



HoxD10 gene delivery using adenovirus/adeno-associate hybrid virus inhibits the proliferation and tumorigenicity of GH4 pituitary lactotrope tumor cells

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ABSTRACT

Prolactinoma is one of the most common types of pituitary adenoma. It has been reported that a variety of growth factors and cytokines regulating cell growth and angiogenesis play an important role in the growth of prolactinoma. HoxD10 has been shown to impair endothelial cell migration, block angiogenesis, and maintain a differentiated phenotype of cells. We investigated whether HoxD10 gene delivery could inhibit the growth of prolactinoma. Rat GH4 lactotrope tumor cells were infected with adenovirus/adeno-associated virus (Ad/AAV) hybrid vectors carrying the mouse HoxD10 gene (Hyb-HoxD10) or the β -galactosidase gene (Hyb-Gal). Hyb-HoxD10 expression inhibited GH4 cell proliferation *in vitro*. The expression of FGF-2 and cyclin D2 was inhibited in GH4 cells infected with Hyb-HoxD10. GH4 cells transduced with Hyb-HoxD10 did not form tumors in nude mice. These results indicate that the delivery of HoxD10 could potentially inhibit the growth of PRL-secreting tumors. This approach may be a useful tool for targeted therapy of prolactinoma and other neoplasms.

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Pituitary adenomas are typically benign tumors that arise in the sella turcica of the brain. They constitute up to 15% of primary intracranial neoplasms [1]. The clinical manifestations of pituitary adenomas are due to the overproduction of hormones and/or the mass effects from an enlarging tumor within the confined space of the sella turcica. There are five major types of pituitary tumors reflecting the different cell types in the pituitary gland. Prolactinomas are the most common pituitary tumor in adults and account for 60% of all functioning pituitary adenomas [2]. Dopamine agonists are the first choice for treatment of prolactinoma [3], but failure to normalize prolactin levels independently of baseline value is a challenge with these agents [4]. While dopamine agonists or surgery can cure most noninvasive prolactinomas, invasive ones often require additional treatment like radiotherapy [5]. Failure to normalize prolactin levels occurs in about 25% of patients with prolactinoma treated with dopamine agonists, regardless of the size or invasiveness of the tumor [4]. Therefore, the development of new

treatment modalities including gene therapy for prolactinoma is necessary.

Growth factors have been implicated in the pathogenesis of pituitary tumors. These include IL1, IL6, TGF β , and FGF2, which modulate hormone production, angiogenesis, and cell growth of the pituitary [6–8]. These factors also play an important role in pituitary lactotrope hyperplasia and tumor formation [9]. Targeted therapy against these growth factors or their receptors could be an appealing strategy.

Homeobox (Hox) genes are master regulatory genes that direct organogenesis and maintain differentiated tissue function. The overexpression of one of such homeobox gene, HoxD10, has been shown to impair endothelial cell migration and block the angiogenesis induced by VEGF and FGF-2 in chick chorioallantoic membrane [10]. A recent report also revealed that HoxD10 expression is lost during tumor progression in breast and endometrial tumors and HoxD10 induces phenotypic reversion from highly invasive tumor cells to a nonmalignant state [11]. We hypothesized that HoxD10 expression would inhibit the growth of rat lactotrope GH4 tumor cells. In the present study, we used adenovirus/adeno-associated virus (Ad/AAV) hybrid vectors carrying the mouse HoxD10 gene and examined the effects of HoxD10 on GH4 cell proliferation *in vitro* and tumor formation *in vivo*.

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Materials and methods

Recombinant Ad/AAV hybrid vectors. A cassette containing the mouse HoxD10 (1.8 kb) cDNA (Open Biosystems, Huntsville, AL) or the β -galactosidase gene was subcloned into an Ad/AAV hybrid transfer plasmid [12] based on pCDNA3 (Invitrogen, Carlsbad, CA) with the cytomegalovirus (CMV) promoter/enhancer and a simian virus (SV) 40 polyadenylation (p(A)) sequence. These Ad/AAV hybrid transfer plasmids were used to generate Ad/AAV hybrid vectors as previously described [13] (Fig. 1). The sequences of the expression cassettes in the Ad/AAV hybrid vectors were confirmed by automated DNA sequencing. The cloned and purified Ad/AAV hybrid vectors were titrated by a plaque assay.

