

# Cellular prostatic acid phosphatase: a protein tyrosine phosphatase involved in androgen-independent proliferation of prostate cancer

Suresh Veeramani<sup>1</sup>, Ta-Chun Yuan<sup>1</sup>, Siu-Ju Chen<sup>1</sup>, Fen-Fen Lin<sup>1</sup>, Juliette E Petersen<sup>1</sup>, Syed Shaheduzzaman<sup>2</sup>, Shiv Srivastava<sup>2</sup>, Richard G MacDonald<sup>1,3</sup> and Ming-Fong Lin<sup>1,3</sup>

<sup>1</sup>Department of Biochemistry and Molecular Biology, College of Medicine, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA

<sup>2</sup>Center for Prostate Disease Research and Department of Surgery, Uniformed Services University, School of Medicine, Bethesda, Maryland 20814, USA

<sup>3</sup>Eppley Institute for Cancer Research, University of Nebraska Medical Center, Omaha, Nebraska 68198, USA

(Requests for offprints should be addressed to M-F Lin, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska 68198-5870, USA; Email: mlin@unmc.edu)

## Abstract

Human prostatic acid phosphatase (PACp) was used as a valuable surrogate marker for monitoring prostate cancer prior to the availability of prostate-specific antigen (PSA). Even though the level of PACp is increased in the circulation of prostate cancer patients, its intracellular level and activity are greatly diminished in prostate cancer cells. Recent advances in understanding the function of the cellular form of PACp (cPACp) have shed some light on its role in prostate carcinogenesis, which may have potential applications for prostate cancer therapy. It is now evident that cPACp functions as a neutral protein tyrosine phosphatase (PTP) in prostate cancer cells and dephosphorylates HER-2/ErbB-2/Neu (HER-2: human epidermal growth factor receptor-2) at the phosphotyrosine (p-Tyr) residues. Dephosphorylation of HER-2 at its p-Tyr residues results in the down-regulation of its specific activity, which leads to decreases in growth and tumorigenicity of those cancer cells. Conversely, decreased cPACp expression correlates with hyperphosphorylation of HER-2 at tyrosine residues and activation of downstream extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) signaling, which results in prostate cancer progression as well as androgen-independent growth of prostate cancer cells. These *in vitro* results on the effect of cPACp on androgen-independent growth of prostate cancer cells corroborate the clinical findings that cPACp level is greatly decreased in advanced prostate cancer and provide insights into one of the molecular mechanisms involved in prostate cancer progression. Results from experiments using xenograft animal models further indicate a novel role of cPACp as a tumor suppressor. Future studies are warranted to clarify the use of cPACp as a therapeutic agent in human prostate cancer patients.

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## Introduction

Human prostatic acid phosphatase (PACp; E.C. 3.1.3.2) is a major phosphatase and a differentiation marker in normal, well-differentiated prostatic epithelial cells (Yam 1974, Vihko 1979, Lin *et al.* 1980). Human PACp is a 100 kDa glycoprotein containing two subunits of approximately 50 kDa each. In the

mid 1930s, Gutman and his colleagues made the noteworthy observation that the activity of PACp is increased in the circulation of patients with prostate cancer, especially those with bone metastasis, compared with healthy adults (Gutman *et al.* 1936). Subsequently, it was established that the PACp activity in the circulation of prostate cancer patients correlates with prostate cancer progression and could serve as an

indicator of the response to treatment (Huggins & Hodges 1941). As a consequence, serum PACP had been studied extensively as a surrogate marker for prostate cancer prior to the establishment of prostate-specific antigen (PSA) as the new standard (Wang *et al.* 1979, 1981, Papsidero *et al.* 1980, Chu & Lin 1998).

There are two forms of PACP in well-differentiated prostate epithelial cells: one remains intracellular, i.e. the cellular form (cPACP); the other is secreted, i.e. the secretory form (sPACP). The two forms of PACP have different isoelectric point (pI) values and exhibit differences in biochemical properties (Vihko 1979, Lad *et al.* 1984, Boissonneault *et al.* 1995). Preliminary studies have revealed that they differ in glycosylation and hydrophobicity, as indicated by differential sensitivity to endoglycosidases and detergent effects on their mobility in gel filtration, fast protein liquid chromatography respectively (R Garcia, X Xia and MF Lin, unpublished data). However, the molecular bases for the physicochemical differences remain to be determined. Both forms of PACP can hydrolyze a broad variety of small organic phosphomonoesters under acidic conditions within the optimal pH range between 4 and 6 (Vihko 1979, Van Etten 1982, Lin *et al.* 1983). Thus, the enzyme is classically referred to as an acid phosphatase (AcP).

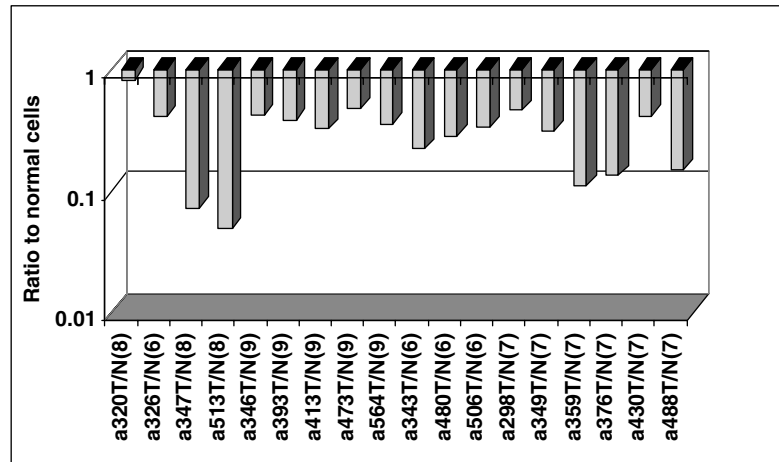
The development of the prostate gland from birth through sexual maturity in the human is mirrored by the changes in cPACP expression. The expression level of cPACP is negligible before adolescence in males. After puberty, the cPACP level increases and reaches a very high level in normal, well-differentiated prostate epithelial cells at approximately 0.5 mg/g wet weight of tissue (Yam 1974, Goldfarb *et al.* 1986). The sPACP is predominantly secreted into seminal fluid at a physiological concentration of approximately 1 mg/ml (Ronnberg *et al.* 1981). Although the serum PACP level is negligible in healthy individuals, its level is elevated in prostate cancer patients and correlates with the stage of prostate cancer. cPACP has been a useful marker for the detection of metastatic prostate cancer, due to its cell-specific expression (Sakai *et al.* 1992). However, the functional role of cPACP in normal and cancerous prostate epithelial cells was not known until a decade ago. Hence, this review focuses on discussing the role of the cPACP-mediated signaling in prostate carcinogenesis and progression.

It should be noted that the expression level of cPACP negatively correlates with prostate carcinogenesis, i.e. its cellular level decreases in prostate cancer cells relative to adjacent non-cancerous cells (Reif *et al.* 1973, Foti *et al.* 1977, Loor *et al.* 1981, Lin *et al.* 2001). Furthermore, the level of cPACP corresponds inversely

to prostate cancer progression, i.e. the higher the grade, the lower the cPACP protein (Abrahamsson *et al.* 1988, Sinha *et al.* 1988, Sakai *et al.* 1991), despite an elevated level of sPACP in circulation. Transcriptome-based tissue microarray analyses using HG U133A GeneChip (Affymetrix, Santa Clara, CA, USA) on 18 laser-captured, micro-dissected, paired normal and cancerous prostate specimens reveal that 100% of advanced prostate cancer specimens of Gleason scores 8 or 9 and 90% of cancer specimens of Gleason scores 6 or 7 have decreased PACP expression compared with the adjacent normal specimens (Fig. 1). Interestingly, the PACP protein isolated from prostate cancer tissues and the circulation of prostate cancer patients exhibits acidic pI values, significantly lower than that of PACP from non-cancerous tissues (Foti *et al.* 1977, Chu *et al.* 1978, Lin *et al.* 1983). Furthermore, the half-life of cancerous PACP in the circulation of experimental animals is longer than that of non-cancerous PACP (Lin *et al.* 1983). The decreased clearance rate of cancerous PACP in those animals was found to be due in part to the differences in post-translational modification, including increased sialylation that is consistent with the low pI values (Lin *et al.* 1980, 1983). Thus, although the level of PACP in prostate cancer cells is decreased (Fig. 1; Hakalahti *et al.* 1993), the elevated serum PACP in prostate cancer patients is apparently contributed by the combined effects of increased tumor mass and the prolonged half-life of serum PACP. Additionally, the loss of membrane polarity, as seen in advanced prostate cancer (Busch *et al.* 2002), may also play a significant role in this phenomenon.

