NEUROENDOCRINE PROSTATE TUMORS MIMIC ENDOCRINE DIFFERENTIATION OF PANCREATIC BETA CELLS IN 12T-10 MICE: FOXA2 AND MASH-1 THE KEY PLAYERS

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ABBREVIATIONS

ACTH	adenocorticotropic hormone
AP	anterior prostate
AP-1	activator protein 1
AR	androgen receptor
ARBS	androgen receptor binding site
ARE	androgen response element
ARR	androgen response region
bp	base pair
Ck	cytokeratin
DBD	DNA-binding domain
DHT	dihydrotestosterone
DLP	dorsolateral prostate
dpc	date post conception
E	embryonic day
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FH	forkhead
Foxa1	forkhead box a1
Foxa2	forkhead box a2
Foxa3	forkhead box a3
GST	glutathione-*S*-transferase
GTF	general transcription factor
hASH-1	human achaete scute homolog-1
HGF	hepatocyte growth factor
HGPIN	high grade prostatic intraepithelial neoplasia
HNF-3	hepatocyte nuclear factor-3
IGF	insulin-like growth factor
IP	immunoprecipitation
LBD	ligand binding domain
LGPIN	low grade prostatic intraepithelial neoplasia
mash-1	mouse achaete scute homolog-1
NE	Neuroendocrine
NEB	Neuroepithelial bodies
NED	Neurendocrine differentiation
NLS	Nuclear localization signal
NSCLC	Non small cell lung carcinoma
NSE	Neuron specific enolase
NTD	N-terminal domain
nt	nucleotide
P	postnatal day
PAP	prostatic acid phosphatase

Pbsn	probasin
PIN	prostatic intraepithelial neoplasia
PMSF	phenylmethylsulfonic fluoride
PrE	prostate epithelium
PSA	prostate-specific antigen
PSA-EP	PSA-enhancer/promoter
SCLC	Small cell lung carcinoma
SDS	sodium dodecyl sulfate
SMA	smooth muscle α-actin
Smad	sma- and mad-related protein 3
SRY	sex-determining region Y
STAT	signal tansducer and activator of transcription
SV	seminal vesicle
TBP	TATA binding protein
Tfm	testicular feminized
Tgf	transforming growth factor
TNT	transcription and translation
TRAMP	Transgenic Adenocarcinoma of the Mouse Prostate
TS	tissue specific
UGE	urogenital sinus epithelium
UGM	urogenital sinus mesenchyme
UGS	urogenital sinus
UR	urethra
VP	ventral prostate

CHAPTER I

GENERAL INTRODUCTION

Prostate is a male accessory exocrine sex gland that contributes proteins, citric acid, and ions to the seminal fluid. Interest in prostate research arises from the fact that it is a frequent site of inflammation, infections, hyperplasia and cancer in the older man. We still lack effective treatments to prostate diseases, as there is a lack of knowledge on the molecular and cellular processes involved in normal prostate development and prostate diseases. It has long been believed that the diseases of prostate recapitulate some of the molecular and cellular events involved in normal embryonic development. It thus, becomes important to understand the normal growth and development of the prostate. Prostate development is hormonally regulated, the key factor being androgens. In the following chapters, I will discuss some of the well-understood facts about prostate development, differentiation and hormonal regulation and some recent findings. I will also share some of the new discoveries made in the field of normal prostate development and prostate cancer.

Prostate development and hormonal regulation

The prostate is a male exocrine gland that is associated with the urethra immediately below the urinary bladder. This gland is found exclusively in mammals and produces many components of semen such as fructose, zinc ions and many other proteins that assist in sperm health and movement. The prostate has a ductal structure and these

ducts lie in a sea of stroma that consists of fibroblasts, smooth muscle cells and mesenchyme. The prostatic ducts have a central lumen that is lined with tall columnar epithelial cells and some basal cells. The number of basal cells in mouse prostate is around 10% of all the epithelial cell population. Interspersed between basal cells are some neuroendocrine cells. Some studies indicate that the basal layer contains some very few and rare prostate stem cells. The ductal structure of the prostate and cell differentiation is initiated and maintained by the androgen signaling.

Prostate cancer and benign prostatic hyperplasia are the two major diseases of the prostate and both are associated with abnormal proliferation of cells. Prostate cancer initially responds to androgen ablation and regresses but with time it grows back as an androgen independent cancer. It thus becomes important to understand the hormonal regulation of normal prostate development, in order to better understand the molecular mechanisms that can lead to hormone refractory prostate cancer.

Prostate induction and ductal morphogenesis

Prostate gland is composed of a complex array of ductal-acinar structures. The prostate is derived from the embryonic urogenital sinus (UGS), a subdivision of the cloaca, which belongs to the endodermal hindgut. The UGS is a midline structure, which is composed of an endodermally derived epithelial layer, and a surrounding mesodermally derived mesenchyme layer (Marker et al., 2003). The UGS arises in male mice at approximately 13 days postconception (dpc) and in humans at about 7 weeks of gestation (Hamilton et al., 1959). (see Figure1-1) The initial event in prostatic morphogenesis, the formation of solid epithelial buds is initiated by the circulating androgen levels. The UGS is composed of urogenital epithelium (UGE) and urogenital

mesenchyme (UGM) (see Figure1-1). Initially, prostatic buds are solid cords of epithelial cells that grow into the UGM in a precise spatial pattern that determines the various lobes of the prostate (Timms, 1994 ; Cunha, 1987).

Most of the prostatic buds in rodents are unbranched at birth. However within the first 2-3 weeks after birth these ducts elongate and bifurcate and form characteristic ductal structures that represent each lobe of the prostate; AP (anterior prostate), DLP (dorsolateral prostate) and the VP (ventral prostate) (Sugimura, 1986). Each of the lobes of the prostate have a characterstic ductal structure as seen in the Figure1-1. These solid canals elongate into the surrounding UGM as a result of high proliferation activity at their tips (Sugimura, 1986). At this stage the epithelial cells of the solid buds are characterized by co-expression of markers for both luminal epithelial and basal cells. Cytokeratin 8 and 18 mark the luminal epithelial cells and cytokeratins 5, 14 and p63 mark the basal cells. These undifferentiated epithelial precursor cells express cytokeratins 5, 8, 14, 18, and p63 (Wang et al, 2001) (see Figure1-2). In rodents, within the first two weeks of birth, the epithelial cells differentiate into basal cells that arrange themselves along the basement membrane in a discontinuous fashion and these cells exclusively start expressing cytokeratins 5, 14 and p63. Concomitantly, tall columnar epithelial cells start expressing cytokeratins 8 and 18 and line the lumen of the prostatic ducts (Hayward, 1996) (see Figure1-2). By now the prostatic ducts have a lumen and are surrounded by luminal epithelial cells, basal cells and a rare cell population called neuroendocrine cells. These are very few cells interspersed between the basal cells and they are believed to maintain the tissue homeostasis. At the same time that the epithelial and basal cells differentiate, the prostatic mesenchyme also differentiates into a layer of smooth muscle cells that

surround the prostatic ducts (Hayward, 1996). The differentiated prostate starts secreting prostate-specific secretory proteins around 12-20 days after the birth in case of rats and mice (Lopes et al, 1996). Probasin (PB) is one of the well known secretory protein in rodents and prostate – specific antigen (PSA) is in humans. Branching morphogenesis is almost complete by 2 weeks after birth in the mouse (Sugimura, 1986).

Cyto-differentiation of the prostate

It is important to understand the stages of cell differentiation in normal prostate epithelium in order to identify the cell type(s) involved in prostate carcinogenesis. The adult prostate consists of mainly four cell types tall columnar secretory epithelial cells, basal cells, neuroendocrine cells and epithelial stem cells. Recent studies have identified cells that express markers for both epithelial and basal cells, referred to as intermediate cells. Androgen deprivation results in prostate regression. Prostate stem cells are of great interest as they are the ones suspected to regenerate the prostate after androgen administration. We will discuss the characteristic properties of each of these cell types in detail.

Luminal cells

The tall columnar secretory luminal cells are androgen dependent cells that require androgens for survival and upon androgen withdrawal undergo apoptosis and die. Theses cells express low molecular weight cytokeratins 8 and 18. These cells are androgen receptor positive and secrete prostate specific proteins like PSA, prostatic acid phosphatase (PAP) and kallikerin-2 into the seminal fluid in humans (McNeal, 1988) (Rittenhouse, 1998). In the case of mice and rats the main secretory protein is probasin (Spence et al, 1989).

Basal cells

The basal layer is believed to be the proliferative compartment of the prostate (Bonkhoff, 1994; Huss, 2004). The number of basal cells in each prostatic ducts is around 10% of epithelial cells. It consists of androgen independent cells that express high molecular mass cytokeratins, Ck5 and Ck14. Other common markers of basal cells are p63 (Signoretti et al, 2000) (a homolog of p53 and the anti-apoptotic protein Bcl-2 (Hockenbery, 1991; McDonnell, 1992). Generally, basal cells are reported not to express androgen receptor (Prins et al, 1991).

Intermediate cells

Luminal and basal cells were identified based on their cell morphology and the cell specific markers. However, studies have shown that some cells in the luminal layer express basal cell markers and cells in the basal layer express luminal cell markers. These studies suggest that prostate epithelium is heterogeneous and the cells that express markers for both luminal and basal cells are referred to as intermediate cells (Bonkhoff, 1994; Bonkhoff, 1996; Yong, 1998).

Prostate epithelial stem cells

The main evidence for the existence of prostate stem cells comes from the experiments performed on rat prostate. After castration 90% of prostate luminal epithelial cells undergo apoptosis leaving behind a thin layer of basal cells (Kyprianou, 1988). After re-administration of androgens the prostate grows back into a fully functional prostate. This suggests that the thin basal layer left after castration contains a cell population that can regenerate the prostate. Attempts to identify and isolate prostate stem cells have been difficult. The most closely accepted stem- cell model is that of an early

basal cell type that expresses Ck5 and 14 and gives rise to differentiated epithelial cells. Collins et al. (2001) have used the basal cell marker CD44 to isolate stem like cells from basal cell primary cultures. They identified a cell population expressing α-2 integrin on the cell surface and demonstrated that only cells expressing α-2 integrin differentiated into functional secretory cells *in vivo* (Collins et al, 2001). There has recently been an enormous increase in interest in stem cell markers in different tissues. Two stem cells markers, Hoechst 33342-excluding side population cells and the cell surface marker, CD133, have been identified in both normal tissues and in cancers and there is now evidence for their expression in the prostate.

Neuroendocrine cells

It is suspected that NE cells play an important role in the prostate carcinogenesis, especially in the development of invasive prostatic carcinomas (di Sant'Agnese, 1984). NE cells are present in all regions of the prostate at birth, but are reported to disappear after birth and reappear at puberty (Cohen, 1991; Cohen, 1993). These cells are sparsely scattered between the basal and luminal layers (Noordzij, 1995). There is no generally accepted theory of the cellular origin of prostatic NE cells. Recent findings suggest that all three-cell types comprising the prostate epithelium (epithelial, basal and neuroendocrine) have a common endodermal origin (Bonkhoff et al, 1994). Aumuller and his co-workers report that prostatic neuroendocrine cells are of neurogenic origin (Aumuller, 1999). Neuroendocrine cells are characterized by the presence of dense cytoplasmic granules responsible for storage and secretion of active substances like serotonin, chromogranin A, thyroid-stimulating hormone like peptide, calcitonin, and parathyroid related protein (Sciarra et al, 2003). NE cells secrete various products that

are essential for growth, differentiation and for maintaining the homeostatic regulation of secretory processes in adult prostate where they function in an autocrine-paracrine manner (Sciarra et al, 2003).

Hormonal regulation of prostate development

The development of the prostate is androgen dependent. The fetal testes start producing androgens before prostatic ductal morphogenesis and continues through their adulthood (Resko, 1978; Pointis, 1980). The first known response to androgens by murine UGS is the expression of the homeobox gene Nkx3.1 in UGE at 15.5 dpc, which is two days before the appearance of prostatic ducts (Sciavolino, 1997). Ablation or surgical removal of testes during ambisexual period inhibits development of prostate. During the postnatal period the prostatic development is also androgen dependent. Castration of neonatal mice or rats inhibits prostate growth and development. This effect can be reversed by administration of androgens (Cunha, 1987). Also administration of exogenous testosterone to immature males, accelerates prostatic growth and it attains maximal size in shorter time (Berry, 1984). Testosterone is the primary androgen produced by the testes but dihydrotestosterone (DHT) is the active androgen responsible for prostatic morphogenesis. DHT is produced by the enzymatic reduction of testosterone by the enzyme 5α-reductase (Wilson, 1971; Wilson, 1981). Androgens act upon developing UGS via intracellular androgen receptors that are expressed prenatally in the mesenchyme and not epithelium (Shannon, 1983;Wasner, 1983; Takeda, 1985). Males, whose androgen receptor are defective or absent, lack male secondary sex organs and develop external genetilia that are feminized. This condition is called testicular feminization (Tfm) and is seen in mouse, rat, human and cow (Griffin, 1984). Tissue

recombination and grafting experiments using UGS from Tfm mice that lack androgen receptors has shown that mesenchymal receptors are required for establishing prostate identity (Cunha, 1978; Bhatia-Gaur et al, 1999) and for stimulating ductal morphogenesis. Epithelial androgen receptors are required for establishing secretory function in the prostate epithelium (Donjacour and Cunha, 1993). The fact that mesenchymal and not epithelial androgen receptors are required for epithelial branching morphogenesis demonstrates that paracrine signals from the UGM mediate the action of androgens on the UGE during prostate development.

Mesenchymal – epithelial interactions in Prostate Development

The interaction between UGM and UGE leads to prostatic development in the presence of androgens. An important relationship between AR and mesenchymal-epithelial interactions is revealed by the expression of AR in the prostate. During prenatal development AR is initially detected only in UGM prior to and during prostatic bud formation. AR is undetectable in growing prostatic buds suggesting that mesenchymal AR is critical in the early phases of prostatic development (Cooke, 1991; Takeda, 1991). During prostatic development the mesenchyme induces epithelial ductal morphogenesis and differentiation. UGM can act as both permissive and instructive inductor. Permissive inductions are those in which mesenchyme permits the epithelium to express its already determined developmental fate. Instructive inductions are those in which mesenchyme induces an epithelium to express an entirely new developmental fate specified by the mesenchyme. The *in vivo* interactions between UGE and UGM were modeled in a series of classical tissue recombination experiments, which were pioneered by Cunha and colleagues. The formation of seminal vesicle and not prostate in tissue recombinants

between UGM and seminal vesicle epithelium is an example of the permissive nature of the UGM. The seminal vesicle epithelium is mesodermally derived Wolffian duct, whose developmental repertoire instructs the formation of epididymis, ductus deferens, seminal vesicle but not prostate (Cunha, 1987). On the other hand tissue recombinants between UGM and bladder epithelium form prostates (Cunha, 1987). This is an example of the instructive nature of the UGM.

Prostate stroma is known to produce at least eight families of growth factors (Wong, 2000) some of which are essential for differentiation and others involved in proliferation and growth inhibition. The five families that are known to be involved in proliferation and growth inhibition are TGF (tumor growth factor), FGF (fibroblast growth factor), IGF (insulin-like growth factor), EGF (epidermal growth factor) and HGF (hepatocyte growth factor).

IGF signalling

IGFs are polypeptide growth factors with an amino acid sequence and functional homology to insulin. These proteins are produced by a variety of tissues, and the regulation of their production and function is extremely complex. There are 2 IGF peptides (IGF-1 and lGF-2), 2 cell surface receptors, and at least 6 specific high-affinity binding proteins that modulate IGF actions. The mitogenic effect of IGFs is due to their ability to facilitate the transfer of cells from the G1 phase to the S phase in the cell cycle. IGFs are produced by the stromal cells and normal epithelial cells, particularly basal cells express IGF-1 receptors suggesting a paracrine pathway. The IGF system has recently been shown to have important mitogenic effects in the prostate and is essential for the development of the prostate (Cohen, 1991). A recent study shows a specific

impairment in gland structure in IGF-1-deficient mice (Ruan et al., 1999). The expression of IGF appears to be regulated with the other growth factor pathways, EGF, FGF, and TGF-ß. Regarding the receptor for IGF, the study of Fiorelli *et al* (Fiorelli, 1991) demonstrated that the IGF-1 receptor is located mainly on basal cells of prostatic epithelium. As for IGF-2 receptor, it has not been reported in either prostatic epithelium or stromal cells.

EGF and TGF-α

EGF and TGF-α are related polypeptides and bind to the same cell surface receptor. Their biological activities involve embryogenesis, cell differentiation and angiogenesis (Ullrich, 1984). Both normal and malignant cells secrete EGF, while TGF-α is predominantly produced by tumor cells. TGF-α is now thought to be produced by a variety of rapidly growing normal tissues (Lee, 1985; Rappolee, 1988). Castration of rats results in a reduction of EGF and TGF-α expression in the ventral lobe, and can be restored by testosterone (Nishi, 1996). Human prostate epithelial cells require EGF in serum-free medium for growth in primary culture (Peehl, 1989), and normal human prostatic fibroblasts can replicate in response to EGF (St-Arnaud, 1988). These findings suggest that EGF may be an important factor in autocrine or paracrine mode of regulation. EGF and TGF-œ exert their effects through their receptor (EGF-R), present in epithelial cells and stroma of male sex accessory organs. Several studies have shown that androgens downregulate EGF-Rs in rat and human prostates (Traish, 1987; St-Arnaud, 1988). Although *in vitro* studies imply that EGF is a potential mitogen for prostatic epithelium but there is no evidence that prostate produced EGF functions as a mitogen for prostatic cells *in vivo*.

TGF-β Signaling-Epithelial-Stromal Communication

5 isoforms of TGF-β have been identified but only types 1, 2 and 3 are present in mammals. TGF-β can play dual roles of both stimulator and inhibitor in male reproductive organs. TGF-βs induce proliferation of mesenchymal cells and inhibit the growth of epithelial cells (Russell et al., 1998). In addition, TGF-β1 has been reported to have an inhibitory effect on rat prostate cells (Cunha, 1992). TGFβ-1, β-2 and β-3 are important for fetal development and are expressed at high levels in 17-day murine urogenital sinus mesenchyme, but not in epithelium. Normal prostate tissue has TGF-β receptors. TGF-β receptors are predominantly expressed in epithelial cells (Kim, 1996). Out of three types of TGF-β receptors identified only type I and type II have a direct role in TGF-β1 signaling. Type II receptor binds to TGF-β and then recruits type I receptor. Type III receptor plays a role in presentation of TGF-ß2. Signaling can occur only as a heterodimeric complex (Wrana et al., 1994).

Prostate Carcinogenesis

Prostate cancer is the most commonly diagnosed cancer in men in the western world and it the second leading cause of cancer deaths in men (Jemal et al, 2005). It is estimated that a man has approximately a 10% chance of developing prostate cancer and a 3-4% chance of dying of prostate carcinoma (Murphy et al, 1997). The annual incidence of prostate cancer appeared to increase and accounted for approximately 28% of all cancers in American men, compared with 18% in 1980 (Murphy et al, 1997). However, this apparent increase was due to detection of prostate cancers by the introduction of PSA testing. Prostate cancer is responsible for the rapid increase in the

death rate, which is predicted to increase by a further 50% over the next 50 years due to the aging population. Based on the cases diagnosed from 1995-2000, about 90% of the cases diagnosed are expected to be detected at the local or regional stage for which the 5-year survival rate in 100% (Jemal et al, 2005). Prostate cancer is a disease with extremely high prevalence relative to its clinical incidence in the population. The huge difference between the incidence and mortality rates of this disease suggests variable behavior of cancer based on individual differences as well as heterogenity within the tumor. Understanding the molecular basis of the variability associated with this disease is very important to better understand and treat prostate cancer.

Serum PSA levels are used as a gold standard for detection of prostate cancer. PSA is an androgen regulated prostate epithelial cell specific secretory protein. Increase in PSA levels is generally associated with prostate cancer. Normal PSA levels do not necessarily mean a disease free state. The first line treatment for patients with prostate cancer is to treat the patients by androgen deprivation. Initially all the tumor respond to androgen withdrawal and undergo regression resulting in shrinkage of tumors and decrease in PSA levels. But eventually around 80% of the tumors re-grow as a more aggressive androgen independent tumors. In some of the patients these tumors grow as very aggressive neuroendocrine tumors, which might go undetectable as they are not associated with increased serum PSA levels. Neuroendocrine tumors readily metastasize and fail to respond to hormonal treatment. In the following sections we will discuss the histology, grade and clinical implications of prostate cancer.

