

# PRL-3 as a target for cancer therapy

## Research Article

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**Abbreviations:** phosphatase of regenerating liver (PRL); protein tyrosine phosphatases (PTPs); Chinese Hamster Ovary (CHO) cells; serial analysis of gene expression (SAGE) technology; human embryonic kidney fibroblasts HEK293; restriction enzyme mediated integration, (REMI)

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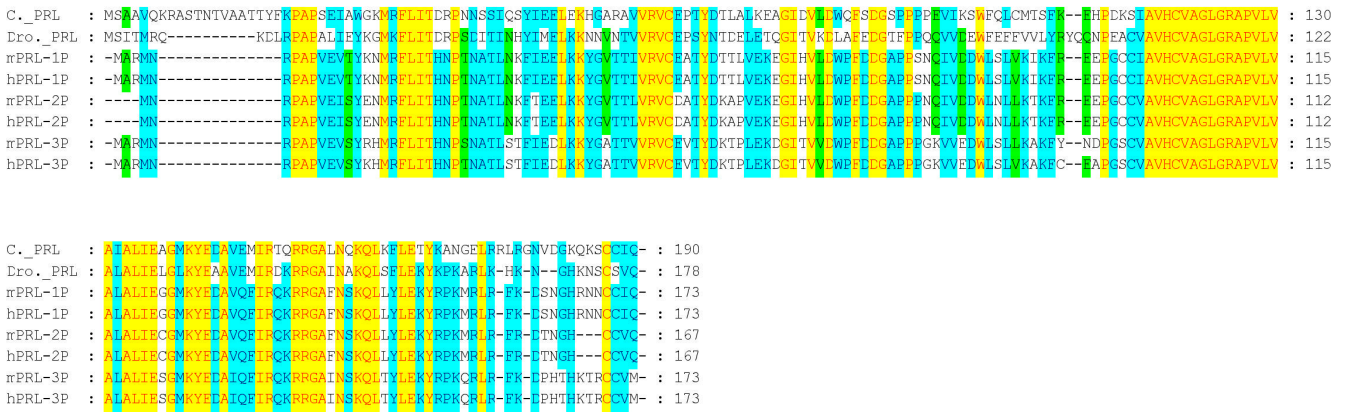
## Summary

A group of protein tyrosine phosphatases (PTPs), the PRL family, has been implicated in the contribution and progression of cancer metastasis. The PRL family consists of three members: PRL-1, -2 and -3. This small (20kD) class of prenylated protein tyrosine phosphatase contains a PTP signature motif (VHCXAGXXR) at their active sites and a catalytic domain, similar to dual specificity phosphatases. The three closely related PRLs share 76-87% identities in their amino acid sequences, with a unique C-terminal prenylation motif and with significant sequence homology to Cdc14p, a mitotic regulator, and PTEN/MMAC1, the tumor suppressor (Zeng et al, 1998a). PRL-1 was identified as an immediate-early gene which was induced in mitogen-stimulated cells and regenerating liver. PRL-3, along with PRL-2, was identified subsequently by using PRL-1 sequence to search mouse EST database. Recently, PRLs have been implicated in the process of oncogenic transformation and cancer metastasis. We suggest that PRLs might be important modulators in the process of cancer metastasis and are therefore potential targets for therapeutic intervention of cancer.

## I. Introduction

PRL proteins are conserved from human to *C. elegans* (Figure 1). We suggested that they have common critical physiological functions, but each might have its own distinct target substrates. PRL-1 is expressed at high levels in proliferating cells and a number of human tumor cell lines, including HeLa (Diamond et al, 1994; Wang et al, 2002). Overexpression of PRL-1 and PRL-2 in epithelial cells results in a transformed phenotype in culture and tumor growth in nude mice (Cates et al, 1996; Zeng et al, 2003). By PCR-based subtractive hybridization, PRL-2 was shown to be upregulated 1.8, 2.7 and 4-fold in advanced prostate cancer cell lines LNCaP, PC-3 and DU-145 respectively, in comparison with normal epithelial cells. The data suggest that PRL-2 is associated with prostate tumor progression (Wang et al, 2001). Recently, by using serial analysis of gene expression (SAGE) technology, researchers (Bert Vogelstein and his colleagues) at the Johns Hopkins Medical Institutes reported that among 144 upregulated genes detected in metastatic colorectal liver samples, PRL-3 was the only gene specifically overexpressed in all 18 metastatic colorectal cancers examined (Saha et al, 2001). We found