### **cPACP: a neutral histidine-dependent protein phosphatase in prostatic epithelium**

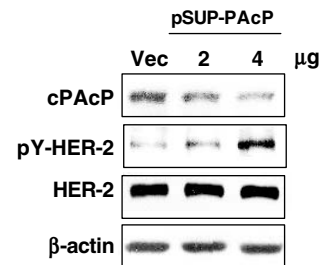
PACP is classically categorized as a histidine-AcP because it uses a histidyl residue as the phosphate acceptor during the hydrolysis of small organic compounds (Van Etten 1982, Ostanin *et al.* 1994, Zhang *et al.* 2001). Results of several studies indicate that cPACP exhibits protein tyrosine phosphatase (PTP) activity. cPACP was shown to be co-purified with the major PTP activity in non-cancerous human prostate tissue (Li *et al.* 1984). The PTP activity of PACP was further supported by studies using purified human PACP protein (Lin & Clinton 1986) and recombinant rat PACP protein expressed in a baculoviral expression system (Vihko *et al.* 1993). RNA interference (RNAi)-mediated knockdown of endogenous cPACP expression



**Figure 1** GeneChip analyses of cancerous and non-cancerous prostate glands show decreased PACp expression in cancerous specimens compared with non-cancerous specimens. Transcriptome-based GeneChip analyses of laser-captured, micro-dissected specimens from paired non-cancerous and cancerous tissues of 18 primary prostate cancer specimens (nine of Gleason scores 6 or 7 and nine of Gleason scores 8 or 9) were performed with HG U133A GeneChips. PACp levels in the cancerous tissues were normalized to the PACp level in adjacent non-cancerous tissues. Decreased cPACp expression was noted in 17 of 18 pairs of matched tumor (T) and non-cancerous (N) specimens. Numbers in parentheses indicate the number of analyses performed on each specimen.

in LNCaP human prostate cancer cells resulted in elevated protein tyrosine phosphorylation, which confirms cPACp as a PTP *in vivo* (Fig. 2). Collectively, the data indicate that cPACp indeed functions as a PTP in prostate epithelial cells.

Significantly, PACp prefers a neutral pH to an acidic environment for the dephosphorylation of tyrosine-phosphorylated epidermal growth factor receptor (EGFR), in contrast to its hydrolysis of small organic compounds (Lin & Clinton 1988). Biochemical characterizations revealed that PACp protein functions as a dual-specificity protein phosphatase *in vitro* and exhibits varying affinities towards the different phospho-linkages, as indicated by the  $K_m$  (association/dissociation constant) values of the dephosphorylation reactions (Wasylewska *et al.* 1983, Lin & Clinton 1986, Chevalier *et al.* 1988, Lee *et al.* 1991). cPACp exhibits  $K_m$  values in the nanomolar range for phosphotyrosine (p-Tyr) linkages in the peptide/protein substrates. This is over 50-fold lower than that for p-Ser/p-Thr linkages in proteins and over several orders of magnitude lower than those for free phospho-amino acids and small phosphorylated organic compounds (Wasylewska *et al.* 1983, Lin & Clinton 1986, Chevalier *et al.* 1988). The low  $K_m$  values indicate that the high-affinity interactions between cPACp and its phosphoprotein substrates may delay the release of the dephosphorylated products and thus could contribute to the low  $V_{max}$  (velocity maximum) values measured for the dephosphorylation



**Figure 2** Transient knockdown of cPACp by siRNA in LNCaP C-33 cells leads to increased tyrosine phosphorylation of HER-2 kinase. Androgen-sensitive LNCaP C-33 cells were transfected with pSUPER vector encoding siRNA oligonucleotides specific to PACp (pSUP-PACp). Control cells were transfected with vector alone (Vec). Knockdown of cPACp in those cells correlates with the increase in the p-Tyr level of HER-2. Phosphorylation was determined in total cell lysates using western blotting with an antibody (4G10) that recognizes phosphorylated tyrosine residues (Upstate Biotechnology, Charlottesville, VA, USA). The protein level of HER-2 was analyzed by rehybridizing the same membrane with HER-2-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) after stripping. β-Actin level was used as a loading control.

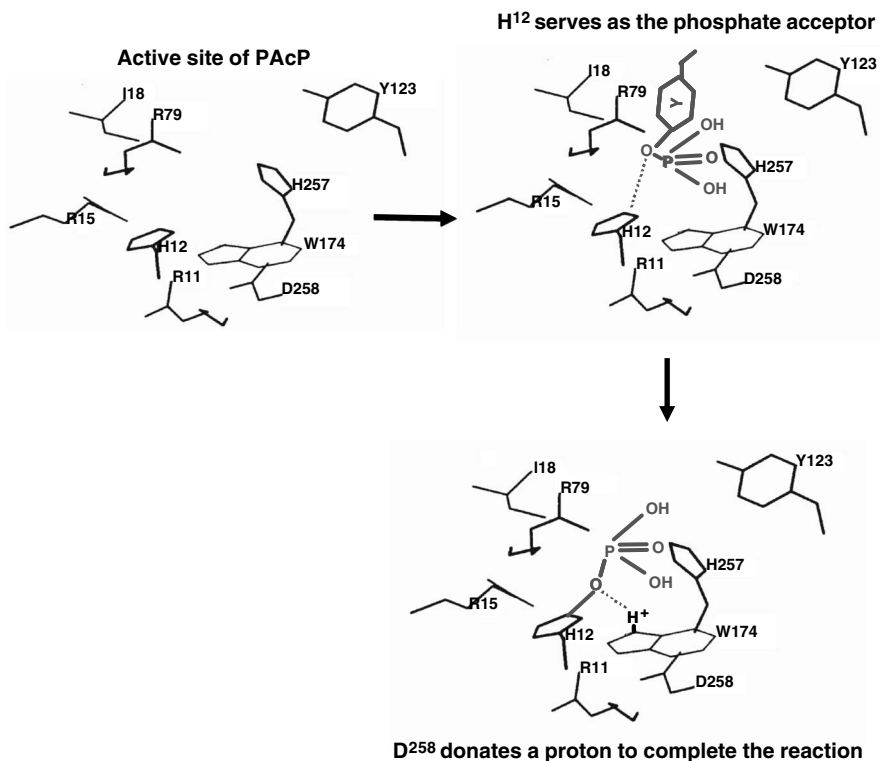
reactions (Lin & Clinton 1986, 1987). The data collectively indicate that cPACp protein could function as a neutral PTP in prostate epithelial cells, while detailed kinetic analyses of the p-Tyr dephosphorylation by PACp would require the identification of its physiological substrate(s) and their specific dephosphorylation sites.

Even though PacP uses tyrosine-phosphorylated proteins as a preferred substrate both *in vitro* (Lin & Clinton 1986, Boissonneault *et al.* 1995, Vihko *et al.* 2005) and *in vivo* (Lin *et al.* 1993a), recent data show that PacP also effectively dephosphorylates lysophosphatidic acid and phosphatidylinositol 3-phosphate (PI3P), thus serving additionally as a lipid phosphatase (Tanaka *et al.* 2004, Vihko *et al.* 2005). The *in vivo* significance of PacP as a lipid phosphatase in prostate carcinogenesis requires further analysis.

