Combining Transfer of TTF-1 and Pax-8 Gene: a Potential Strategy to Promote Radioiodine Therapy of Thyroid Carcinoma

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Running Title: Radioiodine therapy of tumor following TTF-1 and Pax-8 gene cotransfer

Abstract Cotransfer of TTF-1 and Pax-8 gene to tumor cells, resulting in the reexpression of iodide metabolism-associated proteins, such as sodium iodide symporter (NIS), thyroglobulin (Tg), thyroperoxidase (TPO), offers the possibility of radioiodine therapy to non-iodide-concentrating tumor because the expression of iodide metabolism-associated proteins in thyroid are mediated by the thyroid transcription factor TTF-1 and Pax-8. The human TTF-1 and Pax-8 gene were transducted into the human thyroid carcinoma (K1 and F133) cells by the recombinant adenovirus, AdTTF-1 and AdPax-8. Reexpression of NIS mRNA and protein, but not TPO and Tg mRNA and protein, was detected in AdTTF-1-infected F133 cells, following with increasing radioiodine uptake (6.1~7.4 times), scarcely iodide organification and rapid iodide efflux ($t_{1/2}$≈8 min in vitro, $t_{1/2}$≈4.7 h in vivo). On contrast, all of the reexpression of NIS, TPO and Tg mRNA and proteins in F133 cells were induced by the synergetic effect of TTF-1 and Pax-8. AdTTF-1 and AdPax-8 coinfected K1 and F133 cells could effectively accumulate radioiodine (6.6~7.5 times) and obviously retarded radioiodine retention ($t_{1/2}$≈25~30 min in vitro, $t_{1/2}$≈12 h in vivo) (p<0.05). Accordingly, the effect of radioiodine therapy of TTF-1 and Pax-8 cotransducted K1 and F133 cells (21~25% survival rate in vitro) was better than that of TTF-1-transducted cells (40% survival rate in vitro) (p<0.05). These results indicate that single TTF-1 gene transfer may have limited efficacy of radioiodine therapy because of rapid radioiodine efflux. The cotransduction of TTF-1 and Pax-8 gene, with resulting NIS-mediated radioiodine accumulation and TPO and Tg-mediated radioiodine organification and intracellular retention, may lead to effective radioiodine therapy of thyroid carcinoma.

Keywords: TTF-1, Pax-8, radioiodine therapy, thyroid carcinoma

Introduction

Radioiodide therapy is an effective approach for treatment of differentiated thyroid carcinoma and their metastases. Radioiodide therapy is based on the capacity of thyroid carcinoma cells to trap and concentrate iodide, which is completed by the cooperation of thyroid-specific proteins, such as the sodium/iodide symporter (NIS), thyroglobulin (Tg) and thyroperoxidase (TPO).[1] However, to anaplastic thyroid carcinoma and any other nonthyroid carcinoma, which shows no radioiodine accumulation, radioiodide therapy is not suitable to them. It confines the extensive application of radioiodide therapy in clinic. NIS mediated iodide uptake in normal and well differentiated neoplastic thyroid cells. TPO catalyzes oxidation of iodide ion, iodination of tyrosine residues of Tg and coupling of iodotyrosines to generate iodothyronines T3 and T4 and, thus promotes iodide retention within thyroid cells. It is postulated that reexpression of these iodide metabolic proteins would enable

non-iodide-concentration tumor to acquire/restore the capacity of radioiodine concentration, which make it possible to treat non-iodide-concentration tumor with radioiodine.

Transcription factors play a pivotal role in the determination and maintenance of cellular phenotype. The activity of transcription factors is considered as the main switch to regulate gene expression.[2] The expression of those thyroid-specific proteins is controlled by the combination of specific transcription factors with the respective promoters/enhancers of those genes. In thyroid follicular cells, three thyroid-specific transcription factors (TTF) have been identified: TTF-1, TTF-2, and Pax-8, which regulate the transcriptional activity of Tg and other thyroid-specific genes, such as thyroperoxidase (TPO) and thyrotropin receptor (TSHr).[3] TTF-1 is a homeodomain-containing protein expressed in embryonic diencephalons, thyroid, and lung.[4] Pax-8 belongs to the Pax family of paired domain-containing genes and is expressed in the kidney, the developing excretory system, and the thyroid.[5] Three TTF-1 binding sites are present in the Tg and the TPO promoter. TTF-1 could activate transcription from the Tg promoter and, at a much low extent, from TPO promoter in both thyroid and nonthyroid cells.[6] Pax-8 binds to a single site on the Tg and on the TPO promoters and, in both case, the Pax-8-binding site overlaps with that of TTF-1.[7] In addition, TTF-1 could activated the rat NIS promoter by a direct interaction with its enhancer region[8] and, Pax-8 has two binding sites of rat NIS upstream enhancer and plays an important role in the expression of NIS gene.[9] In addition to their expression in the follicular thyroid cells, TTF-1 and Pax8 also expressed in the lung and kidney , respectively. However, the two factors are present together only in the thyroid follicular cell, suggesting that their combination could have a role in the expression of the thyroid-specific phenotype.[10]

Ros and his colleages[11] reported that TTF-1 and Pax8 were expressed in well differentiated thyroid adenomas and that their expression decreased in less differentiated papillary and follicular carcinomas and was lost in undifferentiated anaplastic carcinomas. They also affirmed that the expression of the thyroid specific genes *Tg*, *TPO* and *TSHr* and their transcription factors, TTF-1 and Pax8, was lost in thyroid cells derived from follicular papillary and anaplastic human carcinomas. Fabbro *et al.*[10] found that, in anaplastic thyroid carcinomas, the absence of TTF-1 was associated with the absence of *Tg* gene expression. Both transcription factors directly interact and synergistically activate thyroid-specific transcription.[6,12] TTF-1 and Pax-8 cooperate with several ubiquitous transcription factors, forming complexes on the regulatory region of specific genes.[13,14] Miccadel et al. reported that TTF-1 and Pax-8 cooperatively activated TPO and Tg gene transcription and their synergistic activity require the cross-talk between enhancer and gene prompter.[15] Furthermore, Altmann et al. found that the transcriptional activation of hTg, hTPO and rNIS gene was low in cells expression either hPax-8 or dTTF-1 alone. In contrast, the activation of

hTg promoter and hTPO promoter and, to a lesser extend, of rNIS promoter were significantly activated in cell lines expression both TTF-1 and Pax-8.[16]

To extend radioiodide therapy to undifferentiated thyroid carcinoma and other nonthyroid carcinoma, in the present study we constructed the adenovirus vectors (AdTTF-1 and AdPax-8) for TTF-1 and Pax-8 gene transfer to induce reactivation of endogenous thyroid-specific genes. The ability of TTF-1 and Pax-8 to induce re-expression of thyroid-specific genes, NIS, Tg, and TPO, was investigated in human thyroid carcinoma cells and, then, radioiodine uptake and retention reactivated by TTF-1 and Pax-8 was examined in vitro or in vivo. The results reported here indicated that combining transfer of TTF-1 and Pax-8 gene was an available strategy to promote radioiodine therapy of non-iodide-concentration tumor.

Materials and methods

Cell culture

K1 and F133 cells were purchased from European Collection of Animal Cell Cultures (ECACC, Wiltshire, UK) and HEK293 cells were kindly provided by Pathology Laboratory of West China Hospital, Sichuan University. The human papillary thyroid carcinoma cell line K1 has very low iodide uptake and NIS mRNA and protein in it is hardly detectable.[17] The human follicular thyroid carcinoma cell line F133 expresses Tg, and TSHR, but not TPO. The cell line does not take up iodide and hNIS mRNA expression in it is hardly detectable, too.[18] All cell lines were grown in DMEM medium, high glucose content (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum, 2mmol/L L-glutamine, 100 IU/ml penicillin G and 100 μg/ml streptomycin sulfate. Cells were maintained at 37°C and 5% CO_2 in an incubator with 95% humidity. The culture medium was replaced every second day and cells were passaged at 90% confluency using 0.05% trypsin (Life Technologies).

Recombinant adenovirus

The full-length human TTF-1 cDNA (nucleotides 83~1288bp, GeneBank Acession Number: NM_001079668) and the full-length human Pax-8 cDNA (nucleotides 167~1519bp, GeneBank Acession Number: NM_003466) were respectively removed from the pMD-18T simple vector (reconstructed by Invitrogen Life Techonlogies) by restriction digestion using KpnI and HindIII, agarose gel purified and ligated into pShuttle plasmid (pAdTrack-CMV) of the AdEasier adenovirus system resulting in pAdTrack-TTF-1 and pAdTrack-Pax-8. Subsequently, homologous recombination of pAdTrack-TTF-1/pAdTrack-Pax-8 plasmid and pAdEasy-1 plasmid were performed in the bacteria BJ5183 *Escherichia coli*. The recombined adenovirus pAdTTF-1 and pAdPax-8 plasmid were agarose-gel purified and

confirmed by PCR and DNA sequencing. The pAdTTF-1 and pAdPax-8 were digested by 4 mg PacI and, then they were packaged using Lipofectamine2000 (Invitrogen, Carlsbad, CA, USA) and respectively transfected into HEK293 cells according to the standard procedure. AdTTF-1 and AdPax-8 virus propagated in HEK293 cells. After two-step purification on CsCl gradients, viral stocks were desalted using Pharmacia G50 columns (Orsay, France) and frozen at 80℃ in 10 mM Tris-HCl (pH 7.5) containing 2.5% glycerol. Viral titers were determined by plaque assays using cultured HEK293 cells and were expressed as PFU/ml.

Analysis of mRNA by RT-PCR

Total RNA was prepared from cell lines using Trizol (Invitrogen) and quantitated spectrophotometrically. RT-PCR was performed using 2 μg of total RNA. The initial reverse transcription was at 42℃ for 1 h in 20 μl solution containing M-MLV Reverse Transcriptase 100 U (Toyobo Co. Ltd., Osaka, Japan), 4 μl 5×first brand buffer, 0.1 mM oligo (deoxythymidine) 18 primer, 2 μl dNTPs mix (10 mM of each), 1 μl DTT (0.1 μl) and 6.0 μl ddH_2O. cDNA aliquots equivalent to 100 ng RNA were subjected to PCR using Taq DNA polymerase (BioRule Biology Techonlogies, Shanghai, China). The primers and conditions used and the expected sizes of the target genes were shown in Table 1. For target genes amplification, cycling conditions were 5 min at 95℃ for pre-denaturation, 30 cycles of 45 s at 94℃ for denaturation, 30 s at various temperatures, as shown in Table 1 for annealing, 90 s at 72℃ for extension followed by 10 min at 72℃ for final extension. PCR products (25μl) from each reaction were analyzed by 1.8 agarose/ethidium bromide gel electrophoresis. All amplification reactions were performed in triplicate and the relative expression levels were calculated as the density of the product of the respective target genes divided by that of the control gene, β-actin.