Prostate cancer grading

The Gleason grading system is the dominant method used to grade prostatic carcinoma. Dr Donald F Gleason, a pathologist in Minnesota and the members of the Veterans Administration Cooperative Urological Research Group (VACURG), developed this technique. The Gleason grading system is based entirely on the histologic pattern of arrangement of carcinoma cells in H&E-stained prostatic tissue sections. The five basic grade patterns are used to generate a histologic score, which can range from 2 to 10, by adding the primary grade pattern and the secondary grade pattern. The primary pattern is the one that is predominant in area, by simple visual inspection. The secondary pattern is the second most common pattern. If only one group is in the tissue sample, the grade is multiplied by two to get the final score. Five Gleason patterns 1-5 are the ones that are used to analyze the histology (Gleason, 1992). Gleason pattern 1 is referred to a very well-differentiated growth of closely packed cells but separate and uniform. Gleason pattern 2 refers to less well-differentiated masses that do not have as uniform and rounded tumor-stromal boundary as pattern1. Moderately differentiated cells and the most commonly seen pattern in prostatic adenocarcinomas characterize Gleason pattern 3. Pattern 3 has been characterized as having three distinctive appearances, designated as patterns 3A, 3B and 3C. There is an increased aggressiveness of pattern 3 carcinoma proceeding from pattern 3A to 3B to 3C. Gleason pattern 4 is a high grade and poorly differentiated carcinoma with raggedly infiltrative masses of malignant epithelial cells. Pattern 4 is recognized by ragged edges or invasive periphery compared to smooth, pushing borders of pattern 3C. Gleason pattern 5 is the most poorly differentiated pattern

of prostatic carcinoma comprised of raggedly infiltrative sheet-like growth and central necrosis.

Neuroendocrine differentiation in prostate cancer

Neuroendocrine differentiation in prostate cancer is receiving increasing attention in recent years. NE cells are endocrine and sensory cells that share structural, functional and metabolic properties with neurons. These cells are found in normal prostate, prostatic intraepithelial neoplasia (PIN) and prostate cancer (Abrahamsson, 1989; Bostwick, 1994).

Neuroendocrine differentiation is present focally in virtually all cases of prostate cancer. The number of cells in each case varies based on the tissue fixation, sectioning of the tissue, antibody method used and the number of tissue sections examined (Abrahamsson, 1989). Some studies have reported as high as 92% of the cells have NE differentiation (NED) in prostate cancer (Abrahamsson, 1989; Bostwick, 1994). On the other hand, as low as 24-25% of NE cells have been reported by another group (Theodorescu, 1997). The role of NED in prostate cancer is still debatable; some studies have shown that the presence of NE cells in prostate cancer suggests poor prognosis while others were unable to find a correlation between the two. In the next few pages we will discuss the importance of NE differentiation in prostate cancer, characteristics and markers of neuroendocrine differentiation, clinical outcome of cancers with NE differentiation and treatment strategies.

Characteristics of NED in prostate cancer

Pretl in 1944 was the first to describe neuroendocrine cells in the prostate. These cells constitute part of a general endocrine regulatory system defined by Pearse in 1966

(Pearse, 1966). Pearse coined the term APUD that refers to the chemical characteristics of amine precursor uptake and decarboxylation common to the cells of this system. PerAnders Abrahamsson has summarized the histological and cytological patterns of NE cells in the prostate gland as following " Ideally, a NE cell is defined as a cell of neuronal or epithelial type that fulfills all or most of the following criteria: it contains secretion granules; its secretion is essentially directed towards the blood; the secretion granules store peptide hormones and /or biogenic amines, as shown by IHC techniques; it is often argyrophil or even argentaffin, and is immunoreactive to antisera against neuron-specific enolase (NSE) or Chromogranin A or other so called NE markers" (Angelsen, 1997).

Prostatic neuroendocrine cells contain a large variety of neurosecretory granules, suggesting multiple different cell types. Some of them such as serotonin, the Chromogranin family; Chromogranin A, Chromogranin B, and thyroid stimulating hormone-like peptide are found in most neuroendocrine cells, whereas others such as calcitonin, katacalin and calcitonin-related peptide, parathyroid hormone-related peptide (PTHrP), and neurotensin are present in smaller subpopulations of neuroendocrine cells. Also, some peptides are inconsistently present in some neuroendocrine cells, such as bombesin, gastrin-releasing peptide, or somatostatin.

Neuroendocrine products stimulate tumor growth and cell proliferation in an autocrine-paracrine fashion. Neuroendocrine differentiation in prostatic adenocarcinoma usually manifests itself as isolated foci or islands of cells expressing certain neuroendocrine related peptides. As NED increases there is an increase in the number and volume of such areas. This suggests that an alteration in the microenvironment induces neuroendocrine differentiation. The initial change might have occurred because of an

oncogenic effect but these transformed cells now secrete neuropeptides that act in a paracrine fashion on adjacent cells. Neuroendocrine cells are located in close proximity to proliferating cells, as demonstrated by Ki67 immunoreactivity (Bonkhoff, 1991). There are several studies elaborating on the potential role played by serotonin, bombesin-related peptides, parathyroid hormone related protein, neurotensin and calcitonin in the growth, proliferation and invasiveness of prostate cancer.

The function of bombesin-like immunoreactive peptide/gastrin-releasing peptide has been intensely investigated. One of the three known types of bombesin receptors (GRP-R) was detected in human prostate cancer cell lines by RT-PCR (Aprikian, 1996). In addition, that study showed that bombesin was a potent inducer of signal transduction via the bombesin receptor in the androgen insensitive PC3 and DU145 cell lines, but not in LNCaP cell line. This report also demonstrated that bombesin increases the motility and invasiveness through matrigel of the PC-3 cell line a hundred-fold (Aprikian, 1996). Studies have shown that androgen-independent tumor cell lines express GRP receptors and are coupled to calcium signaling during tumor progression (Wasilenko, 1997).

Serotonin, another neuropeptide has been shown to regulate prostate cancer growth. Using cell lines and *in vivo* models Abdul et al (Abdul, 1994) found that serotonin receptor antagonist had anti-proliferative effect on these cell lines. A marked growth inhibition of PC-3 cells in nude mice was also reported. These findings support the presence of serotonin receptors on prostate cancer cell lines and the potential role of serotonin in prostate cancer proliferation.

An antibody directed towards the N-terminal of PTHrP showed selective immunostaining of neuroendocrine cells (Iwamura, 1994). EGF has been shown to be an

inducer of PTHrP expression in addition to its growth factor activity. PSA cleaves PTHrP in a time and dose dependent manner with chymotrypsin-like activity. This has implications for bone metastasis possibly by bone remodeling and reabsorption. Patients with low PSA and high PTHrP often have neuroendocrine cancer and a higher incidence of osteolytic metastasis.

Calcitonin, another neuropeptide produces a proliferative response in LNCaP cells and also shows increased chemotaxsis.

Markers of neuroendocrine differentiation

The measurement of neuroendocrine markers in the blood of patients with prostatic adenocarcinoma is certainly a more objective measure of the neuroendocrine differentiation of tumors, because this would reflect the entire primary tumor population and its associated metastasis. The first studies of serum concentrations of NSE (Neuron Specific Enolase) and chromogranin A in patients with prostatic adenocarcinoma suggested that neuroendocrine differentiation, as reflected by increase in serum concentrations of these neuroendocrine secretory products, correlated with androgen independence and poor prognosis (Kadmon, 1991; Tarle, 1991). The neuroendocrine differentiation of prostatic adenocarcinoma is not suppressed by androgen ablation treatment, which matches with the results of histological studies with Chromogranin A as a neuroendocrine marker (Abrahamsson, 1989; Aprikian, 1993). A study by Deftos et al (Deftos LJ, 1996) and a similar report by Kimura et al (Kimura, 1997) demonstrated that Chromogranin A is a useful serum marker for patients with various stages of prostatic adenocarcinomas. Chromogranin has also been shown as a useful marker in advanced disease (Kadmon, 1991; Tarle, 1991). Cussenot and coworkers (Cussenot, 1996)

performed a study on 135 prostate cancer patients and detected increased serum concentrations of Chromogranin A and NSE in 23 (17%) and 20 (15%) respectively before any endocrine treatment. They speculated that neuroendocrine differentiation may be involved in progression of prostate cancer independent of androgen withdrawal. However, increased serum concentrations of Chromogranin A were consistently found in patients with androgen-insensitive tumors, in agreement with another report by Hoosein et al (Hoosein, 1995). In a study, the number of chromogranin A positive neuroendocrine tumor cells was found to be correlated with serum chromogranin A concentration (Angelsen, 1997). In a two year follow up study, serum concentrations of chromogranin A, pancreastatin, a breakdown product of chromogranin A, chromogranin B, NSE, and PSA were determined in 22 patients with prostatic adenocarcinomas (Angelsen, 1997). In this study only chromagranin B concentrations showed a statistically significant increase, which could be due to an increase in the number of neuroendocrine cells in the tumor (Abrahamsson, 1989) or an increased production of chromogranin B in the neuroendocrine cells (Schmid, 1994). Thus serum markers of chromogranin family of peptides are promising prognostic markers in neuroendocrine differentiation of prostate cancer.

Androgen receptor status in Neuroendocrine cells

Prostatic carcinoma generally remain androgen-dependent in early stages, but invariably relapse to androgen-independent disease after androgen withdrawal (Gittes, 1991). The underlying mechanisms responsible for the progression to androgen insensitivity are poorly understood. It is well established that androgen-dependent growth in prostatic malignancies requires the nuclear androgen receptor and enzyme 5α-

reductase, which coverts testosterone to DHT, the more active and stable form of androgen. Some data suggest that hormone resistant adenocarcinoma continues to express the nuclear AR and 5α-reductase isoenzymes at high levels (Ruizeveld de Winter, 1994; Bonkhoff, 1996). One of the possible mechanisms for this could be AR gene amplification, which is fairly common in recurrent tumors. Immunohistochemical studies have shown that nuclear AR expression is restricted to exocrine cells and that NE tumor cells consistently lack detectable AR reactivity (Bonkhoff, 1993; Krijnen, 1993). This feature of NE cells remains the same both in the primary and the recurrent tumor after hormonal therapy. The absence of AR in NE cells clearly suggests that these cells are androgen insensitive and refractory to hormonal therapy. NE cells lack androgen receptors but they express 5α-reductase isoenzymes at high levels in the poorly differentiated carcinomas and in recurrent disease (Visakorpi, 1995). This suggests that other substrates of 5α-reductase isoenzymes, including corticosterone and progesterone may be involved in prostate cancer (Visakorpi, 1995).

NE differentiation in prostate cancer-Clinical Implications

DiSant'Agnese has classified neuroendocrine differentiation in prostatic malignancies into three forms on the basis of histological features. If we consider clinical and biological aspects of neuroendocrine differentiation of prostatic carcinoma and the fact that neuroendocrine-like cells are usual during progression of the disease, three neuroendocrine phenotypes for the clinical prostatic carcinoma can be distinguished: (1) the rare endocrine paraneoplastic syndromes associated with prostatic carcinoma; (2) prostatic carcinoma without clinical paraneoplastic signs, but with humoral expression of neuroendocrine tumor differentiation, as assessed by plasma neuroendocrine markers

concentration; (3) and prostatic carcinoma without clinical or humoral expression of neuroendocrine markers, but with histopathologic features of neuroendocrine differentiation (Di Sant'Agnese, 1994).

Paraneoplastic syndromes occur in some patients with prostate cancer. The commonest endocrine syndrome associated with prostatic carcinoma is Cushing's syndrome with ectopic secretion of adenocorticotropic hormone (ACTH) or corticotropin-releasing factor (CRF). The first case was reported by Wise et al., in 1965. In a study by Ghali and Garcia (1984), most prostatic carcinoma associated with Cushing's syndrome were poorly differentiated adenocarcinomas. Other histopathological features have been reported in association with inappropriate ACTH expression, such as small cell carcinoma (Wenk, 1977).

Data on the prognostic impact of NE differentiation in prostate cancer are conflicting. Cohen et al in 1991 showed in their study that prostate cancer cases exhibiting greater NE differentiation had a worse outcome than those with a low percentage of NE differentiation (Cohen, 1991). Taplin et al (Taplin, 2005) assessed the significance of NE differentiation using CgA as a marker in patients with prostate cancer treated with transurethral resection of the prostate and androgen ablation. Their results strongly suggested that NE differentiation correlates with a poor prognosis and disease progression. It was also noted that NE differentiation in prostate cancer was associated with an early failure of androgen ablation therapy. Berruti et al (2005) studied 108 patients and concluded that a significant correlation was present between the extent of NE differentiation and either Gleason score or the disease stage, suggesting that the presence of NE features could negatively influence patient outcome. In a study by

Bostwick et al (Bostwick, 1994) 196 patients with node-specific prostate cancer who underwent bilateral pelvic lymphadenectomy and radical prostatectomy were assessed for NE differentiation using immunohistochemical staining for chromogranin and secretogranin. They found that benign prostatic epithelium and primary prostate cancer express a significantly greater number of NE cells than does lymph node metastasis. This suggests that decreased expression of NE markers may be involved in cancer progression. Some of the studies, in contrast, have shown that NE cells have no clinical or prognostic significance (Cohen, 1991; McWilliam, 1997). It has been either found that NE differentiation did not correlate with the pathologic stage or metastasis or did not have independent prognostic significance. The variable prognostic significance of NE differentiation in different studies could be attributed to methodological differences in determining NE differentiation, variances in the interpretation of the results, as the methods presently in use are semi-quantitative and require standardization; differences in the cohorts of patients studied.

Some of the recent work has focused on the malignant NE cells and their property of apoptosis resistance. Fixemer et al (Fixemer, 2002) noted that irrespective of grade, stage and the degree of NE differentiation, apoptotic activity was restricted to exocrine tumor cells and was undetectable in most of the NE tumor cells expressing CgA. This study suggests that prostate cancer cells with NE features escape programmed cell death. Resistance to apoptosis can enhance the malignant potential of tumor cells in various ways, including resistance to drug and hormonal therapies (Hanahan, 2000). It has also been shown that malignant epithelial cells in close proximity to NE cells express the anti-apoptotic protein bcl-2, and normal NE cells and NE-differentiated epithelial cells

express the protein from a recently identified gene encoding another apoptosis inhibitor, called survivin (Ambrosini, 1997). Angiogenesis is the process of forming new blood vessels from the existing ones, which provides blood supply to the tumor cells and facilitates their growth and proliferation. NE cell secretions are directly involved in angiogenesis. High grade prostate cancer with many NE tumor cells show greater neo-vascularization compared to high grade prostate cancer with few NE cells (Grobholz, 2000).

Mouse models of prostate cancer

In order to better understand and treat prostate cancer, arouse the need of developing prostate cancer mouse models. Several prostate cancer models have been developed using the prostate specific probasin gene promoter to target the SV 40 T antigen oncogene to the prostate. Probasin (PB) is a rodent prostate luminal epithelial cell-specific, androgen-regulated gene. The PB promoter has been widely used to make transgenic mice that target genes specifically to the prostate. The small 5'-flanking sPB fragment (-426/+28 bp) (Greenberg, 1994) , a Large (L) PB promoter fragment (~12 kb)(Yan, 1997) and a ARR_2PB promoter construct (Yan, 1997), all target prostate specific gene expression but ARR_2PB gives 20-100 fold higher transgene expression (Zhang et al, 2000). Described below are a few mouse prostate cancer models that are relevant to this study.

TRAMP (Transgenic Adenocarcinoma of the Mouse Prostate) Model

TRAMP mice were generated using the sPB promoter linked to the SV40 Early Region that expresses both the Large T antigen and small t antigen (Greenberg et al,

1995). Pathologists have now published a consensus report for the MMHCC on mouse models of prostate cancer which describes TRAMP prostate tumors as poorly differentiated small cell carcinomas (NE cancer) that express NE markers and show NE metastasis (Shappell et al, 2004).

LADY

The LADY LPB-Tag expresse only the SV40 large T-antigen. The LADY models fall into two classes of tumor types (Kasper et al, 1998). The 12T-7f line develops PIN lesions which progress to localized adenocarcinomas (Masumori, 2004), while the 12T-10 line develops NE prostate cancer and NE metastasis.

NE-10 allografts

An allograft was established by subcutaneously transplanting 12T-10 tumors into a male athymic nude mouse. The NE-10 allograft model gives rise to NE lesions that metastasize to lung or liver (Masumori, 2004).

Androgen receptor biology and transcriptional regulation

Protein structure of androgen receptor

The AR is a member of the nuclear receptor superfamily. These receptors function as ligand inducible transcription factors and mediate the expression of target genes in response to ligands specific to each receptor. Androgen receptor is an 110kDa protein that regulates the expression of its target genes through binding to an androgen response element (ARE). It is now well documented that co-regulatory proteins modulate the transcriptional activity of AR. Coregulators are defined as proteins that interact with

AR to enhance transactivation (coactivators) or reduce transactivation (corepressors) of target genes. AR can be divided into four functional domains: the NH2 terminal transactivation domain, the DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD). Two transcriptional activation functions have been identified on the androgen receptor. An NH2 terminal activation function (AF-1) functions in a ligand-independent manner when artificially separated from the LBD, creating a constitutively active receptor (Jenster et al., 1995). A ligand dependent AF-2 function is located in the LBD and mutation or deletion of the AF-2 domain dramatically reduces transcriptional activation in response to ligand (Danielian, 1992; Barettino, 1994). Since AR has two separate NH2-terminal transactivation domains, it is possible that each domain interacts with different coregulators or transcription factors in a promoter context-dependent manner (Jenster et al, 1995).

The DNA binding domain of AR has two zinc fingers that recognize specific DNA consensus sequences. AR binds as a dimer to the consensus inverted repeat androgen response element, GGTACAnnnTGTTCT, as well as to more complex response elements (Kasper et al, 1994; Zhou et al, 1997; Schoenmakers, 1999; Verrijdt et al, 1999)

The hinge region of the hormone receptor links the DBD and LBD. AR, in common with other steroid receptors, has a ligand-dependent bipartite nuclear localization signal (NLS) located in the COOH terminus of the DBD and the hinge domain (Jenster, 1993; Zhou, et al, 1994). In AR, the NLS is located between amino acids 617 and 633 (Jenster G, 1993; Zhou et al, 1994).

The LBD of AR, in addition to forming the ligand-binding pocket, mediates the interaction between AR and heat shock proteins (Fang et al, 1996). The LBD of AR also interacts with the AR NH2 terminus to stabilize bound androgen (He et al, 1999). X-ray crystallographic studies have shown that the ligand binding pocket if formed by 11-13 α-helices (Williams and Sigler, 1998). Comparison of the crystal structures of receptors in the absence of ligand and in the ligand-bound state show that ligand binding induces a conformational change in which helix 12 and the AF-2 domain fold back across the ligand binding pocket (Bourguet et al., 1995).

Interaction of AR with general transcription factors

Transcription activation by steroid receptors ultimately requires the recruitment of RNA polymerase II to the promoter of target genes. Polymerase II recruitment is mediated through the assembly of general transcription factors to form the preinitiation complex. The first step in the formation of the preinitiation complex is the binding of the TATA binding protein (TBP) near the transcription start site. TBP is a part of a multiprotein complex, transcription factor IID (TFIID). TFIID also contains general and promoter-specific TBP-associated factors. TBP binding induces DNA bending, bringing sequences upstream of the TATA element in closer proximity, enabling interaction between GTFs and steroid receptor coregulator complexes. The ATPase and the helicase are then recruited to pol II to facilitate DNA strand separation before transcription initiation (Heinlein and Chang, 2002).

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Interaction of AR with co-regulators

AR has been found to interact with a number of transcription factors including AP-1, Smad-3, nuclear factor κB (NFκB), sex-determining region Y (SRY), and the Ets family of transcription factors(Matsuda et al, 2001; Chipuk et al, 2002; Yuan et al, 2001). One of the major mechanisms through which co-regulators were thought to function was by forming a bridge between the DNA-bound nuclear receptor and the basal transcription machinery. This is now considered a characteristic of type I co-regulators (Lemon and Tjian, 2000). By stabilizing or recruiting the RNA pol II complex to the target gene promoter, such a co-regulator will be called a co-activator and would enhance gene transcription. Co-regulator mutations that prohibit the appropriate multiprotein complex assembly would be expected to inhibit steroid receptor transcriptional activation in a dominant manner. The type I co-regulator ARA54, a co-activator of AR (Kang et al, 1999), functions as a dimer (Miyamoto et al, 2002). A COOH-terminal truncation of ARA54 and a COOH-terminal truncation carrying a glutamic acid to lysine mutation at amino acid 472 function as dominant negative mutants of AR transcription (Miyamoto et al, 2002). Co-activators may also function by facilitating ligand binding, promoting receptor nuclear translocation or mediate signal transduction.

The members of the steroid receptor co-activator (SRC) family of nuclear co-regulators are the most studied and SRC-1 is a protein that interacts with the LBD and has been shown to increase ligand-dependent transcription of AR (Yao, 1996). The SCR family members recruit the basal transcriptional machinery and function as Histone Acetyl Transferases, thus are considered to be type I co-regulators (Lemon and Tjian 2000; Robyr et al, 2000).

