

Androgen-Independent Growth and Tumorigenesis of Prostate Cancer Cells Are Enhanced by the Presence of PKA-Differentiated Neuroendocrine Cells

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Abstract

The neuroendocrine status of prostatic adenocarcinomas is considered a prognostic indicator for development of aggressive, androgen-independent disease. Neuroendocrine-like cells are thought to function by providing growth and survival signals to surrounding tumor cells, particularly following androgen ablation therapy. To test this hypothesis directly, LNCaP cells were engineered to inducibly express a constitutively activated form of the cyclic AMP-dependent protein kinase A catalytic subunit (caPKA), which was previously found upon transient transfection to be sufficient for acquisition of neuroendocrine-like characteristics and loss of mitotic activity. Clonal cells that inducibly expressed caPKA enhanced the growth of prostate tumor cells in anchorage-dependent and anchorage-independent *in vitro* assays as well as the growth of prostate tumor xenografts *in vivo*, with the greatest effects seen under conditions of androgen deprivation. These results suggest that neuroendocrine-like cells of prostatic tumors have the potential to enhance androgen-independent tumor growth in a paracrine manner, thereby contributing to progression of the disease. [Cancer Res 2007;67(8):3663–72]

Introduction

Neuroendocrine-like cells are found in the normal prostate as well as in benign lesions of the tissue (1–3). However, their numbers increase in neoplastic growths, and their prevalence correlates with tumor size and progression to hormone-resistant states (4–9), suggesting that they may play a role in advancing the disease. A large number of clinical studies have shown that prostatic neuroendocrine-like cells and their associated markers serve as prognostic indicators of poor patient survival (10–16), providing further support for a functional link between neuroendocrine-like cells and tumor progression.

Existing evidence supports a model in which neuroendocrine-like cells arise from within prostate tumors following tumor cell exposure to one or more differentiation factors (2, 6, 17). LNCaP

cells acquire neuroendocrine characteristics in response to agents that increase intracellular cyclic AMP (cAMP) levels, including forskolin or epinephrine (18, 19), long-term androgen ablation (20, 21), and, to a lesser extent, cytokines (22–24). An array of neuroendocrine-specific markers, including S-100, chromogranin A (CgA), and neuron-specific enolase (NSE; ref. 25), is up-regulated in response to these agents. Long-term androgen ablation therapy or castration has also been found to enrich neuroendocrine-like populations within human tumors or xenografts in nude mice, respectively (26–28). Based upon these and other studies, we postulated that cAMP-mediated signaling might be a primary pathway of neuroendocrine differentiation *in vivo*.

Neuroendocrine-like cells in tumors, while themselves non-mitotic, have been found to correlate with increased proliferative activity of adjacent carcinoma cells (4, 17) and to produce neurosecretory growth and survival factors (19, 28–30) that could contribute to the progression of late-stage prostate cancer during aggressive therapy. However, the causal relationship between nondividing, neuroendocrine-like cells within tumors and tumor progression has not been rigorously tested, in part, because the differentiation process is reversible and requires that the differentiating agent(s) be continuously present (19). A logical molecular candidate to replace extracellular differentiation factors was the major intracellular effector of cAMP, cAMP-dependent protein kinase A (PKA). In fact, the generation of neuroendocrine-like cells by cAMP-activating agonists was found to be dependent on PKA (24). In the current study, tetracycline-regulated expression of a constitutively activated form of PKA was found to enhance neuroendocrine-like differentiation of prostate tumor cells as measured by loss of mitotic activity, gain of neuroendocrine-like morphologic characteristics, and secretion of neuropeptides. Most importantly, when cocultured *in vitro* or coinjected into nude mice with prostate tumor cells, these PKA-differentiated neuroendocrine-like cells enhanced anchorage-dependent and anchorage-independent growth and tumorigenicity of the tumor cells, with the most pronounced effects being observed under conditions of androgen deprivation. These results are supportive of the hypothesis that neuroendocrine-like cells of prostate cancer contribute to disease progression by providing growth and survival factors to surrounding cancer cells. The findings also reveal potential new sites of therapeutic intervention (i.e., signaling pathways that regulate neuroendocrine differentiation and growth of tumor cells activated by neuropeptides).

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Materials and Methods

Cell culture. LNCaP cell lines (from Dr. L.W. Chung, Emory University) were grown in T-Medium supplemented with 5% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD) and penicillin/streptomycin, unless otherwise indicated, and maintained at 37°C in a humidified, 5% CO₂ environment (31). PC3 cells (American Type Culture Collection, Rockville, MD) were grown in DMEM (Life Technologies) with 10% FBS and penicillin/streptomycin.

Constructs. To generate a tetracycline-inducible expression vector for the constitutively active PKA catalytic subunit (caCqr), the pTRE plasmid (Clontech, Palo Alto, CA) was first digested with *EcoRI*-*Bam*HI to insert a linker containing *EcoRI*, *EcoRV*, *Xba*I, *Bgl*II, *Xho*I, *Hpa*I, and *Bam*HI sites, yielding pTRE-L1. FLAG-tagged caCqr was then inserted into pTRE-L1 as an *Xba*I-*Hpa*I fragment from an intermediate vector to generate pTRE-FLAG caCqr. Original cloning of caCqr was accomplished by PCR amplification of a *Xba*I-*Xho*I fragment from the MT Cqr-neo plasmid (kindly provided by Dr. G.S. McKnight, University of Washington, Seattle, WA).

Derivation of Tet-inducible cell lines. LNCaP cells inducibly expressing FLAG caCqr were derived according to the manufacturer's instructions using the pTRE-FLAG caCqr plasmid (Clontech). Positive clones were assessed by Western immunoblotting and immunofluorescence, subcloned by limiting dilution, and maintained in growth medium plus 600 µg/mL Geneticin (G418; Life Technologies) and 2 µg/mL puromycin (Calbiochem, La Jolla, CA).

Immunofluorescence and immunohistochemistry. For immunofluorescence analysis, LNCaP cells were plated onto glass coverslips and processed as previously described (32). FLAG-tagged caCqr expression was detected using anti-FLAG antibody, M5 (Sigma, St. Louis, MO), at a 1:500 dilution and Texas Red-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 1 µg/mL in PBS [50 mmol/L Na phosphate, 150 mmol/L NaCl (pH 7.4)] containing 5% heat-inactivated goat serum. Cells were imaged using phase contrast and immunofluorescence optics (Leica, New York, NY).

For immunohistochemical analysis, tumors were excised from mice, fixed in zinc formalin, and embedded in paraffin. Tissue sections were deparaffinized in xylene and alcohol, and endogenous peroxidase activity was quenched by a 30-min incubation in 0.5% hydrogen peroxide/methanol. Following hydration, microwave epitope retrieval was done in 10 mmol/L citrate buffer (pH 6) for 10 min at 1.15 kW. Sections were incubated with biotinylated M5 antibody, and immunohistochemistry was done using the avidin-biotin-peroxidase complex method according to the manufacturer's instructions (Vectastain Elite kit, Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as the chromogen. Sections were counterstained with hematoxylin.

