# CHAPTER III

## PROTEINS DRIVING DEVELOPMENT ALSO DRIVE TUMORIGENESIS

Multiple molecular events control prostate cancer initiation, growth, invasion and metastasis. In spite of the prevalence of PCa, our understanding of the genetic alterations occurring during these processes is limited. In normal mouse prostate development, prostatic bud commitment occurs at embryonic day 16, when signaling centers develop and subsequently invade the mesenchymal pad and induce epithelial cell elements (117, 118). At birth, prostatic buds are rudimentary and morphogenesis of these structures occurs primarily in the postnatal period from 1 to 5 weeks (wks) of age when outgrowth ceases and maturation of the prostate gland is complete (117, 118). At 6 wks of age, the animal is sexually mature and mitotic cells are typically not observed in the growth quiescent adult prostate (117, 118). Numerous prostate cancer models have been generated by disrupting the expression of a gene of interest in transgenic mice (119). Typically, epithelial cells proliferate and develop into lesions ranging from hyperplasia to low- and high-grade intraepithelial neoplasia (LGPIN and HGPIN, respectively), locally invasive carcinoma and/or metastases. Reactive stromal proliferation is also observed (119).

Several developmentally regulated proteins have been implicated in the tumorigenic process. For example, the Nkx3.1 homeobox gene is an early marker of prostate epithelium during embryogenesis and is expressed at all stages of prostate

differentiation (120, 121). The adult prostate continues to express Nkx3.1 and loss of expression promotes prostatic epithelial hyperplasia and dysplasia, which increase in severity with age (120, 121). Notch 1, another developmental protein, is expressed more highly in early postnatal than in adult prostate and is required during branching morphogenesis, growth and differentiation (122, 123). Inactivation of Notch enhances proliferation and impairs differentiation of prostatic epithelial cells. Down-regulation of Notch1 and Hey-1 expression is also observed in human prostate adenocarcinoma samples (122, 123). Sonic hedgehog (Shh) is expressed in developing prostatic epithelium and activates Gli-1 expression in adjacent mesenchyme to promote branching morphogenesis (124). Shh signaling is not restricted to development, being detected in normal and prostate cancer tissues. In the LNCaP xenograft tumor model, Fan and coworkers demonstrated that overexpression of Shh in LNCaP cells increased Gli-1 expression in adjacent tumor stroma and promoted tumor growth (124).

In the present study, we used two-dimensional gel electrophoresis to identify proteins, which may promote PCa development. This proteomics approach allowed us to compare protein expression profiles in control CD-1 developing and adult prostates with those in 12T-7f prostate tumors. The underlying hypothesis of this approach was that proteins, which regulate normal development of prostate, are absent in growth quiescent adult prostate but are re-expressed during tumorigenesis when the prostate is proliferating at a rapid rate. Earlier, we had generated the LPB-Tag transgenic model by using a 10.8 kb rat probasin promoter region to target SV40 large T antigen (small t antigen is deleted) to the mouse prostate (125, 126). The 12T-7f prostate tumors develop in parallel with

sexual maturation, since large T antigen gene is under control of the androgen-regulated probasin promoter, and tumors continue to exhibit a high proliferative index, progressing from Low Grade Prostatic Intraepithelial Neoplasia (LGPIN) to high grade PIN (HGPIN); however, metastasis is rarely seen (126, 127). Using this model, we identified the phosphoprotein stathmin.