that prenylated PRL-1 and -3 are enriched on the plasma membrane (Zeng et al, 2000, 2003). Overexpression of PRL-1 and -3 in Chinese Hamster Ovary (CHO) cells promotes cell migration, invasion and metastasis. Moreover, PRL-1 and -3 overexpressing CHO cells are capable of inducing metastatic tumor formation in nude mice (Zeng et al, 2003). Overexpression of PRL-3 in human embryonic kidney fibroblasts HEK293 cells has also been found to enhance growth rates versus non-transfected cells (Matter et al, 2001). Taken together, these data suggest that the PRL-1, -2 and -3 might be associated with cancer metastasis and act as major players in oncogenic and metastatic processes. How does PRL-3 spur colon cancer metastasis? How might PRLs be involved in the pathways of signal transduction related to cancer development and metastasis? Much remains to be learned from these striking discoveries to answers. Here, we suggest that PRL-3 might serve as one of the important markers to track the events leading to colon cancer metastasis. We speculate that an excess of PRL phosphatases activity could bring about key alterations that would contribute to the acquisition of metastatic properties in tumor cells. PRLs may therefore be potential targets for new cancer therapeutic strategies.



**Figure 1.** Alignment of amino acid sequence of *C. elegans* PRL (C\_PRL), *Drosophila* PRL (Dro.\_PRL), mouse PRLs (mPRL-1, mPRL-2 and mPRL-3) and human PRLs (hPRL-1, hPRL-2 and hPRL-3) with GENE Doc. Program. Identical residues among all these PRLs are shaded in yellow.

## II. Materials and methods

### A. Establishment of a stable cell line expressing myc-PRL-3 and a stable cell pool expressing EGFP-PRL-3

The pStar-myc-PRL-3 and pEGFP-PRL-3 plasmids were constructed and described in our early studies (Zeng et al, 2000 and 2003). CHO-K1 cell line was obtained from the American Type Culture Collection (Manassas, VA) to generate CHO cell line stably expressing PRL-3 (clone 36) in pStar vector (Zeng et al, 1998b) and to generate CHO stable pooled cells expressing PRL-3 in pEGFP-C1 vector (Clontech: <http://www.clontech.com/index.shtml>). Briefly, the cells were transfected with the pStar-Myc-PRL-3 or pEGFP-PRL-3 respectively, cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and selected in 1mg/ml of G418 to establish a stable clonal cell line for pStar-myc-PRL-3 or stable pooled transfectants for pEGFP-PRL-3.

### B. Experimental metastasis and immunoperoxidase

10-week old female nude mice (Jackson Labs, USA) were each injected via the tail vein with myc-PRL-3 expressing cells ( $5 \times 10^5$ ). Mice were sacrificed 25 days after the tail vein injection and all tissues were examined for metastasis. Lungs with metastasis were performed with immunoperoxidase labeling by using a Vectastain ABC kit from Vector Laboratories (Burlingame, CA) according to the procedure provided by the supplier. The c-Myc antibody (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA).

### C. Confocal microscopy

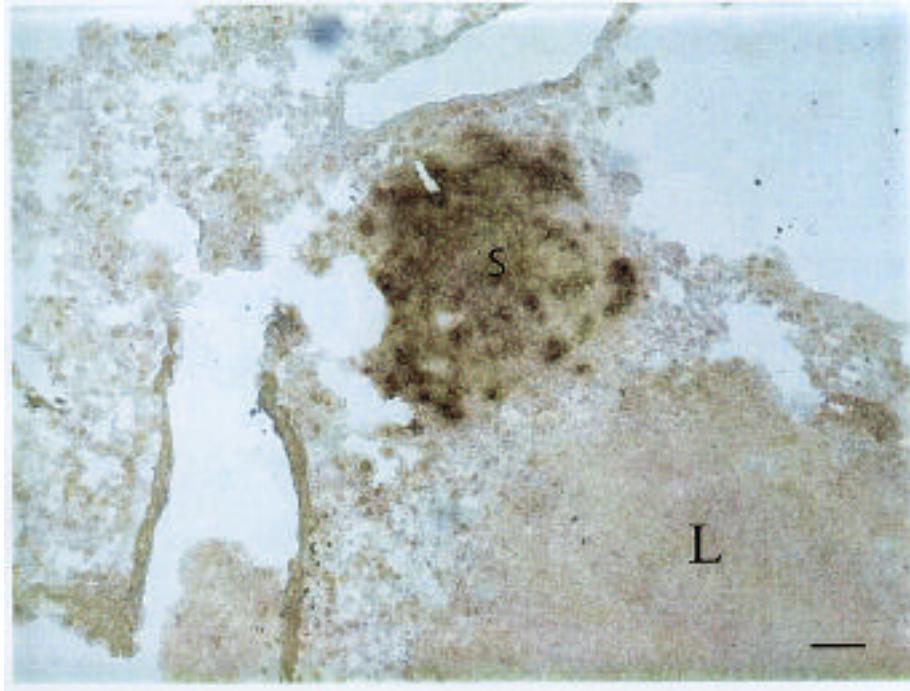
Cells stably expressing EGFP-PRL-3 were seeded onto glass coverslips and grew for 24 hours. Cells were washed twice with PBSCM (PBS containing 1mM of  $MgCl_2$  and 1mM of  $CaCl_2$ ) and then fixed in 3% paraformaldehyde for 20 min at room temperature. After three more washes with PBSCM, cells were mounted onto a glass slide with one drop of anti-fade reagent in PBS glycerol (Biomedica Corp, Foster City, CA), and kept at 4°C in the dark until analysis. Confocal imaging was performed using a laser scanning head (MRC 1024, Bio-Rad Laboratories, GB).

