Inactivation of PITX2 Transcription Factor Induced Apoptosis of Gonadotroph Tumoral Cells

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Nonfunctioning pituitary adenomas (NFPA; gonadotroph derived), even not inducing hormonal hypersecretion, cause significant morbidity by compression neighboring structures. No effective and specific medical methods are available so far for treating these tumors. The pituitary homeobox 2 (PITX2) gene is a member of the bicoid-like homeobox transcription factor family, which is involved in the Wnt/Dvl/β-catenin pathway. PITX2 is overexpressed in NFPA. PITX2 mutations are known to be responsible for Axenfield Rieger syndrome, a genetic disorder in which pituitary abnormalities have been detected. The R91P mutant identified in Axenfield Rieger syndrome is a dominant-negative factor, which is able to block the expression of several pituitary genes activated by PITX2. To better understand the role of Pitx2 on gonadotroph tumorigenesis and to explore new approach for inhibiting tumoral growth, the R91P mutant was transferred via a lentiviral vector in tumoral gonadotroph cells of two kinds: the T3-1 cell line and human adenoma cells. R91P mutant and small interfering RNA directed against Pitx2 both decreased the viability of T3-1 cells via an apoptotic mechanism involving the activation of executioner caspase. Similar effects of the R91P mutant were observed on human gonadotroph cells in primary culture. Therefore, Pitx2 overexpression may play an antiapoptotic role during NFPA tumorigenesis. (Endocrinology 152: 3884–3892, 2011)
PITX2 is rapidly induced by the Wnt/Dvl/β-catenin pathway that stabilizes Pitx2 mRNA by reducing the destabilizing effects of adenylate/uridylate-rich elements binding proteins. In this process of activation, PITX2 contributes importantly to stabilizing its own transcript by modulating the cytoplasmic concentration of a stabilizing adenylate/uridylate-rich elements binding proteins as well as to controlling the turnover of other unstable transcripts (c-jun, cyclin D1, cyclin D2) (2).

PITX2 gene mutations have been detected in patients with Axenfeld Rieger syndrome (ARS), a genetic disorder characterized by ocular anomalies, dental hypoplasia, and mild craniofacial dysmorphism as well as abnormal cardiac, limb, and pituitary development (8, 9). The phenotype of mice with reduced PITX2 levels mimicks ARS (7, 10). R91P (p. Arg91Pro) is one of the PITX2 mutants identified in ARS patients. We have established that this mutation leads to a dominant-negative factor that preserves DNA binding activity but blocks PITX2-activated promoters such as human PRL, human GH, and POU1F1 (the human ortholog of Pit-1) (11).

Human pituitary adenomas account for about 10% of all intracranial tumors. Nonfunctioning pituitary adenomas (NFPA), which do not induce abnormally high levels of hormone secretion, account for about 30% of these tumors. NFPA are benign but cause significant morbidity because they tend to compress neighboring structures. More than 80% of NFPA are thought to be of gonadotropic origin (12, 13). Apart from neurosurgical removal, no specific treatment is available so far for tumors of this kind. Several lines of evidence have indicated that the Wnt/β-catenin pathway may play an important role in pituitary tumorigenesis (14, 15). An increase in the β-catenin nuclear levels has been detected in some human pituitary adenomas. In addition, a decrease in the Wnt inhibitor factor 1 protein level (15) and a high expression level of PITX2 and CYCLIN D1 genes have been found to occur in NFPA (14, 16). In fact, it has been suggested that Pitx2 may be one of the genes involved in the process of carcinogenesis of several tumors (17).

To further determine the role of PITX2 in NFPA tumorigenesis and suggest new approaches for inhibiting gonadotroph tumoral cell viability, it was proposed here to introduce the R91P dominant-negative mutant into gonadotroph tumoral cells via a lentiviral vector. R91P gene expression resulted in a decrease in αT3-1 cell viability, thanks to an apoptotic mechanism involving executioner caspase activation. Similar effects were obtained using small interfering RNA (siRNA) directed against Pitx2. Lenti-R91P also induced a decrease in human gonadotroph tumoral cell viability.

Materials and Methods

Construction and production of lentiviral vectors

Mouse Pitx2, which shows 90% identity with human PITX2, and the dominant-negative form of Pitx2, Arg91Pro (R91P) (11), were inserted into the lentivector plasmid pRLT-PGK-gene-IRES-eGFP-WPRE-sin18 (18). These vectors were named lenti-Pitx2 and lenti-R91P, respectively. The transgenes were placed under the control of the human phosphoglycerate kinase promoter (PGK) and monitored using internal ribosome entry site-enhanced green fluorescent protein (eGFP). A third lentiviral vector, pRLT-PGK-eGFP-WPRE-sin18 containing eGFP under the control of PGK (lenti-eGFP) was used as the control vector (19, 20).

