

CHAPTER VII

CONCLUDING REMARKS AND FUTURE DIRECTION

Stathmin in Prostate Cancer Development and Progression

Androgen deprivation therapy is the most used treatment of *de novo* or recurrent metastatic PCa. Unfortunately, patients with advanced metastatic disease develop androgen-independent or hormone-refractory PCa and will ultimately succumb to their disease. A number of genetic and epigenetic mechanisms have been implicated in this process including activation of AR by amplification, mutations or growth factors, over-expression of oncoproteins, or emergence of androgen-independent cell populations (potentially from prostatic stem cells) that confer resistance to androgen deprivation therapy (140). We discovered that stathmin expression was elevated in normal developing mouse prostates and 12T-7f tumors compared to growth quiescent adult prostates (Figs. 9-11). The pattern of this expression was similar to that of proteins such as Nkx3.1, Shh and Notch1 which have been implicated in the development of PCa. In PCa, Nkx3.1 and Notch1 expression are lost (120, 121). Shh expression however, is increased in PCa (120, 121). Increased stathmin expression was also detected in human PIN and adenocarcinoma (Fig. 13). Thus, the expression pattern of stathmin parallels that of developmental factors such as Shh and suggests that stathmin may exhibit functions similar to Shh in normal prostate development and PCa.

The ontogeny of stathmin expression during normal mouse prostate development

is significantly different from that of 12T-7f tumor development and progression. Stathmin expression is highest early during normal branching morphogenesis and declines steadily to barely detectable levels in the growth quiescent prostate (Fig. 11). Postnatally, the prostatic buds grow out through mesenchymal/epithelial interactions (177) until outgrowth ceases and the gland is fully developed at 5 weeks of age (117, 118). The increased levels of stathmin expression at 2 and 3 wks of age imply that it may play a role in during branching morphogenesis. In contrast, stathmin levels appeared elevated at all time points during tumor development. However, increase in these levels was biphasic, with an initial increase from 2 to 4 wks and a second lesser increase, from 5 to 10 wks of age. The mechanism for inducing the dramatic rise in stathmin levels at 4 wks is not clear. In murine embryo fibroblast Val5 cells, induction of p53 gene resulted in a 70-90% reduction in stathmin mRNA levels (178). Furthermore, p53 regulates stathmin expression by repressing stathmin promoter activity (179). Thus, it is conceivable that loss of p53 activity or decreased p53 expression during development upregulate stathmin expression and thereby promote cell cycle progression. Similarly, increased stathmin expression in Gleason 5 tumors would correlate with loss of functional p53, an important but relatively late event in prostate cancer progression.

The probasin promoter is regulated by androgens and therefore probasin-driven transgene expression is up-regulated early in prostatic development. In ARR₂PB-Cre mice, androgen-regulated Cre expression in prostate is detected as early as 2 weeks of age (180). In the same way, chloramphenicol acetyl transferase (CAT) expression occurs as early as 2 weeks of age and continues to increase in parallel with sexual maturation in

the LPB-CAT model (181). Expression of prostatic large T antigen (Tag) is also under control of the probasin promoter and results in the sequestration of p53 and Rb (125). Thus, the spike in stathmin expression at 4 weeks may coincide with the loss of functional p53 through binding to the Tag protein. A p53-independent mechanism may also, in part, regulate stathmin expression since NeoTag2 cells express 1.5-fold more stathmin than NeoTag1 cells although both cell lines have similar Tag expression levels (139).

Friedrich et al. initially reported that stathmin expression increases in poorly differentiated human PCa (182). We performed a more extensive analysis, utilizing a tissue microarray containing tissue cores exhibiting histological changes from Gleason pattern 3 to 5, including benign tissue controls from BPH specimens. Stathmin expression is similar during Gleason patterns 3 and 4, but a significant increase in stathmin levels occurs in Gleason pattern 5 (Fig. 14), suggesting that stathmin expression parallels the development of advanced adenocarcinoma in human PCa. A similar increase is observed in NeoTag2 recombinant tumors where the development of adenocarcinoma is greater than that in NeoTag1 recombinant tumors (Fig. 12). Thus, this *in vivo* model could be utilized to study the mechanisms by which stathmin influences PCa progression to advanced adenocarcinoma.

Stathmin expression was also differentially localized in BPH, PIN and adenocarcinoma. In BPH, stathmin expression occurs in the basal epithelial cell layer whereas in PIN, stathmin was present in both basal and luminal epithelial cells. In

adenocarcinoma, basal cells are absent, but the epithelial cells still stain strongly for stathmin (Fig. 13). These data suggest that stathmin localization to epithelial cells and elevated expression levels represents a more advanced phenotype. Other proteins involved in PCa progression show similar patterns of expression. Matrix metalloproteinase 2 expression has been associated with tumor aggressiveness in prostate cancer. Membrane type 1-matrix metalloproteinase (MT1-MMP), an activator of latent MMP-2 (pro-MMP-2), is differentially localized to basal epithelial cells in benign prostatic glands, whereas secretory luminal epithelial cells are rarely positive. Conversely, luminal epithelial cells show cytoplasmic MT1-MMP staining in HGPIN (183). The mitogen FGF-2 is produced by stromal cells and promotes normal epithelium cell growth. However, in human PCa, epithelial cells acquire the autocrine expression of FGF-2 which may further stimulate cancer cell proliferation, enhance cell motility, and angiogenesis of primary and metastatic cancers (184). Thus, the re-localization of stathmin to secretory epithelial cells may promote tumor aggressiveness and metastasis in PCa progression.