Western blot analysis

Total proteins (50 μg) prepared from transfected F133 cells were denaturated by water bath with 2×SDS gel loading buffer (Tris-HCl (pH 6.8) 100 mM, 4% SDS, 0.2% bromchlorphenol blue, 20% glycerine, 200 mM DTT) for 10 min at 100 ℃ and loaded on Bis-Tris-HCl buffered polyacrylamide gels. After bromchlorphenol blue run away from gel, proteins were transferred onto nitrocellulose membranes by electroblotting. Following blotting, membranes were preincubated for 2 h at room temperature in milk/TBS-T (20 mM Tris, 137 mM NaCl and 0.1% Tween-20, 5% non fat dry milk) to block nonspecific binding

sites. Membranes were then incubated with mouse monoclonal antibody against human NIS(Chemico Chemicals, New Delhi, India, dilution 1:500), mouse monoclonal antibody against human Tg (HuaAn Biotechnology, Hangzhou, China, dilution 1:200), or rabbit monoclonal antibody against human TPO (Bioss Company, Beijing, China, dilution 1:500) for 2 h at room temperature and then overnight at 4 ℃. After washing with TBS-T, horseradish peroxidase-labelled goat-anti-mouse-antibody was applied (dilution 1:2000) for 1.5 h at 37 ℃. Membranes were washed with TBS-T and were exposed to X-ray films (Kodak Biomax MR, Sigma-Aldrich, St Louis, MO, USA) at room temperature for approximately 30s. Prestained protein molecular weight standards (Life Technologies) run in the same gels for comparison of molecular weight and estimation of transfer efficiency.

Iodide uptake and efflux assay

K1 and F133 Cells were plated into 6-well plates the day before infection at a seeding density of 1.0×10^6 cells per well, to reach 50%~70% confluence for infection. Cells were washed twice with PBS and incubated in serum-free medium. The virus at MOI (appropriate multiplicity of infection) in 1 ml serum-free medium were added to each well for 2 h, and then the serum-free medium were changed into complete medium. After infection for 48 h, cells were washed twice with PBS and incubated in 1 ml serum-free DMEM containing 3.7KBq ^{125}I, with or without 300 μM $NaClO_4$. Following incubation at 37℃ for 5~120 min, the medium containing ^{125}I were removed and the cells were washed twice with PBS. The cell-associated radioactivity at different time point was measured with a γ-counter.

Iodide efflux studies were performed after K1 and F133 cells were infected with adenoviral as described above. Cells in per well were washed twice with PBS and incubated in 1 ml serum-free DMEM containing 3.7KBq ^{125}I at 37℃ for 1 h. Cells were washed twice with PBS, and then 1 ml serum-free DMEM without radioactivity was added per well. The serum-free DMEM was replaced every 5 min for 30 min and the radioactivity of ^{125}I in the collected medium was measured with a γ-counter. After the last time point, trapped ^{125}I were removed from cells and were measured with a γ-counter. Total radioactivity at the beginning of the efflux study (100%) were calculated by summing radioactivity of collected medium at different time point and final radioactivity of cells.

Radioiodine organification assay in vitro

K1 and F133 cells were infected with adenovirus for 24 h as previously described. Methimazole (MMI, 500 μM), a TPO-specific inhibitor that acts by uncoupling

TPO-catalyzed oxidative iodination, was added in medium. After incubation in MMI-containing medium for 24 h, cells were then incubated in 1 ml DMEM (without serum) containing 3.7KBq ^{125}I at 37 ℃ for 1 h. Medium containing ^{125}I was removed, and cells were washed twice with PBS. Proteins in the cell lysates were precipitated by the addition of 0.5 ml 40% trichloroacetic acid (TCA; final concentration, 20%). Precipitated proteins were collected by centrifugation at 3300 ×*g* for 30 min and were washed twice with PBS. Radioactivity in the pellets was measured with a γ-counter.

Clonogenic assay in vitro

K1 and F133 cells were infected with adenovirus for 48 h as previously described. Cells were washed twice with PBS and incubated with 37KBq ^{131}I in serum-free medium. Following incubation with radioiodine for 7 h, cells were washed twice with PBS, trypsinized and plated in six-well plates (10^3/well). After 1~2 weeks, cell colony development, cells were fixed with methanol and stained with crystal violet (250 ml containing 0.5g crystal violet, 25 ml 40% formaldehyde, 50 ml ethanol, and 175 ml H_2O), and colonies containing more than 50 cells were counted. The percentage of survival represents the percentage of cell colonies after ^{131}I treatment, compared with no ^{131}I intervention.

Biodistribution of $Na^{125}I$ in vivo

The experiments involving animals were performed in compliance with the current version of the national law on the Protection of Animals. One million F133 cells were subcutaneously injected into either thighs of 6-wk-old BALB/c nude mice. When tumors reached approximately 1cm in diameter by 2~3 wk after injection, AdTTF-1, AdTTF-1 and AdPax-8, or AdCMV (1.0×10^9 PFU in 100 μl PBS) were injected into the tumors for 3 days. $Na^{125}I$ (7.4×10^4 KBq) were injected via tail vein. The mice were sacrificed at 1, 2, 4, 8 and 12 h after ^{125}I injection. Tumor, blood, and selected tissues (heart, lung, spleen, liver, kidney, muscle, brain, bone, skin, stomach, intestine, thyroid gland) of the mice were dissected, blotted dry, weighted, and measured by γ-counting. Results are expressed as the percentage of injected dose per gram (%ID/g) of tissue. Each experiments were performed in triplicate.

Tumor imaging

Adenovirus were injected into the tumor on the thigh of nude mice as previously described. The tumor-bearing mice were injected with 18.5 MBq of $Na^{131}I$ via tail vein. The tumor-bearing mice were imaged with a γ-camera equipped with a low-energy, high-resolution pinholes collimator (Philips Medical Syst., Milpitas, CA, USA) at 2, 4, 8, 12

and 24 h after $Na^{131}I$ injection. Per image was acquired with 256×256 matrix, 2 times magnify, at least 100K total counts.

Statistical analysis

All experiments were carried out in triplicates or more under the same conditions. Results are presented as means±SD. Statistical significance was tested using Student's t-test. $P<0.05$ was considered statistically significant.

Results

Expression of NIS, Tg and TPO mRNA induced by AdTTF-1 and AdPax-8

To investigate the effect of TTF-1 and Pax-8 on the expression of NIS, Tg and TPO mRNA, F133 cells were infected with 50 MOI AdTTF-1, 20 MOI AdPax-8 and 30 MOI AdTTF, or 20 MOI AdCMV (control adenovirus). After 2h of infection with adenoviral vectors, cells were incubated in adenovirus-free medium for 48 h. Based on RT-PCR analysis, AdTTF-1 alone could promote the expression of NIS mRNA in F133 cells and it had little effect on the expression of Tg and TPO mRNA. Comparatively, not only the expression of NIS mRNA but also Tg and TPO mRNA in F133 cell were activated in F133 cells coinfected with AdTTF-1 and AdPax-8 (Fig. 1). These findings indicate that TTF-1 specifically promoted the expression of NIS mRNA and, TTF-1 and Pax-8 could synergistically reactive all of the expression of NIS, Tg and TPO mRNA in F133 cells.

AdTTF-1 and AdPax-8 induced expression of Tg and TPO proteins

To analyze whether NIS, Tg and TPO mRNA reactivated by TTF-1 and Pax-8 are translated into proteins, NIS, Tg and TPO proteins in F133 cells coinfected with AdTTF-1 and AdPax-8 were determined by Western blot analysis. Forty-eight hours after infection with adenoviral vectors as previously described, total proteins in F133 cells were abstracted to Western blot analysis (Fig. 2). In AdTTF-1-infected F133 cells, NIS proteins were increased, but Tg and TPO proteins were very low and hardly detectable. In contrast, all of the expression of NIS, Tg and TPO proteins were increased in AdTTF-1 and AdPax-8 coinfected F133 cell (Fig. 2). These results clearly showed that NIS mRNA promoted by TTF-1 and, NIS, Tg and TPO mRNA promoted by TTF-1 and Pax-8 in F133 cells are all translated into proteins.

AdTTF-1 and AdPax-8 induced iodide uptake

Because TTF-1 and Pax-8 could synergistically promote NIS expression in F133 cells,

we speculated that radioiodine uptake increased in cells infected with AdTTF-1 and AdPax-8. To analyze the function of NIS protein induced by TTF-1 and Pax-8, we measured the radioiodine uptake in K1 and F133 cells infected with AdTTF-1 and AdPax-8. K1 and F133 cells were infected with adenoviral vectors as previously described. Iodide uptake studies were performed after incubating the cells in serum-free DMEM containing 3.7KBq ^{125}I. Radioiodine uptake in AdCMV-infected K1 and F133 cells were faint and showed little change even after 2 h incubation with ^{125}I. Comparatively, radioiodine uptake in AdTTF-1-infected or AdTTF-1 and AdPax-8-coinfected K1 and F133 cells were rapidly increased and reached maximum at 30 min, thereafter, they gradually decreased as time passing. (Fig. 3).

AdTTF-1 and AdPax-8 coinfected K1 and F133 cells showed a relative higher radioiodine uptake than that in K1 and F133 cells infected with AdTTF-1 alone. At 30 min after incubation with ^{125}I, radioiodine uptake in K1 and F133 cells infected with AdTTF-1 was respectively 10597±648 cpm (cells per well) and 12445±513 cpm, and it increased about 6.1-fold and 7.4-fold over that in AdCMV-infected K1 and F133 cells, respectively (P<0.05). Meanwhile, at 30 min after incubation with ^{125}I, radioiodine uptake in AdTTF-1 and AdPax-8-coinfected K1 (13103±1503 cpm) and F133 cells (14398±237 cpm) increased about 6.6-fold and 7.5-fold over that of AdCMV-infected K1 and F133 cells, respectively (P<0.05) (Fig. 3). The iodide accumulation in K1 and F133 cells induced by AdTTF-1 and AdPax-8 were almost completely inhibited by sodium perchlorate. These results suggest that TTF-1 and Pax-8 could synergistically promote radioiodine uptake in thyroid carcinoma cells by inducing the expression of NIS protein.