Lentiviral particles were generated as previously described (20) using each lentivector plasmid, the vesicular stomatitis virus-G-encoding plasmid pMDG, and the packaging plasmid pCMVdeltaR8.91 (PlasmidFactory, Bielefeld, Germany). The supernatants containing the lentiviral particles were collected 24 and 48 h after the transfection step and concentrated by centrifugation using ultracentrifugation methods (vivaspin 20; Sartorius Stedim Biotech, Aubagne, France), aliquoted, and stored at −80 C (20).

Titration of the three lentiviral vectors was performed on HeLaT cells (cervical cancer cells) as previously described (20). The percentage of transduced cells was determined using fluorescence-activated cell-sorting methods (FACS; Becton Dickinson). The viral titer was calculated as quoted, and stored at 8 C/ml of polybrene. Seventeen hours after the infection step, the medium was changed in both the infected and non-infected wells. This point in the procedure was taken to be the infection d 1. To determine the transduction efficiency of the
lentiviral vectors, FACS analysis was performed. In all these experiments, the results obtained with lenti-R91P or lenti-Pitx2 infected cells were compared with those obtained with lent-iEGFP infected cells and noninfected cells. **Silent interfering RNA**

The Two Silencer Select Predesigned siRNA was used to target endogenous Pitx2 in αT3-1 cells (s71669 and s71671; purchased from Applied Biosystems, Courtaboeuf, France). Twenty-four hours after plating the cells into 12- or 24-well plates (to obtain 30–50% confluence at the transfection step), αT3-1 cells were transfected in triplicate with 100 nM per well of each siRNA using lipofectamine 2000 (Invitrogen, Cergy Pontoise, France). Nontransfected wells were incubated with lipofectamine, using the same procedure. To check the transfection efficiency, Silencer Select glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA was used as the control substance and named control GAPDH siRNA. Moreover, in cell cycle analysis, FAM control siRNA was used as the control substance and named control GAPDH siRNA. Nontransfected wells were incubated with lipofectamine, using the same procedure. To check the transfection efficiency, Silencer FAM-labeled negative control siRNA (named FAM control siRNA; Applied Biosystems) was used. Forty-eight hours after the transfection step, cells were treated with trypsin and collected in tubes containing 800 μl of culture medium containing propidium iodide (0.5 μg/ml; BD PharMingen, San Jose, CA) and run on a FACS apparatus. Silencer Select gceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA (Applied Biosystems) was used as the control substance and named control GAPDH siRNA. Moreover, in cell cycle analysis, FAM control siRNA was also used.

To test the efficiency of Pitx2 siRNA on Pitx2 mRNA, Chinese hamster ovary (CHO) cells that do not endogenously express Pitx2 were used. These cells were stably transfected with pTarget-luc-Pitx2 (Stratagene, Massy, France) containing the luciferase reporter gene fused with the coding sequence of the Pitx2 gene. Pitx2 siRNA were transfected using the same procedure as above.

**RNA extraction and real-time PCR**

Mouse (m) Pitx2 mRNA expression was monitored by performing real-time PCR during the lentiviral infection and siRNA transfection experiments. Six days after the infection step or 48 h after the transfection step, total mRNA were extracted using the RNeasy microkit (QIAGEN, Courtaboeuf, France) and reverse transcribed into cDNA (25). The mPitx2 mRNA was quantified using the sybr green assay (purchased from QIAGEN). This assay recognizes endogenous and transgene mPitx2 mRNA. To quantify human (h) PITX2 mRNA, the 5′ exoneuclease (Taq man) assay was used. The following primers and probes were designed to specifically recognize hPITX2: hPitx2 probe, 5′-cga cat gta ccc agg cta ttc cta caa cag-3′; hPitx2S, 5′-tcc atc ggg ctc atg cag c-3′; and hPitx2R, 5′-gga tag gga ggc tgc tgg-3′ (Invitrogen). The mcMyc RNA was quantified using a sybr green assay (purchased from QIAGEN).

