Neuroendocrine-like prostate cancer cells: neuroendocrine transdifferentiation of prostate adenocarcinoma cells

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Abstract

Neuroendocrine (NE) cells represent a minor cell population in the epithelial compartment of normal prostate glands and may play a role in regulating the growth and differentiation of normal prostate epithelia. In prostate tumor lesions, the population of NE-like cells, i.e., cells exhibiting NE phenotypes and expressing NE markers, is increased that correlates with tumor progression, poor prognosis, and the androgen-independent state. However, the origin of those NE-like cells in prostate cancer (PCa) lesions and the underlying molecular mechanism of enrichment remain an enigma. In this review, we focus on discussing the distinction between NE-like PCa and normal NE cells, the potential origin of NE-like PCa cells, and *in vitro* and *in vivo* studies related to the molecular mechanism of NE transdifferentiation of PCa cells. The data together suggest that PCa cells undergo a transdifferentiation process to become NE-like cells, which acquire the NE phenotype and express NE markers. Thus, we propose that those NE-like cells in PCa lesions were originated from cancerous epithelial cells, but not from normal NE cells, and should be defined as 'NE-like PCa cells'. We further describe the biochemical properties of newly established, stable NE-like lymph node carcinoma of the prostate (LNCaP) cell lines, transdifferentiated from androgen-sensitive LNCaP cells under androgen-deprived conditions. Knowledge of understanding NE-like PCa cells will help us to explore new therapeutic strategies for treating PCa.

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Introduction

Neuroendocrine (NE) cells are the third cell type in the epithelial compartment of normal prostate glands in addition to basal and secretory epithelial cells (Abrahamsson 1999). Though prostatic NE cells were described decades ago, their functional roles in prostate, especially in prostate cancer (PCa) progression, have received attention only recently. In normal prostate, NE cells are apparently involved in regulating epithelial cell growth and differentiation in an androgen-independent (AI) manner. In prostate carcinoma, the NE cell population, as characterized by the expression of NE markers, in tumor foci is increased, which evidently correlates with tumor progression, poor prognosis, and the hormone-refractory stage. The role of NE cells in PCa

progression is suggested by the fact that those cells express and secrete a variety of neuropeptides which have mitogenic effects on adjacent cancer cells and thus can contribute to the AI proliferation of PCa cells (di Sant'Agnese 1992, 1998, di Sant'Agnese & Cockett 1996). Further studies also show that NE differentiation is found in the metastatic lesion and significantly associated with the mortality of PCa patients (Cheville *et al.* 2002, Roudier *et al.* 2003). Although the origin of NE cells and the molecular mechanism of NE cell enrichment during PCa progression are not fully understood, accumulated evidence including *in vitro* cell culture, *in vivo* animal model, and clinical sample analyses together indicate that PCa cells can undergo a transdifferentiation process to become NE-like cells, which

acquire the NE phenotype and express NE markers. Thus, we propose to define those NE-like cells in PCa lesions as ‘NE-like PCa cells’.

In this review, we focus our discussion on the potential origin of NE-like PCa cells and the molecular mechanism of NE transdifferentiation. In the first section, we discuss the differences between NE-like PCa and normal NE cells in their biochemical properties and origins. In the second section, we present data from *in vitro* and *in vivo* studies suggesting the multipathway of NE transdifferentiation occurring in PCa cells and discuss their underlying molecular mechanisms. In the final section, we describe the biochemical properties of newly established NE-like LNCaP stable subclone cells which represent a useful model mimicking clinical NE-like cells in prostate tumors. Knowledge of NE-like PCa cells will provide us with new insights for developing novel therapeutic strategies for treating PCa.

NE-like PCa versus normal NE cells
Differences in biochemical characteristics between NE-like PCa and normal NE cells

Several reviews have extensively described the characterizations of prostatic NE cells and their functions in both physiological and pathological states (di Sant’Agnese 1992, 1998, di Sant’Agnese & Cockett 1996, Abrahamsson 1999, Sciarra *et al.* 2003, Vashchenko & Abrahamsson 2005). However, an important question of whether NE-like PCa cells differ from normal NE cells is less addressed. While several studies in PCa archival specimens have shown that NE-like PCa cells share similar biochemical properties with NE cells in the normal prostate, including the expression of NE markers as well as the lack of AR and PSA, these reports also suggest several differences existing between NE-like PCa and normal NE cells (Table 1; Abrahamsson 1999, Vashchenko & Abrahamsson 2005). First, immunohistochemical studies have shown that NE cells in the normal prostate gland express cytokeratin 5 (K5), a basal cell marker (Schalken & van Leenders 2003, Hudson 2004); while NE-like PCa cells exhibit characteristics of luminal secretory cells by expressing prostatic acid phosphatase (PAP; Huang *et al.* 2006) and K18, a luminal cell-specific cytokeratin (van Bokhoven *et al.* 2003, Vashchenko & Abrahamsson 2005). Huang *et al.* (2006) further showed that CgA-positive NE-like cells in PCa archival specimens are positively stained with antibodies (Abs) against luminal secretory cell-associated cytokeratins; while they are negative for staining with Abs against basal cell markers, including high molecular weight cytokeratin and p63 (Wojno &

Table 1 Comparison of normal neuroendocrine (NE) and NE-like prostate cancer (PCa) cells in prostate

Similarities
Lack of AR and PSA expression
Autocrine/paracrine effects
Non/low-proliferative activity
Neuron-like morphology ^a
Expression of NE markers
NSE
CgA
CgB
Serotonin
PTHrP
NT
Bombesin
Dissimilarities (normal NE versus NE-like PCa cells)
Expression of basal cell markers versus luminal secretory cell markers
Non-aggressive versus highly aggressive
No Bcl-2 expression versus Elevated Bcl-2 expression
No AMACR expression versus AMACR expression

NSE, neuron-specific enolase; CgA, chromogranin A; CgB, chromogranin B; PTHrP, parathyroid hormone-related protein; NT, neurotensin; Bcl-2, B cell lymphoma protein 2; AMACR, α -methylacyl-CoA racemase.
^aSome reports show no neuron-like phenotype, i.e., without processing, in NE-like PCa cells.