### Structural analysis of cPacP

Sequence homology analyses of PacP revealed that it contains neither the PTP signature motif, C(X)<sub>5</sub>R(S/T), nor the extended active site signature sequence for the dual-specificity phosphatases, VXVHCXXGXXRS(X)<sub>5</sub>AY(L/I)M (Vihko *et al.* 1988, Roiko *et al.* 1990, Jackson & Denu 2001, Rudolph 2002). Initial structural analyses on the disulfide linkage showed that each of the two subunits of PacP

contains three disulfide bonds: Cys<sup>129</sup> to Cys<sup>340</sup>, Cys<sup>183</sup> to Cys<sup>281</sup> and Cys<sup>314</sup> to Cys<sup>319</sup> (Van Etten *et al.* 1991). Nevertheless, analysis of the three-dimensional structure of the PacP crystal indicated that only four out of six cysteine residues in each subunit form two disulfide bonds and Cys<sup>183</sup>, which bears a free sulfhydryl group, is located in the interior pocket of the active site of PacP (Schneider *et al.* 1993). Chemical titration experiments confirmed that PacP has two reactive sulfhydryl groups (Ostanin *et al.* 1994). It was thus hypothesized that Cys<sup>183</sup> is needed for the PTP activity of PacP. Further studies indicated that the imidazole ring of His<sup>12</sup> and the carboxyl group of Asp<sup>258</sup> of PacP act as the phosphate acceptor and the proton donor respectively, during the hydrolysis of its small phosphomonoester substrates (Fig. 3; Ostanin *et al.* 1994, Porvari *et al.* 1994). Using site-directed mutagenesis, it was subsequently determined that His<sup>12</sup> and Asp<sup>258</sup> of PacP, but not Cys<sup>183</sup> or Cys<sup>281</sup>, are required for its PTP activity (Zhang *et al.* 2001). The H12A and D258A mutants of human PacP did not exhibit



**Figure 3** Schematic representation of the mechanism of the PacP-mediated phosphotyrosine dephosphorylation reaction. A simplified depiction of the active site of PacP is shown in the figure. Histidine (H12) and aspartate (D258) residues have been shown to be critical for the tyrosine dephosphorylation reaction by PacP. His<sup>12</sup> serves as the phosphate acceptor and Asp<sup>258</sup> serves as the proton donor for the hydrolysis and release of the phosphate moiety from the enzyme (Schneider *et al.* 1993, Ostanin *et al.* 1994, Zhang *et al.* 2001). Molecules depicted in red represent the phosphotyrosine residue in HER-2 protein. Dotted lines indicate the active reaction between the p-Tyr moiety and specific amino acid residues of cPacP.

**Table 1** cPacP expression and the growth of different prostate cancer cells

cPacP expression negatively correlates with the tyrosine phosphorylation level of HER-2 and the growth rate of different prostate cancer cell lines. AR-positive prostate cancer cells expressing cPacP are androgen-sensitive; while PacP-null, AR-positive prostate cancer cells are androgen-independent.

	MDA <sup>-</sup> PCa 2b	LNCaP C-33	PC-3	DU 145
cPacP expression <sup>a,b</sup>	++++	+++	–	–
pY-HER-2 level <sup>a,b</sup>	+/-	+	+++	+++
AR	++	+++ / + + + +	+	–
Cell growth (Doubling time in hours)	61–73 <sup>c</sup>	60 <sup>d</sup>	33 <sup>e</sup>	34 <sup>f</sup>
Androgen sensitivity <sup>a,b</sup>	++	+++	–	–

– Unpublished observations.

<sup>a</sup>Lin *et al.*, 1998.

<sup>b</sup>Meng *et al.*, 2000.

<sup>c</sup>Navone *et al.*, 1997.

<sup>d</sup>Horoszewicz *et al.*, 1983.

<sup>e</sup>Kaighn *et al.*, 1979.

<sup>f</sup>Stone *et al.*, 1978.

significant (detectable) AcP or PTP activities, whereas the C183A and C281A mutants essentially retained both activities completely (Zhang *et al.* 2001). It should be noted that Asp<sup>258</sup> is conserved in the PTP family (Ostanin *et al.* 1994). The side chain of this Asp of PacP may serve as a proton donor during the dephosphorylation reaction (Porvari *et al.* 1994), similar to Asp<sup>129</sup> of the low-molecular-weight AcP, which has been reclassified as a member of a sub-family in the PTP superfamily (Ostanin *et al.* 1994, Zhang *et al.* 1994). Trp<sup>106</sup> and His<sup>112</sup> residues of PacP may be responsible for the dimerization of the molecule, which greatly enhances its phosphatase activity (Porvari *et al.* 1994, Wasylewska *et al.* 2003). These results indicate that PacP represents a novel PTP that does not belong to the classical cysteine PTP family, yet uses the same active site as well as the catalytic mechanism of AcP to execute its PTP activity.

### cPacP: a negative regulator of cell proliferation

The expression of cPacP inversely correlates with prostate cell proliferation. In normal, well-differentiated human prostate epithelial cells, cPacP expression is very high in accordance with the slow growth rate of those cells (Yam 1974, Isaacs 1983, Goldfarb *et al.* 1986). The inverse correlation between cPacP expression and cell proliferation is also evident in canine primary prostate epithelial cell culture, i.e. the cPacP level is low in the rapidly growing cells, while its expression is greatly elevated when the cells are growing slowly under confluent conditions (Chevalier *et al.* 1981, Dionne *et al.* 1983). In prostate cancer

specimens, the cPacP level is decreased in cancerous prostate cells, when compared with the adjacent non-cancerous cells (Reif *et al.* 1973, Foti *et al.* 1977, Vihko *et al.* 1980, 1981, Loor *et al.* 1981, Hakalahti *et al.* 1993, Lin *et al.* 2001). The decrease in cPacP level correlates with the poor differentiation of high-grade prostate cancer (Abrahamsson *et al.* 1988, Sinha *et al.* 1988, Sakai *et al.* 1991). It was thus proposed that prostatic epithelial cells that have a low level of cPacP expression are at a high risk of carcinogenesis (Reif *et al.* 1973).

It could also be argued that the decreased cPacP expression in poorly differentiated prostate cancer cells is due to the nature of PacP as a differentiation antigen, i.e. decreased PacP expression is a consequence of the loss of differentiation of prostate cancer cells. However, existing evidence suggests that this may not be the case. Among different human prostate cancer cell lines, cPacP level inversely correlates with cell proliferation (Table 1) (Lin *et al.* 1992). LNCaP and MDA PCa2b cells, which express endogenous cPacP, grow much more slowly than PC-3 and DU 145 cells, which lack PacP expression (Table 1; Lin *et al.* 1992, Meng & Lin 1998, Veeramani *et al.* 2005). Growth stimulation of LNCaP cells by various factors, including dihydro testosterone (DHT) and epidermal growth factor (EGF) treatment, is accompanied by decreased cPacP activity (Lin *et al.* 1992, 1994). Conversely, under non-permissive growth conditions, cPacP level is elevated and cell growth is diminished (Lin *et al.* 1992). Furthermore, prolonged passage of LNCaP and MDA PCa2b cells in culture results in a decrease in their cPacP expression, correlating with an increase in cell proliferation (Fig. 4; Lin *et al.* 1998,

Igawa *et al.* 2002, Veeramani *et al.* 2005). Importantly, ectopic expression of cPacP using a full-length cDNA in PacP-null prostate cancer cells decreases their growth rate (Fig. 4; Lin *et al.* 1994, 1998, Zhang *et al.* 2001). Conversely, transfection of PacP-positive LNCaP cells with anti-sense PacP cDNA results in approximately 30% decrease in PacP activity which correlates with the enhancement of the growth rate of those cells (Fig. 5). The role of PacP in prostate cell growth has been validated by experiments with PacP knock-out mice in that they showed increased epithelial cell proliferation within the anterior and dorsolateral lobes of prostate, which later developed into invasive adenocarcinomas (Vihko *et al.* 2005). Taken together, it is evident that cPacP plays a critical role in regulating the proliferation of human prostate epithelial cells that leads to the generation of prostate cancer.