### D. Production and analysis of transgenic tadpoles

A modified restriction enzyme mediated integration (REMI) method was used to generate transgenic *Xenopus laevis* tadpoles (Duncan B et al, 2000). Briefly, 2.5  $\mu$ l containing 100-250 ng of the construct was incubated with 2.5  $\mu$ l containing  $1 \times 10^5$  /  $\mu$ l of sperm nuclei for 10 min at room temperature. The incubation mixture was then diluted to a concentration of one sperm per 10-12  $\mu$ l and injected into de-jellied eggs using constant flow injector (Harvard Apparatus PHD 2000 Infusion) at a flow rate of 0.7  $\mu$ l/min. Normal-looking embryos were collected and incubated. The tadpoles were then screened for the expression of EGFP. Pictures were taken by a digital camera (Nikon Coolpix 995) attached to a fluorescent microscope (Zeiss, M2 Bio Quad).

## III. PRL-3 might help tumor formation in foreign territories

Metastasis is a complex process involving many changes in cell/tissue physiology and gene expression. The major events in the process of metastasis include the ability of tumor cells to leave their sites of primary tumor and enter the circulation to extravagate into a new tissue, begin and maintain growth in this tissue to form preangiogenic micrometastases, and then develop a blood supply to enable the formation of macroscopic tumors (Weiss, 2000; Chambers et al, 2002; Takeda et al, 2002). It is metastasis that defeats oncologists to cure their patients. What invokes tumor cells to dissociate from the primary tumor and migrate to distant tissues is largely unclear. Overexpression of PRL-3 has been found in 100% of 18 colorectal cancer liver metastases examined (Saha et al, 2001). To carry out this process in vivo and to study the role of PRL-3 in the pathways of metastasis, cells from a stable CHO clone overexpressing of PRL-3 were injected into the tail veins of 10-week-old female nude mice, thus introducing these cells directly into circulatory blood system of the animals. We found rapid metastasis in our animal experimental metastasis model (Zeng et al, 2003). The tumor was performed with fresh frozen sections and checked for PRL-3 protein expression level by

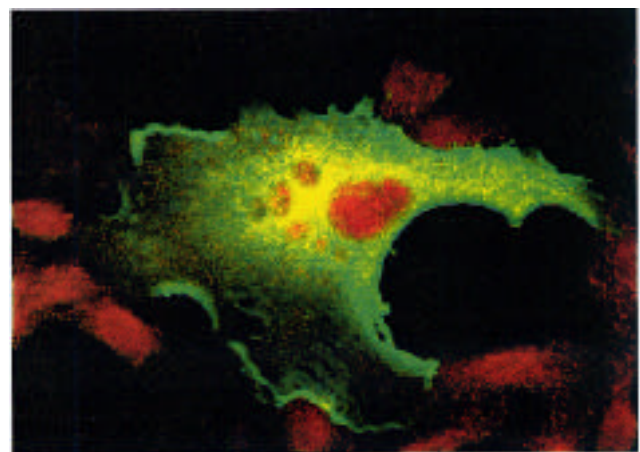


**Figure 2.** Expression of PRL-3 protein in small tumor area (S) but not in large tumor area (L). A cryosection of lung tissue was obtained from 13-week-old nude mouse after 25 days injection with clonal Chinese Hamster Ovary cells overexpression of myc-PRL-3 by the tail vein. The section was precessed for immunoperoxidase labeling using c-Myc antibody (9E10) which was from Santa Cruz Biotechnology (Santa Cruz, CA). Bar represents 20  $\mu$ m.

immunohistochemistry method (ABC). Interestingly, in most cases, PRL-3 was detected in small tumors (**Figure 2**) but not in large tumors. The process of metastasis by overexpression of PRL-3 likely does not require any further inputs from its upstream. These results give us clues that PRL-3 might act as an initiator for the tumor implantation (or perhaps for blood vessel formation) in foreign territory and then pass the job to its downstream effectors.