Several proteins initially thought to be involved in actin-binding have now been found to co-activate AR mediated transcription. Both type I and type II coregulators have been found to be actin-binding proteins. In addition to playing an important role in cell-cell adhesion, β-catenin is a downstream effector of the Wnt signaling pathway that regulates cellular differentiation, proliferation and migration (Willert, 1998). Activation of Wnt pathway results in increase in cytoplasmic β-catenin which forms complex with members of the TCF/LEF (T-cell factor and lymphoid enhancer factor) family of transcription factors in the nucleus and allows transcriptional activation by TCF/LEF (Brannon et al, 1997). This suggests that β-catenin serves as a type I co-regulator. In addition to TCF/LEF, β-catenin has been shown recently to function as a transcriptional coactivator of AR in prostate cancer cells (Truica et al, 2000).

The ligand binding ability of nuclear receptors requires appropriate folding of the receptor. Upon ligand binding, AR dimerizes allowing the NH2 and COOH termini of the receptor to interact. This interaction stabilizes the AR ligand complex. Co-regulators that influence AR protein folding, ligand binding and NH2/COOH terminal interaction could affect AR protein stability and are classified as type II co-regulators. The AR co-activator ARA70 plays a unique role in AR ligand binding. The interaction of ARA70 with DHT-bound AR enhances AR protein stability above DHT binding. In transfection experiments, ARA70 enhances AR transcription in response to the normally weak androgen (Miyamoto, 1998). In addition to enhancing AR transactivation in response to normally weak agonists, ARA70 has also been shown to enable the AR antagonists hydroxyflutamide and casodex to behave as AR agonists (Miyamoto, 1998). This is of

importance in prostate cancer where androgen antagonists are often used as part of androgen ablation therapy.

Transcriptional activity of AR has been found to be influenced by growth factors and cytokines through stimulation of signal transduction cascades. The stimulation of kinase cascades may affect AR transcription through phosphorylation of AR, AR interacting proteins, or co-regulators. Phosphorylation of the transcription factor STAT3 in response to IL-6 allows STAT3 to interact with AR and enhance AR transcription (Chen et al, 2000; Matsuda, 2001).

Hepatocyte nuclear factor (HNF) or Forkhead box A (Foxa) proteins

The hepatocyte nuclear factor proteins were originally found in the liver and were required for the hepatocyte-specific expression of α1-antitrypsin and transthyretin genes, expressed at high levels in the liver (Costa, 1989). All members of this family share a 100 amino acid conserved DNA binding region which is termed as forkhead domain (Lai, 1990). Thus these proteins are also referred as forkhead proteins. The crystal structure of the DNA-binding domain of HNF3γ has been solved (see Figure1-3) and was identified as a variant of the helix–turn–helix motif (Clark et al, 1993). This DNA-binding domain is made up of three α-helices and two characteristic large loops, or 'wings', resulting in the designation of this DNA-binding motif as the 'winged helix DNA-binding domain'. It is assumed that the proteins encoded by the HNF3 genes and the more than 100 related winged helix genes bind to DNA with a winged helix domain similar to that of HNF3γ, although this has not been proved experimentally. Recently, the nomenclature of this gene family has been revised to reflect the phylogenetic relationships between the family members more accurately, and to rectify the considerable confusion caused by conflicting

classification systems. According to the new nomenclature, all vertebrate genes encoding winged helix proteins will be termed *Fox*, for *forkhead box* (Kaestner et al, 2000). Therefore, the genetic loci encoding the three members of this family, HNF3α, β and γ are now known as *FOXA1* (*HNF3α*), *FOXA2* (*HNF3β*) and *FOXA3* (*HNF3γ*), respectively, in humans, and Foxa1 (*Hnf3α*), *Foxa2* (*Hnf3β*) and *Foxa3* (*Hnf3γ*) in mice. Despite the strong sequence similarity in the DNA-binding domain, there is a wide range of DNA sequences recognized by family members. Even amongst Foxa1, Foxa2 and Foxa3 differences in affinities for different oligonucleotides were observed (Lai, 1991). The molecular mechanism by which the FOXA genes control transcription has been elucidated only partially. In addition to its DNA-binding domain, Foxa2 has been shown to contain four *trans*-activation domains (Pani et al, 1992; Qian and Costa, 1995) (domains II–V in Figure 1-3(a)). To date, no co-activators have been identified that might mediate the interaction of these *trans*-activation domains with the basal transcriptional machinery or with histone acetyl transferases. Other functional domains of the Foxa proteins include a bipartite nuclear localization signal (NLS) and two phosphorylation sites for casein kinase I (Qian and Costa, 1995). Interestingly, although the Foxa proteins have been shown to be phosphorylated on serine residues *in vivo*, mutation of the casein kinase I sites had no effect on the *trans*-activation potential of Foxa2 in co-transfection assays (Qian and Costa, 1995).

In addition to promoting transcription of target genes via the aforementioned *trans*-activation domains, the Foxa proteins appear to promote gene activation by altering chromatin structure directly. The structure of the winged helix domain is strikingly similar to that of linker histones H1 and H5 (Clark et al., 1993). The function of the linker

histones is to restrict the DNA on the nucleosome surface (Zhou et al., 1998). Therefore, the presence of linker histones leads to transcriptional inactivation (Steinbach et al, 1997; Lee and Archer, 1998). The Foxa proteins can bind to DNA on the nucleosome core and displace the linker histone (Cirillo et al, 1998). Thus, the net result of Foxa binding to specific enhancers might be to de-compact chromatin and to facilitate binding of other transcription factors. Multiple genes encoding hepatic and pancreatic enzymes, serum proteins and hormones (glucagon) have been shown to contain Foxa-binding sites as assessed by DNA-binding assays and co-transfection experiments using artificial reporter constructs.Foxa1, a2 and a3 are produced at the onset of pancreatic development in the foregut endoderm (Monaghan et al, 1993). Expression of all Foxa mRNAs persists into adulthood, and Foxa2 protein has been localized to most pancreatic islets cells and some, but not all, acinar cells (Cockell et al, 1995; Wu et al, 1997). Most important in this context, analysis of the *Pdx1* (*IPF1* in humans) promoter identified Foxa-binding sites surrounding position −2000 of the mouse (Wu et al, 1997), and position −5920 of the rat *Pdx1* promoter (Sharma et al, 1997). Additional support for the relevance of these binding sites comes from analysis of *cis*-regulatory elements of the *Pdx1* gene in transgenic mice. A transgene containing a 0.68-kb fragment of the promoter including the Foxa-binding site directed expression of the β-galactosidase reporter to all islets in neonates and to β cells in adult mice. Furthermore, the pattern of Foxa2 expression in the pancreas is similar to that of *Pdx1* (Wu et al, 1997), and embryoid bodies lacking Foxa2 express somewhat lower levels of *Pdx1* mRNA (Gerrish et al, 2000). Taken together, these studies indicate that Foxa2 is a direct upstream activator of *Pdx1* and places Foxa2 at the top of the transcription factor hierarchy in the pancreas. In mice Foxa2 is first

expressed in the endoderm progenitor during gastrulation (E6.5), immediately followed by the expression of Foxa1 (E7-8) and Foxa3 (Sasaki and Hogan, 1993). The targeted null mutation of Foxa2 gene results in embryonic lethality due to absence of endodermal progenitor cells (Weinstein, 1994), In contrast to the Foxa2-null mice, embryos carrying homozygous null mutations for Foxa1 or Foxa3 develop normally to term and show no obvious morphological liver phenotype changes. However, transcription of several Foxa target genes is reduced by 50–70% in the Foxa3 mutants (Kaestner et al, 1998). The reduced expression of the genes encoding gluconeogenic enzymes observed in these animals leads to moderate hypoglycemia after prolonged fasting (Shen et al, 2001), indicating that Foxa3 normally functions in the maintenance of euglycemia. Mice homozygous for a null mutation in the winged helix transcription factor Foxa1 showed severe postnatal growth retardation followed by death between postnatal day 2 (P2) and P12 (Kaestner et al, 1999; Shih et al., 1999).

Expression of Foxa transcription factors in developing mouse prostate

Foxa genes are observed in early mouse embryo development and are expressed during the formation of the definitive endoderm with Foxa2 activated first followed by Foxa1 and finally Foxa3. In adult Foxa proteins are expressed in endodermally derived tissues such as liver, lung, pancreas and intestine and play important roles in regulating gene transcription (Kaufmann, 1996; Kaestner et al, 2000). Foxa1 gene is highly expressed in the rodent prostate (Peterson, 1997) and has been shown to be regulated by androgens in the adult (Kopachik, 1998). Recent studies have shown that Foxa1 is expressed in the murine urogenital sinus epithelial cells from Embryonic day 17 and continues to be expressed throughout life time (Mirosevich, 2005) (see Figure1-4(a)).

Foxa2 expression on the other hand was observed in the epithelial cells expressing cytokeratin 14 and was localized to the tips of growing prostatic buds. Foxa2 expression was seen from E17 to day 2 after birth (Figure1-4(b)). Androgen receptor was expressed at similar levels in both epithelial buds and urogenital sinus epithelium.(Mirosevich, 2005) In the adult mature prostate only a few cells at the interface of epithelium and mesenchymal cells were seen to be positive. This observation suggests that both Foxa1 and Foxa2 play important but different roles in prostate development. And the loss of either Foxa1 or Foxa2 during prostatic development could lead to an abnormal prostate.

Recent work by Gao et al has shown that Foxa1 is critical for normal prostatic development (Gao et al, 2005). In this study, the UGS from the Foxa1 knockout animals was re-grafted into nude mice and the tissue was analyzed using different markers for prostate epithelium. The results suggest that the developing prostate was captured in an immature state where cells did not differentiate into distinct epithelial or basal cells; the cells expressed both basal cell and luminal epithelial cell markers (Gao et al, 2005) consistent with the solid cords seen in developing prostate. These immature cells expressed high levels of Foxa2, further suggesting that it is captured in early development stage. This observation also suggests that although Foxa1 and Foxa2 are closely related members of the same family of transcription factors, they have discrete functions and that Foxa2 could not compensate for the loss of Foxa1. There were no ductal structure and the cells appeared like solid cords. Staining for SMA (smooth muscle actin) revealed an expansion in smooth muscle layer that immediately surrounds the epithelial cords, suggesting mesenchymal hypercellularity (Gao et al, 2005).

Expression of Foxa proteins in prostate cancer

Our group has extensively studied the expression of Foxa1 and Foxa2 in mouse models of prostate cancer. A recent report by Mirosevich et al has shown that Foxa1 is expressed in prostatic intraepithelial neoplasia (PIN) and in metastatic lesions from both 12T-7f and 12T-10 LADY models (Mirosevich, 2006). Foxa1 is also expressed in the subcutaneous grafts and metastatic lesions of NE-10 allograft model. On the other hand Foxa2 expression was observed only in the 12T-10 and NE-10 allograft models (Mirosevich, 2006). The prostate and the metastatic lesions from these two models expressed synaptophysin, a marker for neuroendocrine differentiation. This suggests that Foxa2 expression is confined to neuroendocrine tumors of the prostate. Prostate from 12T-7f mice develop non-neuroendocrine PIN lesions that are negative for Foxa2 expression. This study also showed that the Foxa2 is also expressed in the human neuroendocrine prostate cancer samples (Mirosevich, 2006).

Molecular Pathways to neuroendocrine differentiation

Neuroendocrine prostate carcinoma is highly malignant, rapidly progressive and problematic to treat since it is insensitive to androgen blockade. The fact that neuroendocrine cell population exists in majority of the prostate tumors, makes it important to study the mechanism that causes NED. Existing as a minor component in most tumors, the greater the proportion of prostate carcinoma cells that have a neuroendocrine phenotype, the greater the likelihood that the tumor will progress (Abrahamsson, 1999; di Sant'Agnese, 2001). Thus, understanding the biology and growth control mechanisms of the NE variant is of great importance to general oncology.

Another rationale behind understanding the molecular nature of NE cells is the fact that they have a potential to metastasize. From a minority of prostate carcinomas that are histologically "conventional" ductal prostate cancers, some metastases exhibit a NE phenotype (Roudier et al, 2003). Understanding neuroendocrine biology will lead to better treatment options of these metastasis.

Further, the understanding of neuroendocrine prostate cancer biology is important since this phenotype is androgen insensitive. The issue of treating androgen insensitive tumors is a major challenge for recurrent prostate cancer. Although neuroendocrine differentiation is just one of a number of possible mechanisms by which prostate cancers acquire or evolve to an androgen insensitive state (Craft et al, 1999; WallÈn, 1999; Yeh et al, 1999), it is a state that offers the potential for targeted therapy. For example, the same systematic regimen used to treat neuroendocrine variant of lung carcinoma, more commonly known as "small cell undifferentiated carcinoma", may be effective in treating the neuroendocrine variant of prostate carcinoma.

The origin of neuroendocrine cells of the prostate is still unknown. As discussed before, some people believe that they originate from the neuronal cells while others think they are endodermal in origin as other cell types of normal prostate gland. In the following section I will discuss the possible pathways that can lead to neuroendocrine differentiation in the prostate. Prostatic endocrine cell differentiation follows the same gene expression pattern as the islet cell differentiation in the pancreas. Prostatic neuroendocrine differentiation can be caused by activation of the pro-neuronal gene mash-1 (achaete-scute homolog-1) or the pro-endocrine gene, ngn3 (neurogenin-3) or by activation of both mash-1 and ngn3. Studies done on endocrine differentiation of

pancreatic endocrine cells have shown that loss of notch signaling increases expression of both pro-neuronal gene mash-1 and pro-endocrine gene, ngn3. Once neurogenin-3 has been activated in a progenitor cell in the pancreas, that cell is fated to become an endocrine cell. Also, it has been shown that notch signaling is lost in lung neuroendocrine cancer, which is accompanied with an increase in mash-1 in neuroendocrine lung tumors. All these studies point to the likely role of these pathways in the neuroendocrine differentiation of the prostate tumors as well. In the next few pages I will elaborate on each of these pathways, as they appear in pancreatic endocrine differentiation and their possible role in prostate NED. Further we will discuss how these pathways can converge and lead to NED in the prostate.

Notch signaling

In mammalian tissue development, generation of cell type diversity as well as normal morphogenesis involves the cell-cell interaction process, so called "lateral inhibition". During this process, early-differentiating cells send a signal to the neighboring cells to inhibit them from differentiating into the same cell types. The transmembrane protein Notch plays an essential role in the lateral inhibition process. Differentiating cells express the Notch ligands (Delta, Jagged, Serrate) on the cell surface and activate Notch signaling of the neighboring cells. Notch activation inhibits cellular differentiation, thereby maintaining dividing precursor cells and enabling the inhibited cells to adopt different cell types at later stages. In the absence of the Notch signaling, dividing cells decrease and cells differentiate into early born cell types resulting in disorganized tissues. Thus, the Notch-dependent lateral inhibition is critical to maintain correct tissue morphogenesis and cell type diversity. The inhibition of differentiation by

Notch is brought about by basic helix-loop-helix genes, Hes-1 and Hes5 (mammalian homologues of Drosophila hairy and Enhancer of split). These genes act as transcriptional repressors.

Cleavage and translocation of Notch

Notch is a transmembrane protein with epidermal growth factor (EGF) repeats in the extracellular domain and ankyrin repeats in the intracellular domain. There are four different but related Notch genes (Notch 1 to 4) in mice, which show distinct expression patterns. This Notch molecule is activated by its ligands expressed by neighboring cells. When Notch is activated by its ligand, the intracellular domain (ICD) of Notch is likely to be cleaved by γ-secretase (Schroeter et al., 1998; Chan, 1999). After cleavage of Notch by γ-secretase, Notch ICD is then translocated into the nucleus where it forms complex with the DNA-binding protein RBP-J (Nishimura et al, 1998). This complex binds to the RBP-J binding sites in the Hes1 promoter and causes up-regulation of Hes-1. When RBP-J binding sites in the Hes-1 promoter were disrupted, the complex cannot interact with the promoter and Hes-1 up-regulation is completely abolished. Hes-1 is known to inhibit neuronal differentiation (Ishibashi, 1994), thus the above data suggests that the Hes genes mediate Notch-induced inhibition of cellular differentiation. Although four notch genes have a conserved structure, recent analysis indicated that Notch1 and Notch3 are functionally different. While Notch1 ICD up-regulates Hes-1 promoter activity, Notch3 ICD does not.

Hes-1 and Hes-5: transcriptional repressors

Hes-1 and Hes-5 encode bHLH factors that repress transcription mediated by the co-repressor Groucho. Both Hes-1 and Hes-5, which can bind to the N box sequence

(CACNAG), have the four-amino-acid sequence WRPW at the carboxyl terminus. The co-repressor Groucho, which actively represses transcription, interacts with this WRPW sequence (Paroush et al, 1994). Thus, in association with Groucho, Hes-1 and Hes-5 directly bind to N box and actively repress gene expression. Known targets of Hes-1-mediated silencing include mash-1/hASH-1 (mammalian achaete-scute homolog-1) in the nervous system and lung. Hes-1 similarly inhibits neuroendocrine differentiation in the gut and pancreas.

Loss of Notch signaling in neuroendocrine tumors

Notch signaling is lost in lung and intestinal neuroendocrine tumors. Neuroblastomas also show loss of Notch signaling. In this section we will discuss the role of Notch signaling in fetal NE cell development followed by its role in neuroendocrine tumors.

Fetal lung development is very similar to prostate development. In both cases, a primitive endodermal bud surrounded by mesenchyme progressively grows and undergoes branching morphogenesis. Cytodifferentiation takes place and generates the principal lung cell types, ciliated and mucus secreted goblet cells, Clara cells, and interspersed NE cells. Mash-1 first becomes detectable at approximately E13.5 in neuroepithelial bodies (NEBs), clusters of NE cells frequently located at branch points of large and medium-sized airways (Borges et al, 1997). Mash-1 immunoreactivity coincides with NE markers such as synaptophysin and CGRP (Borges et al, 1997; Ito et al, 2000). Later in gestation, solitary mash-1-staining pulmonary NE cells can be seen. Mash-1 mutant mice lack any evidence of either NEBs or isolated NE cells (Borges et al, 1997; Ito et al, 2000). Interestingly, overall lung size is unchanged in the mash-1 mutant

mice, suggesting that, loss of NEBs does not lead to a lung proliferative defect (Ito et al, 2000). The importance of mash-1 in lung NE development is further confirmed by the findings of Ito et al using Hes-1 knockout mice (Ito et al, 2000). Hes-1 is a principal effector of Notch pathway and is expressed in non-NE cells in the airway epithelium. In lungs of Hes-1 K/O mice, mash-1 expression is enhanced, together with NeuroD, a second neural bHLH protein normally undetectable in fetal lung (Ito et al, 2000). Hes-1 mutant lungs show 10-fold more abundant NE cells and a significant reduction in Clara cells (Ito et al, 2000). Clearly the notch signaling regulates differentiation of an airway epithelial precursor cell with potential for either NE or Clara cell phenotype. In the case of pancreas and gut development, Notch signaling inhibits early commitment of progenitors to an endocrine lineage through suppression of NeuroD as well as neurogenin3 (Apelqvist, 1999; Jensen, 2000).

One of the most distinctive features of Small Cell Lung Carcinoma (SCLC) is the expression of NE phenotype including hormones such as ACTH, vasopressin, calcitonin, CGRP, secretory proteins including synaptophysin and chromogranins. SCLC tumors are considered as poorly differentiated NE cancers in contrast to typical and atypical bronchial carcinoid tumors. Conserved pathways from fetal nervous system development are clearly essential in allowing for NE differentiation in normal lung and in lung cancer. One important hallmark of NE regulation is the transcription factor mASH-1/hASH-1 (Borges et al, 1997). hASH1 expression is tightly linked to the NE phenotype in lung cancer. hASH1 expression can be seen in virtually all classic SCLC lines as well as typical and atypical bronchial carcinoid lines, and in many NSCLC lines with NE features. Many classic SCLC lines lack evidence of Notch pathway activation such as

Hes-1 expression and hASH-1 promoter inhibition, whereas NSCLC lines more frequently express these activities (Chen et al, 1997). Several pieces of evidence point to a critical pathogenic role of hASH-1 in SCLC. Studies utilizing antisense oligonucleotides directed at hASH-1 suggest that depletion of this factor represses classic NE markers in cultured SCLC cells (Borges et al, 1997). In addition to this, overexpression of hASH1 in airway epithelial cells of transgenic mice, in concert with SV40 Large T antigen, is sufficient to induce aggressive lung tumors with a NSCLC-NE phenotype (Linnoila et al, 2000). In this study, in the absence of Large T-antigen extensive airway epithelial proliferation occurs but no NE trans-differentiation was observed. Bitransgenic mice, with Large T-antigen exhibit widespread hyperplasia of NE-reactive epithelial cells and adenocarcinomas with NE features (Linnoila et al, 2000). A plausible interpretation is that constitutively expressed hASH-1 cooperates with p53 and pRB loss (due to T-antigen) to promote NE tumors in the lung. SCLC shares the classic APUD phenotype with other tumors of endocrine system including medullary thyroid carcinoma, paraganglioma and carcinoid tumors of the foregut (lung, thymus and pancreas) and midgut (duodenum, jejunum and ileum). hASH-1 expression appears to be an important feature of all these tumors. Recently, Gordon and colleagues have demonstrated exceptionally high level expression of mash-1 in a mouse model of prostatic NE carcinoma. This mouse model expresses SV40 Large T-antigen to prostate neuroendocrine cells using cryptdin2 gene promoter (Hu et al, 2002).