Cell culture and infection with recombinant Ad/AAV hybrid vectors. GH4 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM/F12 + 10% FBS supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin and were incubated at 37 °C and 5% CO₂.

The transduction efficiency of the Ad/AAV hybrid vectors in the GH4 cells was tested using Hyb- β Gal. β -Galactosidase expression was detected in 95–100% of GH4 cells at 48 h after infection with Hyb- β Gal at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell (data not shown). Therefore, subsequent experiments were performed using similar amounts of Hyb-HoxD10.

The effect of HoxD10 expression on GH4 cells in vitro. We investigated the effect of HoxD10 expression on GH4 cell growth using a non-radioactive cell proliferation assay (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega, Madison, WI). Cells were seeded in 96-well plates at a density of 8×10^3 cells/well and infected on the following day with Ad/AAV vectors at different MOIs (5, 10, and 25 PFU/cell). The media were replaced at 5 h after infection and every two days thereafter. Octaplicate wells were assayed for viable cell density on day 6. The relative density was calculated as the absorbance at 490 nm divided by the absorbance for uninfected cells (means \pm SD).

Forty-eight hours after infection of GH4 cell, isolation of total RNA and RT-PCR were performed as described previously [14] to measure the expression of HoxD10 and FGF-2. PCR conditions and oligonucleotides used for PCR amplification can be provided upon request. Western blot analysis using lysates from GH4 cells was also performed using anti-cyclin D2 (M-20, Santa Cruz Biotechnology) as described previously [15].

Transcriptional activity of HoxD10 on cyclin D2 promoter. The human cyclin D2 promoter (1302 bps) [16] was inserted into multiple cloning sites of pGL3Basic vector (Promega) to generate CD2p-luc. Transfections of 293FT cells with 500 ng of CD2p-Luc constructs and 50 ng of HoxD10 expression plasmid were performed using the calcium phosphate method. Cells were harvested 48 h later, and luciferase activity was assayed and normalized as described before [17]. The results were averaged from three independent experiments and plotted as means \pm SD for quadruplicated wells.

The effect of HoxD10 on growth of GH4 tumors in nude mice. GH4 cells were infected with 10 PFU/cell of Ad/AAV hybrid vector and

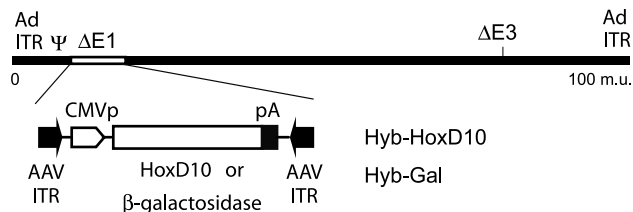


Fig. 1. Structure of the adenovirus/adeno-associated virus (Ad/AAV) hybrid vectors. In the hybrid vector, the expression cassette containing HoxD10 or β -galactosidase is flanked by AAV-ITR. ITR, inverted terminal repeat; CMVp, cytomegalovirus promoter; Ψ , packaging signal.

incubated at 37 °C for 24 h. Cells were collected, washed twice with PBS, resuspended in media and injected (2×10^6 cells) into the flanks of adult (8-week-old) athymic female nude mice