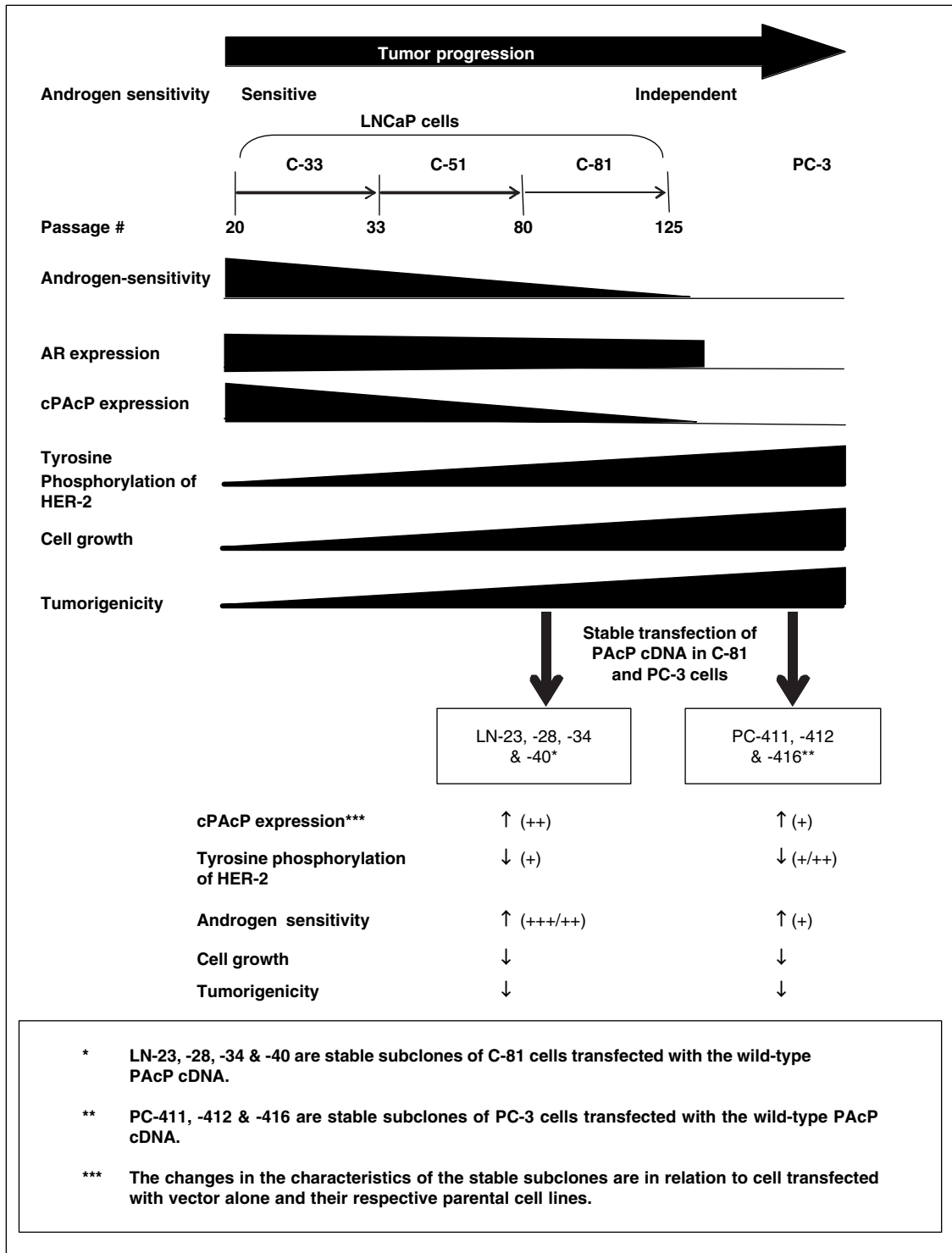
### **HER-2/ErbB-2/Neu (HER-2): a substrate of cPacP in prostate cancer cells**

Several lines of evidence suggest that cPacP functions as a neutral PTP in prostate cancer cells. In non-cancerous human prostate tissue, cPacP represents the major PTP activity; although other PTPs are also present (Li *et al.* 1984, Valencia *et al.* 1997, Lin *et al.* 2001). In sections of normal canine prostate tissue, the level of p-Tyr is always lower in the secretory epithelial cells that express a higher level of PacP, than in the non-secretory basal epithelial cells that do not express PacP (Landry *et al.* 1996). In canine prostate secretory epithelial cells treated with PTP inhibitors — e.g. orthovanadate — the p-Tyr moiety was greatly increased, which indicates the impressive dominance of the PTP activity in regulating tyrosine phosphorylation of proteins in differentiated non-cancerous prostate epithelial cells (Tessier *et al.* 1989, Bourassa *et al.* 1991). Additionally, the overall tyrosine kinase specific activity in PacP-null DU 145 cell lysates is higher than in PacP-expressing LNCaP cell lysates (Lin *et al.* 1986, Lin 1991, Lin & Meng 1996). The high tyrosine kinase specific activity of DU 145 cell lysate, but not that of the LNCaP cells, is sensitive to pre-incubation with purified PacP protein, and this decreased kinase activity in PacP-treated DU 145 cell lysates is not merely due to the hydrolysis of tyrosine kinase products by added PacP. This notion is further supported by the observation that purified PacP protein can directly reduce the specific activity of immunoprecipitated EGFR and HER-2 (human epidermal growth factor receptor-2) kinases through dephosphorylation of their p-Tyr residues (Lin *et al.* 1986, Lin & Clinton 1987, 1988, Lin 1991, Meng & Lin

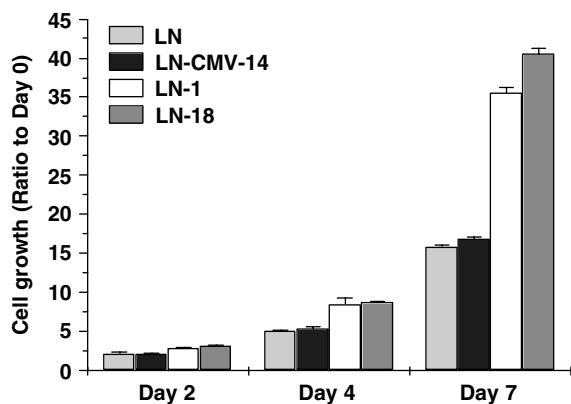
1998). These *in vitro* data collectively implicate cPacP in regulation of the activity of tyrosine kinases.