The mRNA levels measured in αT3–1 cells were normalized to the mouse β-actin mRNA levels (using the β-actin sybr green assay purchased from QIAGEN). The relative quantification was performed using the 2^ΔΔCt method (26). The mRNA levels present in human cells were normalized to the human β-glucuronidase (βGus) mRNA levels (27). For quantifying hPITX2 and mPitx2 in these cells, mRNA standard curves were draw up using plasmid-γ dilutions, checked by performing sequencing and linearized. The plasmid-γ of βGus was purchased from Ipsogen (Marseille, France). The results of hPITX2 and mPitx2 quantification in NFPA were expressed as copy of gene per copy of βGus ratios.

**Western blotting**

The levels of expression of PITX2 and R91P mutant were checked by performing Western blotting 6 d after the infection with 5 MOI lenti-Pitx2 or 5 MOI lenti-R91P as previously described (28). Total denatured proteins (50 μg) were separated on 12% SDS-PAGE gels and transferred to polyvinyl difluoride membranes (PerkinElmer, Courtaboeuf, France). Immunodetection of PITX2 was performed using a Pitx2-specific antibody (Department of Pediatrics, University of Iowa, Iowa City, IA) (29) and using a bovine antirabbit IgG coupled to peroxidase as the secondary antibody (1:5000; Tebu-Bio Sa, Le Perray en Yvelines, France). Blots were developed with a chemiluminescent detection reagent (Immobilon Western chemiluminescent horseradish peroxidase substrate; Millipore, Molsheim, France). The intensity of the bands on the films was quantified using a densitometric system of analysis, the National Institutes of Health Image program, version 1.59 (Agfa Arcus II scanner; Agfa-Gevaert N.V., Mostel, Belgium), in which the background intensities were subtracted. The values given below are the means obtained after quantifying the specific bands, expressed as arbitrary OD units.

**Cell viability**

Cell viability was determined using an indirect method of estimating the number of viable cells, based on a luminescent cell viability assay (CellTiter-Glo; Promega Corp., Charbonnieres-
les-bains, France), in line with the manufacturer’s instructions. The results were expressed as the mean rate of cell viability inhibition vs. noninfected cells. A direct cell count was also performed on αT3-1 cells using a microscopic counting method based on a Thoma cell.

**Cell death analysis**

The cell cycle distribution was analyzed using flow cytometry methods in lentiviral infection and in siRNA transfection experiments (25). DNA cell distribution histograms were analyzed using the CellQuest Pro software program (Becton Dickinson). Ten thousand events were acquired for each analysis. Each experimental condition was assayed in triplicate.

To determine the mechanism involved in cell death, DNA fragmentation was analyzed on αT3-1 and human tumoral gonadotroph cells by performing terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) using the ApopTag Red in situ apoptosis detection kit (Chemicon International Inc., Temecula, CA). Each experimental condition was assayed in triplicate. Apoptotic cells were then viewed and scored manually with a Leica/Leitz (Darmstadt, Germany) DMRB microscope using a PL fluotar ×100 objective. The percentage of TUNEL-positive cells was evaluated on the basis of more than 3,000 counted cells in 70–120 successive fields.

**Detection of caspase activity**

The executioner caspase activities were measured in infection as well as in siRNA transfection experiments using the Caspase-Glo 3/7 assay (Promega) containing the substrate tetrapeptide sequence DEVD, which is specific to caspase-3 and -7. Each experimental condition was assayed in triplicate wells. Caspase activity was normalized to the cell number determined by performing the Celltiter-Glo assay (Promega). To confirm the involvement of caspase, caspase-3 and -7 inhibitor [DEVD-CHO (100 μm) Calbiochem, Nottingham, UK] was added on wells infected and noninfected. The number of cells was determined using a luminescent cell viability assay after 9 d (Celltiter-Glo 3/7 assay (Promega) containing the substrate tetrapeptide sequence DEVD, which is specific to caspase-3 and -7. Each experimental condition was assayed in triplicate.

**Statistical analysis**

The results obtained are presented here as means ± SEM. The statistical significance was determined by performing the Wilcoxon nonparametric test between paired groups and the Mann-Whitney nonparametric test between unpaired groups. Differences were taken to be statistically significant at a probability level of $P < 0.05$.