Proliferation assays. The D29 clone, which inducibly expresses caCqr, was seeded in 10-cm dishes with or without 2 µg/mL doxycycline in growth medium. After 24 h, serum-free RPMI 1640 (Life Technologies) was added in the presence or absence of doxycycline. Five days after plating, the conditioned medium was collected from D29 cells and centrifuged to remove cells and debris. PC3 or LNCaP cells that were plated the day before were rinsed with PBS, and fresh D29 conditioned medium was added. Cells were incubated at 37°C for 48 h and counted with a hemacytometer.

The neurotensin receptor antagonist SR48692 was a gift from Sanofi-Aventis (Malvern, PA). The bombesin receptor antagonist (D-Phe⁶,Leu¹³-*p*-chloro-Phe¹⁴)-bombesin(6-14) and PTHrP receptor inhibitor (Nle^{8,18},Tyr^{3,4})-PTH (3-34) amide were obtained from Bachem (King of Prussia, PA). Neurotensin, bombesin, and PTHrP inhibitors were used at working concentrations of 0.1, 1, and 0.16 µmol/L, respectively. Inhibitors were added to PC3 or LNCaP cells 1 h before addition of D29 conditioned medium and were present for the entire 48-h incubation period afterward. The c-Src inhibitor PP2 (Calbiochem) was used at 10 µmol/L; cells were preincubated with PP2 for 15 min before and during the 48-h incubation period.

Bromodeoxyuridine labeling. Bromodeoxyuridine (BrdUrd; Sigma) labeling was done by adding, during the last 14 h of incubation, 100 µmol/L BrdUrd to the culture media. BrdUrd incorporation into DNA

was assessed by immunofluorescence microscopy of paraformaldehyde-fixed cells using anti-BrdUrd-FITC antibody (Boehringer-Mannheim, Indianapolis, IN) as described by the manufacturer. The mitotic activity of each treatment population was calculated as the mean ± SE percentage of cells that were BrdUrd positive for three independent experiments.

Immunoblotting. After treatment, cells were washed with PBS and lysed in HO buffer containing protease inhibitors as previously described (33). Lysates were clarified by centrifugation at 10,000 × *g* for 10 min at 4°C and subjected to SDS-PAGE and electrophoretic transfer to nitrocellulose. Mitogen-activated protein kinase (MAPK), NSE, and FLAG-tagged PKA were immunoblotted with monoclonal antibodies 1B3B9 (34), anti-NSE (DAKO, Carpinteria, CA), and M5, respectively. Primary antibodies were visualized by binding of horseradish peroxidase-conjugated sheep anti-mouse IgG or Protein A (Amersham Life Sciences, Inc., Cleveland, OH), as appropriate, and detected with chemiluminescence. All immunoblots shown are representative of at least three independent experiments.

Colony formation in soft agar. Anchorage-independent growth was measured as previously described (35), with modifications. Briefly, various ratios of parental LNCaP cells and caCqr-expressing cells were mixed together, suspended in T-Medium containing 0.5% agarose and 5% fetal bovine serum, and layered over a 1% agarose plug in T-Medium containing 5% FCS in a 60-mm dish. Doxycycline was added at 1 µg/mL media to both plug and soft agar layers. Cells were incubated for 17 days, during which time 3 mL conditioned medium from either differentiated caCqr-expressing clones or undifferentiated clones was added at 3- to 4-day intervals to the soft agar layer. Where appropriate, doxycycline was also replenished every 3 to 4 days to the soft agar layer. Colonies were stained overnight at 37°C with 1 mL of a 500 µg/mL solution of ρ-iodonitrotetrazolium violet (Sigma), and those composed of more than 25 cells were counted using EagleSight colony-counting software (Stratagene, La Jolla, CA).

ELISA assays. Conditioned medium from D29 cells was analyzed for bombesin and neurotensin by ELISA assay kits (Phoenix Pharmaceuticals, Belmont, CA) according to manufacturer's instructions. ELISA plates were read at an absorbance of 450 nm using an MRX microplate reader (Dynex Technologies, Inc., Chantilly, VA).

Tumorigenicity in nude mice. Parental LNCaP cells were mixed at various ratios with caCqr-expressing cells and injected s.c. in a 100 µL volume containing 50 µL Matrigel (BD Biosciences, Bedford MA) into each rear flank of 28- to 42-day-old NIH BALB/c AnNCr-nu nude mice (*nu/nu*; National Cancer Institute, Bethesda, MD). Cells were mixed in ratios of 0:*X* (caCqr cells/LNCaP cells), 0.3:*X* (caCqr/LNCaP), and *X*:*X* (caCqr/LNCaP), where *X* = 2.5 × 10⁶ cells. Doxycycline was added to the drinking water of animals 2 weeks after inoculation at a final concentration of 200 µg/mL and replenished every 3 to 5 days. Animals were weighed once weekly, at which time tumor volume was measured with the aid of electronic digital calipers (OEM Pro Am Tools, Ottawa, Canada). Tumor volume was calculated by averaging the length and width of each measurement and dividing by 2 to obtain a value for the radius (*r*), which was then inserted into the volume formula (4/3π*r*³). For androgen-independent studies, animals were castrated after tumors reached ~100 mm³ and showed increased growth for two consecutive weeks. Castrations were done as described (36). Animals were euthanized when body weight dropped by more than 20% or tumor diameter exceeded 15 mm.

Prostate-specific antigen monitoring. Animals were inoculated with a mixture of caCqr and LNCaP cells in Matrigel at ratios of 0:*X* (caCqr/LNCaP) and 0.3:*X* (caCqr/LNCaP). Doxycycline treatment was initiated 2 weeks after inoculation as described above. Blood samples were obtained weekly thereafter via the tail vein, and serum prostate-specific antigen (PSA) levels were determined by immunoassay (Abbott IMX, Montreal, Quebec, Canada).