Epstein 1995, Signoretti *et al.* 2000). Secondly, compared with normal NE cells with non-proliferating and non-tumorigenic activities, NE-like PCa cells, such as small cell carcinoma cells being completely NE differentiated (Yao *et al.* 2006), are highly aggressive and exhibit tumorigenic activity (Oesterling *et al.* 1992). Thirdly, while NE cells from normal prostate and benign prostatic hyperplasia do not express anti-apoptotic protein B cell lymphoma protein 2 (Bcl-2) (Xue *et al.* 1997), studies in archival PCa samples show a correlative relationship between the expression levels of Bcl-2 and neuron-specific enolase (NSE; Segal *et al.* 1994). Fourthly, α -methylacyl-CoA racemase (AMACR), an enzyme involved in the β -oxidation of fatty acids, is only expressed in NE-like PCa cells and non-NE prostate tumor cells but not in normal prostatic cells including NE cells (Huang *et al.* 2006), suggesting the close linkage between NE-like PCa and prostate carcinoma cells. Finally, histological studies have clearly shown that normal NE cells exhibit two distinct morphologies which have a complex appearance with irregular dendrite-like processes extending between adjacent epithelial cells (di Sant’Agnese 1992), while some NE-like PCa cells may lack the typically neuron-like processes and are morphologically similar to the surrounding carcinoma cells (Xing *et al.* 2001). These data together suggest that NE-like PCa cells are clearly distinguished from NE cells in the normal prostate.

Origin of NE-like PCa versus normal NE cells

The distinctions between NE-like PCa and normal NE cells may be attributed to their origins. As shown in Fig. 1, results of studies indicate that normal NE cells, as basal and luminal epithelial cells, are originated from stem cells in the basal layer (Hudson 2004, Long *et al.* 2005). It is also possible that normal NE cells represent an independent cell lineage from the neurogenic origin, differing from secretory and basal cells (Aumuller *et al.* 1999). Thus, the origin of NE cells in normal prostate glands requires further investigations.

Similarly, the origin of NE-like PCa cells remains controversial. It is proposed that NE-like PCa cells share the same origin with normal NE cells and are differentiated from the intermediate stem cells (Bonkhoff *et al.* 1995, Bonkhoff 1998). The differentiation processes of those stem cells may be aberrantly regulated under pathological conditions like androgen ablation, resulting in an abnormally increased NE cell population. Although this stem cell-based hypothesis is attractive, it requires further validation. Alternatively, accumulated evidence suggests that adenocarcinoma cells can undergo a transdifferentiation process to become NE-like cells, which acquire a similar phenotype to normal NE cells and express several NE markers. Importantly, they still retain some epithelial characteristics (Fig. 1; Vashchenko & Abrahamsson 2005). This notion is supported by the observations that NCI-H660 and PSK-1 cells, two human prostatic small cell carcinoma cell lines, express K8/18 of luminal cell markers, but

not K5/14 of basal cell markers (van Bokhoven *et al.* 2003). Furthermore, Bcl-2 expression is found in all specimens examined for prostatic small cell carcinoma (18/18; 100%), whereas its expression is observed in two out of ten cases (20%) of prostate adenocarcinoma (Yao *et al.* 2006) and no expression in normal NE cells (Xue *et al.* 1997), which suggests the correlative relationship of NE-like PCa cells and adenocarcinoma cells. In addition, NE differentiation can be seen in the bone metastatic lesion but not within the primary tumor foci, suggesting the occurrence of transdifferentiation process in metastatic PCa cells (Cheville *et al.* 2002, Roudier *et al.* 2003). Importantly, results of genetic analyses on clinical archival specimens further reveal that NE-like PCa cells share essentially identical allelic profiles with non-NE PCa cells, but not that of normal prostatic epithelium or normal NE cells (Sauer *et al.* 2006). These results collectively provide the evidence that NE-like PCa cells are originated from cancerous luminal epithelial cells, which undergo a transdifferentiation process (Fig. 1), and thus these cells should be defined as ‘NE-like PCa cells’, but not ‘NE cells’.

Multipathways involved in NE trans-differentiation of PCa cells

Androgen depletion-induced NE differentiation

Androgen ablation therapy is the main option for treating metastatic PCa; however, androgen withdrawal also contributes to increased NE

Figure 1 Proposed model of the origin(s) of NE-like PCa cells.

differentiation in PCa (Ismail *et al.* 2002). It has been shown that patients who had received a longer course of androgen ablation therapy would have higher levels of NE differentiation. Ito *et al.* (2001) reported that in archival specimens from 137 PCa patients, 70.5% of 68 patients who had received hormonal therapy for more than 13 months exhibited NE differentiation, higher than 44% of 25 patients with hormonal therapy less than 12 months and 31.8% in 44 patients without therapy. A similar result was also shown in Hirano *et al.* (2004) study, which supports the notion that androgen ablation therapy increases NE differentiation of PCa cells.

To support the observations in clinical specimens, androgen withdrawal-induced NE differentiation of PCa cells is also seen in *in vitro* cell culture (Table 2) and *in vivo* animal model (Table 3) studies. The initial study revealed that androgen-sensitive (AS) LNCaP cells exhibit the capability of being transdifferentiated into an NE-like phenotype in response to androgen deprivation (Shen *et al.* 1997). Our data further showed that androgen-reduced media can induce NE transdifferentiation of AS LNCaP cells, and these cells acquire the neuronal phenotype and express multiple NE markers, including NSE, CgA, gastrin, and neurotensin (Zelivianski *et al.* 2001, Yuan *et al.* 2006). This NE transdifferentiation induced by steroid-reduced (SR) medium is reversed by the addition of DHT. Conversely, Casodex, an androgen antagonist, can compete out DHT effect and restore NE transdifferentiation. These results suggest that androgen-activated AR signaling suppresses NE differentiation. This notion is supported by the observation that knockdown of AR in AS LNCaP cells induces NE transdifferentiation, indicated by acquiring NE-like morphology and expressing NE markers (Wright *et al.* 2003).