#### **IV. PRL-3 might have a role in cardiac hypertrophy**

Human PRL-3 has been reported to play a physiological role of preventing the tyrosine phosphorylation of p130<sup>cas</sup> and has an effect on the mobilizing of intracellular calcium in response to Angiotensin-II in HEK 293 cells (Matter et al, 2001). The PRL-3 was preferentially expressed in adult and fetal hearts at all parts except aorta (Li and Zeng, unpublished data). Transgenic mice overexpressing PRL-3 in the heart show overt cardiac hypertrophy and reduced cardiac function associated with impaired calcium handling (Kadambi et al, 2000). Cardiac hypertrophy involves an increase in size and mass of individual cardiomyocytes without an increase in cell number. We discovered that overexpression of the PRL-3 in CHO cells caused dramatically enlargement in sizes of some transfected cells (**Figure 3**). The studies suggest that PRL-3 may have a physiological role in maintaining normal functions of the heart. Overexpression of the PRL-3 might be involved in the progression of cardiac hypertrophy.



**Figure 3.** A giant cell overexpressing the EGFP-PRL-3. A CHO cell overexpression of PRL-3 dramatically enlarged in size. Bar, 20  $\mu$ m

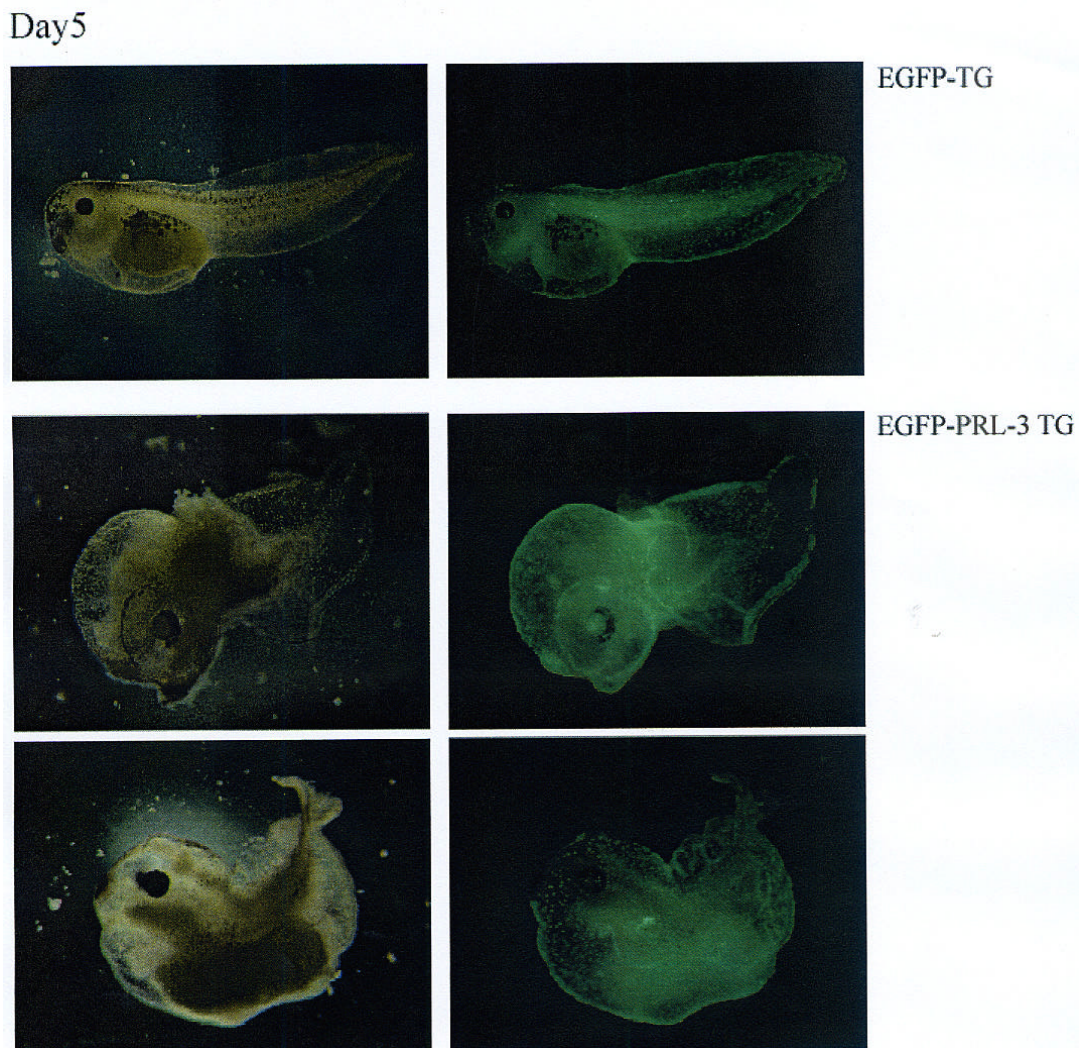
## V. PRL-3 might play an essential role in embryonic development