Several studies have indicated that stathmin promotes cell growth and tumorigenesis. Loss of stathmin expression in K562 leukemic cells abrogates anchorage - independent cell growth and causes growth arrest (108). Similarly, loss of stathmin expression in LNCaP cells have been reported to cause growth inhibition, cell cycle arrest at the G₂-M phase, increased apoptosis and decreased clonogenicity (109). *In vivo*, antisense inhibition of stathmin has been shown to result in inhibition of tumorigenicity of leukemic cells (108).

Stathmin promotes microtubule destabilization by complexing with two molecules of dimeric $\alpha\beta$ -tubulin (101). Phosphorylation of Ser16 and Ser63 reduces tubulin binding and inhibits stathmin-induced destabilization of microtubules *in vitro* (185). Thus, it is possible that in prostate cancer cells, differential Ser16 and Ser63 phosphorylation influence microtubule reorganization and differentially regulate protein kinase activity in response to androgen or antiandrogen treatment.

The anti-microtubule drug Taxotere inhibits microtubule assembly and blocks cell cycle progression similar to that seen with Taxol treatment. It has been used as chemotherapeutic agents in breast, ovarian and PCa patients. Therefore, anti-stathmin strategies combined with anti-microtubule drugs may represent a potent anti-cancer strategy since both therapies target the same microtubule pathway. Furthermore, stathmin antisense molecules have been reported to sensitize K562 cells to Taxol treatment (110). Combinatorial strategies that target microtubule function and prevent tumor growth for treating PCa have not yet been evaluated in the clinic. Understanding the molecular mechanism leading to activation of stathmin expression would facilitate in designing specific combinatorial therapeutic strategies for the treatment of PCa.

Protein phosphorylation can be dysregulated in various pathological conditions and this can modulate key functions such as activity, localization, stability and conformation. Tyrosine phosphorylation of AR in LNCaP cells by Epidermal Growth Factor (EGF) regulates AR transcriptional activity (186). EGF-induced tyrosine phosphorylation of AR regulates nuclear localization of AR and promote AR-dependent

growth of C-81 prostate cancer cells in an androgen-deprived environment (186).

Phosphorylation of serine residues modulates stathmin function. We have demonstrated the serine residues of stathmin are differentially phosphorylated, with Ser63 being primarily phosphorylated in the androgen-sensitive LNCaP cell line and Ser16 being predominantly phosphorylated in androgen-independent PC-3 and DU145 cells (Fig. 15). This differential phosphorylation suggests that the PKA signaling pathway may be more important in androgen-dependent PCa whereas PAK1/ Ca²⁺/calmodulin dependent kinase may play a role in hormone resistant PCa. Interestingly, Ser16 phosphorylation in LNCaP cells increased following androgen or antiandrogen treatment and treatment with DHT and antiandrogen in combination increased Ser16 phosphorylation synergistically (Fig. 16). Up-regulation of Ser16 phosphorylation was also observed in androgen-independent PC-3 and DU145 in the absence of any hormonal treatment (Fig. 15), suggesting that this increase may be a mechanism by which androgen independence develops. Inhibition of stathmin phosphorylation has been reported to promote G₂/M arrest in leukemic K562 cells (132).

In summary, our study indicates that stathmin is localized to luminal secretory cells in PCa and that increased stathmin expression occurs in more advanced PCa. We also demonstrate that stathmin is differentially phosphorylated in androgen-sensitive and androgen-independent cell lines and this phosphorylation is modulated by androgen and anti-androgen treatment. It remains to be determined which pathways, alone or in combination, are activated by modulating stathmin phosphorylation status and whether

this confers survival advantages on cells by promoting cell cycle progression and the development of androgen independence.

Stathmin Regulates Epithelial Cell Homeostasis

We have identified stathmin as a regulator of Smad-independent TGF- β responses. This protein does not influence Smad2/3 phosphorylation. Instead, stathmin blocks p38MAPK phosphorylation, thereby promoting E-cadherin expression and juxta-membrane localization while suppressing vimentin expression. With TGF- β 1 treatment, p38MAPK is phosphorylated and stathmin expression is abrogated, resulting in decreased E-cadherin expression and translocation to the cytoplasm. Concomitantly, vimentin levels rise and EMT develops. However, blocking phosphorylation of p38MAPK in stathmin siRNA-transfected DU145 or expressing stathmin in stathmin-negative NMuMG cells prevents the emergence of EMT despite TGF- β 1 treatment. Interestingly, stathmin-deficient mice develop tubular fibrosis and renal failure (187), in keeping with the function of EMT in fibrosis. Thus stathmin appears to function as a gatekeeper in maintaining a normal epithelial cell phenotype.