AdTTF-1 and AdPax-8-induced radioiodine organification

Because AdTTF-1 and AdPax-8 could induce the expression of Tg and TPO proteins (Fig. 2) in F133 cells, We try to further investigate whether iodide in K1 and F133 cell infected with AdTTF-1 and AdPax-8 would organized by the catalysis of TPO. We pretreated AdTTF-1 and AdPax-8 infected K1 and F133 cells with the TPO inhibitor MMI, and then exposed cells to ^{125}I. As predicted, AdTTF-1-infected K1 and F133 cells showed no apparent increase in intracellular protein-bound radioiodine. In contrast, AdTTF-1 and AdPax-8 significantly enhanced radioiodine organification in K1 and F133 cells. The protein-bound radioiodine in AdTTF-1 and AdPax-8 coinfected K1 cells was 11576±857 cpm, and it was about 13.1-fold and 4.3-fold higher than that of AdCMV-infected K1 cells (820±175 cpm, P<0.01) and AdTTF-1-infected K1 cells (2170±648 cpm, P<0.01), respectively. Meanwhile, the protein-bound radioiodine in AdTTF-1 and AdPax-8 coinfected F133 cells coinfected was 11995±865 cpm, and it was about 11.1-fold and 5.3-fold higher than that of AdCMV-infected F133 cells (990±181 cpm, P<0.01) and AdTTF-1-infected

F133 cells (1893±535 cpm, $P<0.01$), respectively. The protein-bound radioiodine induced by AdTTF-1 and AdPax-8 were very sensitive to MMI pretreatment and it were almost completely inhibited by MMI ($p<0.01$). These results demonstrated that TPO induced by AdTTF-1 and AdPax-8 catalyzed iodide organification in K1 and F133 cells (Fig. 4).

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AdTTF-1 and AdPax-8 induced the prolongation of iodide efflux

We have demonstrated that AdTTF-1 and AdPax-8 could promote iodide uptake (Fig. 3) and induce iodide organification (Fig. 4) in K1 and F133 cells. To determine whether TTF-1 and Pax-8 cotransfer could prolong radioiodine retention in K1 and F133 cells, we performed a radioiodine efflux assay. AdTTF-1 and AdPax-8 infected K1 and F133 cells were exposed to radioiodine, and the release of radioactivity into the medium was monitored every 5 min. There was a rapid efflux of radioactivity from K1 and F133 cells infected with either AdTTF-1($t_{1/2}\approx8$ min) or AdCMV ($t_{1/2}\approx6$ min), and cellular radioactivity was almost completely released into the medium over 30 min. In contrast, iodide efflux was prolonged in AdTTF-1 and AdPax-8 coinfected K1 and F133 cells ($t_{1/2}\approx25$ min and 30 min, respectively) (Fig. 5). These results indicate that TTF-1 and Pax-8 cotransfer could effectively inhibit iodide efflux and prolong radioiodine retention in K1 and F133 cells.

Clonogenic assay in vitro

We want to know therapeutic effect of radioiodine on K1 and F133 cells cotransfected with AdTTF-1 and AdPax-8 because AdTTF-1 and AdPax-8 induced iodide uptake and prolonged iodide retention in K1 and F133 cells (Fig 3 and Fig 5). After ^{131}I treatment, clonogenic assay were performed and results are shown in Figure 6. Following exposure to ^{131}I, about 20% of AdCMV-infected K1 or F133 cells were nonselectively killed. After ^{131}I treatment, the survival rate of AdTTF-1-infected K1 and F133 cells were 40% and 43%, respectively, and that of AdTTF-1 and AdPax-8 coinfected K1 and F133 cells were 21% and 25%, respectively, which were apparently lower than that of AdCMV-infected K1 or F133 cells ($p<0.05$). Moreover, the survival rate of K1 and F133 cells coinfected with AdTTF-1 and AdPax-8 were obviously lower than that of K1 and F133 cells infected with AdTTF-1 ($p<0.05$). These data demonstrate that coinfection with AdTTF-1 and AdPax-8 obviously enhanced the effect of radioiodine therapy of K1 and F133 cells.

TTF-1 and Pax-8 induce iodide accumulation in vivo

We demonstrated that AdTTF-1 and AdPax-8 could promote radioiodine accumulation and prolong radioiodine retention in K1 and F133 tumor cells in vitro. In order to extend those results to an in vivo model, biodistribution of ^{125}I was performed on F133-bearing mice. The quantitation of the ^{125}I uptake (%ID/g) in the tumors and other

tissues was evaluated at 1, 2, 4, 8 and 12 h after ^{125}I administration. The biodistribution data of radioiodine in F133-bearing mice after intratumoral injection of AdTTF-1, AdTTF-1 and AdPax-8, were summarized in Figure 7. The AdCMV-infected F133 tumor accumulated a lower amount of the radioiodine than did AdTTF-1-infected F133 tumor or AdTTF-1 and AdPax-8 coinfected F133 tumor. The radioactivity in AdCMV-infected tumor was faintly increased, thereafter, it gradually decreased. The ^{125}I uptake in TTF-1-expressing F133 tumor quickly increased in 2 h after ^{125}I administration. The ^{125}I concentration in TTF-1-expressing tumor was 7.61±1.17 %ID/g and 4.24±1.44 %ID/g at 2 and 4 h after ^{125}I administration, respectively. After that, ^{125}I concentration in it rapid decreased. At 12h after ^{125}I administration, only 0.14±0.04 %ID/g radioiodine remained in it. In contrast, the ^{125}I concentration in AdTTF-1 and AdPax-8 coinfected F133 tumor rapidly increased in 1 h, after that, it gradually decreased. The ^{125}I concentration in F133 tumor coinfected with AdTTF-1 and AdPax-8 tumor was 9.42±0.83 %ID/g and 8.32±1.14 %ID/g at 2 h and 4 h after ^{125}I administration, respectively. At 12h after ^{125}I administration, 5.64±0.67 %ID/g radioiodine still remained in it, which were obviously higher than that in AdTTF-1-infected tumor (P<0.05). The average biological half-life of radioiodine in F133 tumor infected with AdCMV, AdTTF-1, AdTTF-1 and AdPax-8 was 3.75 h, 4.7 h and up to 12 h, respectively.

All of other organs accumulated slight radioiodine, no more than 5.8 %ID/g (intestine) at all time, except thyroid and stomach. The tumor/blood and tumor/muscle ^{125}I uptake ratios of AdTTF-1-infected F133 tumor were 1.81±0.46 and 3.59±0.87 at 8 h after ^{125}I administration, respectively, which were higher than that of AdCMV-infected F133 tumor (0.68±0.16 and 1.34±0.41, respectively) (P<0.05). Comparatively, the tumor/blood and tumor/muscle ^{125}I uptake ratios of F133 tumor coinfected with AdTTF-1 and AdPax-8 were 3.42±0.51 and 11.09±1.14 at 8 h after ^{125}I administration, respectively, which were higher than that of AdTTF-1- or AdCMV-infected F133 tumor (P<0.05). These data demonstrate that AdTTF-1 and AdPax-8 promoted radioiodine accumulation and prolonged radioiodine retention in F133 tumor in vivo.

Tumor Imaging

Radioiodine uptake in tumor was determined using a γ camera at 2, 4, 8, 12 and 24 h after ^{131}I injection (Fig. 8). AdTTF-1-infected F133 tumors accumulated ^{131}I rapidly and were early visualized at 2 h after ^{131}I administration. However, they quickly attenuated after 4h and were hardly visible at 12 h. In contrast, the F133 tumors coinfected with AdTTF-1 and AdPax-8 began to show at 2 h and they were still visualized at 24h after ^{131}I administration. Some normal organs expressing NIS (including those of thyroid, stomach) and involved in iodide elimination (bladder) showed clearly.

Discussion

Distant metastasis is the first primary cause of death to most patients with cancer, because no durative therapy for metastases. In contrast, DTC, even with distant metastases, can be effectively treated by radioiodine because of the unique ability of carcinoma cells to concentrate iodine from plasma. The special property of DTC makes it to be one of the most manageable cancers.[19] It was postulated that reexpression of iodide-metabolic relative proteins would restore/acquire iodine uptake in cancer and enable them be treated by radioiodine. Mandell et al. initially proposed NIS-based radioiodine concertrator gene therapy for extrathyroidal cancer.[20] It has shown that the transfer of NIS gene confers increased radioiodine uptake in thyroid cancer[21], breast cancer[22], hepatoma[23] and prostate cancer[24], et al. These studies demonstrated that NIS-transducted tumor cells could significantly accumulated radioiodine, and consequently, be selectively killed by radioiodine in vivo and in vitro.[20, 24~26] However, rapid radioiodine efflux form NIS-transfected cells may impact the antitumor efficacy of this therapeutic strategy.

All of the expression of iodide-metabolic relative proteins such as NIS, Tg and TPO in thyroid are regulated by TTF-1 and/or Pax-8. Maybe it is a possible strategy to simultaneously stimulate the expression of NIS, Tg and TPO in tumor cells and, to promote radioiodine uptake and prolong iodide retention in tumor cells at one time, by transfer TTF-1 and/or Pax-8 gene. Our group has found that Pax-8 could activate the expression of TPO and Tg proteins in human thyroid carcinoma cells. The radioiodine uptake was partially restored and $T_{½}$ of ^{131}I in thyroid carcinoma cells was apparently prolonged by Pax-8 (data in manuscript). In the present report, we further co-transferred TTF-1 and Pax-8 gene by adenovires vectors and discovered that TTF-1 alone could specially induced the expression of NIS and the synergetic effect of TTF-1 and Pax-8 could simultaneously induce the reexpression of NIS, TPO and Tg proteins in human thyroid carcinoma F133 cells. TTF-1 and Pax-8 cotransducted K1 and F133 cells could accumulated more radioiodine and obviously retarded radioiodine efflux than TTF-1-transfected K1 and F133 cells in vitro and in vivo. Consequently, the effect of radioiodine therapy of TTF-1 and Pax-8 cotransfected K1 and F133 cells was better than that of TTF-1 transfected cells.