Studies on *in vivo* animal models validate *in vitro* observations that castration of mice bearing AS PC-295 or PC-310 xenograft tumors promotes their NE differentiation, indicated by the elevated expression of NE markers (Jongsma *et al.* 1999, 2000). Similar results were also observed in castrated animals bearing xenografts of AS LNCaP (Burchardt *et al.* 1999) and CWR22 cells (Huss *et al.* 2004) as well as castrated TRAMP mice (Kaplan-Lefko *et al.* 2003). Consistent to clinical observations, data from *in vitro* and *in vivo* studies collectively support the notion that androgen depletion induces transdifferentiation of AS adenocarcinoma cells to become NE-like cells in PCa lesions.

Mechanisms of androgen depletion-induced NE differentiation

Receptor protein tyrosine phosphatase α (RPTP α)-mediated signal pathway

RPTP α is a member of the transmembrane subfamily of PTPs and is widely expressed in mammalian tissues (Sap *et al.* 1990). In addition to the regulation of integrin signaling and potassium channel activity, RPTP α plays a critical role in neuronal differentiation (Pallen 2003). In AS LNCaP cells, the expression of RPTP α at both mRNA and protein levels is upregulated by androgen depletion, correlating with NE differentiation and NSE expression (Zelivianski *et al.* 2000, 2001). Furthermore, addition of androgens abolishes steroid depletion-induced RPTP α elevation, while Casodex can compete out androgen effects and restores RPTP α as well as NSE expression (Zhang *et al.* 2003). Further studies show that even in the regular culture condition containing androgenic activity, increased expression of RPTP α by cDNA transfection in AS LNCaP cells results in elevated levels of multiple NE markers, including NSE, chromogranin A (CgA), CgB, parathyroid hormone-related protein (PTHrP), and NT as well as acquired NE-like phenotype, suggesting a role of RPTP α in NE differentiation of AS PCa cells (Zhang *et al.* 2003, Yuan *et al.* 2006).

The molecular mechanism of RPTP α -induced NE differentiation in AS PCa cells is closely associated with the activation of c-Src and ERK/MAPK (Zhang *et al.* 2003, Yuan *et al.* 2006). This notion is supported by observations that elevated RPTP α expression in LNCaP cells leads to c-Src activation, while the expression of Y789F-RPTP α , a mutant of RPTP α which is devoid of interaction with c-Src, does not have an effect on c-Src activation and fails to induce NE marker expression as well as NE-like morphology (Yuan *et al.* 2006). Similarly, increased RPTP α expression in LNCaP cells also leads to ERK/MAPK activation, correlating with elevated expression of multiple NE markers, while this RPTP α -effect is abolished by PD98059, an MEK inhibitor (Zhang *et al.* 2003, Yuan *et al.* 2006). Importantly, PP2, an inhibitor of c-Src family kinases, can effectively abolish RPTP α -induced ERK/MAPK activation as well as NE marker expression, and thus blocks SR medium-induced NE-like morphology in LNCaP cells (Yuan *et al.* 2006). These results collectively suggest that in androgen depletion condition, RPTP α is elevated and constitutively activates the c-Src-MEK-ERK/MAPK signal pathway,

Table 2 *In vitro* studies of neuroendocrine (NE) differentiation of prostate cancer (PCa) cells

Induction	Cell types	NE-like morphology	Growth arrest	Marker expression	PSA/AR expression	Biological phenotypes	Paracrine (CM) effects	R/IR	References
Androgen depletion									
SR medium	LNCaP	Yes	Yes	NSE/S-100/Bombesin	Decreased PSA/AR	ND	ND	R	Shen <i>et al.</i> (1997)
	LNCaP (C-33)	Yes	ND	NSE/CgA/GS/NT	Decreased PSA	ND	ND	ND	Zelivianski <i>et al.</i> (2001)
	LNCaP (C-81)	ND	ND	No effect	Decreased PSA	ND	ND	ND	Zhang <i>et al.</i> (2003)
	LNCaP (C-33)	Yes	Yes	NSE	ND	ND	ND	ND	
	LNCaP	ND	ND	NSE/CgA	ND	ND	ND	ND	Yang <i>et al.</i> (2005)
	LNCaP	ND	ND	NSE	ND	ND	ND	ND	Burchardt <i>et al.</i> (1999)
cAMP-related									
db-cAMP	LNCaP (C-33)	Yes	ND	No effect	Increased PSA	ND	ND	ND	Zelivianski <i>et al.</i> (2001)
db-cAMP + IBMX	LNCaP (C-81)	ND	ND	NSE	Decreased AR	ND	ND	ND	Bang <i>et al.</i> (1994)
	LNCaP	Yes	ND	CgA	ND	ND	ND	ND	
db-cAMP + IBMX	PC-3M	Yes	Yes	NSE/CgA/S-100	ND	ND	ND	ND	Cox <i>et al.</i> (1999)
	LNCaP/C4-2	Yes	Yes	NSE/Serotonin	ND	ND	ND	R	
Forskolin + IBMX	LNCaP	Yes	Yes	NSE, secreted PTHrP/NT	ND	ND	ND	R	Deeble <i>et al.</i> (2001)
Epinephrine	LNCaP	Yes	Yes	NSE	ND	ND	ND	R	
	LNCaP	Yes	Yes	NSE, secreted PTHrP/NT	ND	ND	ND	ND	
Epinephrine + IL-6	LNCaP	Yes	Yes	NSE/ β III tubulin, secreted PTHrP/NT	ND	ND	ND		
Cytokines									
IL-6	LNCaP	Yes	ND	NSE	ND	ND	ND	ND	Qin <i>et al.</i> (1998)
	LNCaP	Yes	Yes	NSE	ND	ND	ND	ND	Mori <i>et al.</i> (1999)
	LNCaP	Yes	Yes	NSE/CgA	ND	ND	ND	ND	Spiotto & Chung (2000a, b)
	LNCaP	Yes	Yes	β III tubulin	ND	ND	ND	ND	Deeble <i>et al.</i> (2001)
	LNCaP (C-33)	Yes	ND	NSE/CgA/GS/NT	Decreased PSA	ND	ND	ND	Zelivianski <i>et al.</i> (2001)
	LNCaP (C-81)	ND	ND	No effect	Decreased PSA/AR	ND	ND	ND	Wang <i>et al.</i> (2004b)
	LNCaP	Yes	Yes	NSE/ β III tubulin	Decreased PSA/AR	Decreased invasiveness	Inhibiting growth	ND	