**Results**

**Lentiviral transduction efficiency and Pitx2 and R91P mRNA and protein expression in αT3-1**

Six days after infection with lenti-eGFP, lenti-Pitx2, or lenti-R91P at 5 MOI, the mean percentage of αT3-1 expressing eGFP was $71.5 ± 6.5, 61.7 ± 8$, and $63.6 ± 8\%$, respectively. In noninfected cells, no eGFP-expressing cells were detected (data not shown). Endogenous Pitx2 mRNA expression was detected in the αT3-1 cell line and did not differ significantly between lenti-eGFP-infected cells and noninfected cells (mean relative Pitx2 mRNA levels: $1.2 ± 0.77$ and $1.3 ± 0.89$, respectively). The Pitx2 mRNA levels increased approximately 4.6-fold after infection with lenti-Pitx2 and 7.8-fold after infection with lenti-R91P (Fig. 1 A). This difference between the levels of

![FIG. 1. Lentiviral transduction efficiency by lenti-Pitx2 or lenti-R91P vectors in αT3-1 cells. A, Pitx2 mRNA levels in αT3-1 cells. Results of real-time PCR performed 6 d after infecting cells with lenti-eGFP, lenti-Pitx2, or lenti-R91P at 5 MOI and on noninfected cells. Primers of mPitx2 recognized endogenous mPitx2 and transgenic wild-type and mutant mPitx2. Results are expressed as relative Pitx2 mRNA levels normalized to β-actin mRNA. *, $P < 0.05$ vs. noninfected cells as well as lenti-eGFP-infected cells. B, Western blotting analysis of PITX2 and β-actin proteins in αT3-1 cells. Total extract from cells was analyzed 6 d after the infection step at 5 MOI. Noninfected cells (line 1), lenti-Pitx2-infected cells (line 2), and R91P-infected cells (line 3) are shown. PS, Protein standard. Bars on the right indicate apparent molecular weights in kilodaltons. C, Quantified specific immunoreactive bands corresponding to PITX2 and normalized to the level of β-actin. Results are expressed as arbitrary OD units. D, mcMyc mRNA levels in αT3-1 cells. Results of real-time PCR performed 6 d after infecting cells with lenti-eGFP, lenti-Pitx2, or lenti-R91P at 5 MOI and on noninfected cells. Results are expressed as relative mcMyc mRNA levels normalized to β-actin mRNA. *, $P < 0.05$ vs. noninfected cells as well as lenti-eGFP-infected cells.]
PITX2 mRNA expression after lenti-PITX2 and lenti-R91P infection was not statistically significant.

On Western blot, in noninfected cells (Fig. 1B, line 1), one band was detected at around 32 kDa, corresponding to endogenous PITX2 protein. In lenti-PITX2 infected cells (Fig. 1B, line 2) and lenti-R91P infected cells (Fig. 1B, line 3), only one band was detected at the same molecular weight. Quantification of the bands normalized to the level of β-actin protein showed that PITX2 protein expression increased considerably (~4.5 fold) after infection by lenti-PITX2 or lenti-R91P (Fig. 1C). These data showed that these lentiviral vectors efficiently transduced the αT3-1 cell line in vitro and strongly induced the expression of the respective transgene: PITX2 wild-type and R91P mutant.

To validate this model, the impact of wild-type and dominant negative transgenic PITX2 was analyzed on usual PITX2 target as cMyc (30). In αT3-1 cells, the transfer of wild-type PITX2 increased the cMyc mRNA levels (~197 ± 5%), whereas the R91P mutant decreased it (~56 ± 7%, Fig. 1D).

**R91P induced a decrease in αT3-1 cell viability**

The number of cells present in wells infected with lenti-eGFP was similar to that observed in noninfected wells (Fig. 2A). In wells infected with lenti-R91P, the number of cells decreased significantly in comparison with lenti-eGFP-infected wells. The mean rates of decrease were 16.5 ± 3.35, 42 ± 7, and 57 ± 10% 3, 6, and 9 d after infection, respectively (Fig. 2A). These data clearly show that lenti-R91P has negative effects on αT3-1 cell viability. Lenti-PITX2 also induced a slight decrease in cell viability, which reached significance level only after 9 d after infection (mean rate of decrease in the number of lenti-PITX2 cells vs. lenti-eGFP cells: 18.1 ± 5%, P < 0.02). All these results obtained using an indirect method (CellTiter-Glo; Promega) were confirmed by performing a direct cell count (data not shown).

**αT3-1 cell death induced by R91P involved an apoptotic mechanism**

To determine the pathway whereby R91P induced a decrease in the number of cells, the cell cycle was analyzed using flow cytometry methods. In lenti-R91P-infected cells, the proportion of the cells in the subdiploid sub-G1 phase (corresponding to cell death) increased significantly...
in comparison with the noninfected cells (Fig. 2B). A little but significant decrease in the proportion of lenti-R91P-infected cells was observed in the G0/G1 phase, whereas the proportion of cells in the S or G2-M phases did not differ significantly. The proportions of the other three categories of cells (lenti-eGFP, lenti-Pitx2, noninfected cells) were similar in each phase of the cell cycle. These data suggest that lenti-R91P transfer into αT3-1 cells decreased the number of cells by promoting apoptotic cell death.