Pancreatic endocrine cell differentiation

The pancreatic islet cells seem to have more in common with neuronal cells than with their neighboring exocrine and ductal cells. Pancreatic endocrine cells express many

genes originally described in neurons. This is further supported by the fact that pancreatic endocrine development utilizes many transcription factors originally described in the neural development. Some of the examples are Nkx2.2 and Nkx6.1 (Madsen, 1997; Sander et al, 2000) and pro-endocrine bHLH genes neurogenin3 and neuroD1 (Naya et al, 1997; Apelqvist, 1999; Schwitzgebel et al, 2000). The differentiation of the endocrine cells in the developing pancreatic endoderm is reminiscent of the process by which neurons are specified in the developing ectoderm. In the neuronal differentiation notch signaling represses the default activation of the neuronal differentiation in most of the cells. The Notch signaling mediates its function through the expression of hairy/enhancer-of-split transcription factors. Once notch signaling is active, the HES factors are turned on and these inhibit pro-neuronal genes (Jan, 1993). In a similar fashion, notch signaling determines which cells in the developing pancreas will activate the endocrine differentiation program (Apelqvist, 1999; Jensen, 2000). Analogous to this, there is loss of Notch signaling in prostate neuroendocrine tumors. The pro-endocrine genes and the pro-neuronal genes are expressed in these tumors because of loss of Notch signaling and loss of inhibitory effects of Hes-1. The downstream transcription factors involved in pancreatic endocrine cell differentiation are seen to be expressed in prostate neuroendocrine tumor cells in a sequential fashion and this will be discussed in detail in chapter 4.

Hes-1, the downstream target of Notch signaling in the pancreas, inhibits the expression of the pro-endocrine bHLH transcription factor neurogenin3 (Jensen, 2000). Animals lacking neurogenin3 fail to develop any endocrine cells (Gradwohl et al., 2000), while uniform ectopic expression of neurogenin3 throughout the pancreatic epithelium

causes premature differentiation of entire pancreas into endocrine cells (Apelqvist, 1999; Schwitzgebel et al, 2000). Together these studies demonstrate that neurogenin3 expression, permitted by loss of Notch signaling, is both necessary and sufficient for initiating endocrine differentiation. Once neurogenin3 is activated in the progenitor cell, that cell is fated to become an endocrine cell, but it may become any of the four possible endocrine cell subtypes based on other factors that might control this decision. As of now, there is no data that demonstrates that any of these factors are either necessary or sufficient to control endocrine cell fate decisions. These factors contain homeodomains and can be divided into early factors (pax4, Nkx2.2 and Nkx6.1) that are co-expressed with neurogenin3 in endocrine progenitor cells. And the late factors are pax6, isl1, brn4 and PDX1 that are found in more mature cells.

Nkx2.2 is expressed in the pancreatic bud until E13 when it becomes localized to the neurogenin3-expressing progenitor cells. Nkx2.2 also persists in many of the matured endocrine cells including all β-cells. Mice lacking Nkx2.2 have a complete absence of insulin-producing cells. In the absence of Nkx2.2 beta cells are specified but are unable to differentiate to mature insulin-producing cells (Sussel et al, 1998). The absence of Nkx6.1 in the islets of animals lacking Nkx2.2 suggests that Nkx2.2 lies upstream of Nkx6.1. Studies of the Nkx6.1 gene promoter suggest that Nkx2.2 may directly regulate Nkx6.1 expression (Watada et al, 2000).

Control of neurogenin3 expression

Neurogenin3 is a key transcription factor for differentiation of the endocrine pancreas. The expression of neurogenin3 is regulated by hairy and enhancer of split (HES-1-type proteins, which are defined as anti-neural bHLH genes. Neurogenin3

promoter contains at least three HES-1 binding sites adjacent to the TATA box sequence, as confirmed by electrophoresis mobility shift assay, and expression of HES-1 strongly inhibits Ngn3 gene promoter activity in a manner that depends on the sequence containing the HES-1 binding sites (Lee et al, 2001). As discussed, HES-1 expression is regulated by Notch signaling. If the Notch signaling is functional it causes the activation the HES-1 gene in adjacent cells resulting in inhibition of the pro-neural gene, Ngn3 and inhibits endocrine differentiation in the adjacent cells (Figure 1-5). A similar mechanism of lateral inhibition, where cells that start to differentiate inhibit their neighboring cells from entering the differentiation pathway, applies to neuronal differentiation. The Notch-Hes-Ngn3 pathway seems to be important for both endocrine and neuronal differentiation.

In addition, to HES-1, the neurogenin3 promoter contains binding sites for several other transcription factors expressed in the endoderm including HNF1, Foxa and HNF6 (Jacquemin et al, 2000; Lee et al, 2001) (see Figure 1-5). Genetic evidence in mice supports a role for HNF6 as an upstream activator of neurogenin3 expression. HNF6 was originally identified as a regulator of hepatic genes including Foxa2 (Samadani and Costa, 1996; Rausa et al, 1997). The neurogenin3 promoter contains HNF6 binding sites and can be activated by HNF6 in cultured cells (Jacquemin et al, 2000). Mice lacking HNF6 have severely reduced neurogenin3 expression in the developing pancreas, and a severely reduced number of endocrine cells at birth (Jacquemin et al, 2000). Lee et al. identified several HNF-3 binding sites within the 5.7-kb human ngn3 promoter. They tested two f the very prominent binding sites at -3687 bp and -200 bp by EMSA and found that both sites bind *in vitro* produced HNF3β with high affinity(Lee et al, 2001). In

addition, co-expression of HNF3β can activate the ngn3 promoter in transiently transfected 3T3 fibroblast cells (Lee et al, 2001)

<u>Regulation of gene expression by Ngn3</u>

In order to better understand the function of neurogenin3, it is essential to identify the factors that it regulates. NeuroD1 is one of the candidate factors. The promoter fragments of NeuroD1 contain E boxes where Ngn3 can bind and activate the promoter (Huang et al, 2000). Another factor that may be a target of Ngn3 is the paired homeodomain transcription factor Pax4. HNF4 binding site, HNF1 binding site and E box are located around -1900 bp of the Pax4 promoter. HNF1 and Ngn3 can bind to their binding sites and can physically interact and recruit a transcriptional co-activator to activate the Pax4 gene expression (Smith et al, 2003). Nkx2.2 is one of the possible direct downstream targets of Ngn3. Expression of Nkx2.2 promoter depends on the Foxa-binding site and E box, which are located proximal to the transcription initiation site. Foxa2 and Ngn3 or NeuroD1 can bind to each site to interact and synergistically activate the promoter (Watada et al, 2003). This suggests that Ngn3 or NeuroD1 cooperates with Foxa2, in order to recruit a transcriptional co-activator and activate the expression of Nkx2.2.

The other candidate that is influenced by Ngn3 is Ngn3 itself. Unlike Pax4 and Nkx2.2 promoter, Ngn3 itself represses the Ngn3 promoter. This repressor effect seems to be mediated by direct competitive binding of another strong transcription factor, because Ngn3 itself does not show transcription repressor activity (Smith et al, 2004).

Hypothesis

The endodermal fork head transcription factor Foxa1 is expressed in the mouse prostate epithelial cells from E12 through the adult prostate. Foxa2, a closely related member of Foxa1 is expressed only at the tips of growing prostatic buds in the normal prostate. Foxa2 expression in never detected in the adult prostate epithelial cells. Foxa2 expression is detected in the prostatic neuroendocrine tumor cells of the 12-T10 mouse prostate and in the TRAMP neuroendocrine prostate tumors. The prostatic adenocarcinoma from the 12-T7f mice never expresses Foxa2. This lead us to hypothesize that Foxa2 plays an important role in the development of neuroendocrine prostate tumors. Foxa2 expression may result in the expression of some key downstream transcription factors that can in turn lead to neuroendocrine differentiation. We hypothesized that the loss of Foxa2 in the TRAMP mice (develop NE prostate tumors) would prevent the formation of neuroendocrine tumors or would result in the formation of adenocarcinoma in place of NE tumors. In order to test this hypothesis we performed the experiments outlined in the chapters III and IV.

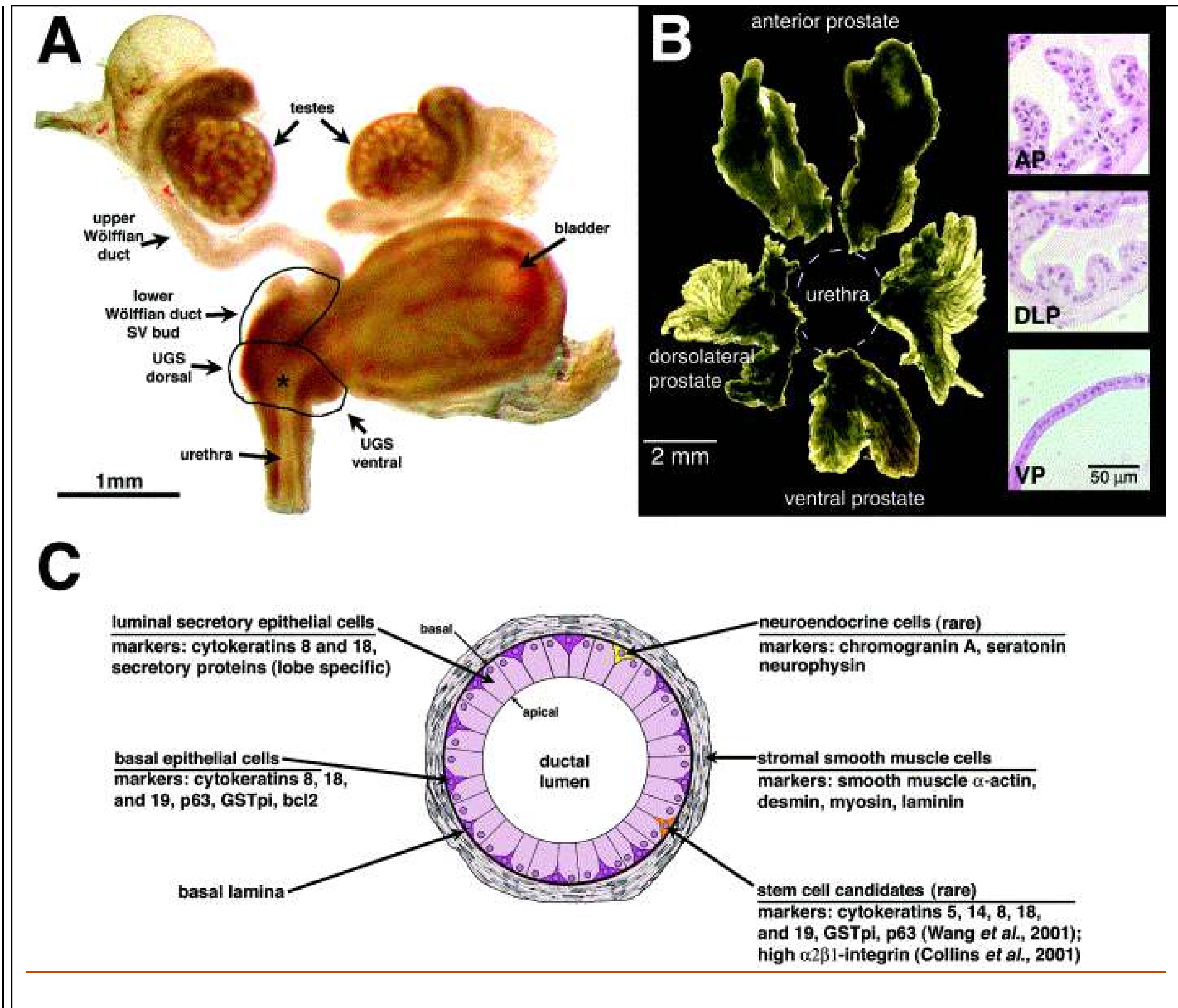


Figure 1-1: Morphological and cellular features of the prostate gland.

(A) The prostate develops from the urogenital sinus (UGS), which is located at the base of the developing bladder. At this stage, the urogenital sinus epithelium is visible as a dilation of the urethra surrounded on the dorsal and ventral sides by condensed urogenital sinus mesenchyme (darker areas). (B) The lobes of the adult mouse prostate are shown together with hematoxylin and eosin-stained sections of prostatic ducts from each lobe (B, inset micrographs). Each lobe has a distinct shape and histologic appearance. (C) A diagram of a ductal cross-section is shown (C) with labels indicating cell types that are present in prostatic ducts including luminal secretory epithelial cells, basal epithelial cells, neuroendocrine cells, stromal cells, smooth muscle cells, and stem cell candidates. Beneath the label for each cell type is a list of differentiation markers commonly used to distinguish these cell types.

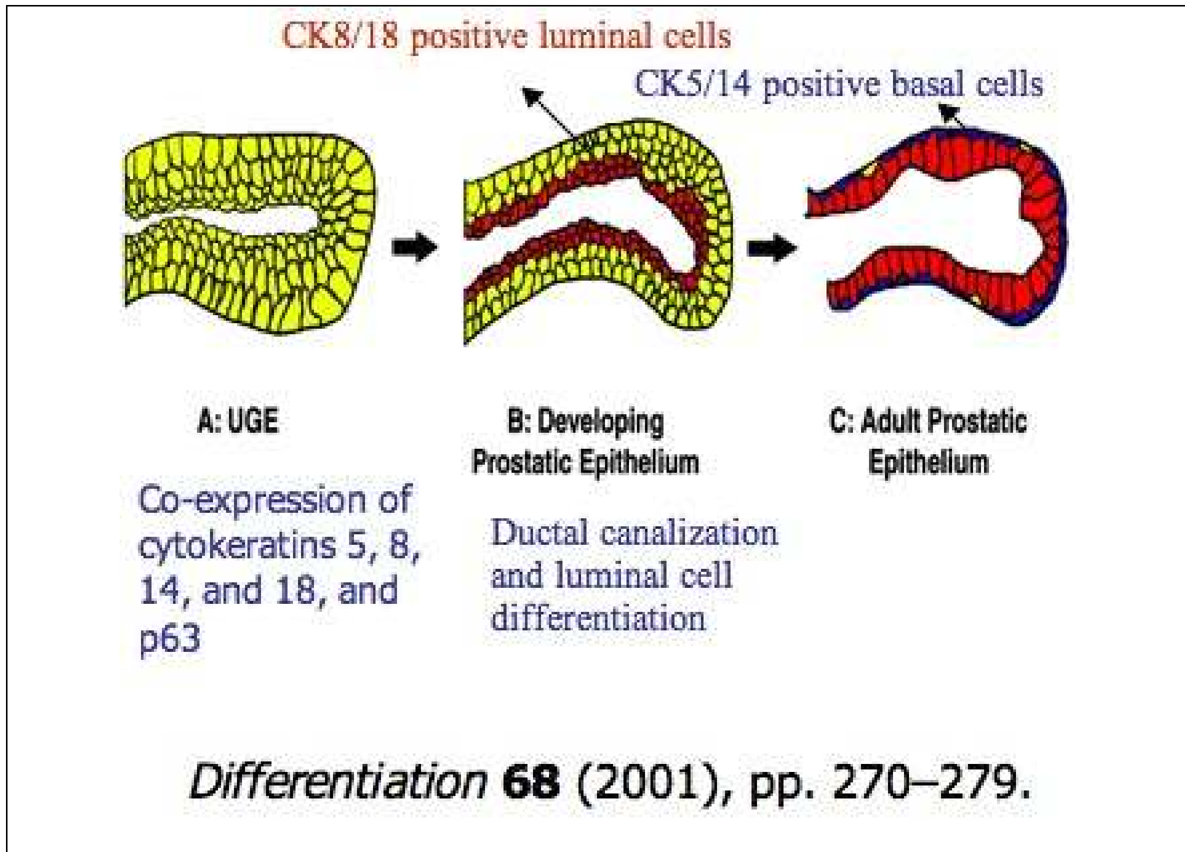


Figure 1-2: Cell lineage relationships in adult prostate

Definitive basal cells (CK5/14/p63/CK19/GSTpi positive/ CK8/18 negative) are *blue;* definitive luminal cells (CK8/18 positive/ CK5/14/p63 negative) are *red;* embryonic-like cells co-expressing both luminal and basal cell markers (CK8/18/14/5/p63/CK19/GSTpi positive) are in *yellow*. In urogenital sinus epithelium, the epithelial cells co-express all markers (yellow cells), and definitive basal and luminal cells are not seen (no red and blue cells). The developing prostate contains embryonic-like cells (yellow) and some definitive luminal cells (red), but no definitive basal cells. The adult prostate contains definitive luminal cells (red) and definitive basal cells (blue) and the extremely rare embryonic-like progenitor/stem cells (yellow). Appearance of the definitive basal cells is a late event.

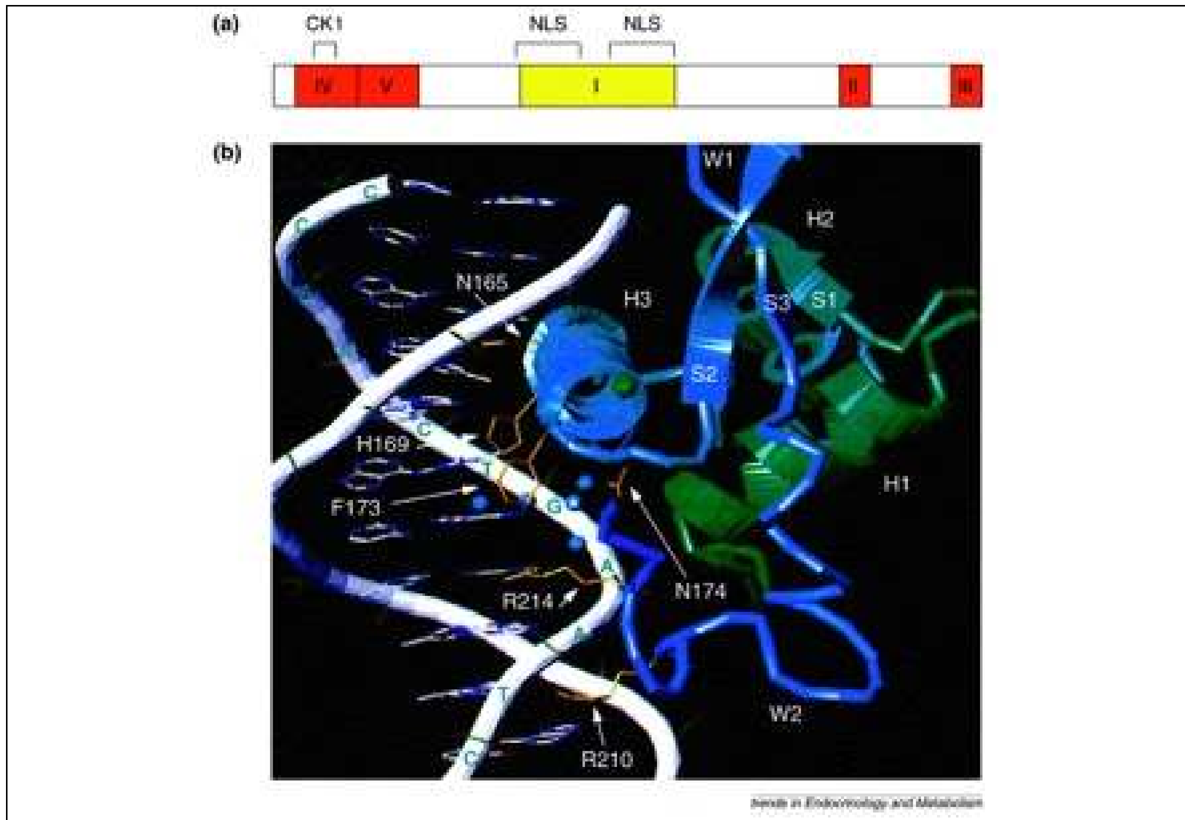


Figure1-3: HNF3γ- DNA interactions

Cartoon of the side chain-base contacts, including α-helix H3 lying in the major groove making direct and water mediated interactions. The Mg^{+2} ion bound at the C- terminus of H3 is depicted as a green sphere, and the water molecules are shown as blue spheres (Kaestner, 2000)

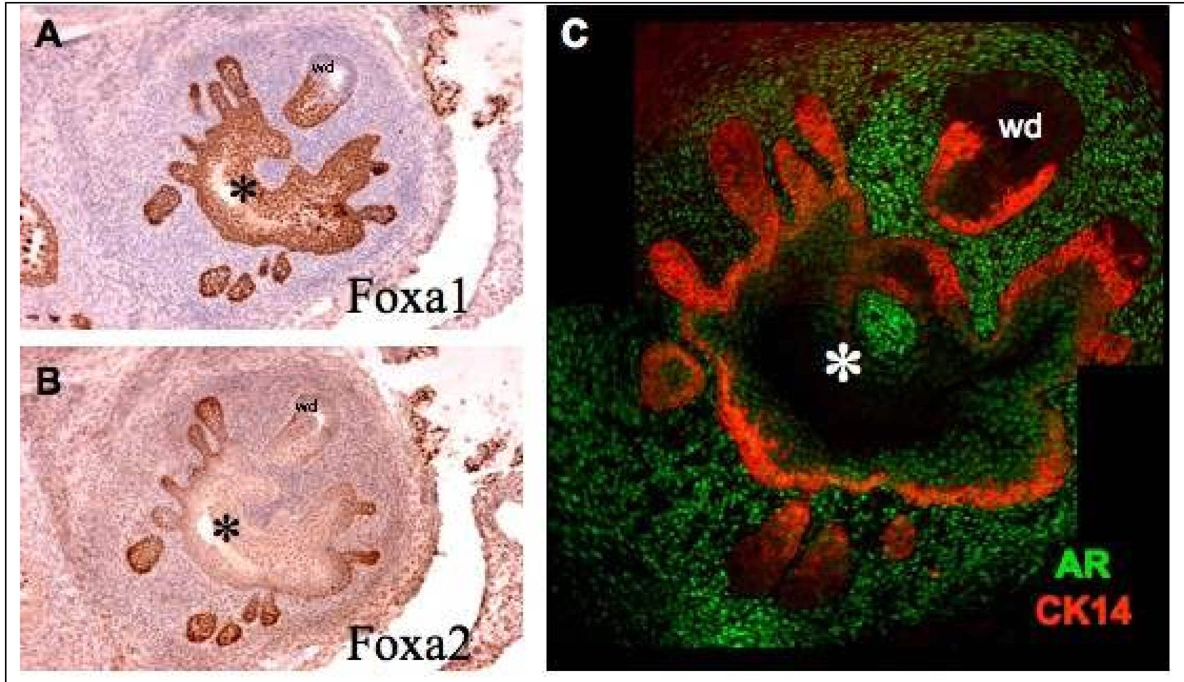


Figure1-4: Foxa, AR, and cytokeratin14 protein expression in E21 developing murine prostate.