Table 2 continued

Induction	Cell types	NE-like morphology	Growth arrest	Marker expression	PSA/AR expression	Biological phenotypes	Paracrine (CM) effects	R/IR	References
IL-1 β /IL-6	LNCaP/DU145	ND	ND	CgA	ND	ND	ND	ND	Diaz et al. (1998)
IL-1 β	LNCaP	Yes	Yes	CgA/Serotonin/K8	Decreased PSA	ND	ND	IR	Chiao et al. (1999)
Protein overexpression									
RPTP α	LNCaP (C-33)	Yes	ND	NSE/CgA/CgB/N-T/PTHrP	ND	ND	ND	ND	Zhang et al. (2003) and Yuan et al. (2006)
PTP1B	LNCaP	ND	No	NSE/CgA	ND	ND	ND	ND	Wu et al. (2006)
PCDH-PC	LNCaP/PC-3	ND	ND	NSE/CgA	ND	ND	ND	ND	Yang et al. (2005)
β -Catenin	LNCaP	ND	ND	NSE/CgA	ND	ND	ND	ND	
MEK (CA)	LNCaP (C-33)	Yes	ND	NSE	ND	ND	ND	ND	Zhang et al. (2003)
PKA (CA)	LNCaP	Yes	Yes	NSE	ND	ND	ND	ND	Cox et al. (2000)
Stat3 (CA)	PC-3	Yes	Yes	NSE	ND	ND	ND	ND	Spiotto & Chung (2000b)
Pharmacological agents									
Silibinin	LNCaP	Yes	Yes	CgA/K8/K18	Decreased PSA	ND	ND	ND	Zi & Agarwal (1999)
Sodium butyrate	LNCaP (C-33)	Yes	Yes	NSE	ND	ND	ND	ND	Zelivianski et al. (2001)
JolkinolideB	LNCaP	Yes	Yes	NSE/K8/K18	ND	ND	ND	ND	Liu et al. (2002)
Genistein	LNCaP	Yes	ND	β III tubulin/CgA/synaptophysin/serotonin	ND	ND	Inhibiting colony formation	ND	Pinski et al. (2006)
Growth factors/neuropeptides									
HB-EGF	LNCaP	Yes	No	NSE	ND	ND	ND	ND	Kim et al. (2002)
EGF	DU145	Yes	Yes	NSE	ND	ND	ND	ND	Humez et al. (2006)
VIP	LNCaP	Yes	ND	NSE	ND	ND	ND	ND	Gutierrez-Canas et al. (2005)
PACAP	LNCaP	Yes	Yes	NT	ND	ND	ND	ND	Farini et al. (2003)

CM, conditioned medium; R/IR, reversible/irreversible phenotype; SR, steroid reduced; CA, constitutively active; K, cytokeratin; LNCaP (C-33), androgen-sensitive LNCaP cells; LNCaP (C-81), androgen-independent LNCaP cells; ND, not determined.

Table 3 *In vivo* studies of neuroendocrine (NE) differentiation of prostate cancer (PCa) cells

Xenograft tumors	Origin of cells	Marker expression	PSA/AR expression	Proliferative index	Other effects	References
Castrated models	PC-295	Metastatic tumor	CgA/SgIII/Bombesin/-Serotonin	Decreased sPSA/AR	Decreased Ki-67 staining	ND
	PC-310	Primary tumor	CgA/SgIII/7B2/PC1/P-AMGRP/VEGF/serotonin	Decreased sPSA	Decreased Ki-67 staining	ND
LNCaP	LNCaP	CgA	ND	ND	ND	Burchardt <i>et al.</i> (1999)
	CWR22	Synaptophysin	ND	Increased Ki-67 staining	ND	Huss <i>et al.</i> (2004)
Transgenic models	G γ /T-15	SV40-Tag transgenic mice tumor	CgA	ND	ND	Metastatic activity
	CR2-Tag	SV40-Tag transgenic mice tumor	CgA/synaptophysin	ND	ND	Metastatic activity
12T-10	LPB-Tag transgenic mice tumor	CgA	Decreased AR	ND	Metastatic activity	Masumori <i>et al.</i> (2001, 2004)
NE-10	12T-10 transgenic mouse tumor	CgA	No AR	ND	Metastatic activity	Jin <i>et al.</i> (2004), Masumori <i>et al.</i> (2004) and Uchida <i>et al.</i> (2006)
					Up-regulation of LNCaP xenograft metastasis/AR growth, PSA secretion and AR expression	

sPSA, secreted PSA; AR, androgen-independent; ND, not determined.

resulting in NE transdifferentiation of PCa cells (Fig. 2).

PTP1B-mediated signal pathway

The expression of PTP1B, a cytosolic PTP, is also found to have an elevated level in NE-differentiated LNCaP cells induced by androgen depletion (Wu *et al.* 2006). In the regular culture condition containing androgenic activity, increased expression of PTP1B in LNCaP cells can lead to NE differentiation. In an androgen-deprived condition, increased PTP1B expression or activity in LNCaP cells synergistically enhances NE marker expression. Conversely, the expression of a dominant-negative mutant of PTP1B blocks the upregulation of CgA expression in response to androgen withdrawal (Wu *et al.* 2006). Thus, PTP1B may play a role in androgen depletion-induced NE differentiation. Consistently, immunostaining of PCa archival specimens revealed the co-expression of PTP1B and CgA in tissues examined (Wu *et al.* 2006). Although the underlying mechanism of PTP1B-induced NE differentiation of PCa cells requires further investigation, PTP1B may activate the transdifferentiation process through upregulating the Ras-ERK/MAPK signaling (Dube *et al.* 2004). Alternatively, PTP1B, similar to RPTP α , can directly activate c-Src as observed in breast cancer cells (Bjorge *et al.* 2000). PTP1B-induced NE differentiation may also be mediated by AKT activation (Wu & Huang 2007).