**Stathmin expression is up-regulated during prostate morphogenesis and tumorigenesis**

In the present study, we used two-dimensional gel electrophoresis to identify proteins which may be involved in promoting PCa development. Our approach was to compare periods in which prostate growth is highest to those in which the prostate is growth quiescent. Since normal prostate development continues postnatally up to 5 wks of age, we selected the 3 wk normal CD-1 prostates as representative of active prostatic development. Fifteen wk CD-1 prostates were chosen to represent mature growth quiescent glands since few, if any, proliferating cells can be detected. The LPB-Tag line 12T-7f mouse PCa model was selected since these tumors undergo rapid proliferation and develop PIN with limited localized adenocarcinoma lesions. Protein lysates were prepared from 3 and 15 wk CD-1 dorsal prostate lobes and compared with those from 15 wk 12T-7f tumors. One hundred micrograms protein lysate from 3 and 15 wk CD-1 and 15 wk 12T-7f tumor tissue were labeled with Cy2, Cy3 and Cy5 respectively, mixed and proteins separated by isoelectric point in the first dimension and molecular weight in the
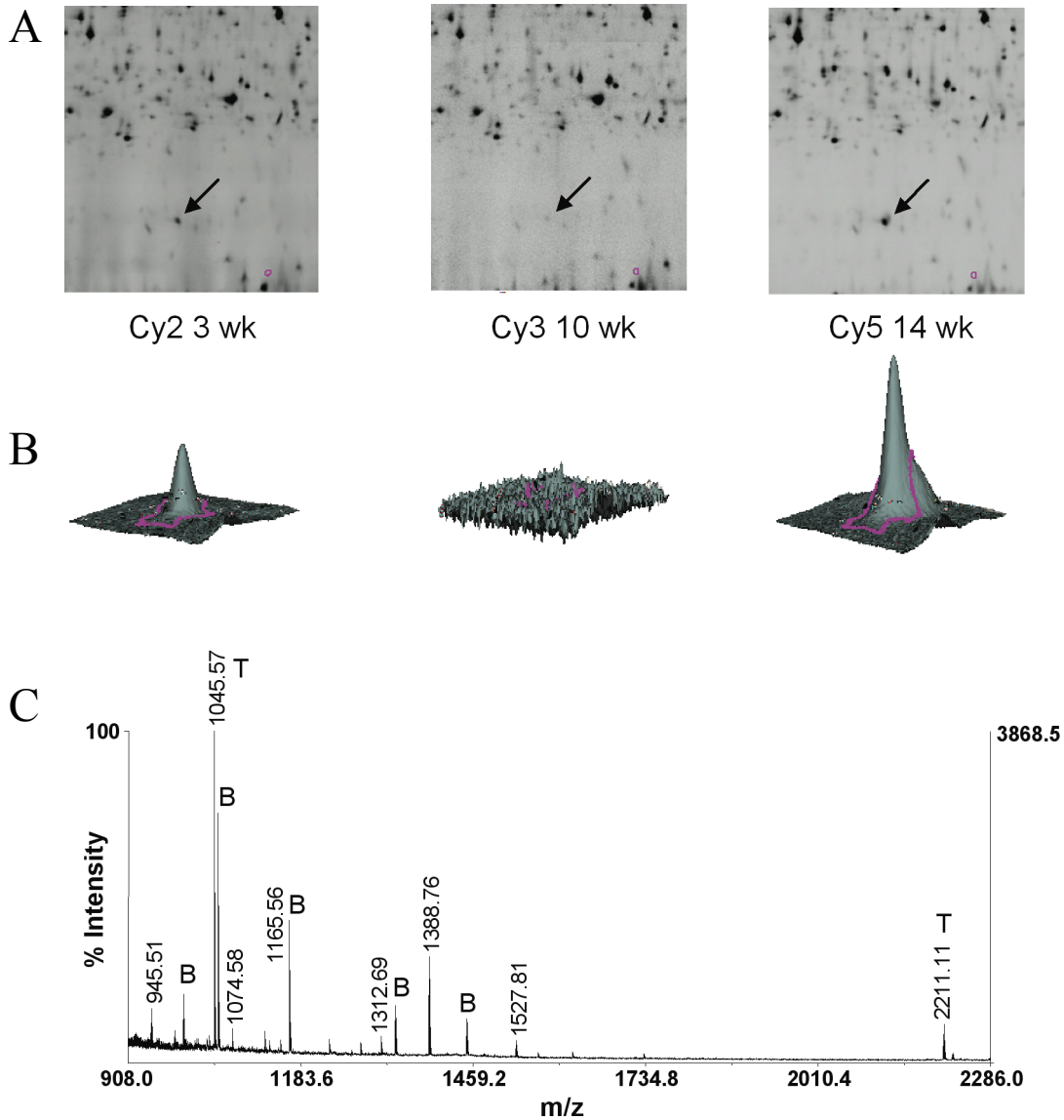
**Figure 9: Identification of Stathmin in normal prostate and prostate tumor development. A.** Two-Dimensional Gel Electrophoresis and Mass Spectrometry of normal mouse prostate compared to 12T-7f prostate tumors. Protein was extracted from dorsal prostate lobes representing the developing normal prostate (3 wk, left panel), the growth quiescent normal prostate (15 wk, center panel) and the progressing tumor (15 wk, right panel). One hundred micrograms of protein from each tissue was labeled with Cy2, Cy3 and Cy5 respectively, mixed and co-resolved by pH in the first dimension and molecular weight in the second dimension. The gel was analyzed under the respective wavelengths and spots selected that represent proteins which are unregulated in the developing normal prostate and in the 12T-7f prostate tumors. The arrow indicates the protein spot-of-interest that was determined to be stathmin. **B.** Graphical representation of stathmin protein levels. Statmin levels in the 3 wk developing prostate increased 2.66-fold compared to the growth quiescent prostate. Stathmin expression in 15 week 12T-7f prostate tumors is elevated 2.31-fold and 5.74-fold compared to normal 3 wk developing and 10 wk adult prostates respectively. **C.** Protein profile of the tryptic peptides. The protein spot representing stathmin was excised, digested with trypsin as described in the Materials and Methods section and separated by MALDI-TOF MS. The tryptic peptides corresponded to stathmin.