Strong Foxa1expression was observed in all epithelial cells of endodermal origin. Foxa2 expression was strongest in the newly forming prostatic buds. AR was strongly expressed in both the urogenital sinus epithelium and mesenchyme (Mirosevich, 2005).

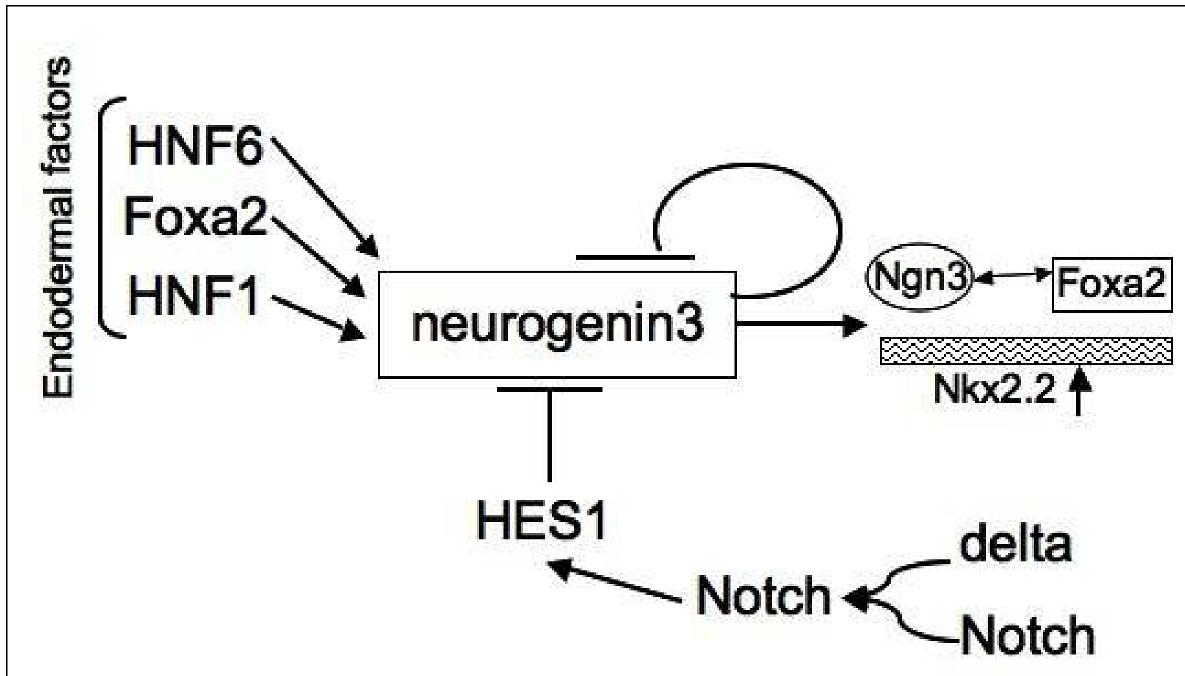


Figure1-5: Cartoon showing the regulation of gene expression of Neurogenin3.

The endodermal factors HNF6, Foxa2 and HNF1 activate neurogenin3 and Notch signaling inhibits neurogenin3. Nkx2.2 is one of the downstream targets of neurogenin3 (Wilson, 2003).

CHAPTER II

MATERIAL AND METHOD

Reporter Plasmids and Expression Vectors

Luciferase reporter plasmids

A 621-bp fragment of PSA minimal promoter (610-11 nt) was amplified by PCR and cloned at the *Sma*I and *Xho*I sites of pGL3-basic luciferase vector (PromegaCorp.), after which an 823-bp upstream enhancer fragment (4758-3935 bp) containing the 4.1/ 3.9 kb PSAcore enhancer region was obtained by PCR and inserted upstream of the PSA promoter at *Sac*I and *Sma*I sites, resulting in PSA-EPLuc reporter construct (EP stands for enhancer/promoter). The primers used for PCR were:

PSA-P-F 5'-CCCGGGTTGGATTTTGAAATGCTA-3'

PSA-P-R 5'-CTCGAGAAGCTTGGGGCTGG-3'

PSA-E-F 5'-GAGCTCCTGCAGAGAAATTA-3'

PSA-E-R 5'-CCCGGGCCATGGTTCTGTCA-3'

Mash-1 Expression vector

Mash-1 was cloned into the lentiviral vector (plenti Erin EGFP) obtained from Dr. Simon Hayward (Department of Urologic Surgery, Vanderbilt University). Mash-1 cDNA was amplified from the RNA obtained from NE-10 tumor. The cloning was a two step process where mash-1 was first cloned into TOPO 2.1 (Invitrogen). The positive

clones were double digested with XhoI/EcoRI to release the insert which was cloned back into plenti Erin EGFP at the XhoI/EcoRI site.

Cell Culture and Transfection Assays

The human prostate carcinoma cell line, LNCaP and the human cervical adenocarcinoma cell line Hela were obtained from American Type Culture Collection (Manassas, VA). All cell lines were cultured as recommended by American Type Culture Collection.

Transient transfection assays were performed using lipofectin reagent (4μl/well) for LNCaP cells and lipofectamine 2000 for Hela cells. The pRL-CMV containing the *Renilla* luciferase reportor gene (Promega, Madison, WI) was used to optimize transfection efficiencies for each cell line. Optimal volumes of liposome and transfection durations were obtained and used to get highest transfection efficiencies. Briefly, cells were seeded at an initial density of 8-10 $\times$ 10^4 /well in 24-well plates one day before transfection. The following morning, cells were transfected with plasmid DNA and lipofectin or lipofectamine 2000 in Opti-MEM I Reduced Serum Medium (Gibco, 31985). Luciferase reporter construct in transfection experiments where AR expression was required, 0.2 μg of rat AR expression vector was transfected for each well. The total amount of plasmid DNA was normalized to 0.8-1 μg /well by the addition of pVZ-1 plasmid. In addition, all samples received 12.5 *ng* /well of pRL-CMV reporter plasmid. After 4-6 h of transfection, the medium was replaced by Minimum Essential Medium (Gibco, 11090) with 5% Charcoal/Dextran Treated Fetal Bovine Serum (HyClone, Logan, Utah) in the presence or absence of 10^{-8} M of R1881 or DHT. Cells were harvested and lysed with 80-µl passive lysis buffers after 24 h of incubation.

Luciferase Reporter Assay

The luciferase activity was determined using the dual luciferase reporter assay system (Promega, E1960) and LUMIstar (BMG lab Technologies, INC., Durham, NC). Background activity of the cell lysate with no DNA transfecton was subtracted from the activities obtained from experimental group. All values were normalized by *Renilla* activity to correct for the transfection efficiency. Results are presented as relative luciferase activities. Each experiment was at least repeated three separate times in triplicate.

Western Blot Analysis

Cell pellets and freshly dissected mouse tissues were collected, sonicated and centrifuged in cold RIPA buffer (1×PBS, pH 7.4, 1% Nonidet P-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM PMSF and 1 × concentration of complete protease inhibitor cocktail). Following the transfer to polyvinylidene fluoride (PVDF) membrane (Invitrogen), membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and 5% skim milk, and incubated with primary antibody (1:1000 dilution: anti-Foxa1, anti-foxa2, anti-mash-1 and anti-NSE, anti-ChrA and anti-ß-actin) for 1 hr with shaking at room temperature. This was followed by thorough washing of the membranes with 1XTBS-Tween. The membranes were incubated overnight with the HRP conjugated secondary antibodies and were washed the next morning and the signal was visualized by enhanced chemiluminescence (ECL) assay (Amersham Pharmacia Biotech).

***In Vitro* Transcription and Translation (TnT) of Foxa1and Foxa2 and Purification of GST-AR Fusion Proteins**

GST-AR fusion proteins were purified as described previously (Snoek et al., 1996). Five mLs of overnight cultures of pGEX-3X-AR plasmids in LB/ampicillin were added into 200 mLs LB/ampicillin in the next morning. Grow the cultures at 37 C until OD600=0.6 (approximately 2 hrs), then induce protein expression with 0.1mM IPTG (20uL of 1M IPTG stock). Continue to grow the bacteria (JM109) at 30 C with shaking for 2 hrs. Spin cultures at 8000 rpm for 10 min at 4 C. Resuspend cells in 2mL Lysis buffer (PBS, protease inhibitors, 100 ug/mL lysozyme) and transfer to 15mL falcon tube. Place on ice for 30 min, then sonicate at maximum setting for 10 seconds then place on ice for 10 seconds. Repeat 5-6 times until solution turns clear. Aliquot into 1.5mL tubes and spin 13,000rpm for 30 min at 4 C to pellet out the cellular debris. Combine the supernatants from each tubes in a 15mL falcon tube that has preswelled glutathione-agarose beads. Incubate with end-over-end rotation for 1hr at 4 C, then spin tubes at 2000rpm for 15 seconds at 4 C. Wash beads with ice cold PBS (with 1× protease inhibitor) for 4 times. Resuspend beads in 10mL PBS with protease inhibitors and store at 4 C for 2 weeks.

To elute the GST-proteins, Spin beads at 2000rpm for 15sec at 4 C and remove supernatant. Add 1mL of elution buffer (20mM HEPES, pH7.6, 150mM KCl, 5mM $MgCl_2$, 1mM EDTA, 0.05% NP-40, protease inhibitor, 0.046g glutathione) and incubate at room temperature with rotation for 15 min. Spin at 2000 rpm for 15 seconds and collect eluate (fraction #1). Repeat elution for 2 times and collect fraction #2 and #3.

After determination of protein concentration, run a SDS-PAGE followed by Commassie Blue staining to check protein quality.

To pre-swell the glutathione-agarose beads (Sigma #G-4501), measure out 0.08g beads for each GST fusion protein. Add 14mL of ice cold PBS and allow beads to swell at least 1 hr on ice. Spin down 2000rpm for 15 seconds and wash beads with PBS once. Spin down again and remove supernatant before adding the GST-protein lysate.

GST-Pull Down Assays

For GST-pull down assays, 50 µl swelled glutathione agarose beads (Sigma G-4510) were incubated with 20 µg GST or GST-AR fusion proteins for each reaction. GST-bound beads were equilibrated with PBS-T binding buffer (1×PBS, pH 7.4, 1% Tween-20, and protease inhibitors) and incubated for 2 h at 4 °C with 5-10 µl products from the TnT reactions. Complexes were washed four times with 1.5 ml of cold binding buffer, heated for 10 min at 70 °C in 1×LDS loading buffer, and separated by SDS-PAGE. Then, anti-Foxa1 and anti-Foxa2 antibodies were used in a standard Western blot to detect proteins that interact with AR *in vitro*.

12T-10 transgenic mouse line

LPB-Tag transgenic mice lines were established with the 5'-flanking region of the rat LPB promoter (-11,500 to +28 bp) (Yan, 1997) linked to the SV40-Tag gene deletion mutant (d1 2005), which removed the expression of the small Tag. Seven transgenic lines were established and maintained in the CD1 mouse strain(Kasper, 1998) . The 12T-10 transgenic line showed the slowest neoplastic prostate growth rate of the seven LPB-Tag

transgenic lines and developed NE features (Masumori et al, 2001). The NE carcinomas occurred in the dorsolateral and ventral lobes and were generally androgen receptor negative. The 12T-10 line develops Low Grade PIN (LGPIN) and high grade PIN (HGPIN) in animals ranging from the age of 2-5 months(Masumori, Thomas et al., 2001). The HGPIN lesions, in mice around 5 months old, began to show NED. Metastases are detected in 6-14 month old animals. The most common sites of metastases are regional lymph nodes, liver, and lung. These metastases are poorly differentiated neuroendocrine cancer (Masumori et al, 2001).

NE-10 allograft model

The primary NE prostate from the 12T-10 transgenic line was implanted subcutaneously (s.c.) in immune-compromised male athymic nude mice. The transplanted tissue was recovered after 18 weeks and a small piece was passed to another nude mice thus maintaining the NE-10 line (Masumori, 2004). The histological analysis of the NE allograft showed the same features as the primary NE prostate from the 12T-10 model (Masumori, 2004). The early passages (p1-15) show metastases to liver and micro metastases to lung, which are histologically similar to the metastasis seen in the 12-T10 mice.

Tissue preparation and processing

Mice were sacrificed by cervical dislocation after the inhalation of an anesthetic agent according to the policy of the Vanderbilt University Animal Care and Use Committee. The prostates were generally dissected into four different lobes (ventral, lateral, dorsal, and anterior lobe) under a dissecting microscope. When it was not possible

to separate the lateral and dorsal lobes, the tissue was taken together as the dorsolateral lobe. Regional lymph nodes, liver, lung, spleen, kidney, bone (lumbar vertebrae), adrenal glands, brain, bulbourethral glands, and s.c. NE-10 tumors were also harvested for histological examination. Tissues were fixed in 10% buffered formalin and processed and embedded in paraffin using standard techniques. Paraffin-embedded tissues were cut at 5 µm, and sections were either stained with Haematoxylin and Eosin (H&E) or with relevant antibodies for immunohistochemical analysis.

Immunohistochemistry

Slides were deparaffinized by immersing in xylene twice for 10 min each and hydrated by immersing in a series of 100%, 95%, 70%, 50% ethanol, and one time in dH_2O for 5 min each. Slides for histological analysis were stained with H&E by standard methods, with generally 3 to 4 sections reviewed per specimen. For Foxa2, synaptophysin, T-antigen, AR, mash-1, Ngn3 and Nkx2.2 immunostaining, antigen retrieval was achieved by micro waving in antigen unmasking solution (Catalog # H-3300, Vector Laboratories, Inc.) for 30 min and the slides were then equilibrated at room temperature for 1 hour. Endogenous peroxidase activity was blocked by peroxidase blocking reagent (Dako) 30 min followed by washing in PBS (pH 7.4). After rinsing with PBS, the slides were placed in blocking solution (goat, horse or rabbit serum as appropriate) for 20 min to block nonspecific binding of antibody to the tissue. Sections were incubated with primary antibody overnight at 4°C. The following primary antibodies were used (with the indicated dilutions in PBS): Foxa2, P19, goat antibody (Santa Cruz Biotechnology Inc., 1:1000); synaptophysin (Cat# 611880, BD Biosciences

Pharmingen, 1:500); T-antigen (SV40 T-Ag, monoclonal mouse IgG (Oncogene, 1:1000); AR, N-20 (Santa Cruz Biotechnology Inc., 1:1000); mash-1 (Cat #556604, BD Biosciences Pharmingen, 1:500), Ngn3 (rabbit, 1:6000) antibody was received from Dr. Michael German Diabetes Center in University of California, San Francisco; Nkx2.2 antibody (mouse, 1:40 dilution, Developmental Studies Hybridoma Bank, the University of Iowa) and p63 (rabbit, 1:200; Santa Cruz, sc-8343). The respective secondary antibodies were used at a dilution of 1:200. Staining was visualized using Vectastain ABC kit (Vector Laboratories, Inc, Burlingame, CA) and 3, 3'-diaminobenzidine tetrahydrochloride (Dako). Slides were counterstained with hematoxylin, dehydrated and cover slipped.

RNA isolation and Reverse Transcriptase-Polymerase Chain Reaction

To further characterize the expression of Hes-1, mash-1, Ngn3 and Nkx2.2 in the mouse neuroendocrine tumors (NE-10), we performed RT-PCR analysis on 10 week old normal CD1 DLP, 10 week old 12T-7F, DLP and prostate neuroendocrine tumors were collected. Total RNA was isolated from the mouse tissues using an RNeasy mini kit (Qiagen Inc, Valencia) with residual genomic DNA were removed by RNase-Free DNase (Qiagen) treatment. One microgram of total RNA was reverse transcribed using Superscript-II^{TM} reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was performed using sense and antisense primers to produce gene specific fragments. The primer sequences for each of the genes are listed below:

Hes-1: sense 5' TGGAAATGACTGTGAAGCACCTCC 3' and antisense 5' ATGATCTGGGTCATGCAACTGGC 3'

mash-1: sense 5’ ACGACTTGAACTCTATGGCGGGTT 3’ and antisense 5’ TGACGTCGTTGGCGAGAAACACTA 3’

Ngn3: sense 5’ TTGAGTCGGGAGAACTAGGATGG 3’ and antisense 5’ TTTGCTGAGTGCCAACTCGCTCTT 3’

Nkx2.2: sense 5’ GGGGTTTTCAGTCAAGGACA 3’ and antisense 5’ CTTTGGAGAAGAGCACTCGG 3’

The conditions for the PCR were: 94°C for 5 min (1 cycle), 94°C for 30sec, 60°C for 1 min, 72°C for 1 min (35 cycles), and 72°C for 10 min (1 cycle). PCR products were analyzed by electrophoresis in 1.5% agarose gels containing ethidium bromide and photographed under UV illumination.

Generation of Foxa2 knock out TRAMP mice

The TRAMP Foxa2 knock out mice were generated by breeding in two alleles of floxed Foxa2 along with one copy of Nkx3.1-Cre in the TRAMP background. The Nkx3.1-Cre mice were obtained from Dr. Michael Shen (Center for advanced Biotechnology and Medicine, Rutgers University) and were in the C57Blk6 background. The Nkx3.1-Cre mice were knock-in mice where one copy of Nkx3.1-Cre was replaced with the transgene. The floxed Foxa2 mice were obtained from Dr. Klaus H. Kaestner (University of Pennsylvania) and were a mixed background of CD1 and C57Blk6. The breeding was a three step process.

1. Two copies of floxed Foxa2 allele were bred into the TRAMP background resulting in TRAMP/Foxa2$^{loxP/loxP}$.

2. Two copies of floxed Foxa2 allele were bred into the Nkx3.1-Cre$^{+/-}$ background resulting in Nkx3.1-Cre/Foxa2$^{loxP/loxP}$.

3. The animals obtained from the above two breeding were bred to each other and the next generation was screened for TRAMP/Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$.

Transduction of NeoTag-1 cells

The 293FT cells were used as a packaging cell line. A day before the transfection the 293 FT cells were plated in 10 cm dish such that they are 90% confluent at the time of transfection. On the day of transfection the culture medium of the cells was replaced with 5ml of growth medium with serum. 9 µg of the ViraPower™ Packaging Mix and 3 µg of pLenti EGFP-mash-1 expression plasmid DNA were diluted in 1.5 ml of Opti-MEM® I Medium without serum. The 293FT cells and the ViraPower Packaging Mix was obtained from Invitrogen (Catalog # K4975-00). Lipofectamine 2000 was added to the DNA mix and incubated for 20 minutes at room temperature to allow DNA-lipofectamine complexes to form. DNA-Lipofectamine™ 2000 complexes were gently added to the 293FT cells and incubated overnight at 37°C in a humidified 5% CO_2 incubator with gentle shaking. The next day the lipofectamine-containing medium was replaced with 10ml of culture medium without antibiotics. Virus containing supernant was collected 72 hours post-transfection and centrifuged at 3000 rpm for 15 minutes pelleting out the cell debris and the supernatant containing the viral particles. The viral stock was diluted to 10^{-4} in a complete growth medium and mixed with polybrene (6 µg/ml). This mix was added on to NeoTag-1 cells plated at 30-40 % confluency. After 24 hours the viral containing medium was replaced with the complete culture medium and the infected cells were

incubated overnight. The infected NeoTag-1 cells were treated with blastocydin (10μg/ml) every 3-4 days and the antibiotic-resistant colonies were selected.