In a previous study, DU145 cells are reported to exhibit actin stress fiber responses after 2 days of TGF- β 1 treatment. However, delocalization of E-cadherin or ZO-1 from the cell–cell junctions does not occur (188). Our study demonstrates that EMT is initiated on Day 3 of treatment and that EMT is complete with 7 days of TGF- β 1 treatment. Similar observations have been reported for KIM-2 and EpH-4 murine mammary epithelial cell lines where EMT is induced with 7 days of TGF- β 1 treatment

(189). Thus, certain events occur during this time period to culminate in the suppression of stathmin expression. Our data indicate that once stathmin expression is decreased or lost, EMT occurs within 18 to 24 h and responsiveness to TGF- β 1 is not required.

The ability of TGF- β to trigger various pathways raises the question of the identity of factors that exert control to prevent or promote EMT. The importance of Smad in EMT is illustrated in primary tubular epithelial cells derived from Smad3 knockout mice which fail to undergo EMT with TGF- β 1 treatment (162). It is well known that TGF- β signalling is involved in inhibiting the growth of normal cells. However, TGF- β may also contribute to tumour progression if cells acquire resistance to the growth-inhibitory effects of TGF- β (190). Treating EpH4 mouse mammary epithelial cells with low doses of TGF-beta cause growth arrest, and the cells maintain their epithelial characteristics. The same treatment causes EMT and invasive growth in Ras transformed EpH4 cells by TGF-beta. After this conversion, the Ras-transformed cells progressively acquire a fibroblastic invasive phenotype that correlates with autocrine TGF- β signaling (191). MAPK activation by oncogenic Ras is essential for EMT and metastasis in this system. Another effector of Ras, PI3 kinase, seems primarily to inhibit induction of apoptosis by TGF- β (192).

TGF- β can also induce epithelial to mesenchymal transdifferentiation (EMT) in mammary epithelial NMuMG cells. In this model, the SMAD and AKT pathways were not essential for EMT, but activation of p38MAPK in cooperation with integrin signalling (193) and activation of the small GTPase, RhoA, or its downstream target

p160^{ROCK}, seemed to be much more important (194). Sustained activation of Raf alone in MDCK cells induces EMT and Raf promoted invasive growth in collagen gels by means of the autocrine production of TGF- β . In this model, activation of Raf led to inhibition of the ability of TGF- β to induce apoptosis, but enhances the positive effects of this molecule on invasiveness (190). *In vivo* mouse models of skin carcinogenesis have also shown that TGF- β has biphasic action during multistage skin carcinogenesis, acting early as a tumor suppressor but later enhancing the malignant phenotype, leading to the formation of spindle-cell carcinoma, the most aggressive form of skin cancer (195). Constitutive activation of c-fos estrogen receptor (FosER) oncoprotein induces EMT in fully polarized mouse mammary epithelial cells (EpH4) (196). This involves loss of E-cadherin expression, nuclear translocation of beta-catenin, and autocrine production of TGFbeta (197). The loss of E-cadherin can contribute to increased LEF/TCF-beta-catenin signalling, which in turn cooperates with autocrine TGFbeta signalling to maintain an undifferentiated mesenchymal phenotype (198). E-box binding zinc finger protein, SIP1, a downstream target gene in the TGF- β -mediated induction of EMT in the NMuMG cell line also inhibited the transcription of E-cadherin genes (199). In our microarray data, SIP1 from the TGF beta pathway was found to be increased more than 20-fold in the EMT cells compared to HPE cells. These studies demonstrate the complexity of interactions between TGF- β and other signaling pathways to bring about EMT. The role of stathmin in these interactions could provide further insight into the evolution of EMT.

In summary, we propose that stathmin is a regulator of epithelial cell homeostasis. Stathmin may exhibit differential function in normal compared to cancer cells, similar to

the dual roles of TGF- β as a tumor suppressor in normal cells and a tumor promoter at later stages of cancer progression (200). Stathmin over-expression has been associated with a number of different cancers including leukemia (201) as well as breast (202), ovarian (97) and prostate cancer (175). Immunohistochemical studies reveal that increased stathmin protein levels correlate positively with poor outcome (203). On the other hand, mechanisms, which disrupt stathmin expression, such as activation of TGF- β 1 signaling, result in destabilization of the cytoskeletal framework of the epithelial cell and progression to EMT. This observation is apparently counter-intuitive, since EMT has been associated with progression to a more aggressive tumor phenotype leading to invasion and metastasis. Thus, on one hand, increased stathmin expression correlates with high grade prostate cancer and on the other hand inhibition of stathmin expression can potentially make the tumor more aggressive. One possibility is downregulation of stathmin expression is required for transformation into more motile EMT cells, which now metastasize to distant organs. Once the invading cells home to a distant organ they may undergo the reverse mesenchymal-to-epithelial transition (MET), re-express stathmin, and grow as epithelial cells. Thus, stathmin represents an attractive target for therapeutic intervention aimed at maintaining a normal epithelial phenotype and controlling tumor spread.