irx5 knockdown had a similar effect on LNCaP cell survival at 96 hours as 10 nmol/L 1,25(OH)2D3 (Fig. 1D). These data suggest that Irx5 may serve as an important mediator for 1,25(OH)2D3 sensitivity in LNCaP cells. Because specific Irx5 down-regulation was found to have substantial inhibitory effects on prostate cancer cells, Irx5 may function as a tumor promoter in these cells. In addition, MCF-7 breast (Fig. 3A) and HCT 116 colon cancer cells (Fig. 3B) transfected with Irx5 siRNA (si2) showed significantly reduced cell numbers at 96 hours compared with transfection with a siRNA control, indicating that Irx5 expression may be important in the regulation of a number of different tumor cells.

**Down-regulation of Irx5 by RNA interference induces apoptosis of LNCaP cells.** To examine the cellular mechanism of cell number reduction by Irx5 knockdown, we determined if apoptosis is induced by Irx5 gene targeting. Log-phase cultures were treated with siRNA Irx5 (si2) or siRNA control and assessed for apoptosis by fluorescent microscopy. Figure 4A shows the difference in morphology between nonapoptotic control cells and apoptotic Irx5 siRNA-transfected cells displaying several apoptotic bodies. Irx5 knockdown resulted in apoptosis of ~15% of the cells at 120 hours compared with controls (Fig. 4B). The induction of apoptosis by Irx5 siRNA was also shown by detection of the PARP cleavage product, a well-known marker of apoptosis (Fig. 4C; ref. 36). These data indicate that Irx5 may use apoptosis as a primary means of regulating cell survival.

**Decreased cell viability induced by Irx5 down-regulation is partially dependent upon p53 gene expression.** Currently a role of Irx5 or other Irx family members in signaling apoptosis has not been elucidated, and little is known about the mechanisms
of apoptotic signaling by other homeobox genes. To study the effect of p53 on Irx5-mediated cell death, we used LNCaP cells that stably overexpress a dominant-negative p53 isoform (LNCaP-248, p53 DN). When Irx5 was inhibited by siRNA interference in cells expressing mutant p53 proteins, PARP cleavage was decreased and delayed compared with LNCaP cells with empty vector controls (LNCaP-EV) that only have endogenous wild-type p53, indicating that the mechanism by which Irx5 knockdown signals cell death is partially p53 dependent (Fig. 5A). Also, the usual reduction in cell numbers induced by Irx5 down-regulation is partially abrogated in cells expressing mutant p53 proteins compared with LNCaP cells expressing p53 wild-type proteins (Fig. 5B; P < 0.05, t test). Furthermore, the wild-type endogenous p53 protein expression is increased (especially at 24 hours posttransfection) as a result of Irx5 knockdown by siRNA (Fig. 5C), further supporting the involvement of p53 in the Irx5 signaling pathway. Finally, when both LNCaP-EV and LNCaP-248 were treated with Tricostatin A, an HDAC inhibitor that induces p53-independent growth inhibition, similar inhibitory effects were observed in both cell lines (Fig. 5D).

Irx5 down-regulation induces more robust apoptosis than treatment with 10 nmol/L 1,25(OH)2D3. As shown above, the growth inhibitory effect of 10 nmol/L 1,25(OH)2D3 on LNCaP cell numbers is similar to that of Irx5 down-regulation at 96 hours. To evaluate the degree of apoptosis induced by Irx5 knockdown and that induced by 1,25(OH)2D3, we first compared the PARP cleavage induced by si3 and 10 nmol/L of 1,25(OH)2D3 (Fig. 6A). The result suggests that targeting Irx5 directly may be a more effective strategy to induce apoptosis than treatment with 1,25(OH)2D3 (Fig. 6A). DNA staining (Hoechst) of 1,25(OH)2D3-treated cells (10 or 100 nmol/L) also suggests that 1,25(OH)2D3 is less efficient at inducing apoptosis than Irx5 down-regulation (Fig. 6B). Targeting Irx5 directly may be a more specific way to induce apoptosis and may prevent possible cross-talk from the induction of multiple pathways by vitamin D.