Results

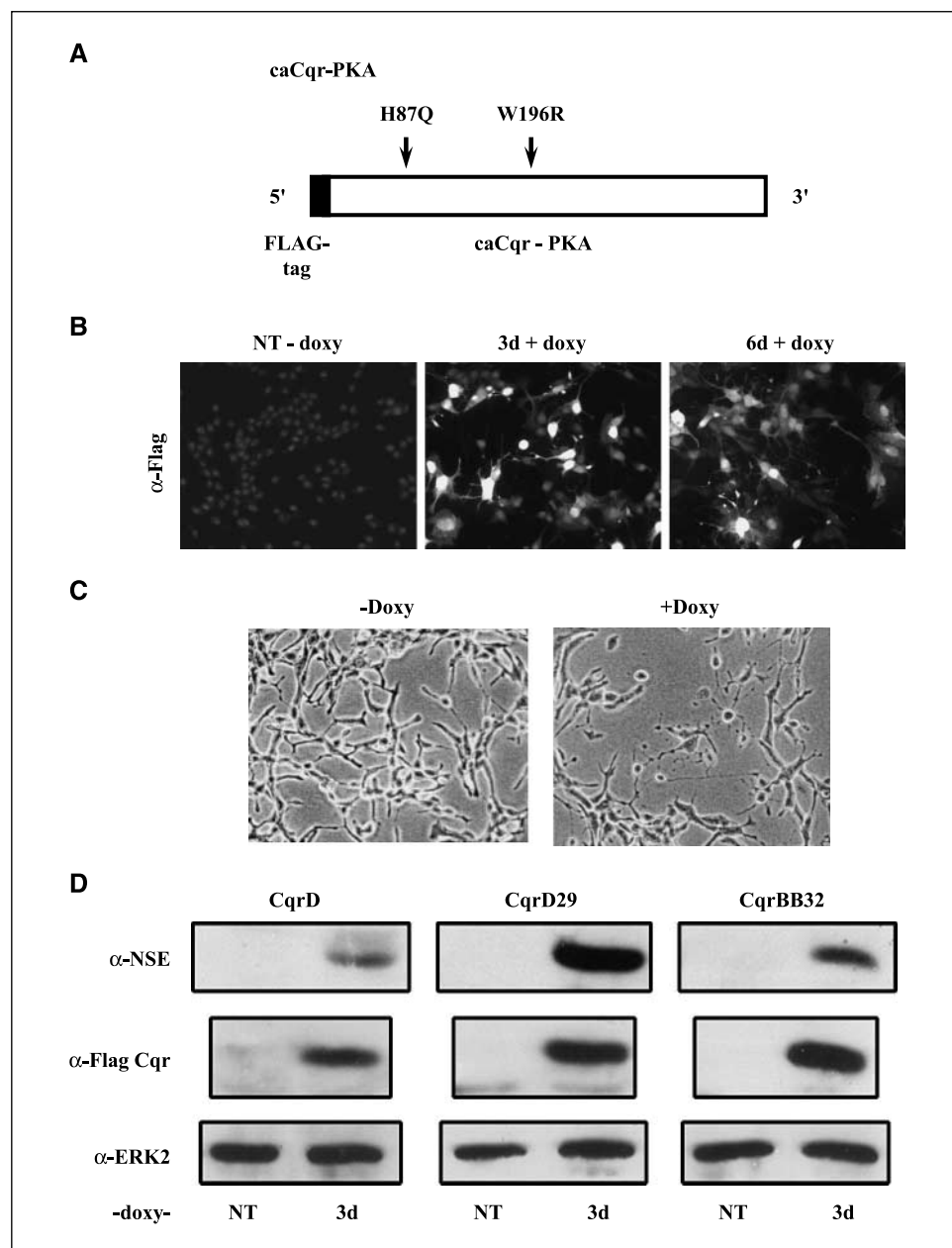
Tetracycline-inducible overexpression of caCqr PKA results in neuroendocrine-like differentiation of LNCaP cells. To test the paracrine hypothesis of neuroendocrine-like cell function, tetracycline-inducible clonal cell lines that conditionally express

the constitutively active, FLAG-tagged caCqr were derived (Fig. 1A). This approach was chosen because it was previously shown that transient overexpression of caCqr in LNCaP cells resulted in neuroendocrine-like differentiation (24), and that neither tetracycline nor doxycycline treatment of parental, untransfected LNCaP cells at the concentrations used had any measurable effects on LNCaP proliferation (data not shown). Figure 1B shows that treatment of a representative clone of caCqr-expressing cells (D29) with 1 μ g/mL doxycycline for 3 to 6 days induced expression of FLAG-tagged caCqr in 70% to 80% of the cells, whereas Fig. 1C shows acquisition of a neuroendocrine-like morphology. Up-regulation of NSE protein levels and induction of FLAG-caCqr expression in three different clones used in subsequent studies are shown in Fig. 1D.

To determine whether caCqr-differentiated LNCaP cells also produced and secreted neuropeptides, ELISAs were done on 5-day

conditioned medium from doxycycline-treated D29 cells. Results indicated that 0.167 ± 0.049 ng/mL bombesin and 0.193 ± 0.020 ng/mL neurotensin were secreted into conditioned medium, whereas media from cells cultured in the absence of doxycycline had 0.064 ± 0.032 and 0.023 ± 0.005 ng/mL, respectively. As an indirect measure of the ability of caCqr-expressing cells to produce these peptides, the ability of specific antagonists of the receptors for bombesin, PTHrP, and neurotensin to inhibit proliferation of PC3 prostate cancer cells induced by conditioned medium from doxycycline-treated D29 cells was tested. Figure 2A and B shows that media from doxycycline-treated cells induced ~ 2 -fold increase in cell number compared with the untreated control, and that antagonists of the bombesin receptor or the PTHrP receptor each partially inhibited the response. We have previously shown a similar response to the neurotensin inhibitor SR48692 (37). Moreover, when antagonists of the bombesin, PTHrP, and

Figure 1. Stable overexpression of caCqr PKA induces a neuroendocrine phenotype in LNCaP cells. **A**, plasmid cDNA encoding a constitutively active mutant of PKA catalytic subunit (caCqr) was stably transfected into LNCaP cells using the Tet-On inducible system, and transfectants were subcloned as described in Materials and Methods. **B**, immunofluorescence microscopy of the caCqr-expressing clone D29 was done using M5 anti-FLAG monoclonal antibody following 3 and 6 d of 1 μ g/mL doxycycline (doxy) treatment in T-medium + 5% FBS. **C**, representative phase-contrast photomicrographs of clone D29 depict cellular morphologies following treatment with vehicle or doxycycline for 5 d. **D**, caCqr-overexpressing stable clones (D, D29, and BB32) were cultured in T-Medium + 5% FBS and treated with vehicle or doxycycline for 3 d. Western blot analysis was done with 50 μ g whole-cell lysate using anti-NSE (top), anti-FLAG (Cqr PKA, middle), and anti-ERK2 (bottom) antibodies. ERK2 represents a loading control. NT, not treated.