To confirm the apoptotic effects of R91P, DNA fragmentation was investigated by performing the TUNEL assay. The number of apoptotic cells was found to be significantly higher in cells infected with lenti-R91P (mean number of apoptotic events 8.48 ± 0.80) than in lenti-eGFP-infected or lenti-Pitx2-infected cells (mean number of apoptotic events 3.98 ± 0.60 and 3.40 ± 0.70, respectively, Fig. 2C, left panels). To determine the apoptotic mechanism involved, the activity of executioner caspases was measured. Lenti-R91P infection significantly increased the basal caspase activity detected in lenti-eGFP cells (rate of increase: 38 ± 8.5%, P < 0.0001), whereas no significant effects were observed after lenti-Pitx2 infection (Fig. 2C, right panels). In addition, the decrease in the number of cells observed in response to lenti-R91P (decrease of 23.5 ± 2.0% vs. lenti-eGFP cells) was reversed by the addition of caspase-3 and -7 inhibitor DEVD-CHO (decrease of 9.0 ± 1.4% vs. lenti-eGFP cells, Fig. 2D).

These data show that lenti-R91P transfer in αT3-1 cells induced cell apoptosis by promoting executioner caspase activity.

**Effects of Pitx2 siRNA on the αT3-1 gonadotroph cell line**

To confirm the decrease in cell viability induced by the Pitx2 protein inactivation and that this decrease involved apoptotic mechanism, two siRNA directed against Pitx2 (which are called siRNA 69 and siRNA 71 here) were used. Using a FAM control siRNA, the proportion of transfected cells was found to be approximately 56.8 ± 7% 48 h after the transfection step. Into CHO cells expressing a transgenic Pitx2, the rates of decrease in Pitx2 mRNA recorded in response to the two siRNA were 86.4 and 72.1%, respectively. In αT3-1 cells, both siRNA decreased the endogenous levels of Pitx2 mRNA expression by 53.8 ± 10% (mean relative Pitx2 mRNA level under siRNA: 0.71 ± 0.26 vs. in nontransfected cells 1.32 ± 0.19) and decreased the endogenous levels of Pitx2 protein expression by 60% (data not shown). With each Pitx2 siRNA, a decrease in the number of cells was observed, whereas no effect on cell viability was found in response to control GAPDH siRNA (Fig. 3A) and FAM control siRNA (data not shown) in comparison with nontransfected cells. The mean percentage decrease in the number of transfected vs. nontransfected cells was 31.0 ± 21 and 29.5 ± 16% with siRNA 69 and 71, respectively (Fig. 3A).

The cell cycle analysis showed that the proportions of the cells in the sub-G1 phase increased significantly in response to each Pitx2 siRNA in comparison with both GAPDH and FAM control siRNA and with nontransfected cells (Fig. 3B), whereas the proportion of the cells in the G0/G1 phase decreased, without reaching the significance threshold. No significant differences were observed in any of the phases in the cell cycle between both control siRNA transfected cells and nontransfected cells. In addition, 48 h after transfection with Pitx2 siRNA, an increase in executioner caspase activity was observed in comparison with the levels detected in nontransfected and control
siRNA transfected cells (mean rate of increase 30.25 ± 18 and 22.7 ± 7% with siRNA 69 and 71, respectively; P < 0.05, Fig. 3C).