Protocadherin-PC (PCDH-PC)-mediated signal pathway

PCDH-PC, a member of the protocadherin gene family encoded on the human Y chromosome, is selectively expressed in hormone-resistant PCa cells (Chen *et al.* 2002, Terry *et al.* 2006). The expression of PCDH-PC is upregulated in androgen-depleted LNCaP cells and in LNCaP xenograft tumors from castrated male mice (Chen *et al.* 2002). Increased expression of PCDH-PC leads to the activation of Wnt signaling, including the nuclear translocation of β -catenin and increased Tcf transcriptional activity (Yang *et al.* 2005). Elevated expression of PCDH-PC by cDNA transfection in LNCaP and PC-3 cells upregulates NSE and CgA expression, while its decreased expression by siRNA oligomers in LNCaP cells abolishes androgen depletion or PCDH-PC-induced NSE expression. Furthermore, NE transdifferentiation of LNCaP cells could be induced by β -catenin cDNA transfection or blocked by its siRNA oligomers (Yang *et al.*

Figure 2 Signal transduction pathways associated with the induction of NE transdifferentiation of PCa cells. The solid line indicates the reported pathways, while the dash line indicates the hypothetical pathways.

2005). These data together suggest that increased PCDH-PC expression in response to androgen deprivation via Wnt signaling plays a role in NE differentiation of PCa cells.

cAMP and its agonists-induced NE differentiation

Several lines of evidence show that elevation of intracellular cAMP leads to NE differentiation of AS and AI PCa cells (Table 2). Bang *et al.* (1994) first showed that increased cAMP, by adding cAMP analogs and phosphodiesterase inhibitors to AS LNCaP or AI PC-3M cell culture medium, induces their NE differentiation by acquiring neuron-like morphology, expressing NE markers, and suppressing cell growth. Further studies showed that elevation of intracellular cAMP by forskolin, an activator of adenylate cyclase, or epinephrine, a β -adrenergic receptor agonist, induces NE transdifferentiation of LNCaP cells (Cox *et al.* 1999). Additionally, androgen depletion also leads to cAMP elevation in AS LNCaP cells, correlated with NE differentiation (Burchardt *et al.* 1999). Nevertheless, the NE characteristics induced by cAMP-elevating agents are fully reversible upon the removal of the stimuli (Cox *et al.* 1999).

Mechanisms of cAMP-induced NE differentiation

Since increased cAMP induces the activation of protein kinase A (PKA) and treatment of epinephrine

also leads to PKA activation (Deeble *et al.* 2001), it is thus proposed that PKA is mediating cAMP-induced transdifferentiation of PCa cells. This notion is supported by the observation that overexpression of PKA catalytic subunit in LNCaP cells leads to NE differentiation, while expression of a dominant-negative mutant of PKA blocks NE differentiation induced by cAMP-elevating agents (Cox *et al.* 2000). Since ERK/MAPK signal pathway crosstalks with cAMP/PKA-regulated proliferation and differentiation in several cell types (Stork & Schmitt 2002), ERK/MAPK may also play a role in the cAMP-elevating agent-induced NE differentiation of PCa cells. For example, epinephrine and db-cAMP treatments in LNCaP cells lead to ERK/MAPK activation (Chen *et al.* 1999). However, in other studies, the withdrawal of db-cAMP/IBMX from NE-differentiated LNCaP cells results in ERK/MAPK activation (Cox *et al.* 1999). Additionally, epinephrine treatment has no significant effect on ERK/MAPK activity in LNCaP cells, although it can lead to an increased cAMP level, the PKA activation, and the induction of NE differentiation (Deeble *et al.* 2001). The inconsistent observations could be contributed by different experimental conditions. It has been shown that transient cAMP accumulation in LNCaP cells leads to proliferation through PKA-dependent ERK/MAPK activation, while sustained elevation of cAMP level increases

PKA activity resulting in NE differentiation by a PKA-dependent but ERK/MAPK-independent mechanism (Farini *et al.* 2003). The role of ERK/MAPK in cAMP/PKA signaling-mediated NE differentiation in PCa cells requires further investigation.

Cytokines-induced NE differentiation

Cytokines, a diverse group of secreted peptides, function as signaling molecules to mediate immune and inflammatory responses as well as to regulate the proliferation and maturation of immune cells. Importantly, cytokines such as interleukins can also regulate the proliferation and differentiation of non-immune cells, including PCa cells. Results of clinical studies reveal that the serum level of interleukin-6 (IL-6) is elevated in patients with PCa, especially hormone-refractory and metastatic PCa (Adler *et al.* 1999, Drachenberg *et al.* 1999, Shariat *et al.* 2001). Furthermore, the levels of IL-6 and its receptor in PCa tissues are significantly higher than that in normal prostate tissues (Siegmund *et al.* 1994, Giri *et al.* 2001). These results clearly show the correlation of IL-6 expression with PCa progression and thus suggest that IL-6 may serve as a marker for PCa morbidity (Twillie *et al.* 1995). Since elevated IL-6 level is associated with advanced PCa and correlated with increased NE differentiation, it is proposed that IL-6 is one of the important inducers of NE differentiation of PCa cells. Indeed, IL-6 treatment induces NE transdifferentiation of LNCaP cells (Qiu *et al.* 1998), indicated by neuron-like morphology, reduced cell growth, and elevated NE marker expression (Table 2). Furthermore, in the absence of ligand, elevated expression of constitutively active gp130, the signal transduction subunit of IL-6 receptor, in LNCaP cells induces NE transdifferentiation in cell cultures and in animal model studies, indicating IL-6-induced NE differentiation of PCa cells (Palmer *et al.* 2005). Additionally, IL-1 β , a pro-inflammatory cytokine, can also induce NE differentiation of PCa cells (Diaz *et al.* 1998, Chiao *et al.* 1999). These results collectively suggest a role of cytokines in the NE differentiation of PCa cells.