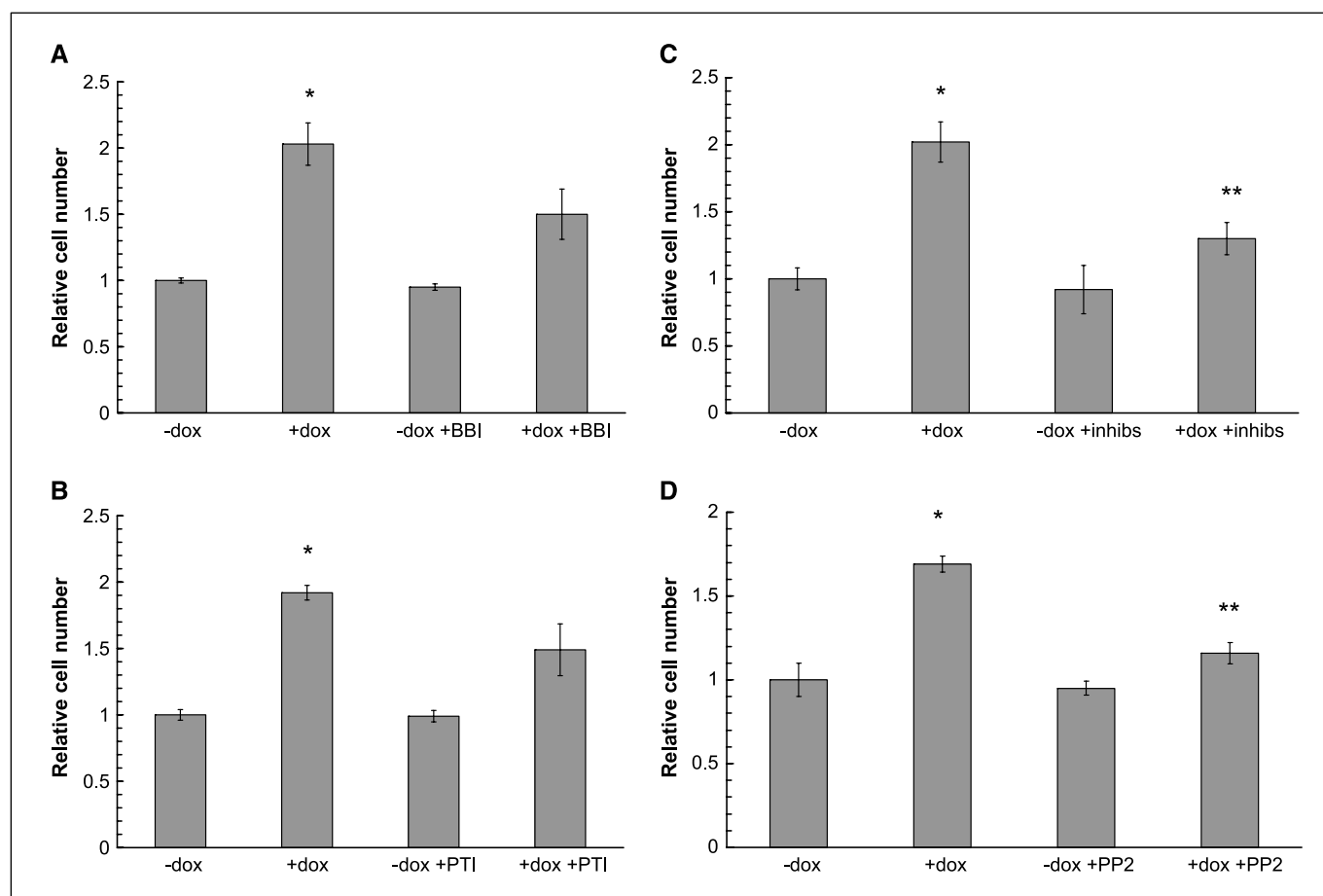


Figure 2. Effect of neuropeptide receptor antagonists or c-Src inhibitor on proliferation of prostate cancer cells induced by neuroendocrine-like cell secretions. D29 cells were incubated in the presence or absence of 2 μ g/mL doxycycline in serum-free medium for 5 d. PC3 (for neuropeptide antagonists) or LNCaP (for Src inhibitor) cells were then pretreated (or not) with inhibitor(s) for 1 h (except PP2, 15 min), incubated with D29 conditioned medium for 48 h \pm inhibitor(s), and counted by hemacytometer. Columns, mean from three independent experiments; bars, SE. *, $P < 0.05$, relative to control; **, $P < 0.05$ relative to doxycycline treated (+dox). A, bombesin antagonist (BBI), 1 μ mol/L; B, PTHrP antagonist (PTI), 0.16 μ mol/L; C, combined BBI, PTI, and neurotensin receptor antagonist SR48692, 0.10 μ mol/L; D, c-Src inhibitor PP2, 10 μ mol/L.

neurotensin receptors were combined, cell proliferation was significantly reduced even further than observed with the individual inhibitors (Fig. 2C). These findings substantiate the ELISA results and also show that neuroendocrine-like cells can exert their growth effects through the concerted action of multiple stimulatory neuropeptides. Interestingly, caCqr-induced neuroendocrine-like cells retained expression of the androgen receptor (data not shown), a finding that contrasts with the situation in human tumors (reviewed in ref. 9) and is inconsistent with the results of Wright et al. (38), who showed that knockdown of the receptor in LNCaP cells triggers neuroendocrine differentiation.

Finally, to determine whether the growth signal(s) mediated by the secreted neuropeptides required transactivation of the epidermal growth factor (EGF) receptor and its synergistic partner c-Src, (as shown in ref. 37 for neurotensin), the ability of the Src family kinase inhibitor PP2 to inhibit the growth of LNCaP cells stimulated with media from doxycycline-treated D29 cells was examined. Figure 2D shows that PP2 significantly inhibited LNCaP proliferation, almost to levels induced by media from doxycycline-untreated D29 cells. A similar result was obtained when PC3 cells were incubated with conditioned medium and PP2 (data not shown). That the EGF receptor is also required for the growth-

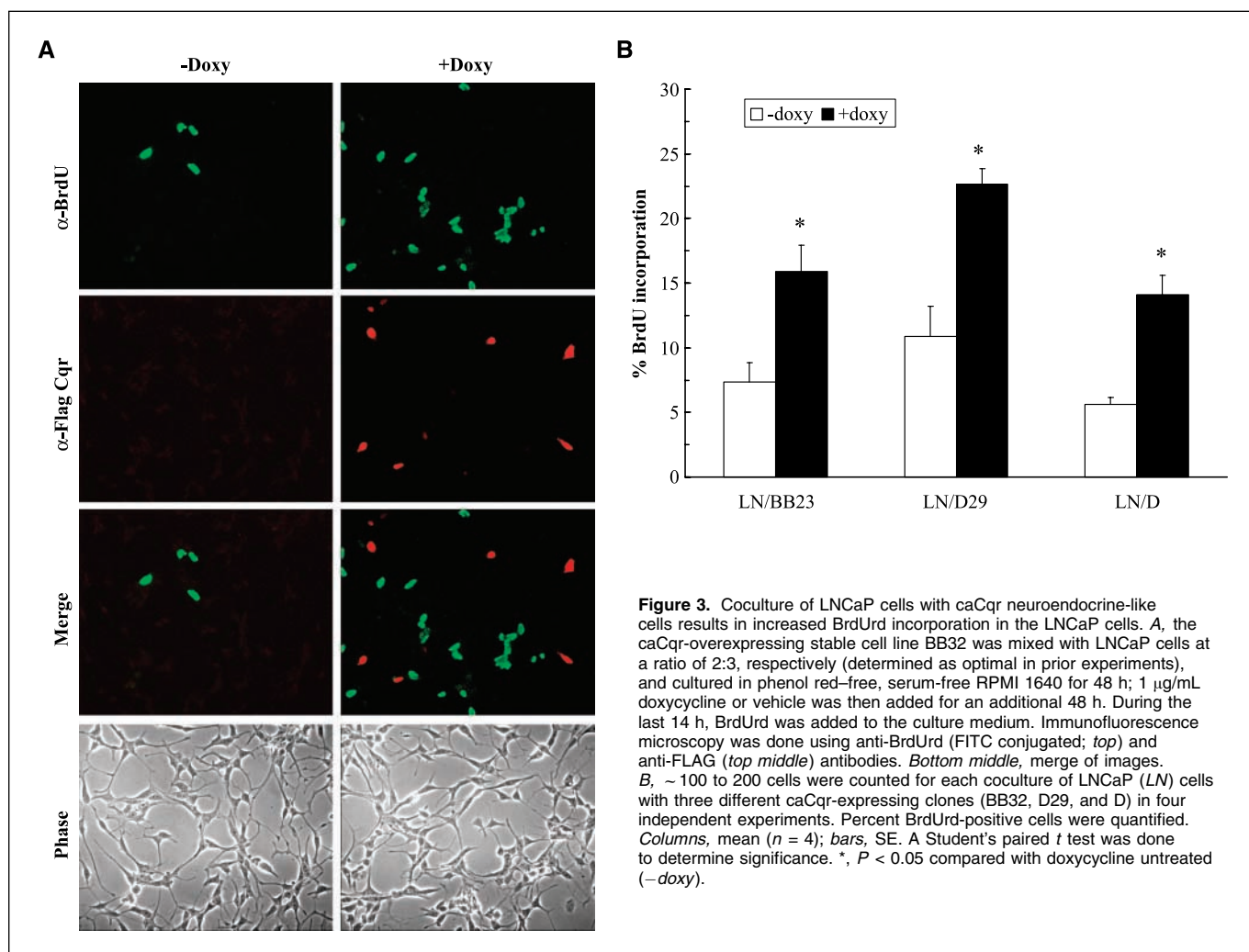
stimulating properties of media from doxycycline-treated D29 cells was shown previously (37). Together, the results shown in Figs. 1 and 2 indicate that doxycycline-inducible overexpression of caCqr is sufficient to induce acquisition of neuroendocrine-like characteristics similar to those observed following cAMP-mediated stimulation (18, 19) and transient overexpression of caCqr (24). Therefore, the Tet-inducible caCqr expression system seemed suitable for studying neuroendocrine-like cells *in vitro* and possibly *in vivo*.