Studies with canine prostate epithelial cells indicated that PTP inhibitor treatment selectively increased the activity of membrane protein tyrosine kinases by inhibiting PTPs such as cPacP and PTP-1B, which were found to be membrane-associated (Boissonneault *et al.* 1995), indicating that cPacP associates with receptor tyrosine kinases. Data from different prostate cancer cell lines show that an inverse correlation exists between the cPacP level and the p-Tyr level of a 185 kDa phosphoprotein. For example, PacP-null prostate cancer cells had a higher p-Tyr level of the 185 kDa phosphoprotein, compared with that of PacP-positive prostate cancer cells (Table 1 and Fig. 4; Lin & Meng 1996, Meng & Lin 1998). This has been further shown in different passages of cells, i.e. high-passage LNCaP (LNCaP C-81) cells, which have a decreased cPacP expression, have an increased level of p-Tyr of a 185 kDa phosphoprotein, compared with that of low-passage LNCaP (LNCaP C-33) cells (Fig. 4). Furthermore, in LNCaP C-33 cells that express endogenous PacP, inhibition of cPacP activity by L(+)-tartrate, a classical inhibitor to PacP, leads to increased tyrosine phosphorylation of a 185 kDa phosphoprotein (Lin & Meng 1996, Lin *et al.* 1998, Meng & Lin 1998). Conversely, liposome-mediated delivery of purified PacP protein into DU 145 cells leads to a decreased specific activity of tyrosine kinases in cell lysates as well as decreased p-Tyr level of a 185 kDa phosphoprotein (Lin *et al.* 1993a, Lin & Meng 1996). Ectopic expression of cPacP in PacP-null prostate cancer cells by cDNA transfection results in decreased tyrosine phosphorylation of a 185 kDa phosphoprotein (Lin *et al.* 1998, Meng & Lin 1998, Zhang *et al.* 2001). This 185 kDa phosphoprotein has been identified as HER-2, a member of the ErbB receptor protein tyrosine kinase family (Meng & Lin 1998). Decreased cPacP expression in LNCaP C-33 cells by means of RNAi-mediated knockdown correlates with increased tyrosine phosphorylation of HER-2 (Fig. 2). Additionally, expression of cPacP by cDNA transfection correlates with decreased phosphorylation of HER-2 protein at specific tyrosine residues, including Tyr<sup>1248</sup>, which leads to a decrease in the extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) activation as well as a reduction in cell proliferation (Zhang *et al.* 2001, Lee *et al.* 2004).

Theoretical and experimental estimates of free energy of binding revealed that PacP has the most favorable binding towards the synthetic peptide that includes 1197–1203 amino acid residues of rat ErbB-2 when compared with several other phosphotyrosine



**Figure 4** Ectopic expression of cPACP expression in AR-positive, PAcP-null cells restores their androgen sensitivity. Prolonged passage of LNCaP parental cells leads to a decrease in the cPACP level, which corresponds to the loss of their androgen sensitivity and an increase in the growth rate and tumorigenicity. Ectopic expression of cPACP in PAcP-null cells such as LNCaP C-81 and PC-3 cells results in the restoration of their androgen sensitivity and a decrease of their growth rate and tumorigenicity (Lin *et al.* 1998, 2001, Meng & Lin 1998, Meng *et al.* 2000).



**Figure 5** Reduction of cPacP expression using anti-sense cDNA transfection leads to increased growth of androgen-sensitive LNCaP C-33 cells. Stable subclones of LNCaP C-33 cells transfected with a cDNA encoding cPacP in the anti-sense orientation (LN-1 and LN-18) were analyzed for growth and compared with parental LNCaP C-33 cells (LN) and a subclone transfected with vector alone (LN-CMV-14). After plating for 72 h, one set of cells was harvested and counted as day 0. Remaining cells were fed with fresh medium. Cell numbers were counted on days 2, 4 and 7 and normalized to day 0. Similar results were obtained in two sets of independent experiments.

peptides from rat EGFR and rat ErbB-2; therefore signifying the specific interaction between HER-2 and cPacP (Sharma *et al.* 2005). Thus, the molecular mechanism by which cPacP regulates cell proliferation is at least in part through the p-Tyr dephosphorylation of HER-2 protein in prostate cells.

### cPacP and HER-2 interactions in the regulation of androgen sensitivity

Prostate cancer cells are initially androgen dependent and, hence, androgen ablation is the primary line of treatment of metastatic prostate cancer. However, prostate cancer progresses to a hormone-refractory state when prostate cancer cells are no longer dependent on androgens to sustain proliferation. The efficacy of treatments at this stage of cancer is limited regardless of different modalities. The molecular mechanisms of androgen independence require further investigation to develop more effective therapeutic approaches. It is well understood now that most of the hormone-refractory prostate cancer cells express functional androgen receptor (AR), which is still required for their androgen-independent growth (Sadi *et al.* 1991, van der Kwast *et al.* 1991, Grossman *et al.* 2001, Debes & Tindall 2002, Huang & Tindall 2002, Zagarra-Moro *et al.* 2002). It is therefore possible that

AR is activated in hormone-refractory prostate cancer cells even in the absence or very low level of androgen during androgen-ablation therapy. It is further hypothesized that AR is activated by phosphorylation in androgen-independent prostate cancer cells even in the absence of ligand binding. This hypothesis suggests that cross-talk between protein kinase signaling and AR could be an important factor for the development of androgen independence in such cells.

Results from several studies collectively indicate that cPacP in prostate cancer cells is involved in determining their androgen sensitivity, although other mechanisms, e.g. AR mutations and/or gene amplification, may also contribute to this mode of regulation. In prostate cancer archival specimens, the expression level of cPacP correlates negatively with the grade of prostate cancer, i.e. the higher the grade, the lower the cPacP expression; it is also well established that hormone-refractory prostate cancers, in general, are of high grades (Abrahamsson *et al.* 1988, Sinha *et al.* 1988, Sakai *et al.* 1991). Our observations from prostate cancer cell lines corroborate the clinical data that androgen-sensitive prostate cancer cells are AR positive and express cPacP, which correlates with slow growth rates. In contrast, androgen-independent cells, regardless of their AR expression level, usually express low/no cPacP and have rapid growth rates (Fig. 4 and Table 1; Lin *et al.* 1992, 1994, 1998). Prolonged passage of androgen-sensitive LNCaP and MDA PCa2b cells in culture is accompanied by a decrease in the endogenous cPacP expression, but not AR expression, which leads to their androgen-independent growth (Fig. 4; Igawa *et al.* 2002, Denmeade *et al.* 2003, Unni *et al.* 2004, F F Lin & M F Lin, unpublished data). Ectopic cPacP expression by cPacP cDNA transfection into those AR-positive androgen-independent prostate cancer cells restores their androgen sensitivity (Fig. 4; Lin *et al.* 1998, 2001, Meng *et al.* 2000). The correlation between cPacP expression and androgen sensitivity in culture is also reflected in xenograft animal models in that prostate cancer cells that express lower cPacP readily form subcutaneous tumors in athymic female mice and castrated athymic male mice, which have a very low level of circulating androgens (Table 2; Lin *et al.* 2001, Igawa *et al.* 2002, 2003, Denmeade *et al.* 2003). The molecular mechanism by which cPacP is involved in regulating androgen-sensitive cell proliferation deserves further investigation.

One possible mechanism by which cPacP regulates androgen sensitivity in prostate cancer cells is through its interaction with HER-2, i.e. the dephosphorylation of HER-2 by cPacP plays a critical role in this mode of



**Table 2** Effect of cPacP expression on the tumorigenicity of different LNCaP cells\*

Tumorigenicity of different LNCaP cells was analyzed by injecting  $1 \times 10^6$  cells with Matrigel (1:1, v/v) subcutaneously in female athymic nude mice ( $n = 5$ ). Tumor volumes were measured at 2 and 4 weeks after injection.

Cell type	Tumor size** (Tumor incidence %)	
	2 weeks	4 weeks
LNCaP C-33	0 mm <sup>3</sup> (0)	300 mm <sup>3</sup> (80)
LNCaP C-81	200 mm <sup>3</sup> (60)	650 mm <sup>3</sup> (100)
LN-23***	0 mm <sup>3</sup> (0)	150 mm <sup>3</sup> (20)
LN-34***	0 mm <sup>3</sup> (0)	100 mm <sup>3</sup> (40)

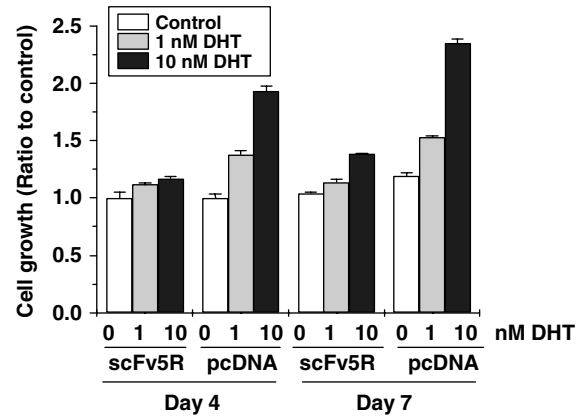
\*Igawa *et al.*, 2003.