Mechanisms of IL-6-induced NE differentiation

IL-6-induced NE differentiation may be mediated by Stat3 transcription factor. Initial studies showed that the transcriptional activity of Stat3 is required for IL-6-induced growth arrest in LNCaP cells (Spiotto & Chung 2000a), in addition to the involvement of other pathways (Mori *et al.* 1999). Since growth arrest and differentiation are associated processes, Stat3 may also

mediate IL-6-induced NE differentiation in PCa cells. Consistently, stable expression of the wild-type Stat3 in PC-3 cells results in the formation of neurite extensions, elevated NSE expression, and growth arrest (Spiotto & Chung 2000b). Furthermore, LNCaP cells stably expressing a dominant-negative mutant of Stat3 fail to undergo NE transdifferentiation process in the presence of IL-6, suggesting the critical role of Stat3 in IL-6-induced NE differentiation (Spiotto & Chung 2000b). Interestingly, IL-6-mediated Stat3 phosphorylation, nuclear translocation, and NE differentiation are closely associated with cholesterol-rich membrane lipid rafts. In LNCaP cells, the disruption of lipid rafts inhibits IL-6-mediated effects, probably because IL-6 receptors predominantly localizes to the lipid raft membrane compartment (Kim *et al.* 2004). Additionally, tyrosine kinase Etk/Bmx has been shown to play an important role in IL-6-induced NE differentiation (Qiu *et al.* 1998). Etk/Bmx may activate c-Src, Stat3, and/or PI3K for NE differentiation (Spiotto & Chung 2000b, Tsai *et al.* 2000, Chau *et al.* 2005). Alternatively, c-Src can activate the Etk/Bmx-Stat3 pathway (Tsai *et al.* 2000). Further investigation is apparently needed.

Clinical relevance of IL-6-induced NE differentiation

IL-6-induced NE differentiation has been clearly demonstrated in LNCaP cells in culture; however, the role of those IL-6-induced NE-like cells in clinical prostate tumor progression is less understood. Studies showed that IL-6 inhibits the growth of LNCaP xenografts, despite the fact that those tumors exhibit NE characteristics including elevated levels of NSE and β -III tubulin (Wang *et al.* 2004a). Furthermore, co-culture of IL-6-induced NE-like cells with LNCaP, PC-3, or DU145 cells leads to cell cycle arrest and DNA synthesis inhibition (Wang *et al.* 2004b). These results suggest that IL-6-induced NE differentiation leads to the growth inhibition of surrounding PCa cells, probably via the release of inhibitory factors by IL-6-induced NE-like cells (Wang *et al.* 2004b). While the underlying mechanism still requires further investigations, these results are opposite to the observations in clinical specimens that the NE cell-proximal PCa cells exhibit higher proliferation indices than the distal PCa cells (Bonkhoff *et al.* 1995). The data suggest that clinical NE cells in PCa lesions secrete growth stimulatory factors to enhance proximal PCa cell proliferation. It should also be noted that IL-6 exhibits a growth stimulatory effect on the primary prostate epithelial cells as well as a cell line derived from a

precancerous lesion and high-grade prostatic intraepithelial neoplasia (Giri et al. 2001, Liu et al. 2002b). The contradictory effects of IL-6 on both PCa cell proliferation and NE differentiation may result from the bifunctional character of IL-6. It has been proposed that the paracrine effect by IL-6 leads to the growth inhibition and NE differentiation, while the autocrine effect by IL-6 causes the growth stimulation (Lee et al. 2007). Evidently, the role of IL-6-induced NE differentiation of PCa cells in clinical PCa progression under AI condition requires further clarification.

Other factors in NE differentiation

Results from studies clearly suggest multiple pathways for inducing NE transdifferentiation of PCa cells (Table 2 and Fig. 2; Zelivianski et al. 2001). For example, HB-EGF, an EGFR ligand serving as a mitogenic and survival factor for PCa cells, can also promote NE differentiation *in vitro* and *in vivo* (Adam et al. 2002, Kim et al. 2002). HB-EGF activates EGFR via ERK/MAPK to induce NE differentiation of LNCaP cells (Kim et al. 2002). Similarly, activation of EGFR by EGF can also induce NE differentiation of DU145 cells although the underlying molecular basis remains unclear (Humez et al. 2006). Contrarily, treatment of genistein, a classical tyrosine kinase inhibitor, has been shown to be associated with NE differentiation of LNCaP cells, which is correlated with the activation of Stat3 and ERK/MAPK (Pinski et al. 2006). Additionally, treatment of genistein abolishes EGF-induced NE differentiation of DU145 cells (Humez et al. 2006), and conditioned media from genistein-induced NE-like LNCaP cells have an inhibitory effect on the growth of PC-3 and DU145 cells (Pinski et al. 2006). Vasoactive intestinal peptide (VIP) is a neuropeptide and can also promote NE differentiation of LNCaP cells (Juarranz et al. 2001). Further studies reveal that VIP-induced NE differentiation is mediated by the activation of multiple signaling molecules, including PKA, ERK/MAPK, and PI3K (Gutierrez-Canas et al. 2005). Interestingly, intracellular calcium level is also associated with the development of NE phenotype in PCa cells (Vano-verberghe et al. 2004). Calcium chelator BAPTA/AM inhibits VIP-induced NE phenotype in LNCaP cells (Collado et al. 2005), while increased expression of calcium channel proteins in LNCaP cells promotes NE differentiation (Mariot et al. 2002). The treatments of some anticancer agents, such as jolkinolide B and silibinin, are also reported to be correlated with the induction of NE differentiation in LNCaP cells (Zi & Agarwal 1999, Liu et al. 2002a). The data collectively

support the notion that NE differentiation of PCa cells is mediated by multiple signal pathways, in part due to the heterogeneity of PCa cells (Zelivianski et al. 2001), and PCa cells can indeed transdifferentiate into NE-like cells.