caCqr-mediated neuroendocrine-like differentiation induces increased mitotic activity in prostate tumor cells. Because conditioned medium from neuroendocrine-like cells only modestly stimulated prostate cancer cell proliferation (Fig. 2; ref. 37), we postulated that physical contact between neuroendocrine-like cells and tumor cells may be needed for efficient mitotic stimulation. To address this possibility, coculture experiments were done. Two thousand caCqr-positive cells and 3,000 LNCaP cells were plated together in six-well dishes, incubated with or without doxycycline in the presence of BrdUrd, and scored for the number of BrdUrd-positive cells, as a measure of mitotic activity. caCqr-expressing cells in the population were identified by anti-FLAG immunofluorescence. Figure 3A shows a representative immunofluorescent

image of a culture that contained 40% caCqr-expressing cells of clone BB32 and 60% LNCaP, in both the doxycycline-induced and uninduced conditions. Quantitation of these and four additional experiments is shown in Fig. 3B. The BB32 clone was found to induce a >2-fold increase of BrdUrd incorporation in the cocultures when caCqr expression was induced with doxycycline, compared with cells cultured in the absence of doxycycline. Analogous results were observed with cocultures containing caCqr-expressing clones D and D29 (Fig. 3B). In all three cocultures, cells that stained positively for FLAG-caCqr were distributed randomly through the microscopic fields and rarely (<0.5%) stained positively for BrdUrd incorporation. Similar results were obtained when caCqr-expressing clones were cocultured with PC3, androgen-independent cells, although basal levels of BrdUrd incorporation were higher, resulting in smaller differences between induced and uninduced cultures (data not shown). Taken together, these data indicate that LNCaP-derived neuroendocrine-like cells induce an increase in the mitotic activity of surrounding prostate tumor cells, similar in extent to that observed with conditioned medium and consistent with the paracrine hypothesis.

Anchorage-independent growth of LNCaP cells is stimulated by caCqr neuroendocrine-like cells. To test the possibility that caCqr neuroendocrine-like cells could affect the anchorage-

independent growth of LNCaP cells, mixtures of LNCaP and caCqr-expressing cells were examined for their ability to form colonies in soft agar. In preliminary experiments, a ratio of 2.5:1.0 LNCaP/caCqr-expressing cells was found to form the greatest number of colonies in the presence of doxycycline; thus, this ratio was used in subsequent experiments. LNCaP and caCqr-expressing cells were cultured alone or in combination, at a ratio of 2.5:1, respectively, in the presence or absence of doxycycline. Figure 4 shows that no significant effect of caCqr-expressing cells on the colony-forming ability of cocultured LNCaP cells was observed in the absence of doxycycline (LN/D29 or LN/BB32), compared with that expected from the additive growth of each cell type grown independently (LN + D29 or LN + BB32). However, in the presence of doxycycline, a striking increase in soft agar colony formation in cocultures was observed. Specifically, LNCaP cells, cultured alone, were unaffected by doxycycline and produced ~700 colonies when 25,000 cells were seeded. D29 and BB32 seeded alone at 10,000 cells per plate and treated with doxycycline produced ~50 colonies, indicating that inducible neuroendocrine-like cells would contribute <0.5% of the total soft agar colonies formed in coculture. When D29 and BB32 were mixed with LNCaP cells at identical plating densities as when seeded alone and treated with doxycycline, 1,100 and 1,050 colonies were produced, respectively, yielding statistically



significant increases in both instances ($P < 0.05$), compared with the sum of LNCaP alone (700 colonies) + D29 alone ([50]) = ([750]) or the sum of 700 LNCaP + ~ 50 BB32 alone (~ 750), respectively. Representative examples of culture dishes for each of the variables are shown below the graph in Fig. 4. These data indicate that the presence of caCqr-expressing neuroendocrine-like cells resulted in increased soft agar colony formation by LNCaP cells that was not attributable to growth of the caCqr neuroendocrine-like cells themselves.

Tumor growth, FLAG-Cqr expression, and PSA levels in intact male mice. LNCaP and caCqr-expressing cells were injected alone or together into each hind flank of athymic mice at a ratio of 0.3X:X (caCqr cells/LNCaP), where $X = 2.5 \times 10^6$ cells. Figure 5A shows that the volume of tumors formed by LNCaP cells coinjected with caCqr cells (clone D29) was only modestly greater than and not significantly different from tumors formed by LNCaP cells alone. In addition, neither the frequency nor the rate of tumor formation was appreciably altered by the presence of caCqr cells. Similar results were observed when mice were coinjected with caCqr and LNCaP cells at a ratio of X:X (data not shown). Injection of D29 cells alone induced few tumors (3 of 15 compared with 100% for the other two groups of mice) of very small size (<20 mm³ compared with 400 mm³ of coinjected animals), consistent with the previously observed decrease in mitotic activity of these cells in the presence of doxycycline.

To verify that animals injected with FLAG-caCqr cells still expressed this protein during tumor formation, tumors removed at the termination of the experiment were analyzed by Western immunoblotting. Figure 5B (lane 6) shows FLAG-Cqr expression in

tumors from animals coinjected with 0.3X:X caCqr cells/LNCaP and treated with doxycycline, but not in tumors from LNCaP or caCqr cells alone that underwent doxycycline treatment, or from any doxycycline-untreated animals. Densitometric analysis revealed a ~3-fold increase in FLAG antibody reactivity in lane 6 versus lane 2, when levels of FLAG-caCqr were normalized to the MAPK loading control. Immunohistochemical analysis substantiated the presence of FLAG-positive cells in tumors of coinjected mice, which were absent in tumors derived from LNCaP cells alone (Fig. 5C). The frequency of FLAG-positive cells ranged from 0% to 15%, with most samples showing 2% to 3% positivity and the highest levels in tumors of the X:X mice.