In this study, we discovered that AdTTF-1 specially activated the expression of NIS in F133 cells, and which were further promoted in TTF-1 and Pax-8 cotransfected F133 cells. Accordingly, the radioiodine uptake was obviously increased in both AdTTF-1-infected and AdTTF-1 and AdPax-8 coinfected K1 and F133 cells. Altmann et al. found that TTF-1 has no induction or a low induction of transcriptional activation of NIS promoter. On the other hand, the activation of rNIS promoter (up to 6-fold) were activated in cell lines expression

both TTF-1 and Pax-8.[16] However, Furuya et al. found that no detectable NIS expression was show in thyroid carcinoma (BHP18-21v) cells, regardless of whether they infected with AdTTF-1. But AdTTF-1 induced a small, but significant, iodide accumulation, that was inhibited by sodium perchlorate in BHP18-21v cells.[27] Schmitt et al. also reported that TTF-1 had no influence in the activation of NIS promoter and Pax-8 had a moderate stimulating effect (3 folds) on the NIS promoter in Hela and COS-7 cells.[28] To explain this observations, it could be speculated that the expression of NIS activated by TTF-1 had the cell-specificity because Endo T et al. observed that TTF-1 can bind to a minima 5'-flanking sequence region (between -291 and -134 bp) of NIS gene extracts from a rat thyroid cell line, FRTL-5 cells, but not BRL-3A rat liver cells, forming a specific DNA-protein complex and causing a significant increase in the transcriptional activity of the promoter of rat NIS gene.[8] Moreover, the study that TTF-1 is a phosphoprotein and phosphorylation is reduced in the Ha-ras traformed thyroid cells, where TTF-1 is unable to activate transcription from the Tg promoter, suggests a possible mechanisms whereby differential phosphorylation of TTF-1 in different cell types could be responsible for its promoter selection.[29]

The therapeutic efficacy of radioiodine is dependent on the radiation dose delivered to the target tissue and, the radiation dose delivered to a tumor is dependent on two factors: the radioactive concentration of ^{131}I and the effective half-life ($T_{½}$) of ^{131}I in the tumor. $T_{½}$ of ^{131}I in thyroid carcinoma, which had response to ^{131}I therapy, was 78.8 h. The effect of ^{131}I therapy was not satisfactory if $T_{½}$ of ^{131}I was shorter than 45.8 h.[30] Rapid radioiodine efflux after sufficient iodide uptake is probably the main problem of NIS-transduction. Although the tumors efficiently concentrated iodide after NIS gene transfer, no effect of ^{131}I on tumor growth was observed because the rapid iodide efflux from the tumor did not allow the delivery of a radiation dose sufficient to inhibit cell growth.[21, 31, 32] Thus, to efficiently therapy tumor with ^{131}I by gene transfer, it is mandatory to increase the retention time of iodide in the tumor. An appealing strategy is to mimic the situation existing in the thyroid, i.e., to organify the iodide taken up by the tumor. TPO is the main enzyme to catalysis iodide organification in thyroid. Any iodide not organified by TPO will rapidly efflux from thyroid follicular cells.[33] Some researchers try to inhibit iodide efflux by reexpressing TPO in tumor cell. Haberkorn et al. found that the accumulation of iodide was not significantly enhanced and there was no correlation between hTPO expression and enzyme activity in human anaplastic thyroid carcinoma cells transfered human TPO gene. They presumed that the transduction of hTPO gene was not sufficient to restore iodide trapping in non-iodide-concentration tumor cells.[34] After that, Huang et al. transfected non-small cell lung cancer cells with human NIS and TPO genes. They discovered that the combination of NIS and TPO gene transfection, with resulting in NIS-mediated iodide uptake and TPO-mediated organification and intracellular retention of radioiodine, might lead to more

effective radioiodine therapy of tumor.[35] However, Bland et al. reported that an increasing of iodide organification could be observed in rat thyroid FRTL-5 cells coinfected with both AdNIS and AdTPO in the presence of exogenous hydrogen peroxide, but the levels of iodide organification obtained were too low to significantly increase the iodide retention time in the target cells.[36] In the current study, the expression of Tg and TPO in F133 cell, not like that of NIS, was not promoted by Ad-TTF-1. Besides, while combining with Pax-8, the activating effect of TTF-1 on the expression of Tg and TPO was apparently enhanced. The results of radioiodine organification assay and radioiodine efflux analysis demontrated that, following with the expression of Tg and TPO, radioiodine organized and radioiodine retention time obviously prolonged in Ad-TTF-1 and Ad-Pax-8 coinfected F133 cells, comparing with that in Ad-F133 or Ad-CMV infected F133 cells. Altmann et al. also reported that TTF-1 could not activate Tg and TPO promoter, but the activation of hTg promoter (up to 23-fold) and hTPO promoter (up to 28-fold) were significantly activated in cell lines expression both TTF-1 and Pax-8.[16] These results indicated efficient iodide organification and retention induced by TTF-1 and Pax-8.

We hope that the efficacy of radioiodine therapy for TTF-1 and Pax-8 cotransfected tumor would improved because the sufficient iodide accumulation and retarded iodide retention were observed in TTF-1 and Pax-8 cotransfected K1 and F133 cells. The data from biodistribution of radioiodine in vivo showed that TTF-1 and Pax-8 cotransducted cancer could accumulate significantly more radioiodine than parental tissue, and comparatively, Ad-TTF-1 and Ad-Pax-8 coinfected F133 and K1 cells were selectively and efficiently killed by radioiodine in vitro. The effective half time of radioiodine in F133 tumor infected Ad-TTF-1 and Ad-Pax-8 was about 12 h, which was obviously shorter than the mean effective half time of radioiodine in DTC (78.8h).[37] We speculated it was due to the defect of transfer gene technology itself, because it is unlikely to achieve 100% transfection rate in vivo. The powerful gene delivery systerms toward clinical application must be performed in further studies. Besides, the combinatorial strategy should be considered to improve the efficacy of radioiodine therapy for tumor following gene transfer.

Another approach involved the use of more powerful and suitable radionuclides transported by NIS, such as ^{188}Re-perrhenate or ^{211}At rather than ^{131}I. ^{188}Re-Perrhenate is a β-emitter and has a shorter half life ($T_{1/2}$) and is effective over a wider range than ^{131}I.[38, 39] ^{188}Re-perrhenate has been proposed to use in the treatment of NIS expressing tumors as an alternative to ^{131}I because ^{188}Re-perrhenate exhibited NIS-dependent uptake into the mammary tumor, and dosimetry calculations in the mammary tumor demonstrate that ^{188}Re-perrhenate was able to deliver a dose 4.5 times higher than ^{131}I.[38] Moreover, in NIS-transfected prostate cancer, tumor absorbed dose for ^{188}Re

was 4.7-fold compared with ^{131}I and therapeutic effect of ^{188}Re in larger tumors was superior to that of ^{131}I.[37] Besides, ^{211}At is regard as a promising radionuclide for cell-targeted radiotherapy owing to a combination of favourable properties, including short half-life (7.2 h) and decay via a bibranch pathway emitting two α-particle types (6.8 MeV mean energy), leading to deposition of high energy over a short distance (55~88 μm mean tissue range). ^{211}At uptake is shown to be NIS-dependent, with characteristics similar to ^{131}I uptake.[40] NIS-expressing tumor cells could effectively accumulate ^{211}At and tumor absorbed dose for ^{211}At was significantly higher than that for ^{131}I (3.5 Gy/MBq$_{tumour}$ for ^{131}I and 50.3 Gy/MBq$_{tumour}$ for ^{211}At).[41] In this study, radioiodine rapidly flowed into tumor cells infected AdTTF-1 and AdPax-8 after iodide administration. We speculate that ^{188}Re and ^{211}At might be used to therapy tumors expressing TTF-1 and Pax-8 with superior therapy effect compared as ^{131}I, and which need be demonstrated by further studies.

A better understanding of the mechanisms governing iodide transport in the cells may provide better solutions to efficiently improve iodide retention. Indeed, an increased iodide retention time could be obtained by specifically blocking the mechanism controlling iodide efflux. Pendrin is a glycoprotein protein located at the apical membrane of thyrocytes, which is thought to be involved in iodide efflux from the intracellular compartment to the follicular lumen in the thyroid gland.[42] Other proteins (SLC5A8 and chloride channel 5 ClCn5) have been proposed to mediate apical iodide efflux.[43,44] Functional studies performed in *Xenopus* oocytes and polarized Madin-Darby canine kidney (MDCK) cells clearly demonstrate that SLC5A8, originally designated as human apical iodide transporter (hAIT)[43], does not mediate iodide uptake or efflux.[45] Localization of the *ClCn5* protein at the apical membrane of thyrocytes and a thyroidal phenotype of the *ClCn5*-deficient mice that is reminiscent of Pendred syndrome suggest that ClCn5 could be involved in mediating apical iodide efflux or iodide/chloride exchange.[44] This possibility has, as of yet, not been demonstrated by further experimental data.

In conclusion, TTF-1 can specially activate the expression of NIS, resulting in an increasing radioiodine accumulation in K1 and F133 cell. However, no detectable induction of Tg and TPO proteins expression is observed in TTF-1 tansducted F133 cell, following limited iodine organification and retention. Comparatively, the synergy of TTF-1 and Pax-8 can simultaneously activate the expression of NIS, Tg and TPO proteins in F133 cells, and

induces more significant radioiodine accumulation and obviously radioiodine retention than TTF-1 alone. TTF-1 and Pax-8 cooperatively promote the therapeutic effect of radioiodine on thyroid carcinoma cells. The cotransduction of TTF-1 and Pax-8 gene is a feasible strategy to promote the effect of radioiodine therapy on non-iodide-concentrating tumor. Further studies will concentrate on enhancing the effect of radionuclide therapy conducted by TTF-1 and Pax-8 gene cotransfer through using more powerful therapeutic radionuclides, such as ^{211}At and ^{188}Re.

Conflict of Interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of this paper.

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Table 1. Primer sequences, conditions, and product sizes for the RT-PCR

Primer	Primer Squences	Annealing Temperature	Product Sizes
NIS-FP	5’-ctctctcagtcaacgcctctgg-3’	57℃	316 bp
NIS-RP	5’-cccttgaccaagttgtcatccag-3’		
Tg-FP	5’-ggctaatgctacatgtcctg-3’	57℃	231bp
Tg-RP	5’-gcttctgttggagatgctgg-3		
TPO-FP	5’-gtctgtcaggctggttatgg-3’	57℃	242bp
TPO-RP	5’-caatcactccgcttgttggc-3’		
β-actin-FP	5’-ctggcaccacaccttctacaatg-3’	56℃	250bp
β-actin-RP	5’-cctcgtagatgggcacagtgtg-3’		

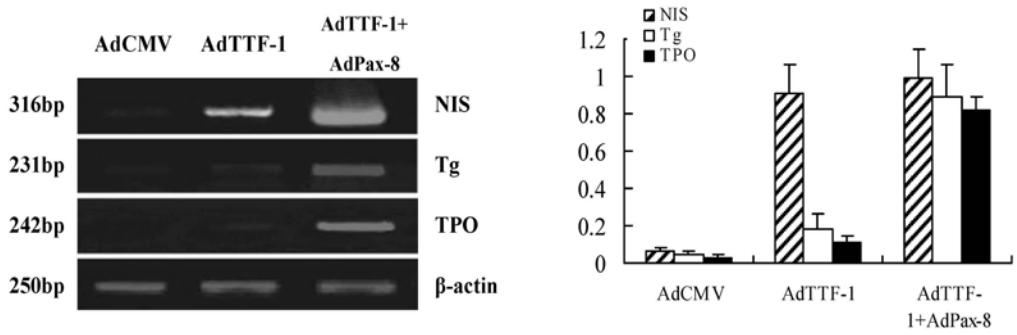


Fig 1. Expression of NIS, Tg and TPO mRNA in F133 cells induced by AdTTF-1 and AdPax-8. The mRNA levels of thyroid-specific genes in F133 cells infected with AdTTF-1 and AdPax-8 were investigated by RT-PCR analysis.