Summary

Although several studies have shown interesting results regarding the NE transdifferentiation of PCa cells in cultures as well as in animal models, it remains for further investigations to determine whether those transdifferentiated NE-like cells exhibit the similar biochemical properties as that of clinical NE-like PCa cells. For example, the biochemical information of those transdifferentiated NE-like cells, such as the expression profiles of NE markers, the paracrine effect and the tumorigenic activity, is limited. It is because most of transdifferentiation processes induced by various reagents are transient and cells can fully revert to their original phenotype in the absence of inducers. As such, they are apparently different from those terminally differentiated clinical NE-like PCa cells; nor can they be used for studying the interaction between NE-like PCa and prostatic carcinoma cells. To elucidate the molecular basis of NE differentiation and their roles in PCa progression, stable NE-like PCa cell lines of clinical relevance are imperatively needed.

An NE-like PCa cell model

Establishment and characterization of NE-like LNCaP subclone cells

To investigate the molecular mechanism of NE differentiation and its role in PCa, we established NE-like PCa cell lines by culturing AS LNCaP cells in an androgen-depleted condition, resembling clinical androgen ablation therapy. By prolonged culturing in an androgen-reduced medium, AS LNCaP cells transdifferentiate into NE-like cells and several subclone cells grew to become independent cell lines. These NE-like LNCaP subclone cells exhibit a neuronal morphology, differing from AS LNCaP parental cells (Zhang et al. 2003, Yuan et al. 2006). Importantly, these NE-like LNCaP subclone cells share the same genetic profile with AS LNCaP parental cells (Yuan et al. 2006), corresponding to the observation that NE-like PCa cells in tumor lesions have essentially identical genetic profiles with adjacent exocrine PCa cells (Sauer et al. 2006).

Expression of neuron-specific markers and neuropeptides is one of the major hallmarks of NE-like PCa cells in tumor lesions. Studies in three NE-like LNCaP

Table 4 Prostatic neuroendocrine (NE) xenografts and cell models

Name	Origin of cells	NE-like morphology	Marker expression	PSA/AR expression	Biological phenotypes	Paracrine (CM) effects	References
Xenograft models							
UCRU-Pr-2	Primary NE tumor (human)	ND	NSE/somatostatin β -endorphin/ATCH	No PSA/AR	ND	ND	van Haaften-Day <i>et al.</i> (1987) and Jelbart <i>et al.</i> (1988)
WISH-PC2	Primary tumor (human)	ND	NSE/CgA/synaptophysin/-K8/K18/Bcl-2	No sPSA/PSA/AR	Metastatic activity High Ki-67 staining	ND	Pinthus <i>et al.</i> (2000)
LuCaP 49	Metastatic tumor (human)	ND	NSE/synaptophysin/VEGF	No sPSA/AR	75% of cells with Ki-67 staining	ND	True <i>et al.</i> (2002)
SCC cell lines							
NCI-H660	Metastatic NE tumor (human)	ND	NSE/CgA/CgB/K8/K18	No PSA/AR	Metastatic activity	ND	Johnson <i>et al.</i> (1989) and van Bokhoven <i>et al.</i> (2003)
PSK-1	Primary NE tumor (human)	No	NSE/CgA/K8/K18	No PSA/AR	Tumorigenic activity	ND	Kim <i>et al.</i> (2000) and van Bokhoven <i>et al.</i> (2003)
SO-MI	Primary NE tumor (human)	Yes	NSE/CgA	No PSA/AR	ND	ND	Okada <i>et al.</i> (2003)
NE-CS	NE-10 allograft (mouse)	Yes	Secreted serotonin	No AR	Tumor development in nude mice	Up-regulation of cell migration and invasion	Uchida <i>et al.</i> (2005, 2006)
Trans differentiated NE cell line							
NE-like LNCaP sub-clone cells	Androgen depleted-LNCaP (C-33) cells	Yes	NSE/CgA/CgB/NT/PTHrP/-K8/K18	No PSA/AR	Tumor development in nude mice	Up-regulation of AI growth, PSA secretion	Yuan <i>et al.</i> (2006)

CM, conditioned medium; SCC, small cell carcinoma; sPSA, secreted PSA; AI, androgen-independent; LNCaP (C-33), androgen-sensitive LNCaP cells; ND, not determined.

subclone cells have revealed that they differentially express elevated levels of multiple NE markers and neuropeptides, including NSE, CgA, CgB, NT, and PTHrP, compared with LNCaP parental cells (Yuan *et al.* 2006; Table 4). Furthermore, these NE-like LNCaP subclone cells express K8 and K18, two prominent markers for luminal epithelial cells, higher than LNCaP parental cells (Yuan *et al.* 2006). Similarly, in clinical archival specimens, over 90% of serotonin-positive prostatic NE cells and two cell lines derived from prostatic small cell carcinomas express K18 (Xue *et al.* 1997, van Bokhoven *et al.* 2003; Table 4). This observation is in parallel with the expression of PAcP, a prostate secretory epithelium-specific differentiation antigen, in these NE-like LNCaP subclone cells and clinical NE-like PCa cells in prostate carcinomas (Abrahamsson 1999, Yuan *et al.* 2006). Importantly, these NE-like LNCaP subclone cells do not express AR and PSA (Yuan *et al.* 2006), similar to those NE-like PCa cells found in the archival specimens (Bonkhoff 2001, Huang *et al.* 2006). Additionally, these NE-like LNCaP subclone cells express an elevated level of Bcl-2 (Yuan *et al.* 2006), correlated with the observation in clinical small cell carcinoma cells (Yao *et al.* 2006). Significantly, even after a 3-month re-culturing in a medium containing androgenic activity, these NE-like LNCaP subclone cells retain the NE phenotype and express NE markers, indicating a terminal transdifferentiation. Collectively, our NE-like LNCaP subclone cells resemble those NE-like PCa cells in clinical samples and are suitable for further biochemical characterizations.