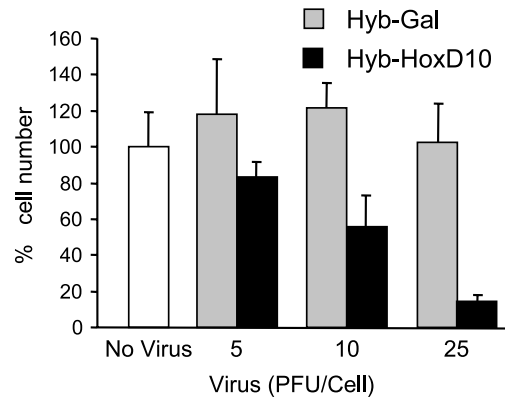


Fig. 2. Effects of HoxD10 expression on GH4 cell growth. Cell proliferation assays were performed to test whether HoxD10 expression affects GH4 cell growth. GH4 cells were infected with increasing amounts of Ad/AAV hybrid vectors and cell growth was assayed at day 6. GH4 cell growth was inhibited by HoxD10 expression at a dosage of 10 or 25 PFU/cell.

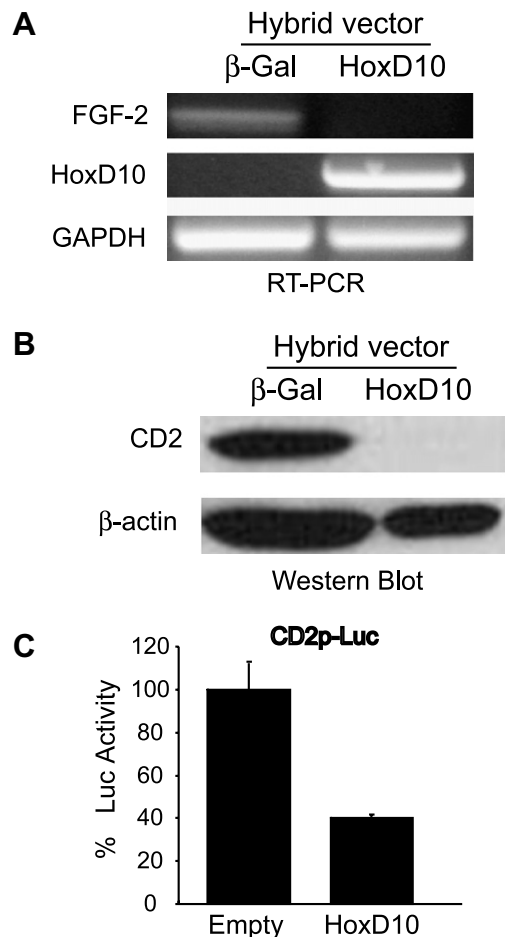


Fig. 3. Effects of HoxD10 on the expression of FGF-2 and cyclin D2. Expressions of FGF-2 ((A) RT-PCR analysis) and cyclin D2 ((B) Western blot analysis) were suppressed in GH4 cells infected with Hyb-HoxD10 (10 PFU/cell). (C) Transcriptional activity of HoxD10 on cyclin D2 promoter was examined using a luciferase reporter system. 293-FT cells were co-transfected with HoxD10 expression vectors and CD2p-Luc. HoxD10 suppressed cyclin D2 promoter by 60%. Luciferase values were normalized by protein measurements. Results were averaged from three independent experiments and plotted as means \pm SD for quadruplicated wells.

(Harlan–Sprague–Dawley, IN). The mice were divided into two groups: (A) Hyb-HoxD10 ($N = 10$) and B) Hyb-Gal ($N = 10$). Animals were examined for tumor formation every 2–3 days, and the size of the tumor was measured in three dimensions with a caliper. Tumor size (cubic millimeters) was calculated using the formula: $(3.14 \times \text{length} \times \text{width} \times \text{depth})/6$. The experiment was terminated 3 weeks the injection because of the large size of the tumors of the control (Hyb-Gal) mice. All studies involving the use of nude mice were approved by the Northwestern University Medical School Animal Care and Use Committee.

Results

HoxD10 inhibits GH4 cell proliferation

The proliferation of GH4 cells was examined after infection with various doses (5, 10, and 25 PFU/cell) of Ad/AAV hybrid vectors. Hyb-HoxD10 infection suppressed GH4 cell growth by 34–80%, depending on the viral dose, whereas infection with Hyb-Gal did not suppress cell growth (Fig. 2).