Irx5 down-regulation increases p21 protein expression and induces G2-M arrest in LNCaP cells. In view of the well-described effects of vitamin D receptor ligands on cell cycle progression, we sought to examine the effect of Irx5 down-regulation on cell cycle progression and p21 expression. In

![Fig. 3. Down-regulation of Irx5 by siRNA results in decreased MCF-7 and HCT116 cell number. MCF-7 (A) and HCT 116 (B) cells were counted 96 h after transfection with Irx5 si2 or control siRNA. The differences between siRNA and control-treated cells are statistically significant in both experiments (P < 0.0001, t test). Results are expressed as mean percentage of control siRNA-transfected cells. Columns, mean of three independent experiments; bars, SD.](image-url)

![Fig. 4. Down-regulation of Irx5 by siRNA induces apoptosis. Log-phase LNCaP cultures were transfected with Irx5 (si2) or control siRNA at the indicated time points and stained with bis-benzimide (Hoechst). A, the difference in nuclear morphology between nonapoptotic (left) and apoptotic (right) cells was assessed by fluorescent microscopy in control and Irx5 siRNA-treated cells. B, Irx5 and control siRNA-transfected cells were harvested at the indicated time points and stained with bis-benzimide. The difference is statistically significant at 72, 96 (P < 0.02), and 120 h (P = 0.001, t test). Cells with at least three nuclear fragments were scored as apoptotic. Points, mean of triplicate experiments; bars, SD. C, knockdown of Irx5 induces PARP cleavage. LNCaP cells were transfected with Irx5 (si2 or si3) or control siRNA as indicated, and induction of apoptosis was shown by detection of the PARP p85 cleavage product by Western blot.](image-url)
addition to apoptosis, down-regulation of Irx5 by siRNA caused an increase in the cyclin-dependent kinase inhibitor p21 protein levels, indicating that Irx5 may also regulate cell cycle progression (Fig. 7A). Interestingly, 1,25(OH)2D3 has been shown to increase p21 protein expression in LNCaP cells. The observed increase in p21 protein levels was not preceded by an increase in p21 mRNA as measured by real-time RT-PCR (data not shown), suggesting that Irx5 may be involved in regulating p21 protein stability rather than gene expression.

To examine more directly the effects of Irx5 on cell cycle regulation, we analyzed the cells by flow cytometry after their transfection with Irx5 si2 or control siRNA. Figure 7B is a representation of the flow data in triplicate. Irx5 knockdown resulted in a significant accumulation of cells in G2-M, both at 48 and 72 hours, confirming that Irx5 knockdown does indeed affect cell cycle progression and indicating that this may be through the regulation of p21 expression. Interestingly, the increase in p21 protein expression observed after Irx5 knockdown is not affected by the absence of functional p53 protein (Fig. 7C) and therefore is not p53 dependent.

Overexpression of Irx5 decreases p21 and p53. To determine the effects of Irx5 overexpression, LNCaP cells were transiently transfected with an Irx5 overexpression vector containing the Irx5 open reading frame or empty vector control. Irx5 overexpression reduced both p21 and p53 protein levels at the indicated time points in LNCaP cells (Fig. 8A and B). This provides further evidence of a role of Irx5 in cell cycle regulation.

Discussion

To our knowledge, this is the first report to show that Irx genes are involved in the regulation of apoptosis and cell cycle arrest in human prostate cancer cells. However, recently, it has been shown that an antisense oligodeoxynucleotide capable of simultaneously inducing the loss of Irx1-3 and Irx5 expression...
in developing lung explants dramatically increased apoptosis of the mesenchymal cells while decreasing cell proliferation (37). Knockdown of \( \text{Ir}x5 \) leads to an increase in p53 protein expression. We were not able to detect specific phosphorylated/activated species of p53 in our parental LNCaP protein extracts but did observe phosphorylation of two specific species in our LNCaP 248 cells, which express high levels of mutant p53 proteins. In the p53 mutant cells, phosphorylation of Ser\(^{15} \), which is thought to be involved in p53 ubiquitination/degradation, was increased by \( \text{Ir}x5 \) knockdown, whereas phosphorylation of Ser\(^{392} \), which is involved in p53-induced transcriptional activation, was increased after transfection with \( \text{Ir}x5 \) si\(^2 \) (data not shown). LNCaP cells expressing mutant p53 proteins also were partially resistant to the growth inhibitory effects of \( \text{Ir}x5 \) knockdown, and PARP cleavage in these cells was delayed and reduced compared with p53 wild-type controls. However, PARP cleavage was increased in the p53 mutant cells after \( \text{Ir}x5 \) knockdown, indicating that at least in part PARP cleavage is p53 independent.