\*\*Tumor size is expressed as the mean tumor volume.

\*\*\*LN-23 and LN-34 are stable subclones of LNCaP C-81 cells transfected with an expression vector encoding the full-length, wild-type PacP cDNA.

regulation (Lin *et al.* 1998, Meng *et al.* 2000, Lee *et al.* 2003). Data from our laboratory clearly show that androgen stimulation of LNCaP C-33 cells results in decreased cPacP activity and concomitant increase in the tyrosine phosphorylation of HER-2, but not ErbB-1/EGFR or ErbB-3, the other ErbB family members in prostate cells. Kinetic studies on androgen action in those cells reveal that cPacP activity is decreased prior to HER-2 activation by tyrosine phosphorylation (Meng *et al.* 2000, Lee *et al.* 2003). The involvement of HER-2 in androgen action is further implicated in androgen-sensitive LNCaP C-33 cells such that treatment with HER-2 inhibitors, including AG879, or expression of a dominant-negative mutant of HER-2 abolishes the androgenic stimulation of cell proliferation (Meng *et al.* 2000, M S Lee & M F Lin, unpublished data). Conversely, inhibition of EGFR with specific inhibitors, such as AG1478, has no effect on androgenic stimulation of prostate cancer cell proliferation, indicating the specificity of HER-2 involvement in androgen action in those cells (Sherwood *et al.* 1998, Meng *et al.* 2000). Furthermore, androgenic stimulation of cell proliferation is abolished in HER-2-impaired LNCaP cells, in which the HER-2 is trapped in the endoplasmic reticulum (ER) by scFv5R (a single-chain anti-HER-2 antibody that contains the ER targeting and retention sequence) (Fig. 6; Qiu *et al.* 1998, Liu *et al.* 2005). Apparently, activation of HER-2 is required for androgenic stimulation of prostate cancer cell proliferation.

Owing to the importance of HER-2 in androgen action, we further hypothesize that an aberrant activation of HER-2 by factors that include decreased cPacP expression can lead to androgen-independent



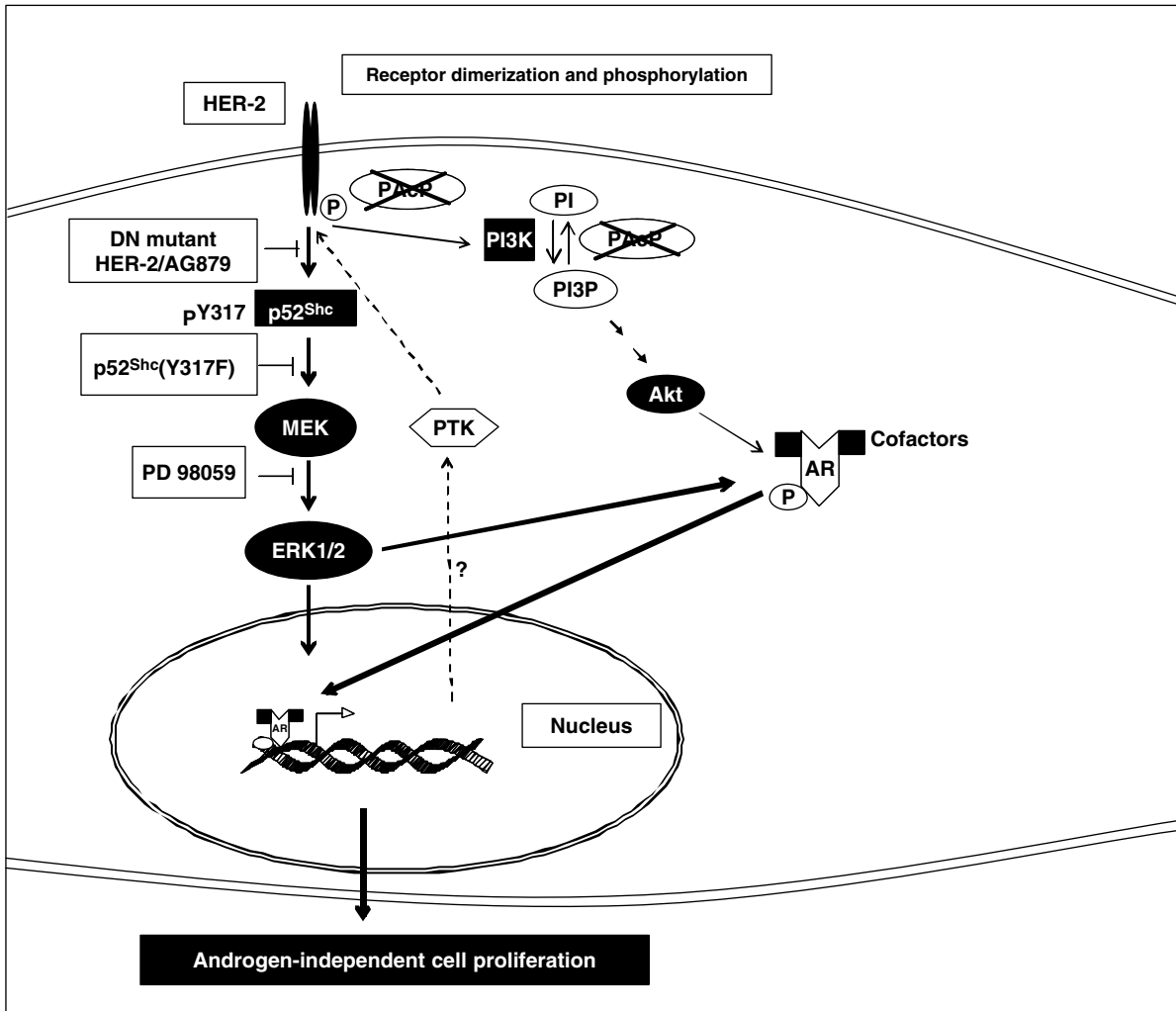
**Figure 6** Reduced HER-2 signaling abolishes androgen-induced proliferation of prostate cancer cells. LNCaP cells, in which HER-2 protein is trapped in the endoplasmic reticulum by a Fab fragment that is specific to HER-2 and tagged with an ER localization signal (scFv5R), are insensitive to androgen stimulation when compared with the subclone transfected with the vector alone (pcDNA). Cell counts at day 4 and day 7 were normalized to the control at day 0. Similar results were obtained in three sets of independent experiments.

prostate cancer growth. This notion is supported by the observation that in androgen-independent LNCaP C-81 as well as PC-3 cells, cPacP expression is low or nil and HER-2 is activated by hyperphosphorylation on tyrosine residues. In those cells, ectopic expression of cPacP by cDNA transfection reduces HER-2 tyrosine phosphorylation, decreases cell proliferation and restores androgen-sensitive cell proliferation, despite the fact that PC-3 cells express a low level of AR (Fig. 4; Lin *et al.* 1998, Meng & Lin 1998, Meng *et al.* 2000). In PacP cDNA-transfected stable subclones of LNCaP C-81 and PC-3 cells, DHT treatment results in decreased exogenous cPacP and increased HER-2 tyrosine phosphorylation with concurrent stimulation of cell proliferation, but not in the control cells transfected with vector alone (Meng *et al.* 2000). The hyperactivation of HER-2 by tyrosine phosphorylation in androgen-independent prostate cancer cells is in keeping with the amplification of HER-2 gene in the hormone-refractory growth of breast and ovarian cancer cells (Press *et al.* 1990, Meden & Kuhn 1997, Press *et al.* 1997, Menard *et al.* 2001) as an alternative mechanism for stimulating the growth of steroid hormone-dependent cancers in the absence of ligand. Hence, it is clear that, despite the requirement for AR, the interaction between cPacP and HER-2 can regulate the androgen sensitivity of the prostate cancer cells. The involvement of HER-2 in hormone-refractory prostate cancer is further supported by similar observations in androgen-independent LAPC4

cells (Craft *et al.* 1999, Mellinger *et al.* 2004). An aberrant regulation of either molecule can contribute to androgen-independent proliferation of prostate cancer cells.