second dimension. The three dyes facilitated: a) visualization of the three individual proteins under their respective wavelengths and b) selection of protein spots differentially over- or under-expressed in the developing normal prostate (3 wk CD-1) and prostate tumors (15 wk 12T-7f) compared to those in the 15 wk growth quiescent adult CD-1 prostate gland. Spots corresponding to these criteria were excised, treated with trypsin, and the tryptic digest analyzed by MALDI-TOF MS. The protein stathmin was identified using this approach (Fig. 9A). Stathmin expression increased 2.66-fold in the developing CD-1 prostate compared to the adult prostate. Stathmin levels were further up-regulated in 12T-7f prostate tumors compared to the CD-1 developing and adult mouse prostate (2.31-fold and 5.74-fold respectively, Fig. 9B). Therefore, stathmin expression followed the anticipated pattern of a developmentally regulated protein whose expression was upregulated in tumor progression. The ion signals at $m/z$ = 945.51, 1074.58, 1165.56, 1312.69, 1388.76 and 1527.81 identified stathmin using a peptide mass mapping strategy that yielded statistically significant search scores and 36% protein coverage (Fig. 9C). Predicted MW and isoelectric point were consistent with the gel region.

Stathmin expression at these time points was further analyzed by IHC to identify the prostatic cells, which express stathmin. Tissue sections from 3 and 15 wk CD-1 prostates and 15 wk 12T-7f prostate tumors were stained using anti-stathmin antibody and analyzed by light microscopy. IHC analysis determined that stathmin is only expressed in luminal epithelial cells and that as expected, the highest levels of expression occur in the 3 wk CD-1 prostate and in 12T-7f tumors (Fig. 10A). Western blot analysis was performed by separating 20μg protein from these samples on 12% SDS-PAGE and