F133 cells were infected with AdTTF-1, AdTTF and AdPax-8, or AdCMV for 2h, then incubated with adenovirus-free medium for 48 h. Total mRNA (100ug) were subjected to RT-PCR using human NIS, Tg, TPO or β-actin primer. NIS, Tg and TPO mRNA levels were normalized with β-actin mRNA levels, based on optical density measurements and compared to β-actin (n=3).

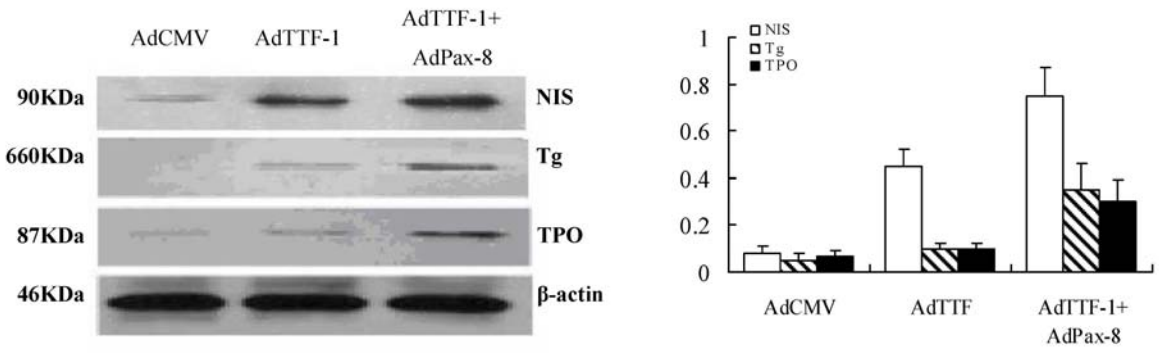


Fig 2. Expression of NIS, Tg and TPO proteins in F133 cells induced by AdTTF-1 and AdPax-8. The protein levels of NIS, Tg and TPO in F133 cells infected with AdTTF-1, AdTTF and AdPax-8, or AdCMV were investigated by Western-blot. F133 cells were infected with AdTTF-1, AdPax-8, AdTTF and AdPax-8, or AdCMV for 2h, then incubated with adenovirus-free medium for 48 h. Total proteins (50 μg) were subjected to Western Blot analysis as described in Methods. NIS, Tg and TPO protein was observed as the major band of molecular weight at 90 kDa, 660 kDa and 87 kDa, respectively. Tg and TPO protein levels were normalized with β-actin protein levels, based on optical density measurements and compared to β-actin (n=3).

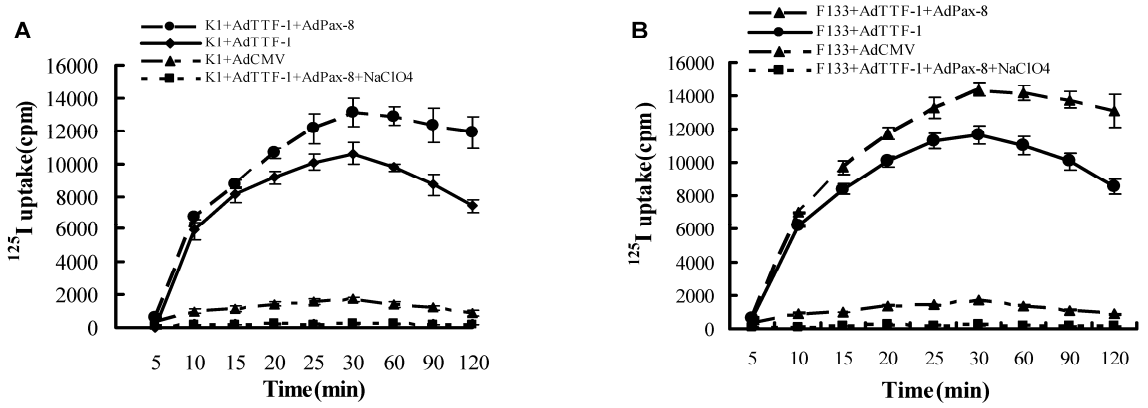


Fig 3. Time course of iodide uptake in K1 and F133 cells induced by AdTTF-1- and AdPax-8. K1 (A) and F133 (B) cells were infected with AdTTF-1, AdTTF and AdPax-8, or AdCMV for 2h, then incubated with adenovirus-free medium for 48 h. All cells were exposed to medium containing 3.7 KBq $Na^{125}I$ with or without 300 μM $NaClO_4$. At various time points, the cells were washed twice and intracellular radioactivity was measured with a γ-counter. Data are expressed as the mean ± SEM (n=6).

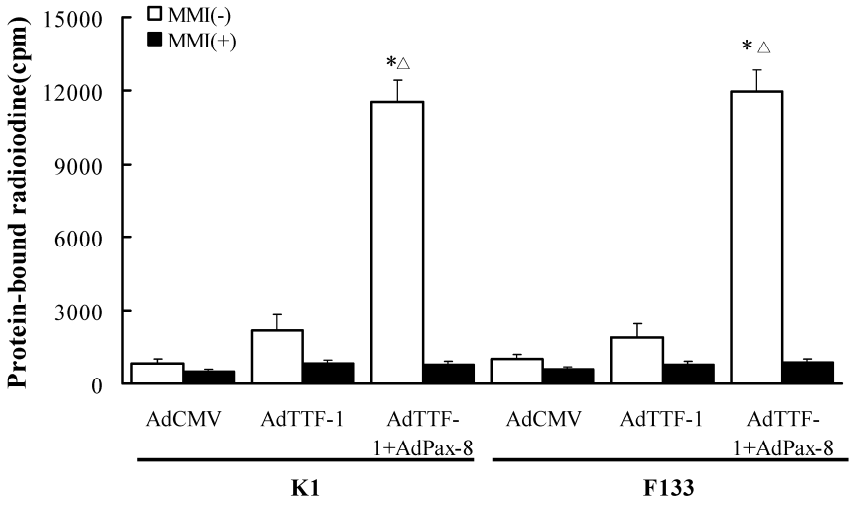


Fig 4. Iodide organification in K1 and F133 cells induced by AdTTF-1 and AdPax-8. K1 and F133 cells were infected with AdCMV, AdTTF and AdPax-8 for 2 h, then incubated with adenovirus-free medium for 24 h. The medium containing ^{125}I were replaced with medium with or without 500 μM MMI for 24 h. Cells were exposed to 3.7 KBq $Na^{125}I$ for 1 h and then the radioactivity of ^{125}I-bound protein in cells was determined by TCA precipitation. Data are expressed as the mean ± SEM (n = 6). *, $P<0.01$, compared with MMI-inhibited cells; △, $P < 0.01$, compared with AdCMV-infected cells.

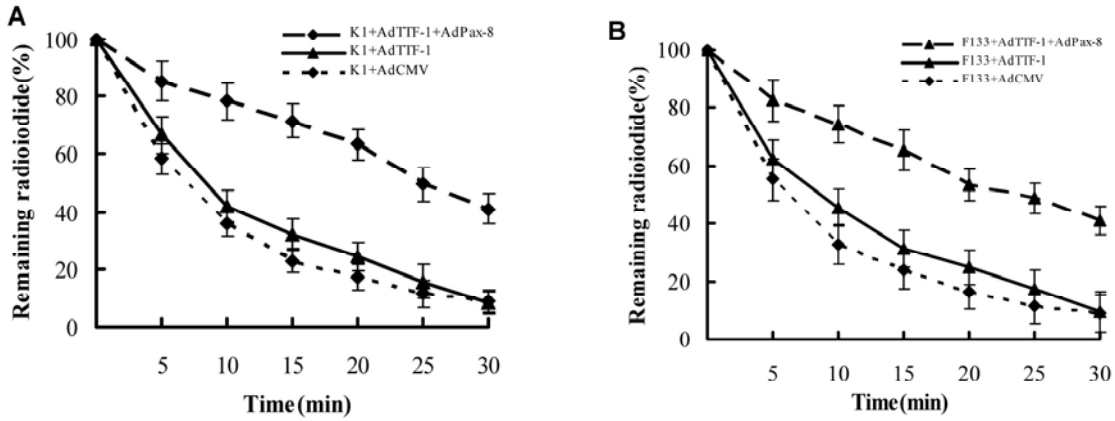


Fig 5. Iodine efflux from AdTTF-1- and AdPax-8 infected K1 and F133 cells. K1 (A) and F133 (B) cells were infected with AdTTF-1, AdTTF and AdPax-8, or AdCMV for 2h, then incubated with adenovirus-free medium for 48 h. All cells were incubated in serum-free medium containing 3.7 KBq $Na^{125}I$ for 1 h. Cells were washed twice with PBS, and the medium was replaced with nonradioactive medium every 5 min. The radioactivity in the replaced medium was measured with a γ-counter. After the last medium was removed, the cells were extracted. The total radioactivity present at the initiation of the efflux study (100%) was calculated by adding the counts in the final cells to the medium counts at various time point. Data are expressed as the mean ± SEM (n=6).