Because tumor volumes of the 0.3X:X group were insignificantly but consistently greater than those of the 0:X group, serum PSA values were measured in repeat experiments to determine whether PSA levels might reflect some metabolic differences between the two groups. Figure 5D shows that significantly higher levels of PSA were found in the 0.3X:X D29/LNCaP group compared with the 0:X D29/LNCaP group at 12 and 13 weeks after tumor cell inoculation, whereas, again, no significant differences in tumor take, rate, or size were noted. Under androgenic conditions, caCqr-expressing cells treated with doxycycline produced similar amounts of PSA as did the parental LNCaP cells (data not shown). However, because caCqr neuroendocrine-like cells are mitotically inactive, and because only 0.3X was used, the contribution of PSA produced by caCqr cells was considered negligible. These data suggest that the increased PSA release, while correlating with enhanced growth, is more likely attributed to increased PSA production than to increased proliferation, and that PSA levels may be a more sensitive

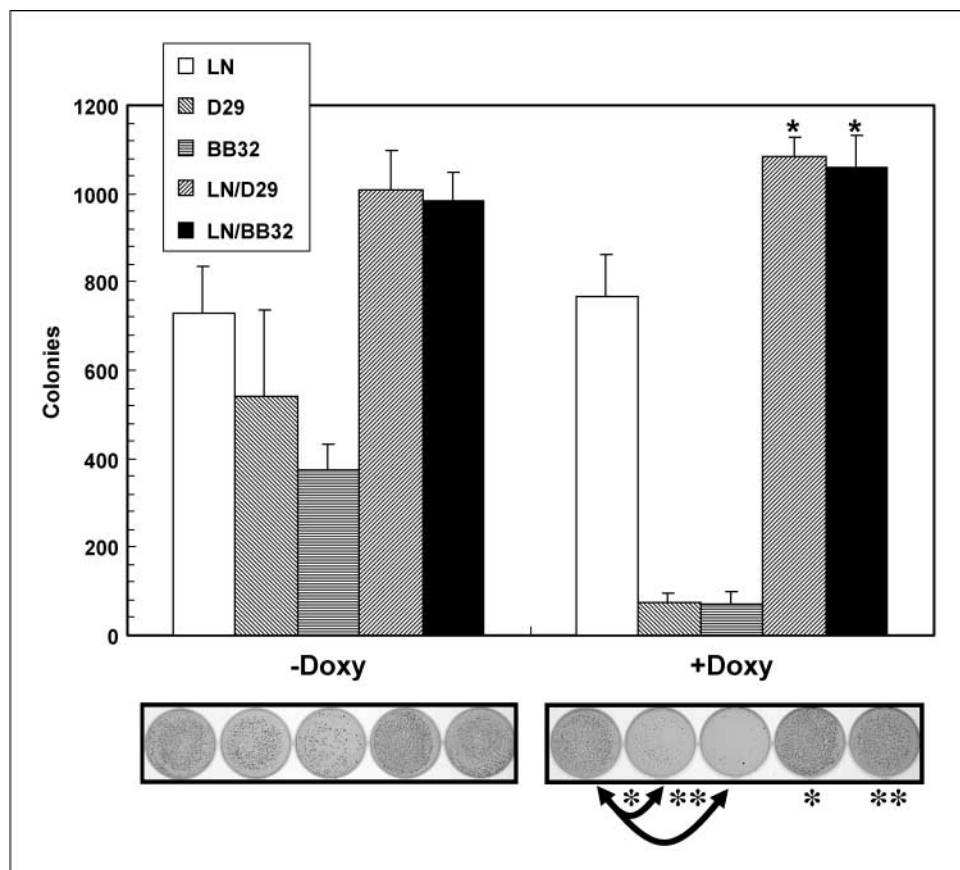


Figure 4. Coculture of LNCaP cells with caCqr neuroendocrine-like cells results in increased soft agar colony formation by LNCaP cells. Cells of caCqr-overexpressing clones D29 and BB32 were seeded individually or with LNCaP cells at a ratio of 10,000:25,000, respectively, and cultured in soft agar for 17 d. LNCaP cells were seeded alone for comparison. The soft agar layer was replenished every 3 to 4 d with conditioned media taken from LNCaP cells or from doxycycline-treated (+Doxy) or untreated caCqr clones (-Doxy). Columns, mean colony number of four independent experiments; bars, SE. Statistical comparisons were done in the doxycycline-treated conditions by adding the number of LNCaP colonies to the caCqr colonies and comparing the sum with the number of colonies formed by the mixture (LN/caCqr). * or **, $P < 0.05$, significant differences. Representative photographs of soft agar plates (bottom) with arrows and asterisks indicating which plates were compared.