**Effects of lenti-R91P on human NFPA**

To test whether these data obtained on murine gonadotroph cell line might be also relevant to the human tumors, the effects of R91P mutant transfer were tested on primary culture of seven human gonadotroph tumors. Lentiviral vectors have been found to efficiently transduce human pituitary tumoral cells in primary cultures, giving about 75 ± 5% transduced cells (20). Endogenous hPITX2 mRNA expression was found in all the tumors tested, and these levels were not affected by infection with any of the present lentiviral vectors [mean hPITX2 mRNA level 33 (5–129) copy/copy βGus]. Transgenic mPitx2 mRNA levels were strongly expressed after infection with lenti-Pitx2 and lenti-R91P [mean mPitx2 mRNA level 319.8 (103–1107) and 304.0 (50–898) copy/copy βGus, respectively] (Fig. 4A). A significant negative effect of R91P on cell viability was observed in the five tumors tested 9 d after infection at 10 MOI (−29 ± 5, −53 ± 2, −34 ± 10, −14 ± 5, and −38 ± 6% for G1, G2, G3, G4, and G7, respectively, P < 0.02 vs. lenti-eGFP cells, Fig. 4B). Moreover, DNA fragmentation was investigated by performing the TUNEL assay on primary culture of three NFPA, 5 d after infection at 10 MOI. The number of apoptotic cells was found to be significantly larger in lenti-R91P infected cells (mean number of apoptotic events: 15.1 ± 3.31) than in lenti-eGFP-infected or lenti-Pitx2-infected cells (mean number of apoptotic events: 6.1 ± 2.29 and 7.8 ± 2.87, P < 0.0001, vs. lenti-eGFP cells, Fig. 4C).

**Discussion**

No completely satisfactory methods are available so far for treating human NFPA. These tumors not inducing hormonal hypersecretion are associated with symptoms resulting from the invasion or compression of the surrounding structures, which in most cases makes it impossible to perform complete neurosurgical removal in the later stages of the disease. Dopamine and somatostatin analogs are rarely efficient means of dealing with tumors of this kind. In a recent reassessment of dopamine agonist efficacy in NFPA, a tumoral reduction was reported to occur in only 27.6% of these tumors (31). Radiotherapy is often used after tumor surgery, but this treatment takes a long time to be effective and there are complications, the most common of which is hypopituitarism (31).

In this context, gene therapy may be a potentially useful alternative method of targeted treatment for NFPA. The dominant negative PITX2 mutant R91P was thought to be a good candidate gene for the following reasons: 1) PITX2 is highly expressed in NFPA (16); 2) this protein is induced by the Wnt/Dvl/catenin pathway, which is involved in pituitary tumorigenesis (14); 3) during pituitary development, gonadotroph cells are the most sensitive cells to decreased levels of Pitx2 expression (7); 4) the R91P mutant is involved in human ARS and its dominant-negative function has been established (11).

In the present study, we established that the R91P dominant-negative mutant strongly decreases the viability of mouse αT3-1 gonadotroph tumoral cells and that of human tumor cells *in vitro*. The cell cycle analysis of αT3-1 cells showed that, under our experimental conditions, the decrease in cell viability of lenti-R91P-infected cells involved a process of cell death due to an increase in the...

**TABLE 1.** Human NFPA hPitx2 mRNA and mPitx2 mRNA expression before and after infection by lenti-eGFP, lenti-Pitx2, and lenti-R91P of seven human gonadotroph adenomas. Results are expressed in Pitx2 copies per 10^7} cells and 304.0 (50–898) copy/copy βGus, respectively
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served a slight but significant decrease in tumors were promising. The results obtained here on human pituitary inactivation is involved in caspase activity has yet to be determined. The mechanism by which Pitx2 dominant-negative R91P or siRNA, resulted from executioner caspase activation. The mechanism by which Pitx2 inactivation is involved in caspase activity has yet to be determined. The results obtained here on human pituitary tumors were promising.

When wild-type PITX2 was overexpressed, we also observed a slight but significant decrease in αT3-1 cell viability but only in the long term. In fact, the activity of Pitx2 is sensitive to gene dosage (7). In mice, up-regulation and inactivation of Pitx2 induce similar ocular disorders (32). Pitx2 also inhibits cell proliferation in HeLa cells that do not express endogenous Pitx2 (33). However, in our experiments the decrease in cell viability was distinctly smaller in response to Pitx2 overexpression than to R91P, and this decrease was not associated with any changes in the basal caspase activities, the cell cycle, and apoptotic events. The exact molecular mechanisms involved in the cell death induced by Pitx2 overexpression still remains to be elucidated.

Apoptosis is an important process in the case of neoplastic lesions, whereas these events are fairly rare in human pituitary tumors. In addition the apoptotic labeling index has been found to be lower in NEPA than in functioning human pituitary adenomas (34). Therefore, the high expression of the PITX2 gene observed in gonadotroph adenomas (16) may play a role in pituitary tumorigenesis via an antiapoptotic effect. The results of this study suggest that dominant-negative PITX2 mutants or Pitx2 siRNA may provide useful new tools for developing targeted strategies for treating tumors in which PITX2 is overexpressed. Animal model of spontaneous pituitary adenomas will be essential for evaluating the lenti-R91P effect in vivo for a potential human application in gene therapy strategy.

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