CHAPTER III

NEUROENDOCRINE DIFFERENTIATION IN THE 12-T10 TRANSGENIC PROSTATE MOUSE MODEL MIMICS ENDOCRINE DIFFERENTIATION OF PANCREATIC BETA CELLS

Introduction

Neuroendocrine differentiation (small cell carcinoma) in prostate cancer is receiving increasing attention in recent years. It is an androgen insensitive tumor and could be one of the many possible mechanisms by which prostate cancers evolve to androgen insensitive state. Neuroendocrine differentiation is present focally in virtually all cases of prostate cancer. The number of cells in each case varies based on the tissue fixation, sectioning of the tissue, antibody method used and the number of tissue sections examined (Abrahamsson, 1989). Some studies have reported as high as 92% of prostate cancers contain some NE cells (Abrahamsson, 1989; Bostwick, 1994). On the contrary, others have reported much lower numbers in the range of 24-25% of the prostate cancers have some NE cells (Theodorescu, 1997). It is important to better understand the role of neuroendocrine differentiation of prostate cancer since the greater the proportion of neuroendocrine phenotype accompanying prostate carcinoma, the poorer the prognosis for the patient and the greater the likelihood that the tumor will progress to androgen independence (Weinstein, 1996; Abrahamsson, 1999; di Sant'Agnese, 2001). Cohen et al in 1991 showed in their study that prostate cancer case exhibiting greater NE differentiation than those with a low percentage of NE differentiation had a worse outcome (Cohen, 1991). Taplin et al (Taplin, 2005) assessed the significance of NE

differentiation using CgA as a marker in patients with prostate cancer treated with transurethral resection of the prostate and androgen ablation. Their results strongly suggested that NE differentiation correlates with a poor prognosis and disease progression. It was also noted that NE differentiation in prostate cancer was associated with an early failure of androgen ablation therapy. Berruti et al (Berruti et al, 2005) studied 108 patients and concluded that a significant correlation was present between the extent of NE differentiation and either Gleason score or the disease stage, suggesting that the presence of NE features could negatively influence patient outcome. Also, some metastases from conventional ductal prostate cancers exhibit a neuroendocrine phenotype (Roudier et al, 2003). Recent study shows that NE-10, a NE allograft model established from the 12T-10 transgenic model of NE prostate cancer, when grafted on mice bearing a LNCaP xenograft, it maintains growth of the androgen dependent LNCaP xenografts in castrated mice (Jin et al, 2004). This study demonstrates that the neuroendocrine tumor secretions have the potential to convert androgen dependent LNCaP cells to androgen insensitive cells (Jin et al, 2004). Understanding the molecular mechanisms that lead to NED would be an important step towards early diagnosis and effective treatment for this aggressive and androgen insensitive form of prostate cancer.

A significant amount of work is done to understand the effects of neuroendocrine differentiation in prostatic carcinoma. There is very little literature talking on the molecular mechanism/s that can lead to prostate neuroendocrine cancer. With the aim to discover the molecular pathways that can lead to NED in the prostate, we have made use of the 12-T10-mouse model. Targeting SV 40 large T-antigen to the luminal epithelial cells of the mouse prostate using the prostate luminal epithelial cell specific probasin

promoter generated this line of transgenic mice (Masumori et al, 2001). Seven transgenic lines were established and maintained in the CD1 mouse strain (Kasper, 1998) . The 12T-10 transgenic line showed the slowest neoplastic prostate growth rate of the seven LPB-*Tag* transgenic lines and developed NE features (Masumori et al, 2001). The NE carcinomas occurred in the dorsolateral and ventral lobes and were generally androgen receptor negative. The 12T-10 line develops LGPIN and HGPIN in animals ranging from the age of 2-5 months (Masumori et al, 2001). The HGPIN lesions, in mice around 5 months old, began to show NED. Metastases are detected in 6-14 months old animals. The most common sites of metastases are regional lymph nodes, liver, and lung. These metastases are poorly differentiated neuroendocrine cancer (Masumori et al, 2001). The results obtained from the 12-T10-mouse line were further confirmed in NE-10 allograft model, where the primary prostates from the 12-T10 mice were subcutaneously injected into the nude mice. This allograft had the same histological features as the primary prostates for the 12-T10 line (Masumori, 2004). They demonstrated metastases to liver and lung with the same NE features (Masumori, 2004).

Earlier work from our laboratory has shown that the endodermal forkhead transcription factor, Foxa2 is expressed at the growing tips of prostatic buds from E18 through E21(Mirosevich, 2005). We have also shown that Foxa2 is only expressed in the neuroendocrine 12-T10 prostates and is never seen expressed in the normal mouse prostate or in the non-neuroendocrine tumors from the 12-T7f mouse prostates(Mirosevich, 2006). In this study we have taken this work further and shown that following Foxa2, a number of other downstream transcription factors involved in pancreatic endocrine ß-cell differentiation are also sequentially expressed in the NE

prostate tumors. Figure 3.1 shows the sequence of transcription factors that are expressed in pancreatic endocrine precursor cells. First, endodermal transcription factors, HNF6, Foxa2 and HNF1 are expressed. These differentiating endocrine cells express Notch ligands (delta and serrate) on the surface and activate Notch signaling in adjacent cells, this prevents neighboring cells from differentiating into the same cell type. Thus Notch signaling maintains a balance in the various cell types within the pancreas. Notch signaling mediates its response through activation of Hes genes (Jan, 1993). Hes genes act as transcriptional repressors and Hes1 inhibits the expression of pro-endocrine gene, Ngn3 (Apelqvist, 1999; Jensen, 2000) (Figure 3-1). Hes1 is known to bind to the N box–related sequence of the mash-1 promoter and repress mash-1 transcription (Chen et al., 1997). Animals lacking Ngn3 fail to develop endocrine cells (Gradwohl et al, 2000) and ectopic expression of Ngn3 causes premature differentiation of pancreas into endocrine cells (Apelqvist, 1999; Schwitzgebel et al, 2000). Once Ngn3 is activated in the progenitor cell, that cell is fated to become an endocrine cell. The Ngn3 promoter contains Hes1 binding sites proximal to the TATA box that inhibit transcription of Ngn3 (Lee et al, 2001). In addition to Hes-1, the Ngn3 promoter contains binding sites for HNF1, Foxa2 and HNF6 (Jacquemin et al, 2000; Lee et al, 2001). Figure 3-1 shows that Nkx2.2 is a downstream target of Foxa2 and Ngn3 which interact synergistically to recruit transcriptional activators to Nkx2.2 gene promoter(Watada et al, 2003). Nkx2.2 is expressed in mature insulin secreting ß-cells of the pancreas

Results

Sequential expression of transcription factors in Neuroendocrine PIN

Immunohistochemical analysis was performed on the NE PIN lesions from the 12-T10 mouse prostate to estimate the expression of Foxa2, mash-1, Ngn3 and Nkx2.2. All of the PIN lesions analyzed were positive for synaptophysin, a marker for NE cells (Figure 3-2C &D). The NE PIN lesions were mainly negative for AR expression (occasionally individual faintly AR positive cells are detected) as shown in Figure 3-2E &F. These PIN lesions did express T-antigen, the transgene used to generate these prostate tumors (results not shown). The PIN lesions were always positive for Foxa2 (Figure 3-2A &B) as indicated by the arrow, mash-1 expression was detected in only about 50% of the samples analyzed (Figure 3-2G &H). In Figure 3-2 the area surrounding the region indicated by the arrow shows a faint positive staining for Foxa2 (Figure 3-2B) and also a decrease in AR expression in these cells (Figure 3-2F). Although these cells did not express synaptophysin and mash-1, the faint positive staining for Foxa2 suggests that the cells might be beginning to differentiate into neuroendocrine cells in which Foxa2 is an early marker Expression of Ngn3 in the normal prostate or during early stages of PIN in the 12T-10 line could not be detected even by RT-PCR. These results suggest that there is a sequential expression of these transcription factors in the NE prostate tumors where Foxa2 and mash-1 are the early markers that get expressed in the PIN lesions and Ngn3 and Nkx2.2 are the late stage markers. Further RT-PCR analysis was performed on the NE-10 tissue to confirm the expression of Hes-1, mash-1, Ngn-3 and Nkx2.2 in the poorly differentiated NE tumors. Figure 3-3A shows that Hes-1

expression is lost in these NE tumors (lane3) compared to the prostate from a normal mice (lane 1) and the non-neuroendocrine 12T-7 prostate (lane 2). mash-1 is expressed only in the NE-10 prostate (Figure 3-3B) (lane3) and the normal mouse prostate (lane 1) and the 12T-7f prostate (lane 2) do not express mash-1. Similarly Ngn3 expression was detected only in the NE tumor (Figure 3-3C: lane 3). Very faint expression of Nkx2.2 (Figure 3-3D: lane3) was detected only in the NE10 sample, suggesting that limited number of cells express it or the expression level is very low. Immunohistochemistry in Figure 3-3 shows an example of HGPIN that has some features consistent with early stage prostate cancer where the ductal structure is still partially retained. The HGPIN cells in this sample are positive for synaptophysin expression (Figure 3-3F), marker for NE cells. These cells also express strong levels of Foxa2 (Figure 3-3G) and mash-1 (Figure 3-3H). AR expression is completely lost in these cells (results not shown). Nkx2.2 expression is not detected in any of these cells (Figure 3-3I).

Dorsolateral prostate of 12T-10 mice expresses pro-endocrine genes

Immunohistochemical analysis was performed on the poorly differentiated NE carcinoma of the dorsolateral prostate from 56 week old 12T-10 mice. The H&E staining (figure 3-4A) shows that the tumor is a poorly differentiated cancer with complete loss of ductal structure. Advanced NE tumors continue to express synaptophysin (Figure 3-4C), a marker of NE cells. In accordance with our previous report (Mirosevich, 2006) the NE cells express Foxa2 (Figure 3-4B). Figure 3-4D shows that the NE cells are positive for large T-antigen, the transgene targeted to the prostate by the probasin promoter. These NE tumors show loss of androgen receptor (Figure 3-4E). Panels F and G of Figure 3-4 show positive immunostaining of the NE tumor for mash-1 and Ngn3 respectively. Foxa2

interacts with Ngn3 or NeuroD1 to recruit transcriptional activators to Nkx2.2 promoter (Watada et al, 2003). Thus, the expression of Nkx2.2 by NE tumor cells is consistent with the expression of known activators of this gene (Figure 3-4H). The homeodomain transcription factor Nkx2.2 is required for the final differentiation of the β-cells in the pancreas and for the production of insulin. The expression of Nkx2.2 is not uniform throughout the NE tumor suggesting that certain cells may lack factors required to activate the Nkx2.2 promoter. The RT-PCR results for Nkx2.2 expression (Figure 3-3D) in the NE tumor shows a very weak expression suggesting that not all the cells express Nkx2.2, also reconfirming the immunohistochemistry results.

The Ventral Prostate of 12T-10 mice does not express Nkx2.2

Panel A of Figure 3-5 shows H&E staining on the serial section of an advanced and poorly differentiated NE tumor derived from the VP of 12T-10 mice. Figure 3-5 shows the expression of Foxa2 (B), Synaptophysin (C), Large T-antigen (D), androgen receptor (E), mash-1 (F), Ngn3 (G) and Nkx2.2 (H) in the serial sections from the VP of 52 week old 12T-10 mice. This NE tumor was positive for Foxa2, mash-1 and Ngn3 but expression of Nkx2.2 was not detected. Lung metastases from the same mouse express Ngn3 but do not express Nkx2.2 while the liver metastasis from this animal did not express either Ngn3 or Nkx2.2 (results not shown). This suggests that the primary NE tumors could have a mixed population of cells; some express Nkx2.2 and some do not as is the case in the DLP in Figure 3-4. It is also possible to have an advanced NE tumor with cells not expressing detectable levels of Nkx2.2 as in Figure 3-5H. This observation suggests that either NE tumors advance to different stages in different mice and/or the

tumors are heterogeneous in nature with different patterns of Ngn3 and Nkx2.2 expression.

<u>Neuroendocrine Liver metastases express Nkx2.2</u>

The 12T-10 mice usually demonstrate extensive liver metastases with only micro metastases to the lung. Immunohistochemical analysis showed that the NE cells that reside in the two metastatic sites express different genes. H&E staining (Figure 3-6A) shows liver metastases with rosette histology, typical pathology of small cell carcinoma/NE cancer. Figure 3-6 shows positive expression of Foxa2 (B), synaptophysin (C) and large T-antigen (D) in a liver metastasis from a 69 weeks old 12T-10 mouse. These metastases lose androgen receptor expression (E). The liver metastases express mash-1 (F) but do not express Ngn3 (G). The loss of Ngn3, a marker for differentiated endocrine cells, suggests that cancers have progressed to poorly differentiated NE tumors. However, the liver metastases can still express Nkx2.2 (H) even in the absence of Ngn3. Based on a 15 mice studied, Nkx2.2 expression was not seen in any of the NE lung metastasis but 33% of liver metastasis were positive for Nkx2.2 expression. Thus, even when mice had an Nkx2.2 positive liver metastasis, the lung metastases in the same animals were negative for Nkx2.2. This demonstrates that the metastases to different sites are different in terms of the gene expression profile. This could indicate that Nkx2.2 is not required for liver metastasis since 66% of the metastasis were negative for this gene. However, cells expressing Nkx2.2 did prefer to grow in the liver and never the lung. None of the liver NE metastases expressed Ngn3, suggesting that the 33% that do express Nkx2.2 must regulate the Nkx2.2 gene by a different pathway.

Neuroendocrine Lung metastases have different gene expression profile compared to liver metastases.

Figure 3-7 shows serial sections of NE lung metastases from a 69 week old 12T-10 mouse that also had liver metastasis (Figure 3-6). The NE lung metastases express Foxa2 (Figure. 3-7B), synaptophysin (Figure. 3-7C), Large T-antigen (Figure 3-7D) and mash-1 (Figure 3-7E). As in the primary NE tumors, lung NE metastases do not express androgen receptor (Figure. 3-7F). Unlike liver metastases, lung metastases always express Ngn3 (Figure 3-7G) but never express Nkx2.2 (Figure. 3-7H). This suggests that although Ngn3 is expressed in the lung metastases, Ngn3 is unable to switch on the expression of Nkx2.2, probably because of the lack of other transcription factors that also control the Nkx2.2 promoter. Alternatively, Nkx2.2 expression may be controlled by inhibitory signals from the surrounding microenvironment.

The neuroendocrine allograft model (NE-10) reconfirms the results seen in 12T-10 neuroendocrine mice model.

The neuroendocrine prostate tumor from the 12T-10 mice was grafted subcutaneously into athymic nude mice. These grafts were harvested and further passaged to establish a transplantable allograft model termed NE-10 (Masumori, 2004).These subcutaneous grafts retained the characteristic rosette pattern of the 12T-10 NE prostate tumors, express the neuroendocrine markers and metastasize to the lung and liver of the nude mice. We checked the expression of the genes involved in pancreatic differentiation in the NE-10 subcutaneous grafts as well as the lung and liver metastases. The subcutaneous graft and the liver metastases from P13 (passage 13) NE-10 tumor were positive for Foxa2 as shown in figure 3-8A and 3-8E respectively. Further, mash-1

expression was seen in both subcutaneous (Figure. 3-8B) and liver metastases (Figure. 3-8F) samples. The subcutaneous tumor expressed Ngn3 (Figure. 3-8C) and Nkx2.2 (Figure. 3-8D) but the liver metastases from the same mice did not express Ngn3 (G) but did express Nkx2.2 (H), consistent with what is seen in the 12T-10 transgenic model. The lung metastases, on the contrary, express Ngn3 and do not express Nkx2.2 (data not shown). Since the subcutaneous NE-10 allograft is heterogenous for the expression of Nkx2.2 but only lung metastases express Ngn3 and only liver metastases express Nkx2.2, this observation again suggests that different populations of NE cancer cells adapt to the microenvironment of the metastatic site. The NE-10 subcutaneous tumor, liver and lung metastases express Large T-antigen and synaptophysin but they do not express the androgen receptor (data not shown). The consistency between the results seen in the 12T-10 and NE-10 model suggests that prostate NED follows the similar molecular pathway as that involved in pancreatic endocrine cell differentiation. To establish that this pathway is not unique to NED of mouse prostatic tumors, the expression of hASH-1 (Figure 3-9A) and synaptophysin (Figure 3-9B) was confirmed in human neuroendocrine prostatic tumors. We have reported that the human NE prostate tumors express Foxa2 and generally show loss or reduction of androgen receptor (Mirosevich, 2006). These results suggest that the human NED of prostate cancer follow the same pathway as the NE mouse models of prostate cancer.

Foxa proteins interact with the DNA binding domain of AR

In vitro GST pull-down assay was performed to confirm the AR/Foxa2 interaction as well as to determine the interacting regions. A full-length Foxa2 protein was synthesized *in vitro*. Five GST-AR fusion proteins containing different AR subdomains

(Figure 3-10A) were purified as described before (Snoek et al, 1996). Figure 3-10B is a GST pull down experiment showing that the AR DBD/hinge region (524-649 amino acids) alone is sufficient to mediate the interaction with Foxa2.

In the experiments, the ARNT/DBD showed a weaker interaction with Foxa1 as compared with ARDBD/Hinge or ARDBD/LBD (Figure 3-10B), suggesting AR N-terminal might have a negative effect on the interaction. Foxa1 has been reported to interact with AR and regulate prostatic gene expression (Gao et al, 2003). To determine whether similar protein interactions also occur between Foxa2 and AR, GST-pull down experiments were performed. *In vitro* translated full length Foxa1 or Foxa2 was incubated with various purified AR-GST fusion proteins in which different domains of AR were fused with GST. Similar to Foxa1, Foxa2 bound specifically to AR-GST protein (Figure. 3-10). Further, the DNA binding domain of AR was required and sufficient for the protein/protein interaction, indicating that Foxa2 interacts with AR through DNA binding domain (Figure 3-10).

Transcriptional Activity of Foxa1 and Foxa2 on Prostate Gene Expression

To determine the role of Foxa proteins in the regulation of prostatic genes, the ability of exogenous Foxa1and Foxa2 to activate prostate-specific antigen gene expression was assessed in culture of non-prostatic and prostatic cells. Transient transfection experiments were performed on Hela and LNCaP cell lines using the PSA promoter-luciferase construct (PSA) co-transfected with various expression vectors (Figures 3-11B,C). The Hela cells used in these experiments have previously been shown to be negative for Foxa1 expression (Gao et al, 2003) and Foxa2 (data not shown). As shown in Figure 3-11B, cotransfection of PSA with androgen receptor markedly

increased luciferase activity in Hela cells cultured with the addition of DHT. The addition of Foxa1 resulted in repression of PSA activity when DHT was present (Figure 3-11B). In contrast, co-transfection of Hela cells with Foxa2 stimulated PSA transcriptional activity with or without androgen receptor and in the presence or absence of DHT (Figure 3-11B). Transient transfection experiments performed in LNCaP cells produced similar results to Hela cells, however, Foxa2 stimulated PSA promoter-luciferase activity to a greater degree, without the additional androgen receptor co-transfection, in the presence or absence of DHT (Figure 3-11C). Additionally, similar luciferase activity results were obtained when the rodent androgen-regulated prostate specific probasin promoter-luciferase construct (Gao et al, 2003) was used instead of PSA in both Hela and LNCaP cell lines (results not shown). These data show that Foxa2, but not Foxa1, can positively regulate androgen-dependent promoters in an androgen-independent manner. In vitro experiments suggest that Foxa2 may be involved in androgen-independent gene regulation. Unlike Foxa1, Foxa2 was able to activate the PSA promoter in an androgen receptor and ligand-independent manner in Hela cells, while overexpression of Foxa2 in LNCaP cells increased the activity of the PSA promoter with or without ligand, and with the endogenous mutated androgen receptor expressed by LNCaP cells. Since Hela cells do not normally express either Foxa1 or Foxa2, transfected Foxa2 did not have to compete with endogenous Foxa1 for the same binding sites on the PSA promoter. However, LNCaP cells have high Foxa1protein levels, which may act to abrogate Foxa2 binding to the same DNA site. Indeed, overexpression of androgen receptor in LNCaP cells suppressed the effect of Foxa2 by direct interaction with Foxa1 (Gao et al, 2003). Although numerous co-factors have been shown to interact with the androgen receptor to

activate or repress PSA promoter activity (Suzuki et al, 2003); to our knowledge, Foxa2 is the first transcription factor demonstrated to activate this promoter without the requirement for the androgen receptor as well as enhance androgen receptor activity in the absence of ligand. As such, Foxa2 provides a potential mechanism for androgen-independent gene activation that occurs when prostate cancer patients fail androgen ablation therapy, and may represent a novel therapeutic target for advanced prostate cancer.