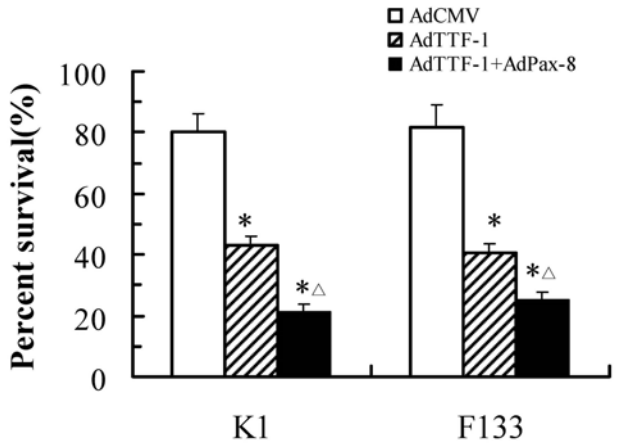


Fig 6. The effect of ^{131}I therapy of K1 and F133 tumors transfected with AdTTF-1 and AdPax-8 *in vitro*. In clonogenic assay in vitro, K1 or F133 cells were infected with dTTF-1, AdTTF and AdPax-8, or AdCMV and then exposed to 37 KBq Na^{131}I for 7 h. Cells were washed with PBS and plated in six-well plates. After 2~3 weeks, cells were fixed with methanol and stained with crystal violet, and colonies containing more than 50 cells were counted. The percentage of survival represents the percentage of cell colonies after ^{131}I therapy, compared with no ^{131}I intervention. Data are expressed as the mean ± SEM (n=3). *, $P< 0.05$, compared with AdCMV-infected cells; $^{\triangle}$, $P < 0.05$, compared with AdTTF-1-infected cells.

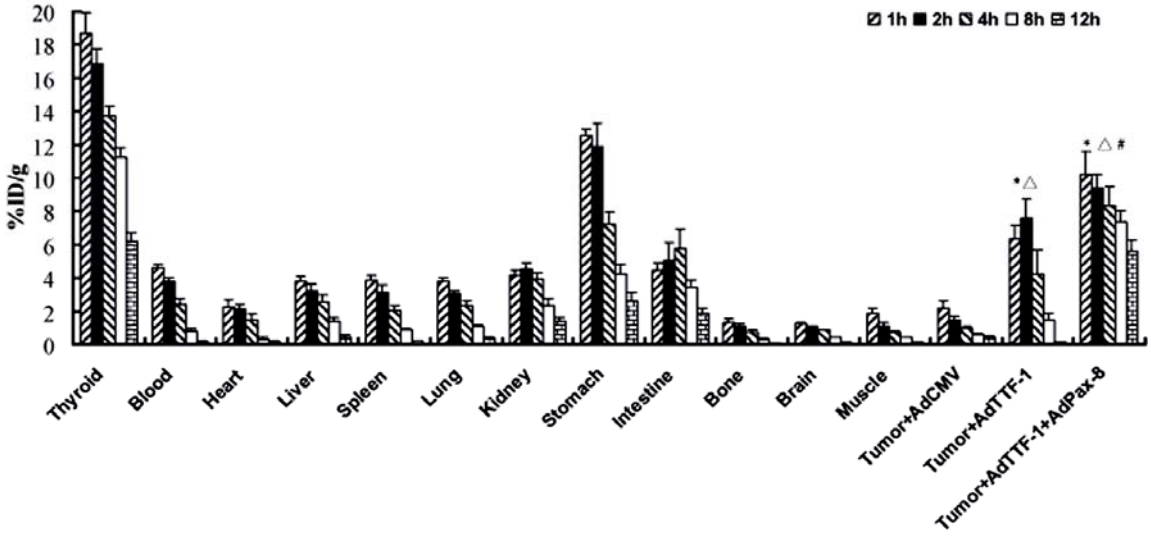


Fig 7. Biodistribution of radioiodine in nude mice bearing F133 tumor cells. After AdTTF-1 and AdPax-8, or AdCMV (1.0×10^9 pfu) were injected into tumors for 3 d, 7.4×10^4Bq Na^{125}I were injected via tail vein. The mice were sacrificed at 1, 2, 4, 8 and 12 h and various tissues (tumor, blood, heart, lung, liver, et al.) of the mice were dissected. The radioactivity of various tissues were measured with a γ-counter. Results are expressed as the percentage of injected dose per gram (%ID/g) of tissue (n=3). *, $P<0.05$, compared with blood; $^{\Delta}$, $P<0.05$, compared with muscle;

#$P<0.05$, compared with AdTTF-1-infected tumor.

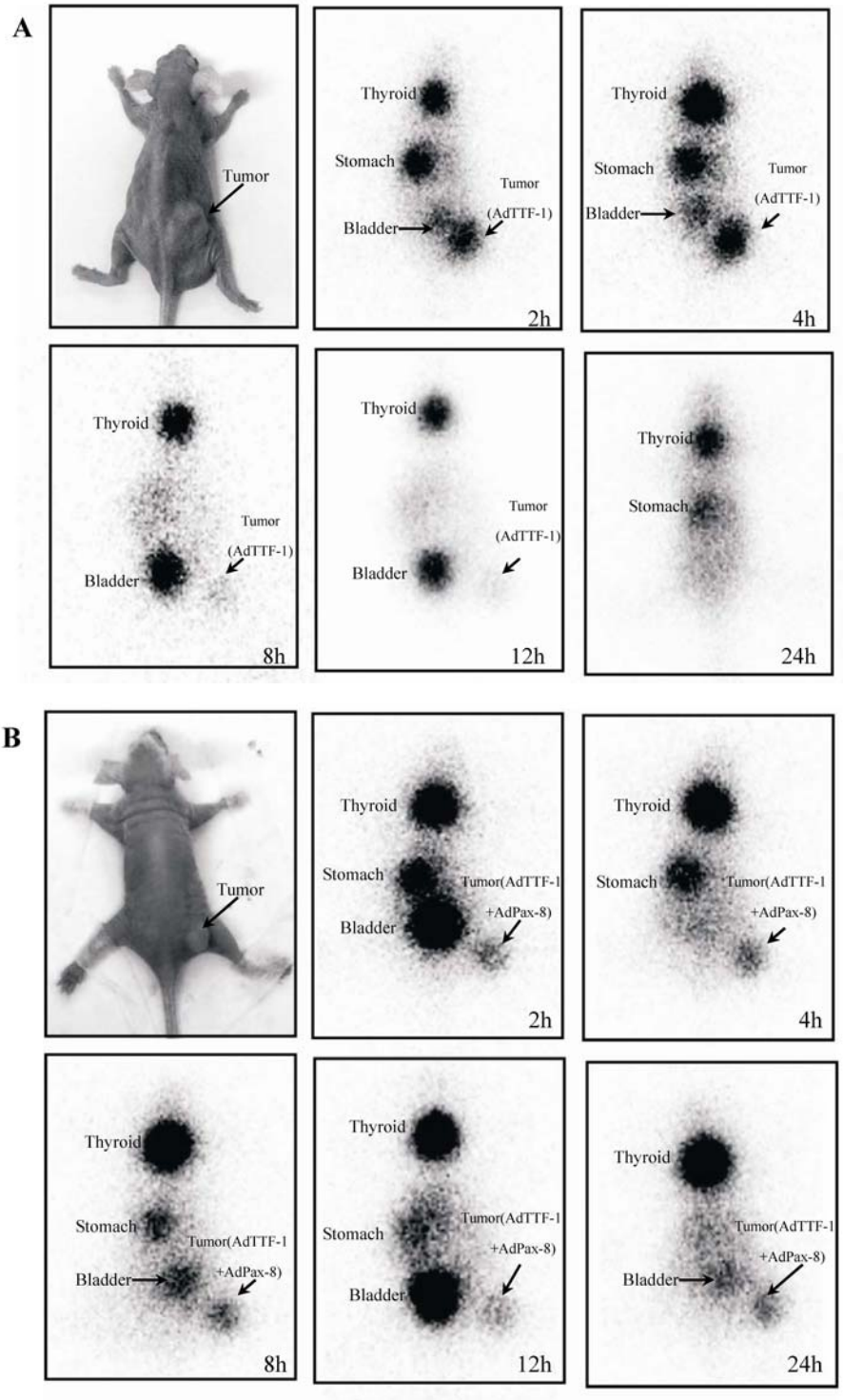
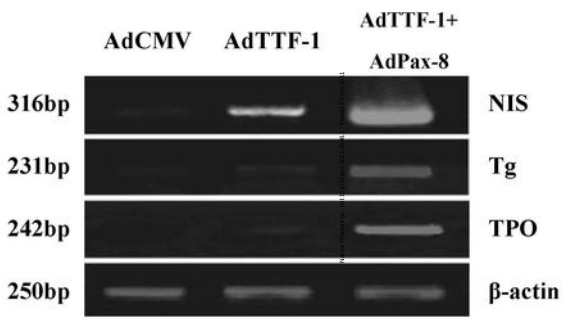


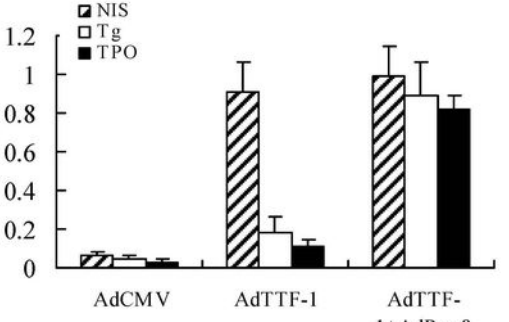
Fig 8. Whole-body scintigraphic images of nude mice transplanted with F133 cells infected with AdTTF-1 and AdPax-8. Tumor imaging studies were performed after AdTTF-1 (A), AdTTF-1 and AdPax-8 (B) were injected into tumors for 3 d. At 2, 4, 8, 12 and 24 h after $Na^{131}I$ (18.5 MBq /rat) were injected i.p., mice were imaged with a γ

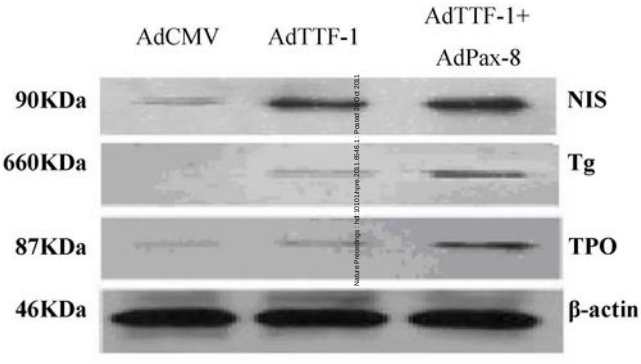
camera.