The protein-tyrosine phosphatases (PTP) superfamily consists of a large group of enzymes that play critical roles in the regulation of cellular growth and differentiation. The balance between phosphorylation and dephosphorylation is a major regulatory mechanism affecting the functions of diverse proteins that participate in many aspects of cellular, physiological, pathogenic processes and embryogenesis. In order to have a quick examination of the possible functions of PRL-3 during embryonic development, we generated transgenic frog embryos overexpressing EGFP-PRL-3 and compared them with control embryos overexpressing EGFP to investigate the outcomes of the animals. *Xenopus* embryos are excellent for analyzing defects in early development (Amaya et al, 1999; Duncan et al, 2000). Overexpression of EGFP-PRL-3 in transparent *Xenopus* embryos allows us to easily trace the green fluorescent protein marker with better spatial and temporal control. A large amount of embryos can be easily monitored and manipulated at the same time. Statistic data can be obtained shortly.

Disturbances in PRL-3 expression in transgenic *Xenopus* embryos showed severe and diverse defects during embryonic development at very early stages. The examples were showed at day 5 (Figure 4). The poor embryonic survival rate of the transgenic animals is due to their death, abnormal growth and retardation. From day 1 to day 5, the total survival rate of the transgenic EGFP-PRL-3 is 8% which is 7.5 times lower than control transgenic EGFP (53%). Our preliminary results therefore support a major physiological role for the PRL-3 during embryogenesis.

## VI. Summary and future perspectives

In summary, PRL-3 has been so far the only phosphatase linked to colorectal cancer metastasis (Saha et al, 2001). Whether PRL-3, perhaps PRL-1 and -2, is also involved in other types of cancer and how these PRLs initiate and maintain the process of metastasis remain as important questions to be solved. The reason that makes scientists believe PRL-3 gene might provide a new therapeutic target for colorectal cancer is that



**Figure 4.** Overexpression of the EGFP-PRL-3 disrupts *Xenopus* embryogenesis at early stages. The EGFP-PRL-3 positive embryos were examined and compared with EGFP control positive embryos on day 5 (stages 41).

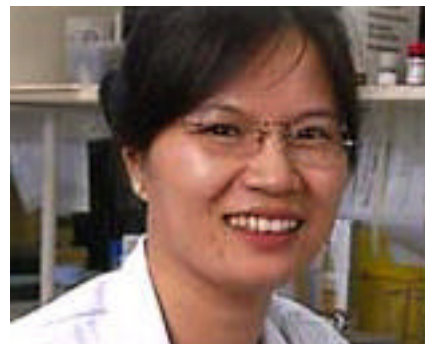
most of the previously described genetic alterations in colorectal cancers involve inactivation of tumor suppressor genes. It is difficult to target with drugs for inactive or absent genes in the cancers (Saha et al, 2001). In contrast, PRL-3 is elevated in cancer cells, which provides an excellent target for drug discovery purposes. By NMR resonance, the PRL-2 and -3 structures have been investigated (Kozlov et al, 2002; Zhou et al, 2003). Understanding of its structure, especially the catalytic domain, may help us to search specific inhibitors for PRL-3 in the treatment of the colorectal metastases. We studied a catalytically inactive PRL-3 (C104S) mutant and found that it reduced effect on promoting cell migration (Zeng et al, 2003). Here, we suggest that the consensus phosphatase motif might potentially be a therapeutic target. We also suggest that specific antibodies against each PRL are needed to distinguish the expression and investigate the individual roles of these closely related PTPs, which in turn may lead to new insights into cancer metastasis. Such antibodies may also provide a platform for novel diagnostic, prognostic or therapeutic approaches. The functions of the PRLs-PTP family in cancer metastasis undoubtedly cannot be ignored.

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