p21 protein expression in both p53 wild-type and dominant-negative mutant cells was similarly increased by the knockdown of \( \text{Ir}x5 \), suggesting that the effect of \( \text{Ir}x5 \) on p21 is p53 independent as well. Multiple studies have shown that p21 is induced by both p53-dependent and p53-independent mechanisms. A number of transcription factors, including Sp1, Sp3, MyoD, STAT proteins, and CAAT/enhancer binding proteins activate p21 transcription via p53-independent pathways (38), and recently, it has been suggested that p21, in addition to inhibiting the cell cycle, also plays a role in regulating cell death (39). Most of this evidence indicates that p21 protects cells against apoptosis, but a number of reports have shown p21 to be proapoptotic in certain models, and studies in p21-deficient mice suggest that p21 is involved in tumor suppression (40). We found that p21 protein expression is up-regulated by \( \text{Ir}x5 \) knockdown while p21 mRNA remains unchanged, suggesting the regulation of p21 by posttranscriptional modifications or reduced ubiquitination. p21 expression has previously been

Fig. 6. 1,25(OH)\(_2\)D\(_3\) induces apoptosis in LNCaP cells. A, LNCaP extracts treated with 10 nmol/L 1,25(OH)\(_2\)D\(_3\) or \( \text{Ir}x5 \) si\(^3\) or control siRNA (C) were probed for cleaved PARP. B, LNCaP cells treated with either 10 or 100 nmol/L 1,25(OH)\(_2\)D\(_3\) were harvested at the indicated time points and stained with bis-benzimide. Cells with at least three nuclear fragments were scored as apoptotic. The differences between 1,25(OH)\(_2\)D\(_3\) and control are significantly different at all time points (\( P < 0.0015 \) for 10 nmol/L at 24 h, \( P < 0.0001 \) at all other time points and concentrations, \( t \) tests). Experiments were carried out in triplicate. Points, mean of triplicate experiments; bars, SD.

Fig. 7. p21 protein expression is increased by \( \text{Ir}x5 \) down-regulation. A, LNCaP cells were transfected with \( \text{Ir}x5 \) si\(^2\) or control siRNA (C) and proteins were harvested at the indicated time points. An anti-p21 antibody was used to probe protein blots. This experiment was carried out multiple times, and a representative Western blot is shown. B, results of flow cytometry at 48 and 72 h, showing the percentage of cells in G\(_1\), S, and G\(_2\)-M as normalized to control siRNA transfected cells. C, increased p21 protein expression after \( \text{Ir}x5 \) knockdown is not affected by the absence of functional p53 protein. Protein extracts from LNCaP pC53\(_{12}p248\) (LNCaP-248) and control pRC-CMV LNCaP (LNCaP-EV) cells transfected with \( \text{Ir}x5 \) si\(^2\) or control siRNA were probed with anti-p21 antibody. All experiments were carried out in triplicate, and representative Western blots are shown. Columns, mean of three independent experiments; bars, SD.
The slow MyHC3 gene is one of the earliest atrial chamber-specific genes expressed during cardiogenesis (23). This suggests that Iroquois proteins may play a role in vitamin D receptor signaling.

Misexpression of many homeobox genes has been detected in a number of cancers. An elegant study by Gidekel et al. showed that the aberrant expression of the homeobox gene Oct-4 increases the tumorigenic potential of embryonic stem cells and is required for maintenance of the malignant phenotype (47). Homeobox genes also seem to be involved in cell differentiation. Xiro1 (an Iro gene identified in Xenopus) represses the orthologue of mammalian Gadd45γ, which antagonizes neuronal differentiation in Xenopus early development (48). Recently, Irx5 expression was identified in postnatal bipolar interneurons during retinal development and may play a role in the differentiation of these cells (49). Homeobox genes also seem to play a role in regulating apoptosis, angiogenesis, and metastasis; however, very little is known about the specific signaling pathways involved (31, 32). In numerous embryonic tissue types, Iroquois gene expression is regulated spatially and temporally by a number of different signaling pathways, including Sonic Hedgehog, Wingless, Notch, epidermal growth factor receptor, and Hox signaling. Interestingly, these pathways have all been implicated in tumorigenesis.

In the present study, we have shown that the homeobox protein Irx5 is down-regulated by 1,25(OH)2D3 in human prostate cancer tissue, in the prostate cancer cell line LNCaP, and in MCF-7 breast cancer cells. Irx5 knockdown in LNCaP cells leads to a reduction in cell number, increased p21 and p53 protein expression, G2-M cell cycle arrest, and increased apoptosis and also results in reduced cell survival in the colon cancer cell line HCT 116 and in MCF-7 cells. Conversely, forced expression of Irx5 increases p21 and p53 expression. These data indicate that Irx5 may be a promising new therapeutic target in cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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