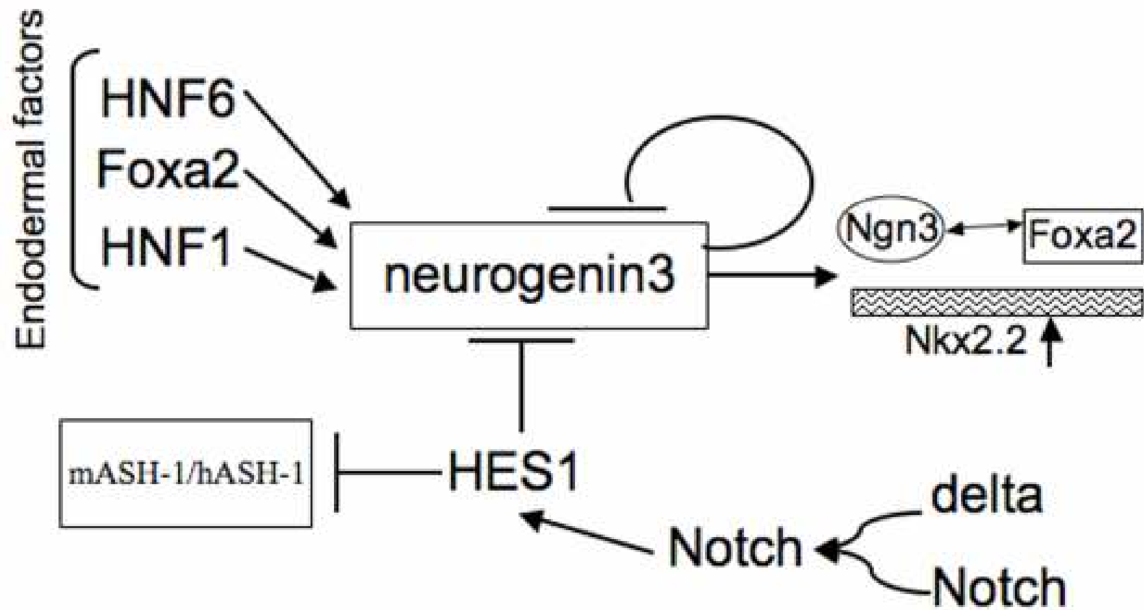


Figure3-1: Diagrammatic representation of gene expression in pancreatic endocrine cell differentiation.

Endodermal factors are the initial ones to be expressed. Notch signaling prevents adjacent cells from differentiating into endocrine cells and maintains normal cell differentiation. Notch signaling mediates its response through Hes-1(hairy/enhancer of split) that represses pro-endocrine gene, neurogenin3 (Ngn3). Hes-1 also down regulates mASH-1. Cells expressing Ngn3 are destined to differentiate into endocrine cells. Ngn3 and Foxa2 interact synergistically to recruit transcriptional activators to Nkx2.2 promoter. Nkx2.2 is the final gene expressed in pancreatic ß-cell.

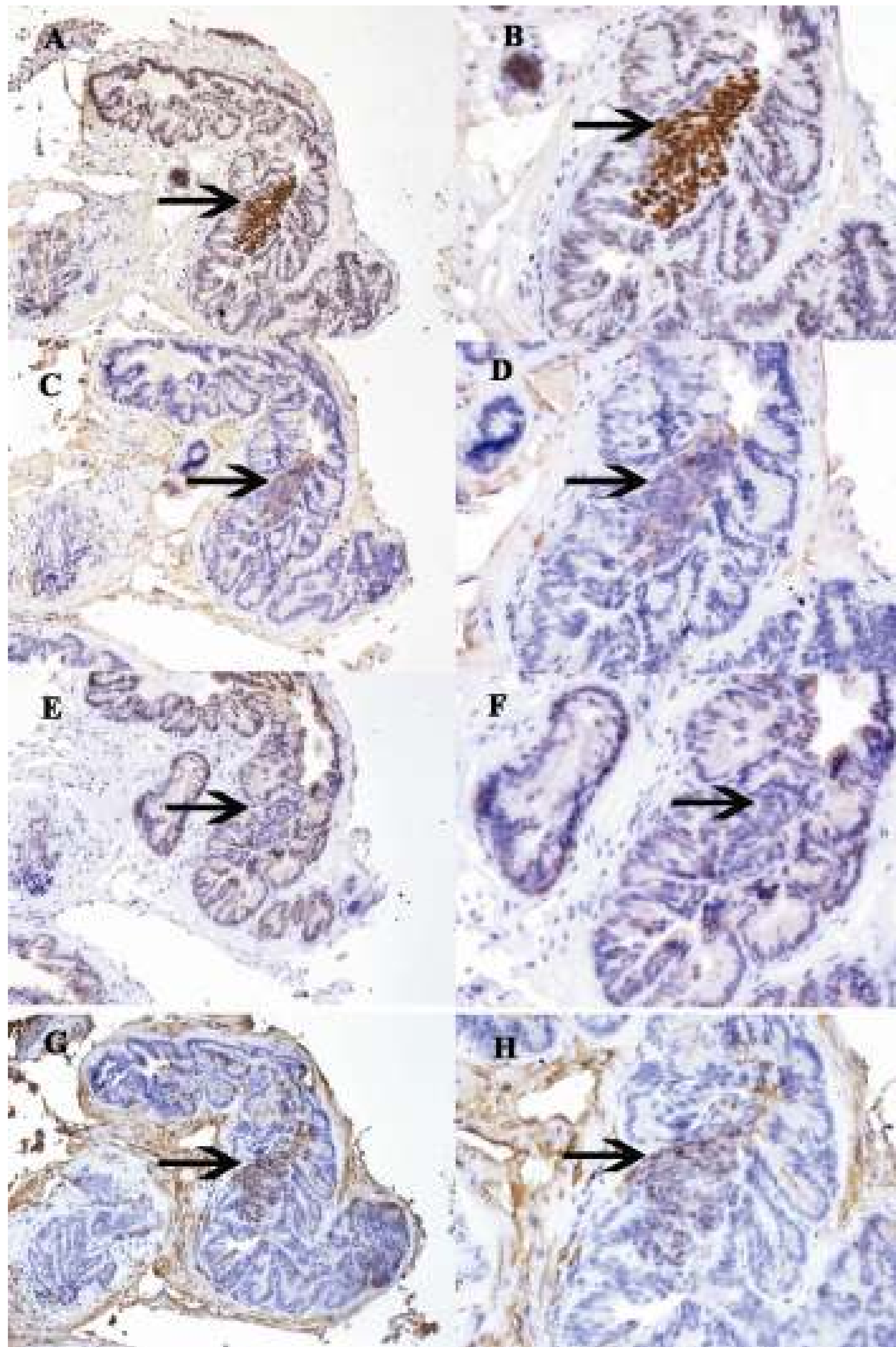


Figure 3-2: LGPIN lesions from 12-T10 mouse prostate.

Imunohistochemistry showing positive expression of Foxa2 (A; 20X) (B; 40X), synaptophysin (C; 20X) (D; 40X), loss of AR expression (E; 20X) (F; 40X) and mash-1 (G; 20X) (H; 40X) expression in the low grade NE PIN lesions.

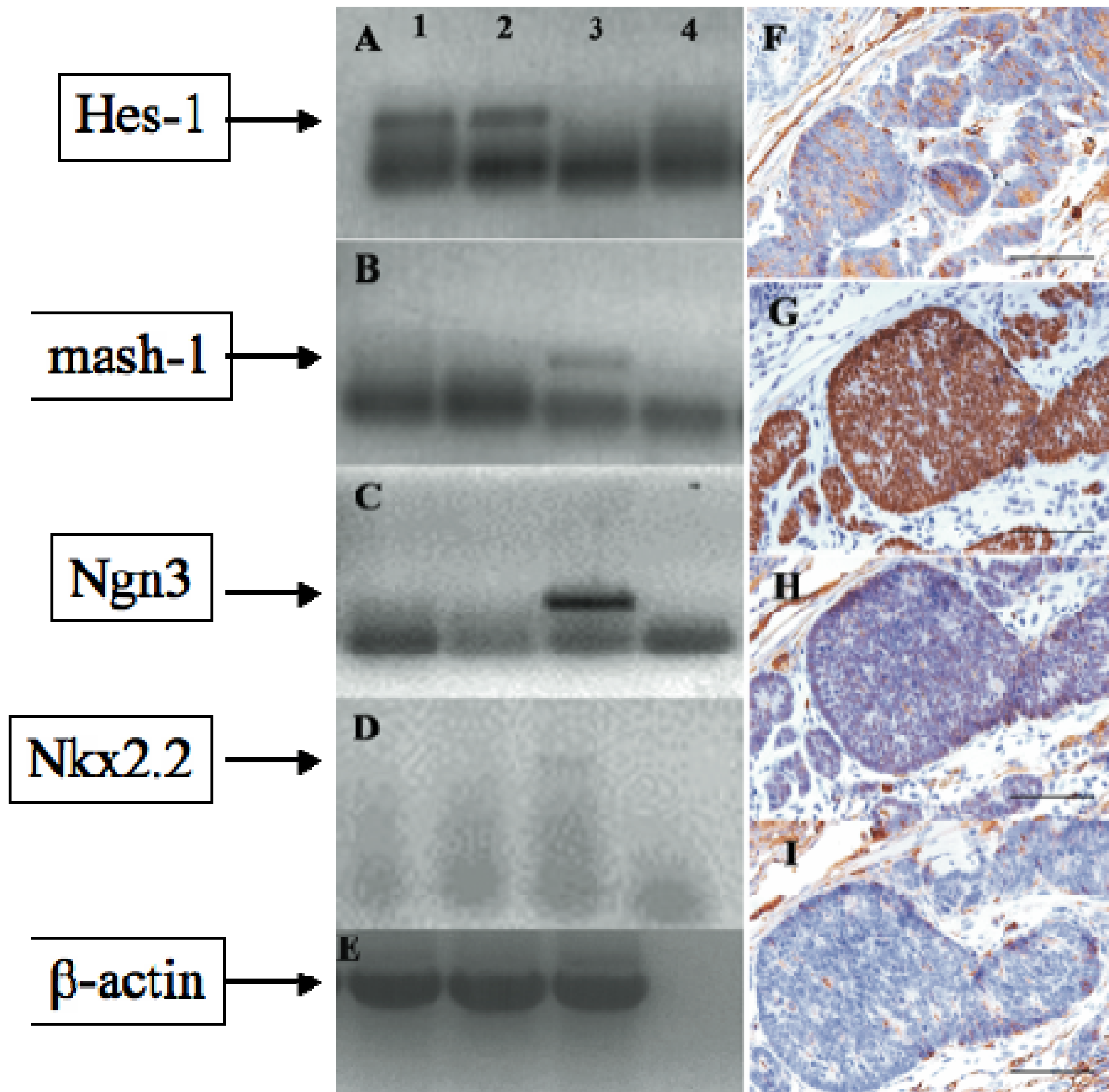


Figure3-3: mRNA expression in NE-10 tumor and immunoistochemistry on high grade NE PIN lesions.

RT-PCR results showing expression of Hes-1 (A), mASH-1 (B), ngn3(C), Nkx2.2 (D) and ß-actin control (E). Lane 1: normal prostate, lane 2: prostate from 12-T7f, lane 3: NE-10 tumor and lane 4: negative control. Immunohistochemistry of the prostate shows expression of Synaptophysin (F), Foxa2 (G), mash-1 (H) and Nkx2.2 in HGPIN lesions indicating neuroendocrine differentiation in the 12-T10 mice line at 50 weeks of age. Bars, 50µm.

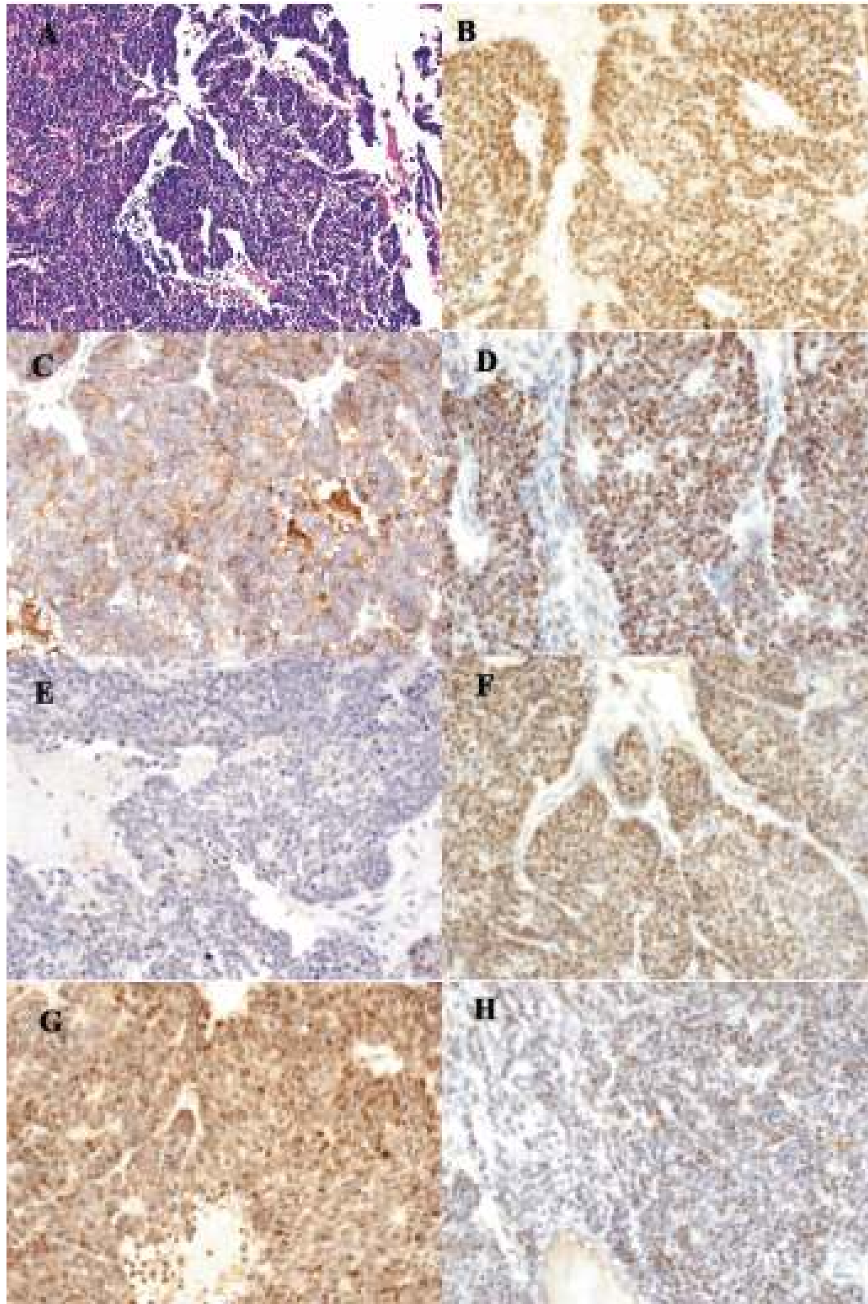


Figure 3-4: **Poorly differentiated NE tumor of dorsolateral prostate**

Immunohistochemical analysis of poorly differentiated dorsolateral prostate of 12-T10 neuroendocrine prostate tumor showing H & E staining (A), positive staining for Foxa2 (B), synaptophysin (C) and T-antigen (D), negative staining of androgen receptor (E), positive nuclear staining for mash-1 (F), neurogenin-3 (G) and a mixed population of cells both positive and negative for Nkx2.2 (H).

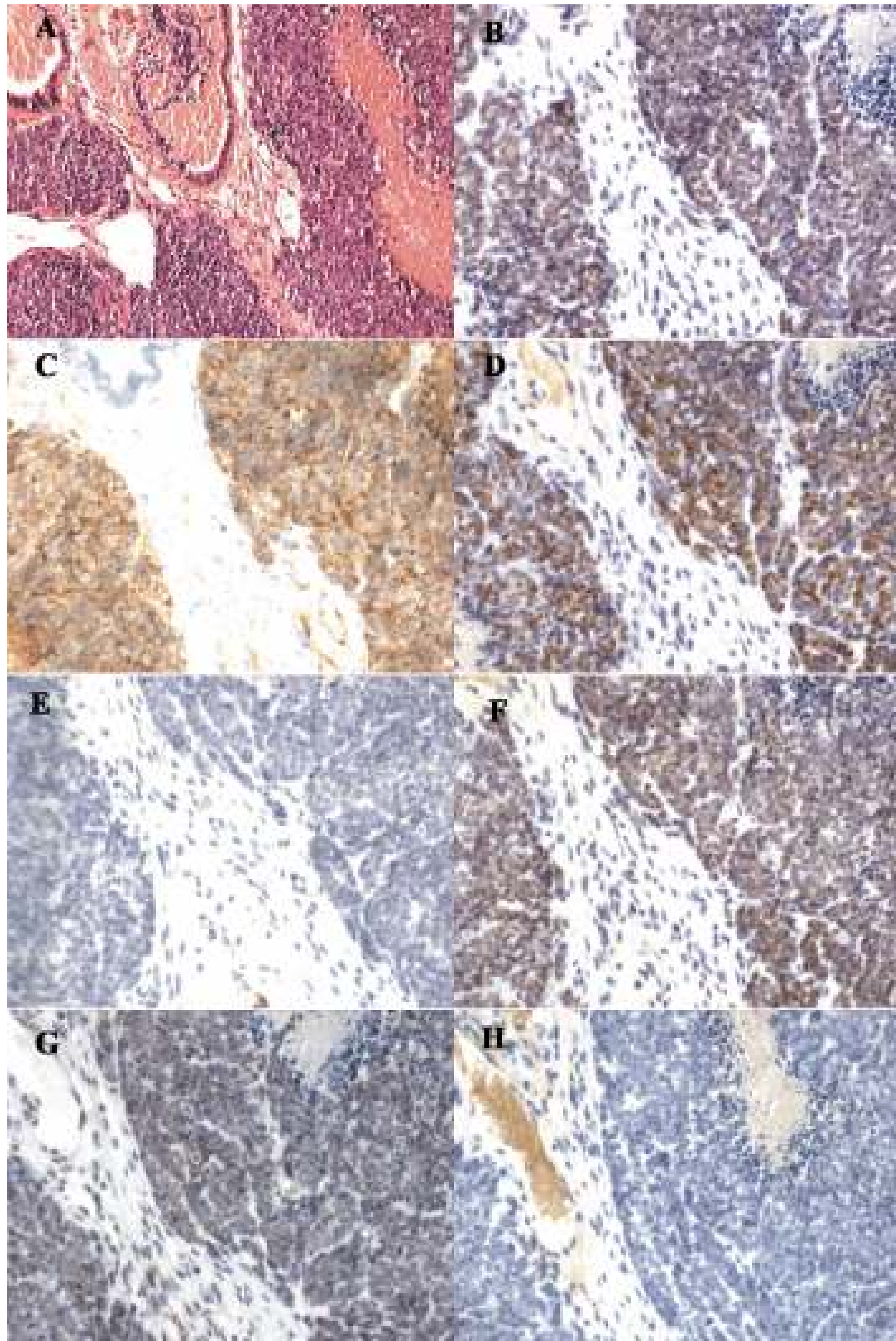


Figure 3-5: **Poorly differentiated NE tumor of Ventral Prostate**.

Serial sections of ventral prostate of 52 weeks old 12-T10 neuroendocrine prostate showing H& E staining (A), positive immunostaining for Foxa2 (B), synaptophysin (C) and T-antigen (D), negative for androgen receptor (E), positive staining for mash-1 (F) and neurogenin-3 (G) and negative for Nkx2.2 (H).