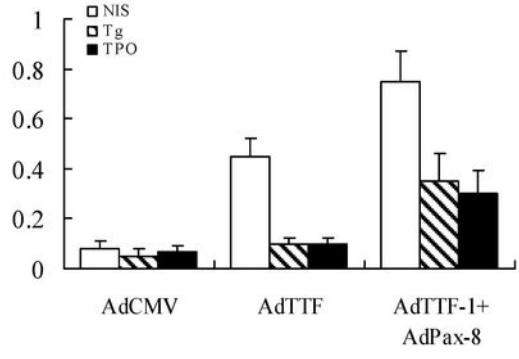
Table 1. Primer sequences, conditions, and product sizes for the RT-PCR

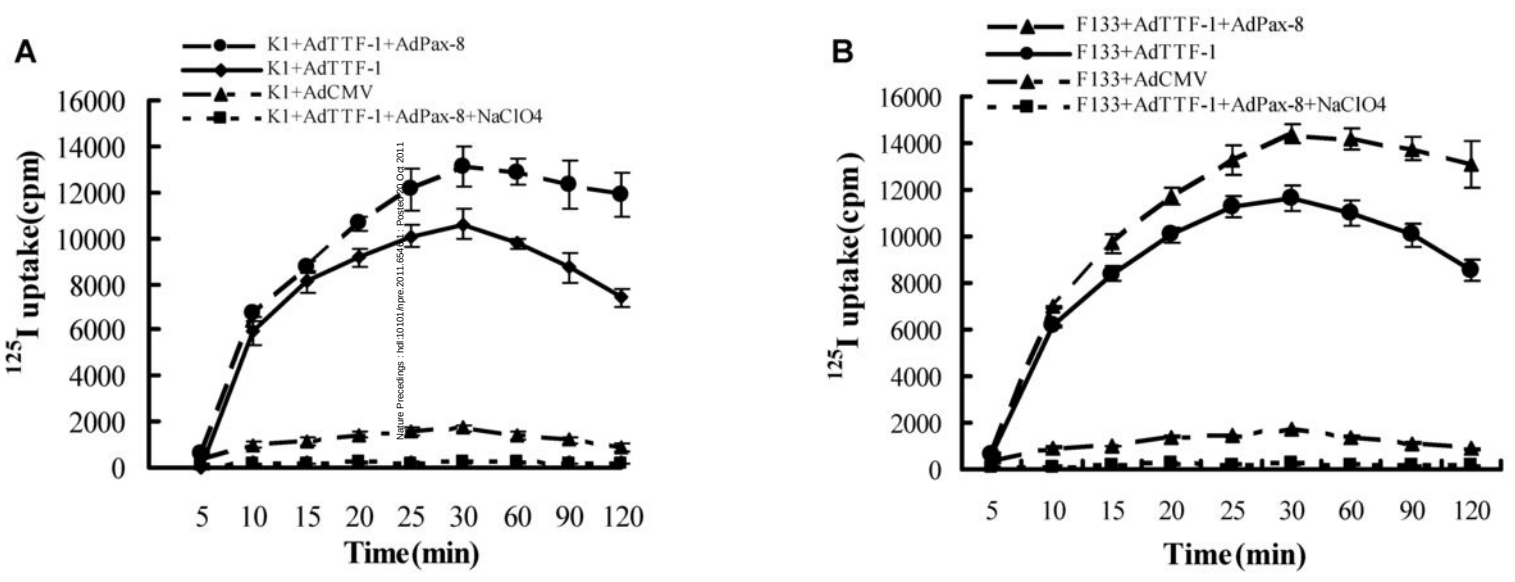
Primer	Primer Squences	Annealing Temperature	Product Sizes
NIS-FP	5’-ctctctcagtcaacgcctctgg-3’	57℃	316 bp
NIS-RP	5’-cccttgaccaagttgtcatccag-3’		
Tg-FP	5’-ggctaatgctacatgtcctg-3’	57℃	231bp
Tg-RP	5’-gcttctgttggagatgctgg-3		
TPO-FP	5’-gtctgtcaggctggttatgg-3’	57℃	242bp
TPO-RP	5’-caatcactccgcttgttggc-3’		
β-actin-FP	5’-ctggcaccacaccttctacaatg-3’	56℃	250bp
β-actin-RP	5’-cctcgtagatgggcacagtgtg-3’		