It is conceivable that in cPacP-null prostate cancer cells, activated HER-2 transmits signals via p52<sup>Shc</sup> to increase downstream ERK/MAPK activity. Activated ERK/MAPK may increase the ligand-independent

activity of AR by phosphorylation that results in elevated androgen-independent prostate cancer proliferation (Fig. 7; Yeh *et al.* 1999, Meng *et al.* 2000, Lee *et al.* 2003, 2004). This activated HER-2 signal can also up-regulate androgen-independent induction of PSA synthesis and secretion in those prostate cancer cells (Yeh *et al.* 1999, Wen *et al.* 2000, Lee *et al.* 2003, 2004). This is particularly evident in HER-2-impaired



**Figure 7** Working hypothesis: loss of cPacP expression in prostate cancer cells results in hyperphosphorylation of HER-2 on tyrosine residues leading to androgen-independent cellular proliferation. Progression of androgen-sensitive prostate cancer cells towards androgen independence is accompanied by early decrease/loss of cPacP, resulting in HER-2 activation by phosphorylation on tyrosine residues. Activated HER-2 can transduce its signals via p52<sup>Shc</sup> (blocked by dominant-negative (DN) HER-2 cDNA transfection or an HER-2 inhibitor, AG879 (Meng *et al.* 2000)) to activate the downstream ERK/MAPK pathway (blocked by p52<sup>Shc</sup> Y317F mutant cDNA transfection or a MEK inhibitor, PD 98059 (Lee *et al.* 2004)). These events could lead to AR phosphorylation and activation, resulting in an increase in androgen-independent cell proliferation. Activated HER-2, via Akt, may also phosphorylate AR (Wen *et al.* 2000). Alternatively, the loss of cPacP expression results in the accumulation of PI3P (Vihko *et al.* 2005), which may lead to activation of the Akt pathway. Solid arrows in the figure indicate one of the major cPacP-regulated pathways in androgen-independent prostate cancer cells with clinical significance; dashed arrows indicate additional pathways that may exist in androgen-independent prostate cancer cells and that need further investigation.

**Table 3** Distribution of the composite scores of HER-2 expression in cancerous and non-cancerous prostate cells

Immunohistochemical analysis was performed in 77, formalin-fixed archival cancerous prostate tissue specimens. Sections containing both benign and malignant cells on the same slide were evaluated by a pathologist for the level of HER-2 expression as measured by intensity and extent of staining. Composite score was derived by multiplying the scores given for the intensity and extent of tissue staining (Lin *et al.* 2001). Results indicated that the level of HER-2 did not differ significantly ( $P=0.11$ ) between the non-cancerous and cancerous prostate cells.

	Composite c-HER-2 expression in prostate cancer cells			Total
	Low	Medium	High	
<b>Composite c-HER-2 expression in non-cancerous cells</b>				
Low	31	14	0	45
Medium	22	8	0	30
High	0	2	0	2
Total	53	24	0	77

LNCaP cells, which showed impaired induction of PSA by androgen (Lee *et al.* 2003, Liu *et al.* 2005). Taken together, these results provide a mechanistic explanation for the apparent paradox arising from clinical observations in advanced hormone-refractory prostate cancer that they express functional AR, but are nevertheless androgen independent. In those cells, the expression of cPacP is decreased, indicating that HER-2 is activated by tyrosine phosphorylation (Lor *et al.* 1981, Lin *et al.* 2001, Pontes *et al.* 1981, Solin *et al.* 1990, Sakai *et al.* 1993), while the HER-2 gene is not amplified (Table 3; Signoretti *et al.* 2000, Osman *et al.* 2001). Furthermore, in prostate cancer specimens, the phosphorylation level of ERK/MAPK is elevated (Gioeli *et al.* 1999, Price *et al.* 1999) with the elevation of serum PSA (Chu & Lin 1998). Thus, down-regulation of tyrosine phosphorylation of HER-2 by cPacP plays a key role in determining the androgen sensitivity of prostate cancer cells.

In addition to mediation by the ERK/MAPK pathway, HER-2 may also transduce its signals through other downstream molecules, such as the PI3K–Akt pathway, to confer androgen-independent cell proliferation. For example, several studies indicate that Akt could phosphorylate AR at Ser<sup>791</sup> under androgen-deprived conditions (Wen *et al.* 2000, Li *et al.* 2001, Ghosh *et al.* 2003). Interestingly, androgen-independent LNCaP C-81 cells have higher Akt activation when compared with the androgen-sensitive

LNCaP C-33 cells. This property inversely correlates with their PAcP expression (Lin *et al.* 2003, Fig. 4). PI3K–Akt is found to be activated in prostate tissue of PAcP knock-out mice (Vihko *et al.* 2005). Thus, loss of PAcP in prostate epithelial cells might also activate the PI3K–Akt pathway, in addition to the HER-2–MAPK pathway, leading to the tumor progression. Collectively, in the absence or presence of low levels of cPacP, activated HER-2 by virtue of its increased tyrosine phosphorylation, can transduce its signals through PI3K–Akt to confer androgen-independent cell proliferation (Wen *et al.* 2000). Alternatively, since PAcP can directly dephosphorylate PI3P, loss of PAcP in prostate cancer cells can lead to the accumulation of PI3P that can activate the downstream Akt (Fig. 7; Vihko *et al.* 2005). Further investigations are required to clarify whether cPacP functions independently of phosphatase and tensin homolog (PTEN) or interacts with PTEN in regulating the Akt pathway. PTEN-independent Akt activation by HER-2 and PAcP may thus play an additional role in androgen-independent proliferation of prostate cancer cells, despite the fact that PTEN may be inactivated by mutation leading to the constitutive activation of Akt (Li *et al.* 1997, Reiss *et al.* 2000). This notion is supported by observations that LNCaP C-33 cells, which have a mutant, non-functional PTEN gene and, hence, express activated Akt, are still androgen sensitive (Lin *et al.* 1998, 2003, Bastola *et al.* 2002). Further studies are needed to define the role of Akt in the development of androgen-independent prostate cancer under clinical conditions. It is also important to identify whether other pathways that, in conjunction with the HER-2–MAPK signaling pathway, may further promote AR activation and hormone-refractory prostate cancer proliferation.

### cPacP: a tumor suppressor phosphatase

Several lines of evidence collectively suggest that cPacP exhibits the characteristics of a tumor suppressor. First, results of clinical studies show that the expression of cPacP is decreased or absent in prostate carcinomas, correlating with their malignancy (Fig. 1; Reif *et al.* 1973, Lin *et al.* 2001). Furthermore, in cancerous tissue, PAcP mRNA exhibits sequence heterogeneity in the coding and non-coding regions (Vihko *et al.* 1988, Van Etten *et al.* 1991, Lin *et al.* 1993b); and cPacP protein in cancerous tissue has distinct biochemical properties, such as different pI values, from non-cancerous tissues (Foti *et al.* 1977, Chu *et al.* 1978, Lin *et al.* 1983). Thus, it is tempting to speculate that the molecular and biochemical

differences between cPacP in cancerous versus non-cancerous prostate cells, which can lead to differences in specificity and kinetics of the enzyme as reported for other PTPs (Wishart *et al.* 1995, Cirri *et al.* 1996), may make a critical contribution to the development of prostate cancer.