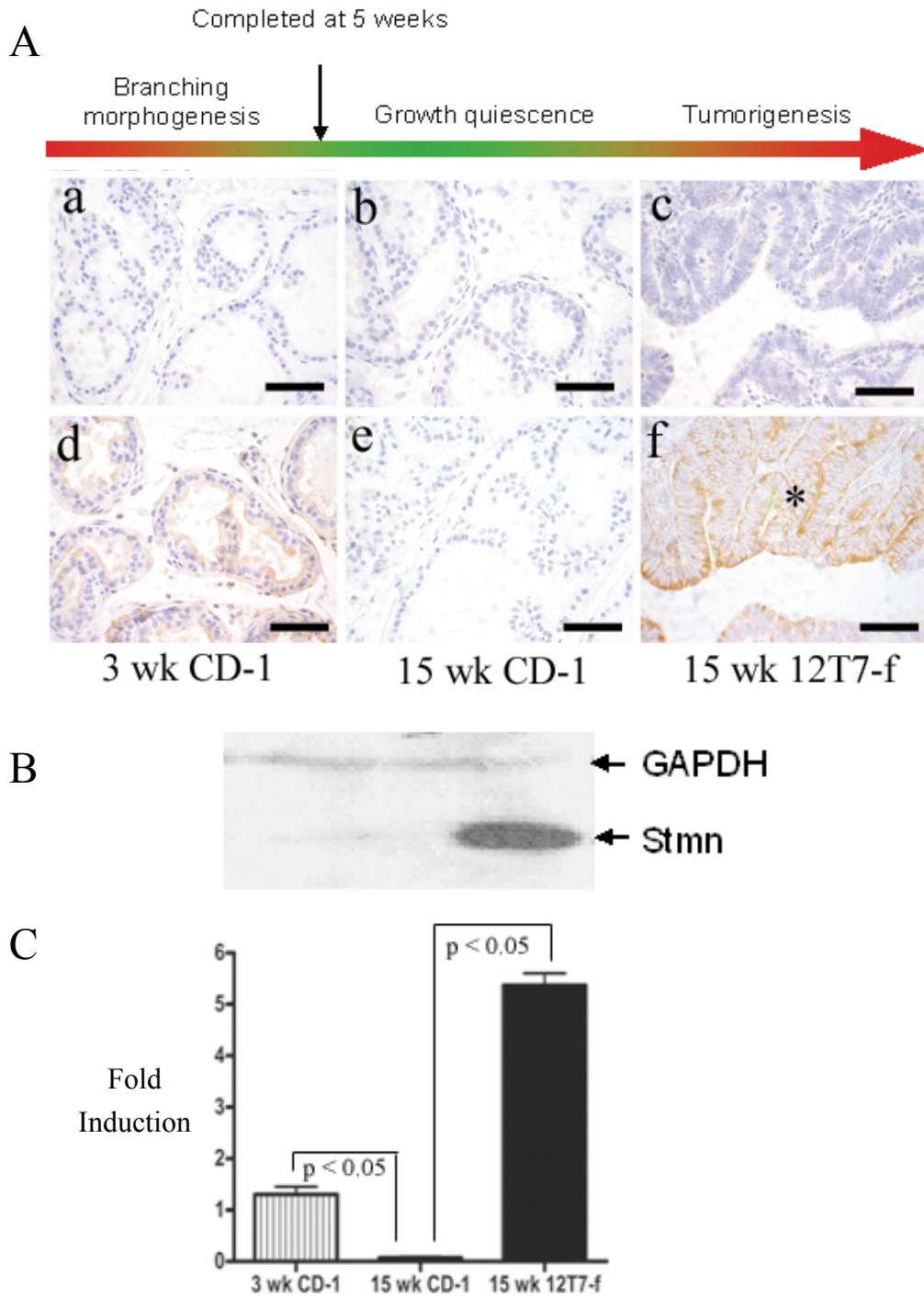**Figure 10: Immunohistochemical (IHC) analysis of Stathmin expression in normal prostate and 12T-7f prostate tumors. A.** IHC Comparison of stathmin expression in CD-1 normal 3 wk developing and 15 week adult prostate with 15 wk 12T-7f prostate tumors. Top Panel: Paraffin embedded sections were probed with HRP-conjugated anti-rabbit IgG as negative control. Scale: 50μm. Lower Panel: Paraffin embedded sections were probed with rabbit anti-stathmin primary antibody and HRP-conjugated anti-rabbit IgG to detect stathmin expression. * indicates PIN lesions. Scale: 50μm. **B.** Western Blot analysis. Protein extracts of the three tissues described in Panel A were separated by 12% SDS-PAGE, transferred to PVDF membrane and probed for stathmin expression using rabbit anti-stathmin primary antibody. **C.** Densitometric analysis of the Western blot presented in Panel B. Densitometry was performed and data was analyzed using ImageJ software. Stathmin expression was normalized to GAPDH. This graph summarizes the data of 4 individual Western blot analyses.

probing the resulting immunoblot with anti-stathmin antibody. Again, stathmin expression was greatest in the 3 wk CD-1 prostate and in 12T-7f tumors (Fig. 10B). Densitometric analysis indicated that stathmin levels in the 3 wk CD-1 prostate increased 2-fold ($p<0.05$) compared to the 15 wk CD-1 prostate and that stathmin expression in 12T-7f tumors increased ~18-fold ($p<0.05$) compared to the 15 wk CD-1 prostate (Fig. 10C). These observations confirm the expression pattern of stathmin generated by two-dimensional gel electrophoresis, namely that stathmin expression is elevated in the developing prostate and that this expression is even greater in 12T-7f tumors.