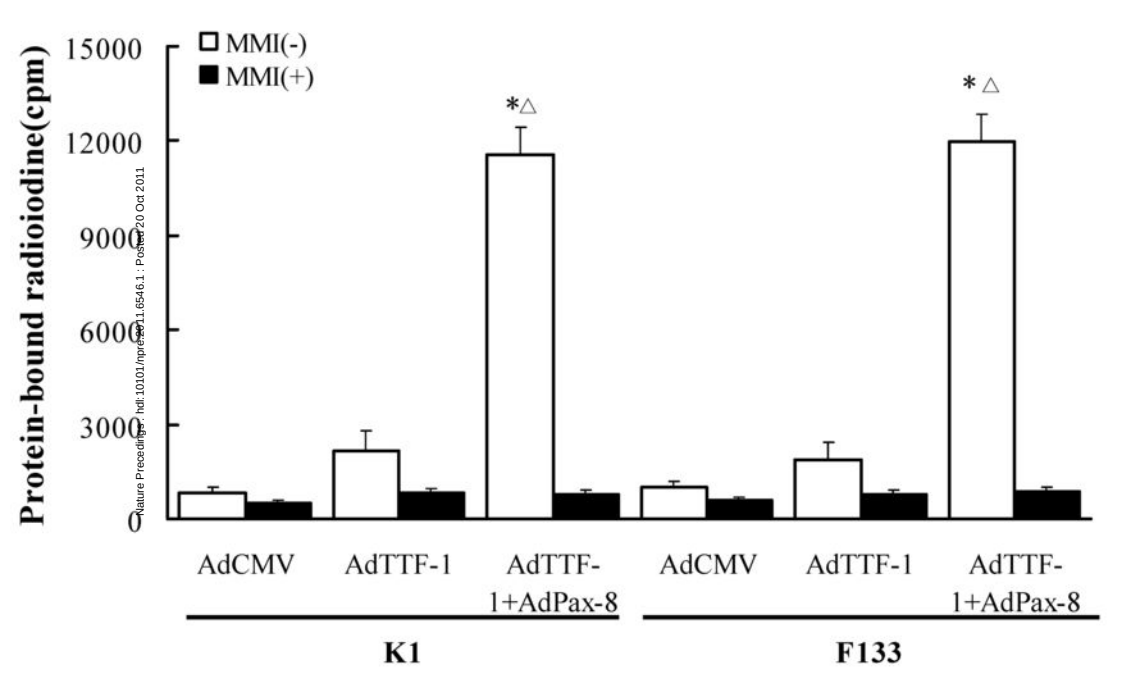

AdCMV
AdTTF-1
AdTTF-1+
AdPax-8
316bp
NIS
231bp
Tg
242bp
TPO
250bp
β-actin

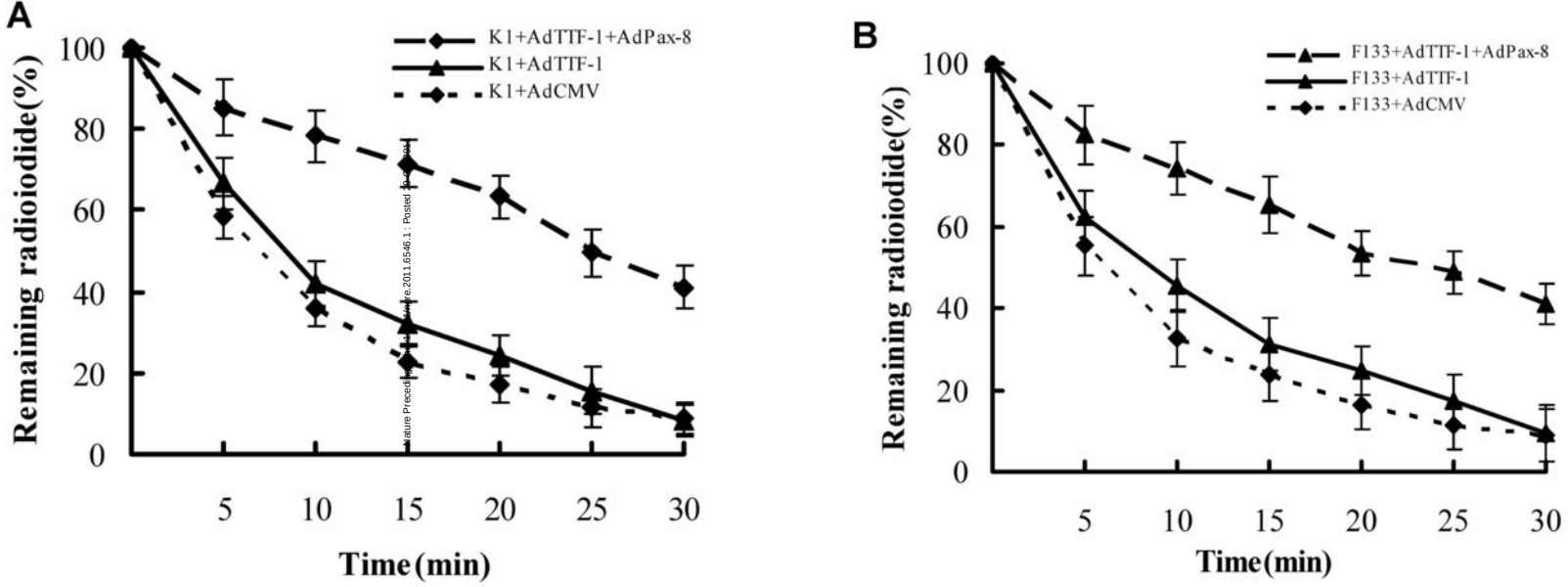

NIS
Tg
TPO
1.2
1
0.8
0.6
0.4
0.2
0
AdCMV
AdTTF-1
AdTTF-1+AdPax-8

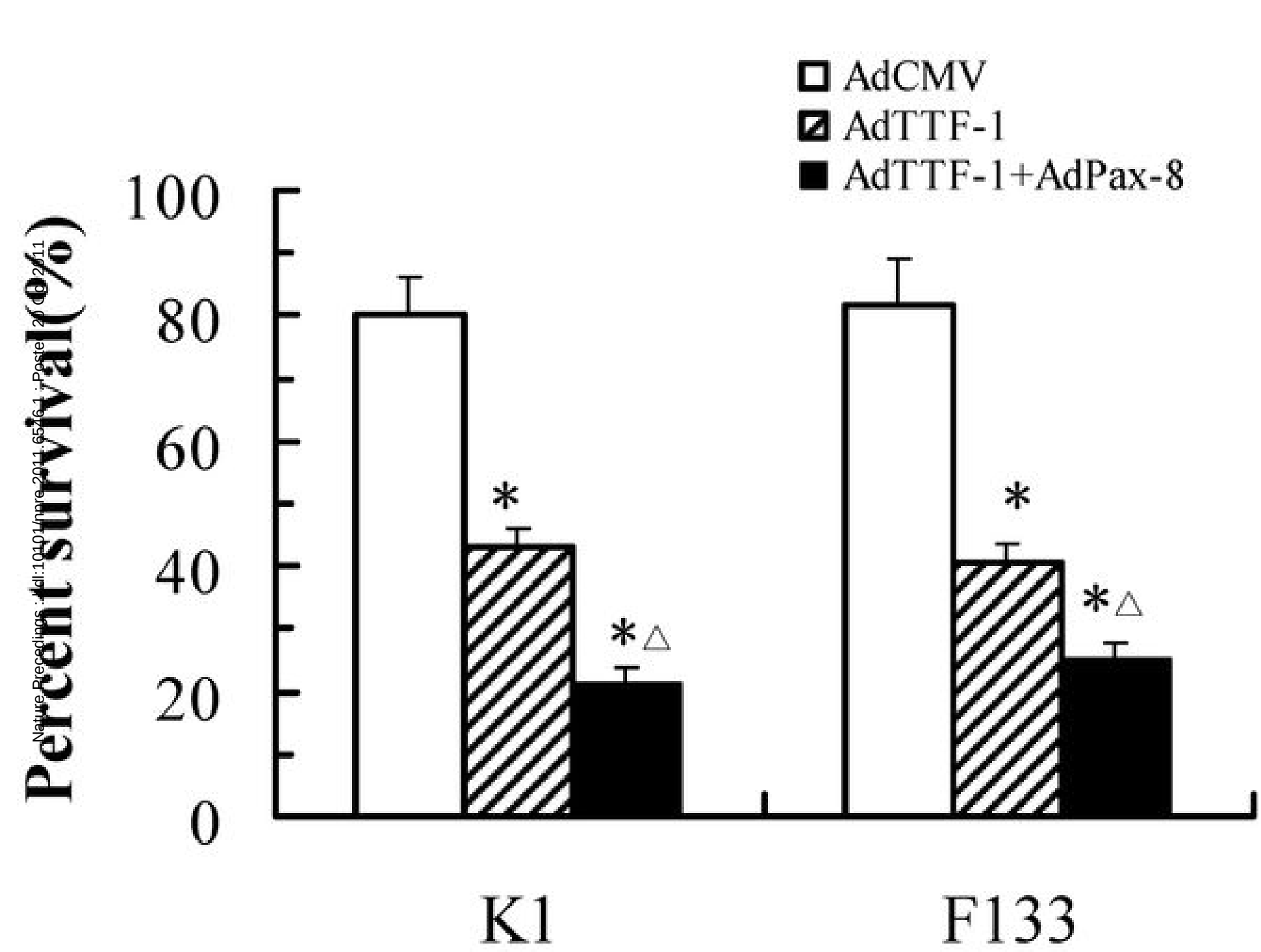

AdCMV
AdTTF-1
AdTTF-1+
AdPax-8
90KDa
NIS
660KDa
Tg
87KDa
TPO
46KDa
β-actin

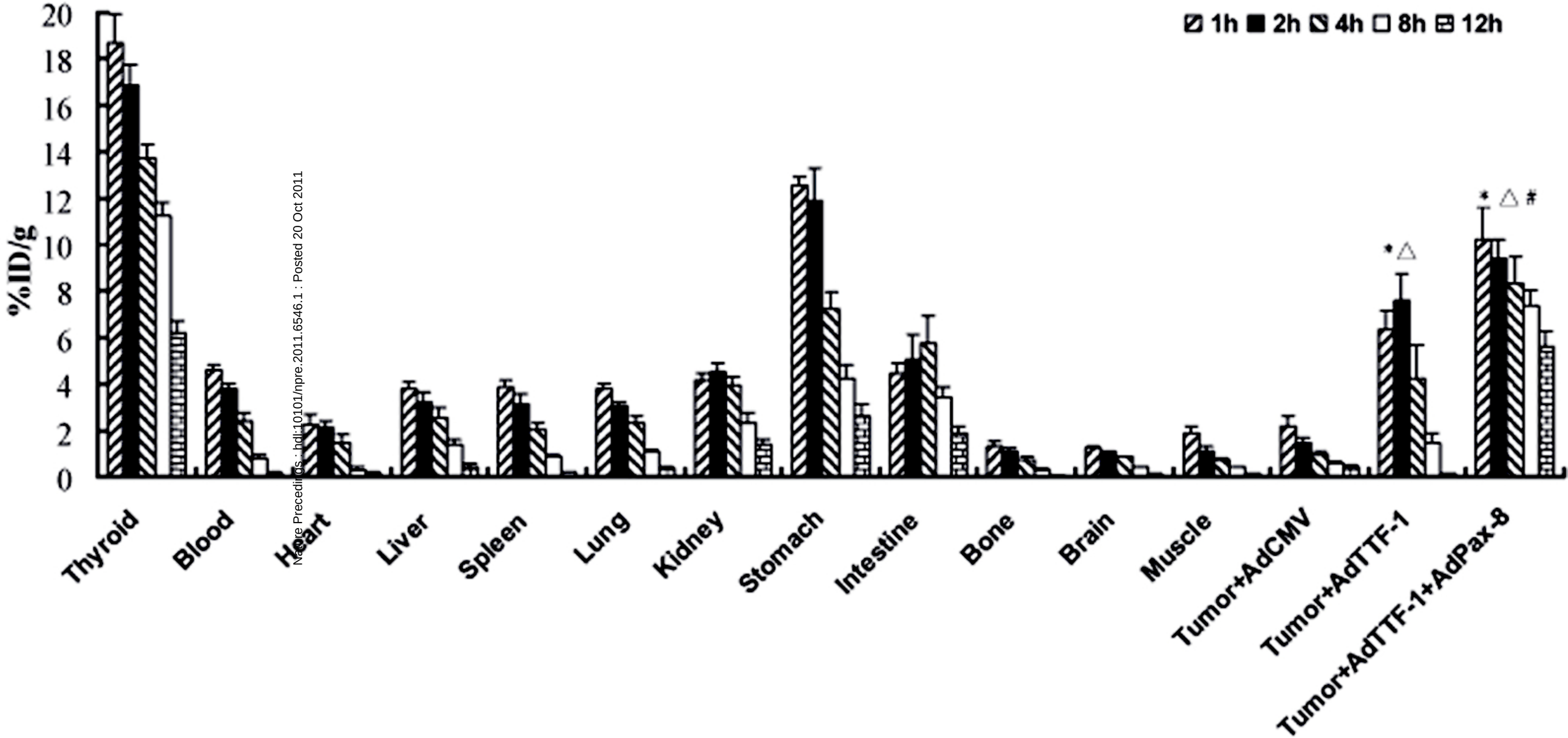

NIS
Tg
TPO
1
0.8
0.6
0.4
0.2
0
AdCMV
AdTTF
AdTTF-1+
AdPax-8

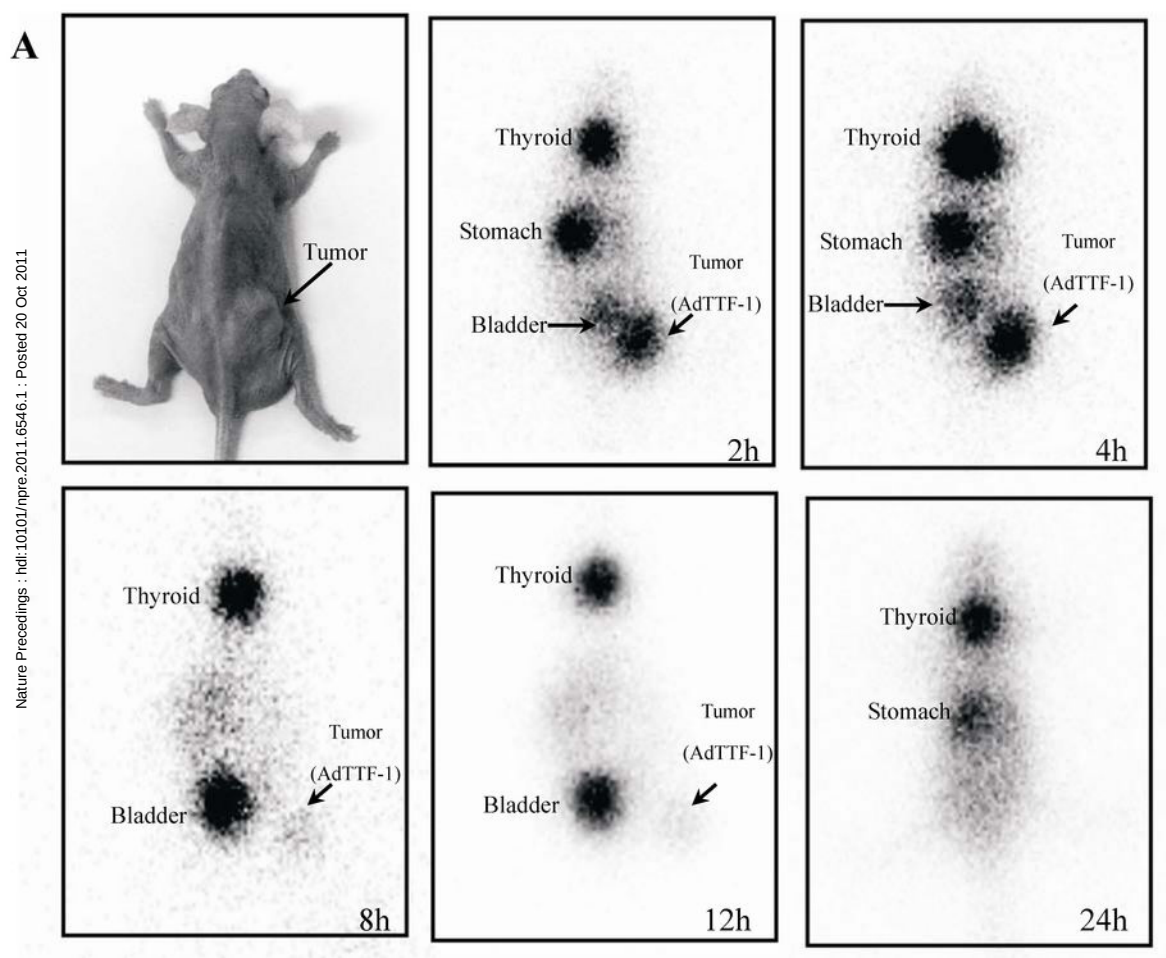





A
Remaining radioiodide(%)
100
80
60
40
20
0
5
10
15
20
25
30
Time (min)
K1+AdTTF-1+AdPax-8
K1+AdTTF-1
K1+AdCMV
B
Remaining radioiodide(%)
100
80
60
40
20
0
5
10
15
20
25
30
Time (min)
F133+AdTTF-1+AdPax-8
F133+AdTTF-1
F133+AdCMV







B