HoxD10 suppresses FGF-2 and cyclin D2 expression

We analyzed whether HoxD10 expression affects the expression of angiogenic factor in GH4 cells. RT-PCR analysis revealed that while FGF-2 mRNA was expressed in the Hyb-Gal-infected GH4 cells, it was absent in GH4 cells infected with Hyb-HoxD10, indicating the inhibition of angiogenic factor expression (Fig. 3A). Because GH4 cell growth was inhibited by HoxD10 expression, cyclin D2 expression was evaluated by Western blot analysis. Expression of cyclin D2 was suppressed in GH4 cells infected with Hyb-HoxD10 but it was not suppressed in Hyb-Gal-infected GH4 cells. We inves-

tigated whether HoxD10 affects the transcription of cyclin D2 using luciferase reporter system. Cyclin D2 promoter activity was significantly suppressed by co-transfection of HoxD10 expression plasmid compared to the control empty plasmid (Fig. 3C).

Effect of HoxD10 on growth of GH4 tumors in nude mice

Based on the findings that HoxD10 inhibits FGF-2 expression and cell growth *in vitro*, we evaluated whether HoxD10 expression may affect tumor formation in nude mice. GH4 cells infected with Ad/AAV hybrid vector were injected into athymic nude mice. In the mice injected with GH4 cells infected with Hyb-Gal, seven tumors developed from 10 injections. Tumor development occurred within 12 days. These tumors grew very rapidly and reached 1.5–2 cm in diameter after 3 weeks. In mice injected with GH4 cells infected with Hyb-HoxD10, tumor formation was not seen until 22 days after the injection (Fig. 4).

Discussion

Gene therapy is an appealing strategy for the treatment of prolactinomas that are resistant to dopamine agonists. Among the gene therapy strategies for the treatment of pituitary adenomas including prolactinoma, acromegaly, and Cushing's disease, the delivery of toxic genes by adenovirus vectors is the most common [13–15,18–22]. Development of gene therapy that targets mitogenic or angiogenic molecules is an area of active research. In this study, we used an Ad/AAV hybrid vector to deliver a novel gene, HoxD10, which acts as either an angiogenic inhibitor or cell cycle inhibitor. Antiangiogenic or antimitogenic gene therapy might require the long-term expression of a therapeutic gene to suppress tumor angiogenesis or growth. Because AAV is capable of genome