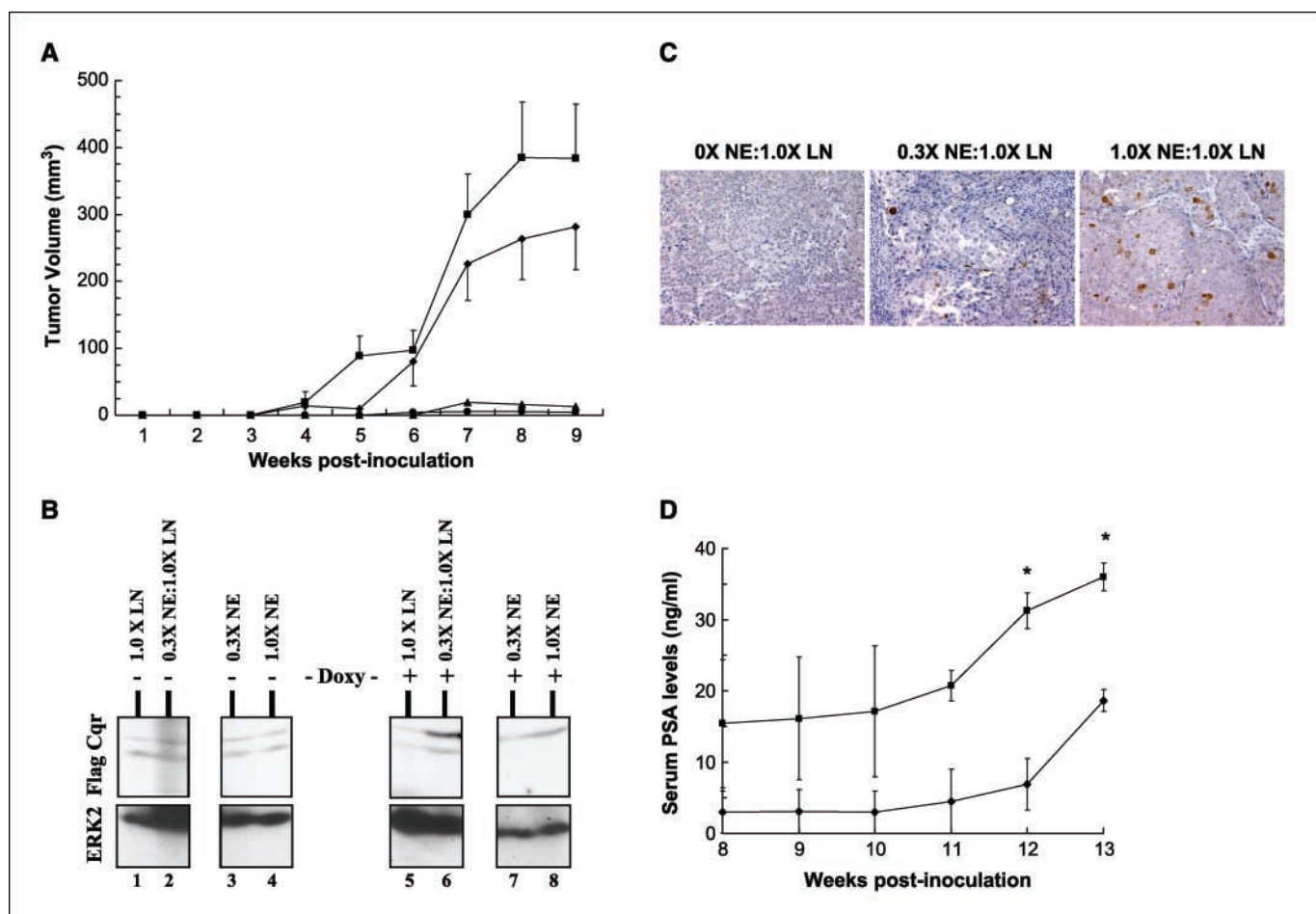


Figure 5. Tumor volume, FLAG-Cqr expression, and PSA levels in intact mice bearing LNCaP tumors. caCqr-expressing D29 or LNCaP cells were injected individually or in mixtures containing the indicated ratios, where $X = 2.5 \times 10^6$ cells. Animals were administered doxycycline in their water every 3 to 5 d, beginning at day 14 after inoculation, unless otherwise indicated. **A**, tumor volume measurements were made in the following groups (parentheses indicate the number of tumors formed per total injection sites): \blacklozenge , 0:X D29/LNCaP (8/8); \blacksquare , 0.3X:X D29/LNCaP (4/4); \blacktriangle , 0.3X:0 D29/LNCaP (1/8); \bullet , X:0 D29/LNCaP (2/7). Points, mean; bars, SE. **B**, portions of tumors removed from animals were combined by group, flash frozen, homogenized in lysis buffer, and sonicated. Tumor lysate (200 μ g; from combined samples) was subjected to immunoblot analysis using an anti-FLAG antibody to detect expression of FLAG-caCqr and an anti-ERK2 antibody to control for protein loading. A background band in the same molecular weight range as FLAG-caCqr was present in all tumor samples. **C**, portions of tumors removed from animals injected with 0:X D29/LNCaP, 0.3X:X D29/LNCaP, or X:X D29/LNCaP were paraffin embedded, sectioned, and stained with anti-FLAG antibody. **D**, experiments were done as described in (A), except that serum PSA levels were measured as an endpoint. Points, mean for three mice per treatment group (both doxycycline treated); bars, SE. A Student's *t* test was used to evaluate statistical significance. \blacklozenge , 0:X D29/LNCaP; \blacksquare , 0.3X:X D29/LNCaP. *, $P < 0.05$, compared with 0:X.

indicator of effects of neuroendocrine-like cells on the LNCaP tumor cells than is tumor growth in androgen-replete conditions.

caCqr neuroendocrine-like cells enhance tumor growth in castrated mice. To reproduce in animals, the androgen-independent conditions used in *in vitro* growth assays, animals bearing tumors were castrated and then monitored for changes in tumor growth. Figure 6A and B shows that in two independent experiments, within a few weeks following castration, tumor growth rates were significantly increased when caCqr cells were present, compared with rates of tumors containing only LNCaP cells. In the absence of doxycycline treatment, no significant rate differences were observed between the tumors containing or lacking caCqr cells (data not shown). In a third independent experiment under the same conditions, the mean tumor volume tripling times were calculated to be 7.9 ± 0.7 , 5.8 ± 0.4 , and 4.0 ± 0.6 weeks for D29/LNCaP ratios of 0:X, 0.3X:X, and X:X, respectively. These results indicate that caCqr neuroendocrine-like cell effects on tumor size may be most significant under reduced androgen conditions and are consistent with the notion that

neuroendocrine-like cells in human prostate cancers contribute to androgen-independent disease.

Immunohistochemical analysis of tissue sections prepared from tumors removed at the end of these experiments was done, but the quality of the images was poor due to the highly necrotic and neovascularized nature of the tumors. Therefore, although no FLAG-positive cells were observed in any of the tumors examined, interpretation was compromised, and no definitive conclusion was drawn regarding their abundance.

Discussion

Human prostatic adenocarcinomas frequently contain neuroendocrine-like cells that increase in number with disease progression, and multiple reports suggest that neuroendocrine-like cells are involved in acquisition of the androgen-insensitive state, a characteristic of late-stage cancers (39). Mechanistically, it has long been postulated that neuroendocrine-like cells secrete factors capable of enhancing the survival or replication of surrounding

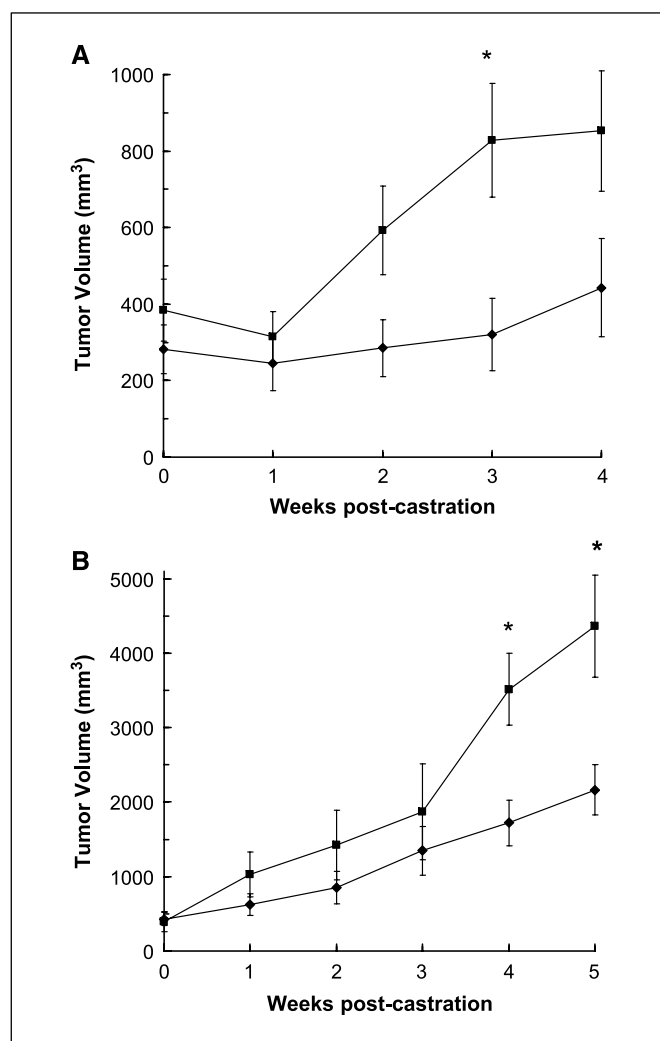


Figure 6. caCqr neuroendocrine-like cells enhance androgen-independent growth of LNCaP tumors in nude mice. caCqr-expressing cells of clone D29 were injected s.c. with or without LNCaP cells in the indicated ratios, where $X = 2.5 \times 10^6$ cells. Animals were administered doxycycline in their drinking water, as described in Fig. 5 legend. Animals that possessed tumors of $\sim 100 \text{ mm}^3$ in volume that had shown growth for two consecutive weeks were castrated (~ 9 wks following tumor cell injection and 5 wks following the onset of tumor growth), and monitoring of tumor growth continued. Points, mean tumor volume calculated from the number of tumors indicated; bars, SE. Two representative experiments. Statistical significance was determined by Student's *t* test. A, \blacklozenge , 0:X D29/LNCaP ($N = 8$); \blacksquare , 0.3X:X D29/LNCaP ($N = 4$). *, $P < 0.05$ relative to 0:X. B, \blacklozenge , 0:X D29/LNCaP ($N = 9$); \blacksquare , X:X D29/LNCaP ($N = 4$). *, $P < 0.05$, relative with 0:X.

tumor cells, but direct causal analysis of neuroendocrine-like cell effects within prostate tumors has been lacking. This study provides that causal evidence, which supports the paracrine hypothesis of neuroendocrine-like cell function in prostate cancer progression.