We subsequently performed real-time RT-PCR to determine the levels of stathmin expression during the continuum for prostatic development from 2 to 5 weeks of age and in the mature prostatic gland (6 and 10 wk of age). The numbers of prostates per time point are described in the Materials and Methods section and the time course for both CD-1 normal and 12T-7f prostate tumor samples are presented in Figure 11. In CD-1 prostates, stathmin expression was highest at 2 wks of age and declined steadily to 5 wks of age. This decrease correlated with completion of branching morphogenesis at 5 wks of age. At 10 wks of age, stathmin expression was below detection limits of the assay, suggesting that high levels of stathmin expression were not required in the growth-quiescent prostate.

By comparison, stathmin expression in 12T-7f tumors was greater in all age groups tested. Stathmin expression decreased from 2 to 3 weeks but then unexpectedly increased 6-fold compared to that in the 3 wk 12T-7f prostate. This dramatic rise in

stathmin expression was statistically significant (p<0.001), since 3 sample groups

consisting of 100 dorsal prostatic lobes/group were analyzed at the 4 wk time point, and
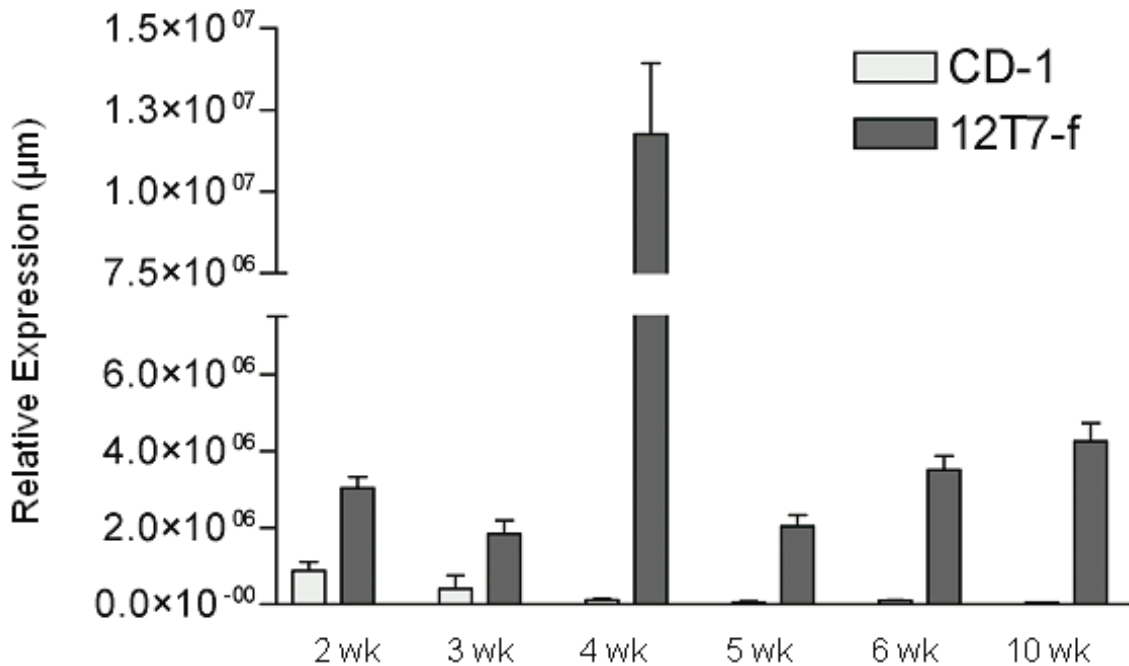


**Figure 11: Real Time RT-PCR analysis of stathmin expression in CD-1 normal and 12T-7f tumor prostates during development.** RNA was extracted from 2, 3, 4, 5, 6 and 10 week old prostates from CD-1 and 12T-7f mice and stathmin expression was quantified by Real Time RT-PCR. Number of mice per group and methodology are discussed in the Material and Methods section.

did not appear to correlate to known events in prostate development. At 5 wks, stathmin

levels decreased to those observed at 3 wks after which a second rise in stathmin

expression occurred and continued up to 10 wks of age (although not to the levels seen at

4 wks).

In summary, the expression pattern in normal CD-1 prostates is distinct from that

in 12T-7f tumors, being highest early on in prostatic development and decreasing to

nearly undetectable levels with age. In contrast, stathmin expression in 12T-7f prostates

appears biphasic with a dramatic increase 4 wks postnatally and a second lesser increase

prior to the completion of prostate maturation at 5 wks of age.