The functional characterizations further reveal the clinical relevance of these NE-like subclone cells. Results of studies in prostatic small cell carcinomas indicate that those clinical NE-like PCa cells are highly aggressive and exhibit a malignant phenotype (Abrahamsson 1999). Corresponding to the clinical observations, our NE-like LNCaP subclone cells exhibit high tumorigenicity. These NE-like subclone cells have a greater tumorigenic activity in xenograft animals and a higher clonogenic activity, but a lower proliferation rate in culture, than AS LNCaP parental cells (Yuan *et al.* 2006). Importantly, conditioned media (CM) by these NE-like subclone cells, but not by LNCaP parental cells, can stimulate the growth and PSA secretion from LNCaP cells under an androgen-reduced condition (Yuan *et al.* 2006), corresponding to the observation that clinical NE-like PCa cells have a mitogenic effect on adjacent cancer cells. Thus, the data collectively suggest that these NE-like LNCaP subclone cells, similar to NE-like PCa cells observed in clinical studies, exhibit a

Figure 3 Biochemical characterizations of NE-like LNCaP stable subclone and small cell carcinoma cells. Androgen-sensitive LNCaP parental cells (LN) were plated and maintained in the regular medium, while two NE-like LNCaP subclone cells, i.e., NE-1.3, NE-1.8, and small cell carcinoma NCI-H660 (NCI) cells were grown in a steroid-reduced medium. An aliquot of total cell lysates was separated by SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting with Abs against RPTP α , phospho-ERK1/2, ERK1/2, NSE, CgB, AR, and cellular PSA (cPSA) respectively. As an internal loading control, the level of β -actin on the same membrane was examined.

malignant phenotype and have a paracrine effect on AI proliferation of PCa cells.

RPTP α signaling in NE-like LNCaP subclone cells

As described earlier that RPTP α plays a role in androgen depletion-induced NE differentiation, RPTP α is highly expressed in these NE-like LNCaP subclone cells and its expression level is associated with increased ERK/MAPK activity as well as elevated NSE expression, compared with LNCaP parental cells (Fig. 3; Zhang *et al.* 2003, Yuan *et al.* 2006). We further compared our NE-like LNCaP subclone cells with NCI-H660 cells, a prostatic small cell carcinoma cell line (Johnson *et al.* 1989). As shown in Fig. 3, NCI-H660 cells express a high level of RPTP α , even higher than both NE-1.3 and NE-1.8 cells, correlated with activation of ERK/MAPK by phosphorylation. As a control, LNCaP parental cells express a low level of RPTP α and exhibit very low ERK/MAPK activity. Furthermore, NCI-H660 cells, similar to NE-1.3 and NE-1.8 cells, expressed elevated levels of NE markers but no AR and PSA expression (Fig. 3).

These results further support the functional importance of RPTP α in NE differentiation of PCa cells.

Conclusions and prospectives

Accumulated evidence has suggested the roles of NE-like PCa cells in PCa progression and has also revealed that their biological characteristics are different from the normal NE cells. Furthermore, NE-like PCa cells are originated from cancerous luminal epithelial cells, but not from normal NE cells, via a transdifferentiation process. This notion is supported by the fact that those NE-like cells in PCa lesions exhibit the same genetic profile as PCa cells in cancerous lesions but not NE cells in normal tissues both from the same specimen (Sauer *et al.* 2006). We propose that those NE-like cells in PCa lesions should be defined as ‘NE-like PCa cells’ to accurately reflect their origin. While several factors may induce NE transdifferentiation via multiple signal pathways, the constitutive ERK/MAPK activation apparently plays a critical role in converging multiple signalings for NE differentiation in PCa cells (Fig. 2). Thus, we propose that activation of ERK/MAPK is one of the major mechanisms in the transdifferentiation of PCa cells into NE-like cells. We further propose that sustained, but not transient, hyperactivation of ERK/MAPK is required to support the NE transdifferentiation process of PCa cells, similar to NE differentiation of PC-12 cells (Marshall 1995). This notion is supported by the observations in NCI-H660 and our NE-like stable subclone cells that exhibit a high and sustained level of ERK/MAPK activity (Fig. 3). In parallel, constitutive activation of ERK/MAPK is observed in PCa archival specimens, including advanced hormone-refractory tumors (Gioeli *et al.* 1999, Price *et al.* 1999). Therefore, ERK/MAPK activation plays an important role in NE transdifferentiation of PCa cells and may serve as a target for developing new therapeutic approaches for PCa therapy.

It should also be pointed out that NE tumors are rare for PCa, and the post-mitotic NE-like PCa cells in tumor lesions may be one or two steps away from becoming NE tumors. The transition from the post-mitotic NE-like PCa cells to highly aggressive NE tumor cells, such as small cell carcinoma cells, may occur after additional genetic alterations. In parallel, the NE-like LNCaP subclone cells were established after growth silence in SR condition. Interestingly, studies from the TRAMP mouse model as well as the Rb/p53-null mice showed that the simultaneous inactivation or knockout of both p53 and Rb genes promotes NE differentiation of PCa (Perez-Stable *et al.*

1997, Zhou *et al.* 2006). While LNCaP cells express the wild-type p53 (Isaacs *et al.* 1991, Carroll *et al.* 1993), NCI-H660, and PSK-1, two human prostatic small cell carcinoma cell lines, exhibit truncated and mutated p53 proteins respectively (van Bokhoven *et al.* 2003). Despite that further investigation on the molecular characterization of those NE-like LNCaP subclone cells is required; it is possible that those NE-like LNCaP subclone cells have acquired additional genetic alterations, such as p53 or Rb mutation/deletion seen in a subpopulation of advanced PCa, which cause the transition from the post-mitotic NE-like PCa cells to highly aggressive NE tumor cells. Thus, our NE-like LNCaP subclone cells provide a useful model to study both NE-like PCa and NE tumor cells.

It is an urgent need to have proper cell lines for studying the biological significance of NE-like PCa cells. Our NE-like LNCaP stable subclone cells acquires many features of clinical NE-like PCa cells, including irreversible NE phenotype, the same genetic profile as the parental PCa cells, expression of multiple NE markers, lack of AR or PSA expression, and high tumorigenicity in xenograft animals. Importantly, the paracrine effect of these NE-like subclone cells on AI proliferation as well as PSA secretion by LNCaP parental cells in SR conditions is in parallel with clinical observations that the proliferation index of cancer cells adjacent to NE-like PCa cells is increased and the circulation level of PSA in a hormone-refractory PCa patient is rebound. In summary, these NE-like LNCaP stable subclone cells can serve as a useful cell model to further explore the molecular mechanism of NE differentiation and its role in PCa for developing targeted therapy.

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