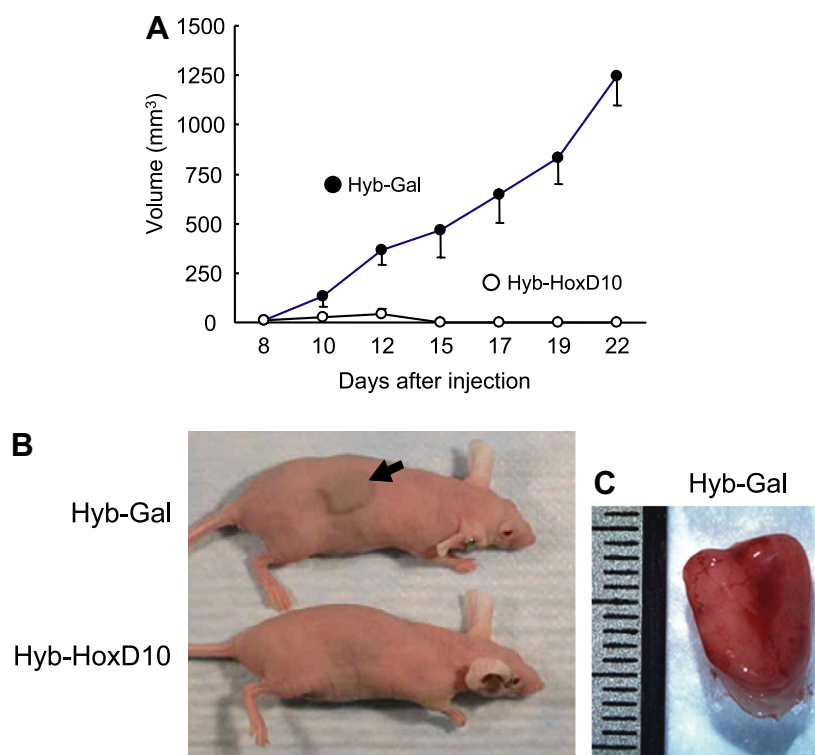


Fig. 4. Inhibition of GH4 tumor formation by Hyb-HoxD10 in nude mice. (A) GH4 cells were infected with 10 PFU/cell of Hyb-HoxD10 or Hyb-Gal. After incubation for 24 h at 37 °C, cells were collected, washed twice with PBS, and injected into the flank of nude mice (1×10^6 cells). The size of the tumor was measured in three dimensions with calipers every 2–3 days for 3 weeks. Each point represents the mean \pm SD of tumor volumes, as described in Materials and methods. (B) A tumor formed in nude mice injected with Hyb-Gal-infected GH4 cells (upper), whereas a tumor did not form in mice injected with Hyb-HoxD10-infected GH4 cells (lower). (C) An excised tumor from a nude mouse bearing a GH4 tumor (Hyb-Gal).

integration [23], sustained expression of therapeutic genes can be achieved, making AAV vectors useful for tumor-suppressing gene therapy.

Angiogenesis has been shown to play an important role in prolactinoma formation and growth [6–8]. FGF-2, 1 of the 19 members of the FGF family, [24,25] is known to play diverse roles in pituitary differentiation and tumor formation [26,27]. FGF-2 is a potent angiogenic and mitogenic molecule *in vivo* and *in vitro* [28], and has been shown to stimulate prolactinoma growth and regulate prolactin secretion [29]. Furthermore, increased expression of fibroblast growth factor-2 (FGF-2) has been demonstrated in lactotrope tumorigenesis [6–8]. Development of treatment modalities that inhibit FGF-2 expression in these tumor cells may provide a new therapeutic approach.

The Hox genes are clustered in four linkage groups (A–D) on four different chromosomes. Among them HoxD3 and HoxB3 are known to promote angiogenesis. HoxD10 is expressed in quiescent endothelial cells but not in tumor-associated angiogenic endothelium. The expression of HoxD10 decreases in angiogenic vessels in the tumor microenvironment [10]. It has also been reported that normal breast epithelial cells and endometrial cells express abundant HoxD10, while expression is lost in carcinogenesis [10,30]. Sustained expression of HoxD10 impairs endothelial migration and blocks angiogenesis induced by FGF-2 and VEGF [10]. In our study, FGF-2 expression was completely inhibited by HoxD10 gene delivery to GH4 cells, suggesting that HoxD10 strongly inhibits angiogenic factors.

Due to the fact that HoxD10 suppressed GH4 cell proliferation in our study and is known to maintain a differentiated phenotype of the cells [10,30], we reasoned that HoxD10 may play a role in cell cycle regulation. As shown in the results, inhibition of cyclin D2 expression was observed in GH4 cells transduced with HoxD10. Transfection study revealed that the suppression of cyclin D2 by HoxD10 was mediated through transcription. It would be interesting to investigate the HoxD10–regulatory element in the cyclin D2 promoter.

Our results show *in vitro* and *in vivo* inhibition of GH4 lactotrope tumor cell growth with HoxD10 gene delivery. HoxD10 suppresses the expression of FGF2, which is known to have mitogenic and angiogenic effects on prolactinoma cells. Although various treatment modalities exist for prolactinomas, our results indicate that delivery of HoxD10 using gene therapy tools has the potential to inhibit the growth of prolactinoma. This approach may also provide a useful tool for targeted therapy of other neoplasms.

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