Secondly, among different prostate cancer cell lines, cPacP level inversely correlates with tumorigenicity and growth rate, both of which are the cellular correlates of cancer and indispensable for tumor progression. It is well known that PacP-null PC-3 and DU 145 cells are highly tumorigenic, as indicated by higher soft agar colony formation and tumor growth in experimental animals, and are rapidly growing compared with the PacP-positive LNCaP and MDA PCa2b cells (Table 1). Furthermore, in those PacP-positive cells, cPacP level decreases upon passage corresponding to the increase in the tumorigenicity as well as the cell growth rate (Fig. 4). Similarly, decreasing expression of endogenous cPacP by transfection of its small interfering (si)RNA or PacP cDNA in the anti-sense orientation into PacP-positive LNCaP cells increases HER-2 tyrosine phosphorylation and cell proliferation (Figs 2 and 5).

Thirdly, ectopic expression of the wild-type cPacP by cDNA transfection into rapidly growing, PacP-null human prostate cancer cells reduces their growth rates and tumorigenicity; as shown in a soft agar anchorage-independence assay and xenograft animal models (Fig. 4) (Lin *et al.* 1994, 1998, 2001, Zhang *et al.* 2001). Stable subclones of LNCaP C-81 cells transfected with the wild-type PacP cDNA are less tumorigenic than cells transfected with vector alone (Table 2) (Igawa *et al.* 2003). The direct tumor-suppressive activity of cPacP has been demonstrated in xenograft animal models in that a single intratumoral injection of the wild-type PacP cDNA into established subcutaneous LNCaP C-81 cell-induced tumors results in dephosphorylation of HER-2 in those tumors with concomitant suppression of their growth (Igawa *et al.* 2003). The expression level of cPacP in those PacP cDNA-injected subcutaneous C-81 tumors was high at 1 week post-injection and the level of cPacP in those tumors gradually dropped almost to the level of controls by 6 weeks, correlating with the tyrosine phosphorylation of HER-2 (Igawa *et al.* 2003). A PTPase activity-dead mutant of PacP exhibits dramatically reduced tumor-suppressive activity in these assays, suggesting that the PTP activity of cPacP, acting via the down-regulation of HER-2 activity, plays a critical role in the tumor suppression (Igawa *et al.* 2003). Since nude mice were used for the above experiments the influence of other factors, such

as T-cell immune response to the injected PacP, could be overruled. Nevertheless, under clinical conditions, for example, within an intact human body, prostate cancer might be exposed to a variety of factors including immunological responses that could operate along with other mechanisms to influence the outcome of cancer. Thus, PacP may be involved in prostate cancer suppression via multiple pathways.

Finally, PacP knock-out mice show increased cell proliferation followed by prostatic intraepithelial neoplasia (PIN) lesions, which later progress into invasive adenocarcinomas (Vihko *et al.* 2005). These observations are collectively consistent with the notion that cPacP functions as a tumor suppressor by dephosphorylating HER-2 (Fig. 2) and that decreased cPacP expression, observed in early stages of prostate cancer, is one of the key determinants for prostate cancer progression.

### Potential applications of cPacP in prostate cancer therapy

cPacP may represent a novel reagent to treat prostate cancer. Studies with experimental animals have indicated that cPacP has a potential therapeutic effect against prostate cancer, i.e. a single intra-tumoral injection of an expression vector encoding the wild-type PacP protein, driven by the CMV immediate-early promoter, results in the suppression of the growth and progression of xenograft prostate tumors (Igawa *et al.* 2003). The restoration of cPacP expression in prostate cancer, for example, may provide a new avenue for treating those advanced human prostate cancers in which the expression of PacP is decreased. This idea is supported by the observations that treatment of androgen-independent LNCaP sublines with vitamin D<sub>3</sub> leads to the suppression of their growth in a steroid-depleted medium (Swamy *et al.* 2004, Murthy *et al.* 2005) that correlates with the elevated level of cPacP expression and decreased HER-2 phosphorylation (Hsieh *et al.* 1996, Blutt *et al.* 2000, Stewart *et al.* 2004). Thus, the impressive ability of cPacP to suppress prostate cancer growth and the tumorigenicity of androgen-independent prostate cancer necessitates further studies on its therapeutic applications.

PacP expression exhibits prostate specificity and thus its promoter elements may be used as a tool for targeting prostate cancer cells. The tissue-specific activity of the PacP promoter has already been reported (Zelivianski *et al.* 1998, 2002, Shan *et al.* 2003). For example, deletion analyses of the PacP promoter revealed that p779 (0 to -779 bp fragment)

has the basal promoter activity and p1356 (0 to –1356 bp fragment) exhibits the prostate specificity (Zelivianski *et al.* 2004). This p1356 promoter fragment is active even in aggressive prostate cancer cells, such as PC-3 and DU 145 cells, although they lack endogenous PACp expression (Zelivianski *et al.* 2002, 2004). Despite the fact that the molecular mechanism by which PACp expression is decreased in prostate cancer remains unknown, one strategy to exploit the tissue specificity of the PACp promoter for anti-tumor therapy is to use an expression vector that encodes a cytotoxic protein under the transcriptional control of the PACp promoter element, for example, p1356. This fragment is active even in advanced prostate cancer cells and can drive the expression of the cytotoxic protein in a prostate-specific manner. Alternatively, a mini-gene construct with PACp cDNA under the transcriptional control of the p1356 fragment may be used to inhibit the growth of prostate cancer cells with minimal side effects. However, further *in vitro* experiments and xenograft animal studies are needed to determine the efficacy of those elements of the PACp promoter prior to their application for prostate cancer therapy in human patients.

Another avenue for the therapeutic application of PACp under clinical conditions is the utilization of the immunogenic property of the PACp protein in prostate cancer patients. This is supported by the findings of active immunization studies for prostate cancer based on prostatic antigens such as PSA and PACp (McNeel *et al.* 2001; for review, see Fong & Small 2003). Patients with metastatic prostate cancer responded to the immunization of dendritic cells pulsed with PACp protein with more than 50% of the patients developing an immune response (Fong *et al.* 2001). Phase 1 and phase 2 trials with dendritic cell-based PACp vaccination for androgen-independent prostate cancer led to a greater than 50% decrease in PSA (Small *et al.* 2000, Burch *et al.* 2004). Further clinical trials on a larger scale are required to assess the efficacy of PACp-based vaccination. Studies are also needed for the potential use of the cancer-associated PACp isolated from prostate cancer patients as an immunogen designed to improve the efficacy of prostate cancer immunotherapy.

## Conclusion and perspectives

The PTP activity of cPACp, which is predominant in well-differentiated secretory epithelial cells of prostate gland, down-regulates HER-2-mediated growth signals. The decrease in cPACp expression during prostate

carcinogenesis and progression results in uncontrolled tyrosine phosphorylation of HER-2, which can lead to increased hormone-refractory growth of prostate cancer cells. This notion is further validated by the observations that the prostate glands of PACp knock-out mice developed PIN lesions that subsequently progressed into invasive adenocarcinomas (Vihko *et al.* 2005). The decreased expression of cPACp is thus implicated as a crucial step of carcinogenesis and/or the progression of human prostate cancer towards androgen independence. Additionally, the polymorphisms in the PACp gene, heterogeneity of the mRNA and the biochemical differences of the protein in prostate cancer versus normal prostate epithelial cells may lead to differences in substrate specificity and kinetics of the enzyme (Wishart *et al.* 1995, Cirri *et al.* 1996), which have led to the hypothesis of possible linkage of PACp isoforms with prostate carcinogenesis. Future studies, however, are required to determine whether these differences could serve as a surrogate marker to distinguish between indolent and aggressive prostate cancers. The recent discovery that vitamin D suppresses prostate cancer cell growth and increases cPACp expression lends further support to the need to identify novel inducers of cPACp expression in the chemoprevention and treatment of prostate cancer. More studies on the basic biochemistry and molecular biology of PACp should provide new insights into its role in prostate cancer suppression and therapy.

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