The paracrine activity of neuroendocrine-like cells and their effects on LNCaP cells have been shown in another mouse model of prostate cancer in which a mouse prostate neuroendocrine tumor (NE-10), derived from the LPB-Tag 12T-10 "Tramp" transgenic mouse, enhanced the growth of LNCaP xenografts in castrated, athymic mice, when injected contralaterally to the LNCaP cells (40). Although the overall results of the NE-10 cell study are similar to those reported here, some characteristics of the caPKA-induced

neuroendocrine-like model seem to more closely resemble those of neuroendocrine-like cells in human prostate cancers than do those of the NE-10 model. For example, it is not clear whether the constellation of factors secreted by the NE-10 cells are similar to or distinct from those secreted by neuroendocrine-like cells in human tumors because characterization of such molecules has not been reported for NE-10 cells. To date, factors produced and secreted by caPKA-induced neuroendocrine-like cells are identical to those produced by neuroendocrine-like cells in human prostate tumors and include the neuropeptides, bombesin, PTHrP, neurotensin, serotonin, etc. (Fig. 2; accompanying text; data not shown; ref. 6).

The mitotic nature of the neuroendocrine-like cells in the two models can also be compared. The caPKA-induced neuroendocrine-like cells are poorly mitotic or nonmitotic (similar to those in human prostatic cancers), whereas NE-10 cells are highly proliferative and representative of pure neuroendocrine tumors derived from other tissues, such as lung. As such, if mixed with prostate tumor cells, NE-10 cells would likely assume a significant volume of the resulting tumor. We believe this not to be the case with the caPKA-induced neuroendocrine-like cells. Although one third or equal numbers of caPKA-induced neuroendocrine-like cells were initially cocultured or coinjected with LNCaP tumor cells (Figs. 3–6), the PKA-expressing cells ceased dividing in the presence of doxycycline and contributed insignificantly to the number of proliferative cells in coculture studies ($<0.5\%$ of total; Fig. 3) or to the volume of tumors formed in coinjected animals (range, 0–15% between tumors and between multiple slices of the same tumor; Fig. 5).

In spite of the fact that the majority of coinjected tumors contained few or no neuroendocrine-like cells, the presence of 15% FLAG-PKA-positive cells in one of the coinjected xenografts suggests that the cells might not be entirely post-mitotic. One reason for this apparently high percentage (given the premise that the neuroendocrine-like cells should be greatly overgrown by the tumor cells) could be uneven exposure of the caPKA-expressing cells to doxycycline or to variable responses of the engineered cells to doxycycline during the long-term experiment. Nevertheless, the frequency and size of the tumors formed by caCqr cells alone in the presence of doxycycline were very small (<20 versus 400 mm^3 in intact animals and $4,000 \text{ mm}^3$ in castrated animals). These data together with the finding that in intact (noncastrated) animals coinjected with caCqr and LNCaP cells, no significant differences in tumor volume between coinjected and singly injected animals were seen (Fig. 5A, \blacksquare and \blacklozenge , respectively) suggest that caCqr cells did not contribute significantly to the tumor mass that formed in coinjected, castrated animals (Fig. 6A and B).

The variation in abundance of neuroendocrine-like cells detected in the PKA-induced neuroendocrine-like cell model is also similar to that of neuroendocrine-like cells found in late-stage human tumors (reviewed in refs. 6, 9, 41). The bulk of the evidence indicates a low occurrence of neuroendocrine-like cells in human primary tumors that increases with androgen-independent growth and cancer progression. However, Roudier et al. found wide variation in neuroendocrine positivity in separate metastases within and among 14 patients who died of prostate cancer metastasis to the bone (41). Although, in most patients $<1\%$ of tumor cells in all metastatic sites expressed CgA, in four patients, 6.5% to 100% of tumor cells expressed CgA in some, but not all sites. Thus, the range of abundance of neuroendocrine-like cells found in the caCqr-NE cell model is well within the range found in human tumors. In summary, whereas the two mouse models for

testing neuroendocrine-like cell function in prostate cancer differ in substantial ways from each other and from the human setting, the outcome of the studies using both models is remarkably similar and consistent with the hypothesis derived from correlative studies of human prostatic adenocarcinoma (i.e., that neuroendocrine-like cells seem to exert their greatest survival and growth effects on prostate tumor cells that are deprived of androgen).

Our use of caPKA to induce neuroendocrine-like differentiation raises the question of whether all neuroendocrine-like cells in human tumors arise from elevated intracellular levels of cAMP and subsequent activation of PKA. This question has not been addressed systematically in human tumors, but anecdotal evidence suggests that it may be one relevant mechanism of neuroendocrine cell induction. First, androgen deprivation of LNCaP cells in culture results in a 9-fold elevation in levels of intracellular cAMP, suggesting that PKA may be activated in these cells under these conditions (27). The cytokine interleukin-6 can also induce a small but significant activation of PKA in cultured LNCaP (24). Additional evidence that PKA may be activated in human prostate cancers comes from the studies of Kvissel et al. (42), who observed that the C β 2 splice variant of the catalytic subunit of PKA is up-regulated in human prostate tumor cells compared with normal prostatic tissue. Although activation of PKA may be one mechanism by which neuroendocrine cells arise, other PKA-independent mechanisms of differentiation are clearly possible, resulting in neuroendocrine-like cells with different molecular

characteristics, as described by Zelivianski et al. (43) and, perhaps, with different functions.

The elucidation of PKA as a key mediator of neuroendocrine-like differentiation both *in vitro* and *in vivo* raises the possibility of therapeutically targeting activated PKA and induction of neuroendocrine-like differentiation in prostate cancer patients. By using the mouse model described in this report, pharmacologic inhibitors of PKA or antagonists of the neuropeptides secreted by neuroendocrine-like cells can be tested for their ability to inhibit tumor growth, survival, and/or metastasis, thus reducing the number or effectiveness of neuroendocrine-like cells. Targeting the signaling pathways of tumor cells responding to neuroendocrine cell-derived factors provides an alternative approach.

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Androgen-Independent Growth and Tumorigenesis of Prostate Cancer Cells Are Enhanced by the Presence of PKA-Differentiated Neuroendocrine Cells

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