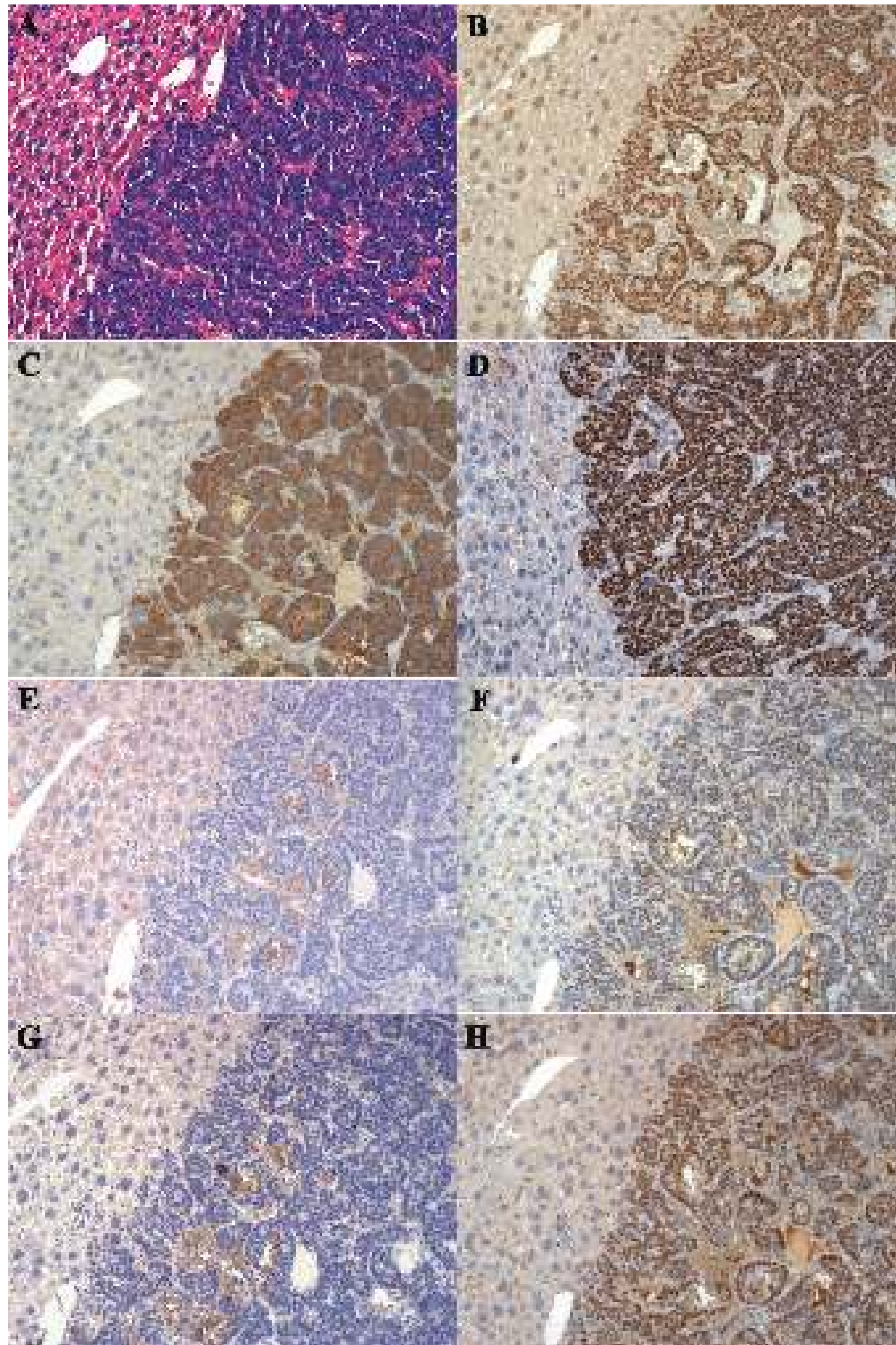


Figure 3-6: **NE liver metastasis from 12-T10 mice**.

Serial sections of neuroendocrine liver metastasis from a 69 weeks old 12-T10 mice showing H &E (A), positive immunostaining for Foxa2 (B), synaptophysin (C) and T-antigen (D), negative staining for androgen receptor (E), positive staining for mash-1 (F), negative for neurogenin-3 (G) and positive nuclear staining for Nkx2.2 (H).

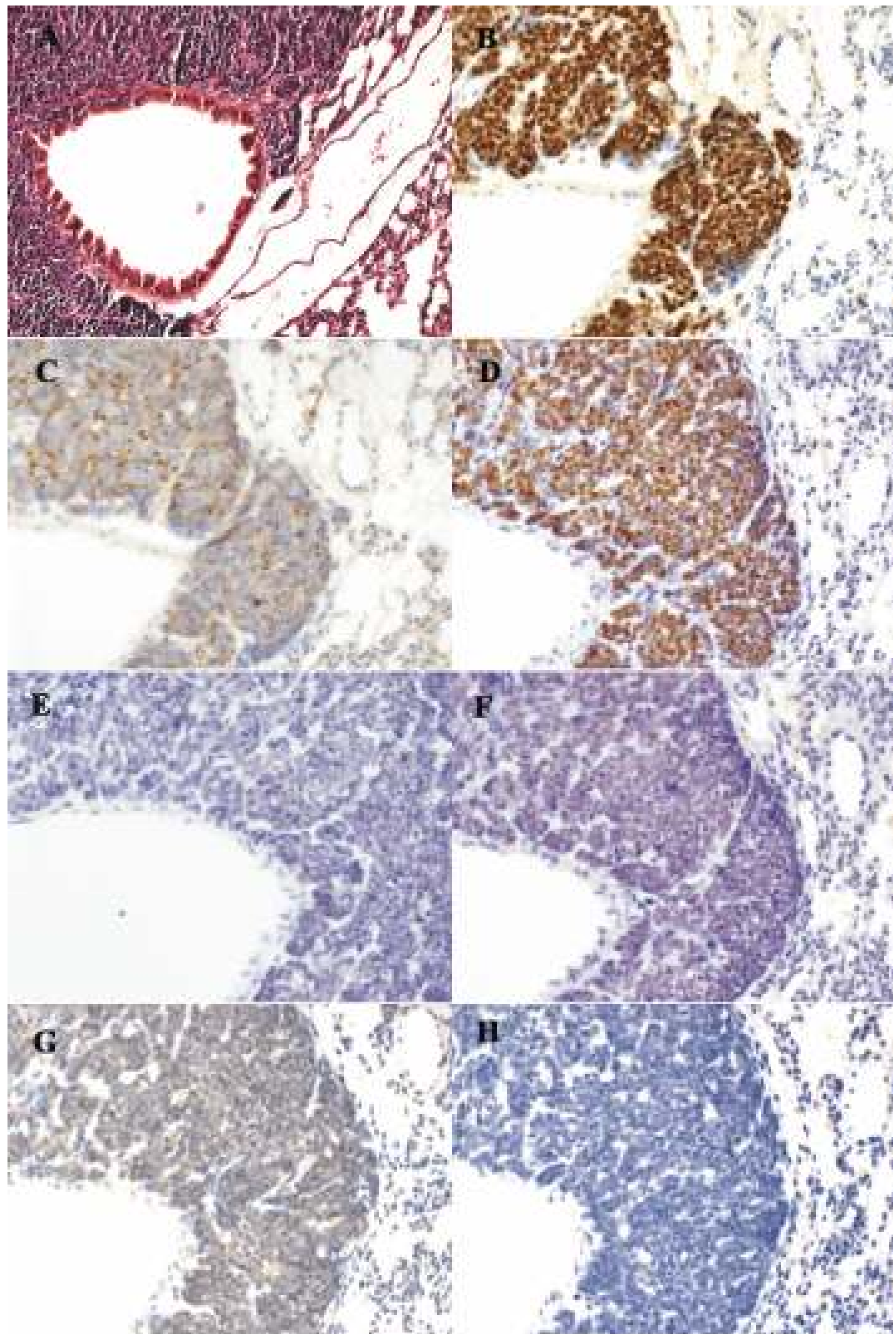


Figure 3-7: **NE lung metastasis from 12-T10 mice**.

Immunohistochemical analysis on serial sections of lung metastasis from a 69 weeks old 12-T10 mouse with H &E staining (A), positive staining for Foxa2 (B), synaptophysin (C), T-antigen (D), negative for AR (E), positive nuclear staining for mash-1 (F) and neurogenin-3 (G) and negative for Nkx2.2 (H).

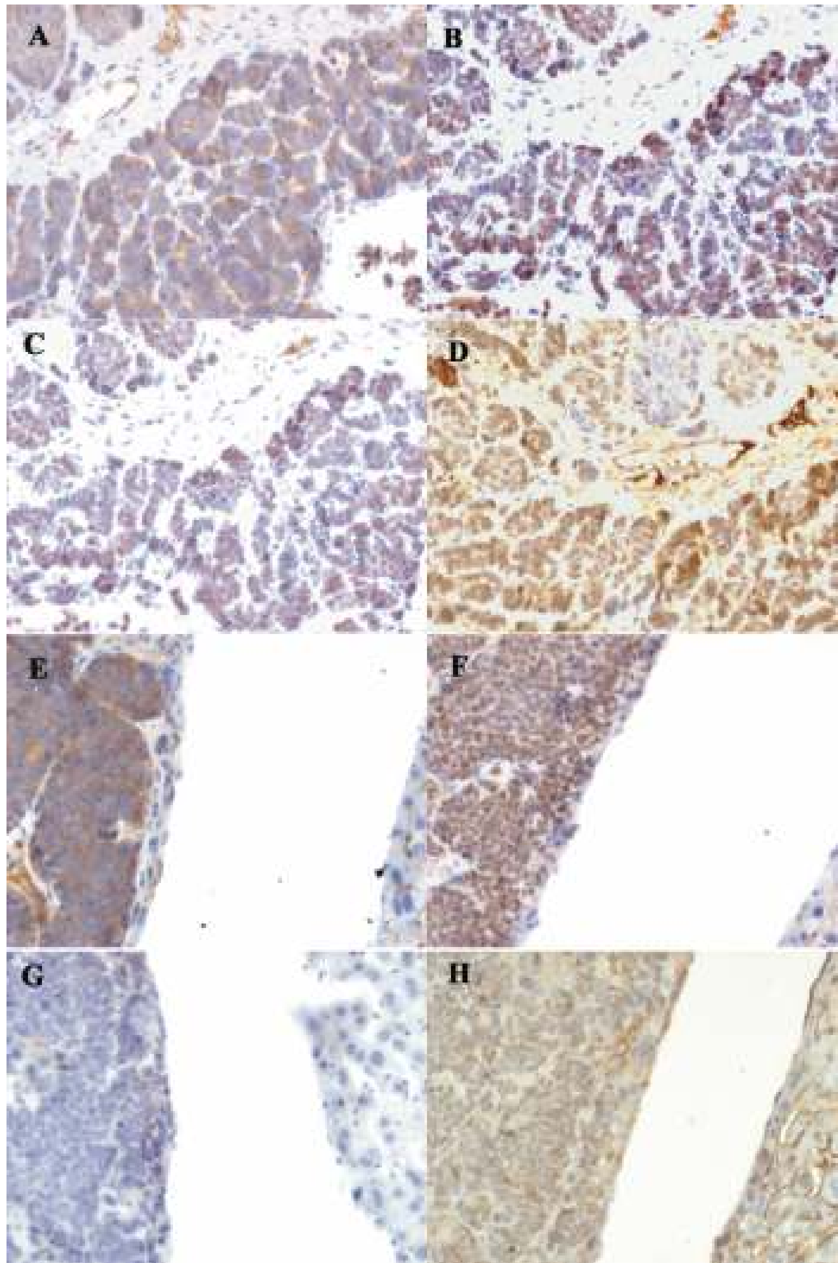


Figure 3-8: **NE-10 subcutaneous graft and liver metastasis from the same mice**.

Serial sections of subcutaneous neuroendocrine tumors from the xenograft model of 12-T10 mice (NE-10) showing positive immunostaining for synaptophysin (A), mash-1 (B), neurogenin-3 (C) and Nkx2.2 (D). Immunohistochemical analysis on the liver metastasis from the same athymic mice showing positive expression for synaptophysin (E), mash-1 (F), negative for neurogenein-3 (G) and positive for Nkx2.2 (H). Both the subcutaneous tumor and the liver metastasis are positive for Foxa2 and T-antigen and negative for AR (results not shown).

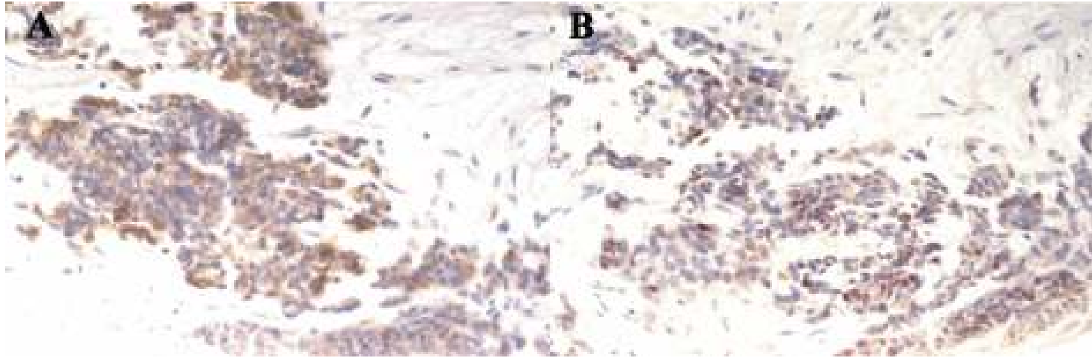


Figure 3-9: **Human NE prostate tumor biopsies**.

Immunohistochemical analysis showing positive expression for Synaptophysin(A) and hASH-1 (B) in neuroendocrine human prostate tumor needle biopsies.

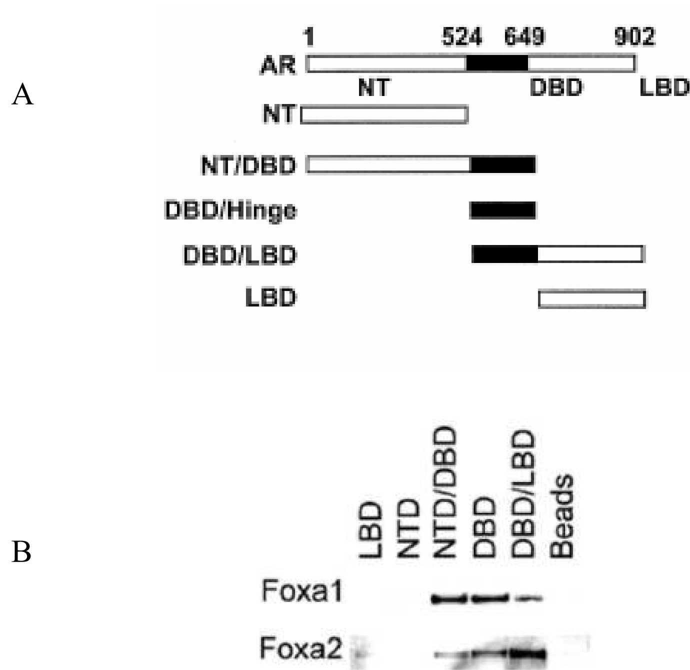


Figure 3-10. GST Pull-down Assays

(A) A schematic diagram showing a series of AR subdomains used in in vitro GST pull-down assays. The AR DBD/hinge region is highlighted in black. (B) GST pull-down assay showing that Foxa proteins interact with the AR. Different domains of AR were fused with GST and used in GST pull-down assays. LBD = ligand binding domain; NTD = N-terminal domain; DBD = DNA binding domain. As with Foxa1, Foxa2 specifically bound to DNA binding domain of AR.

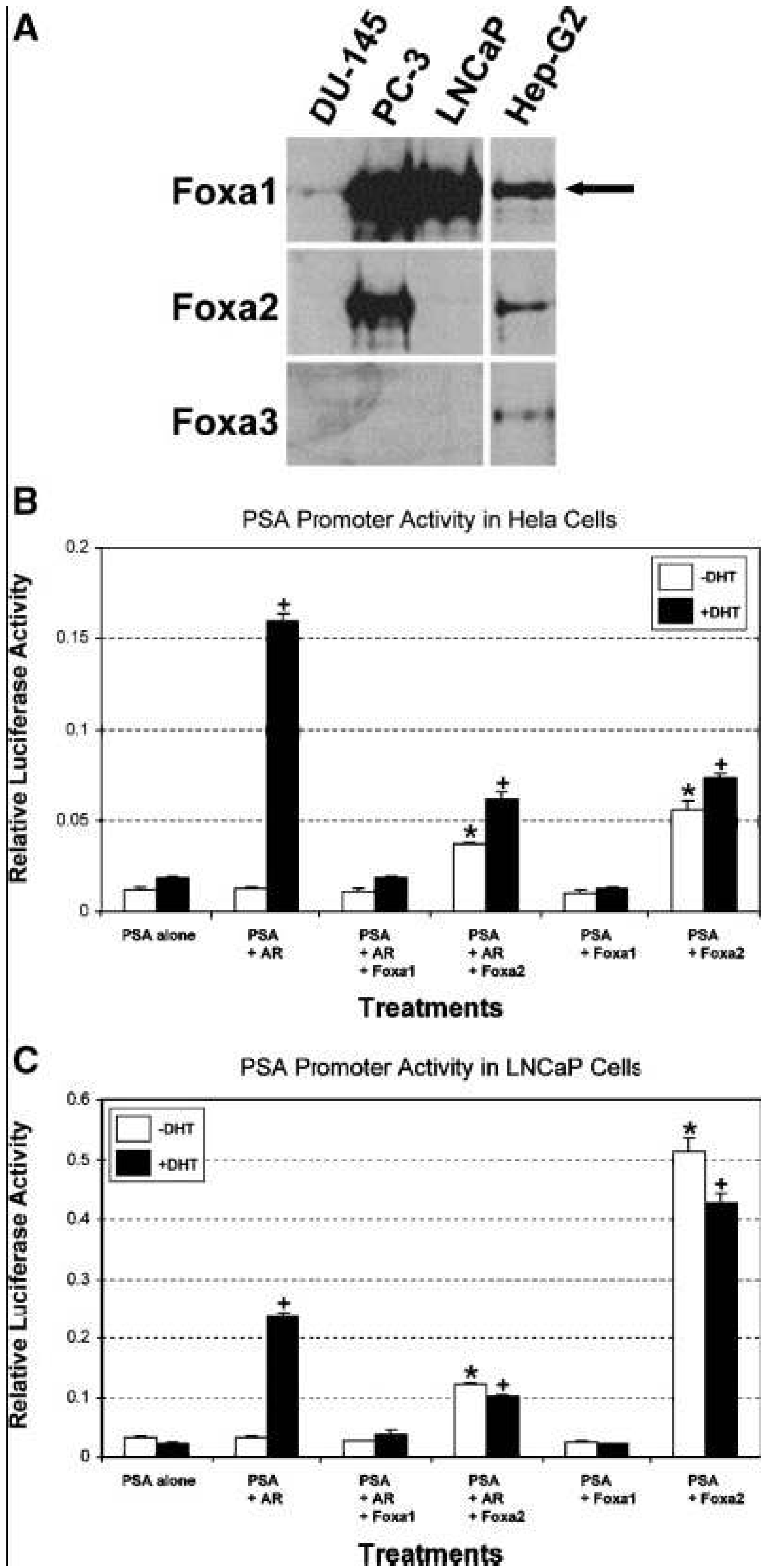

A
DU-145
PC-3
LNCaP
Hep-G2
Foxa1
Foxa2
Foxa3
B
PSA Promoter Activity in Hela Cells
Relative Luciferase Activity
-DHT
+DHT
PSA alone
PSA + AR
PSA + AR + Foxa1
PSA + AR + Foxa2
PSA + Foxa1
PSA + Foxa2
Treatments
C
PSA Promoter Activity in LNCaP Cells
Relative Luciferase Activity
-DHT
+DHT
PSA alone
PSA + AR
PSA + AR + Foxa1
PSA + AR + Foxa2
PSA + Foxa1
PSA + Foxa2
Treatments

Figure 3-11: **Transient transfections showing relative luciferase activity of the PSA-luc reporter construct**.

Differential expression of Foxa proteins in human prostate cancer cell lines and activation of prostate gene transcription. A: Foxa Western blot analysis of prostate cancer cell lines. StrongFoxa1 expression was detected in LNCaP and PC-3 cells (arrow), while Foxa2 was only detected in PC-3 cells. Hep-G2 cells served as a positive control for Foxa expression. B: Hela and(C) LNCaP transient transfection experiments, using the PSA promoter-luciferase construct (PSA) co-transfected with various expression vectors. Results are expressed as means SEM and are presented as relative luciferase activities that are a representative of three independent experiments performed in triplicate. *Significantly increased compared to PSA alone without DHT treatment, $P<0.01$;+Significantly increased compared to PSA alone with DHT treatment, $P<0.01$.Foxa2 was shown to significantly increase PSA gene promoter activity in the absence of DHT, or androgen receptors in both cell lines. (adapted from (Gao et al, 2003; Mirosevich, 2006)

CHAPTER IV

FOXA2 KNOCKOUT IN THE TRAMP NEUROENDOCRINE TUMORS RESULTS IN EXPRESSION OF MASH-1

Introduction

Prostate neuroendocrine tumors or neuroendocrine differentiation in prostatic adenocarcinomas are rarely diagnosed diseases, but once a patient presents with any of them, it is often associated with poor prognosis and no effective treatment. Efforts have been made to better understand the neuroendocrine phenotype but there is still no unified mechanism/gene sequence that can be targeted to get an effective remedy for the NE phenotype. Previous reports from our laboratory have identified Foxa2, an endodermal forkhead transcription factor as being specifically expressed at the tips of growing prostatic buds between E18 through E21 (Mirosevich, 2005). Foxa2 expression is not detected in normal adult murine prostate. We have also reported that Foxa2 is specifically expressed in the mouse and human NE prostate tumor. Non-neuroendocrine tumors of the prostate express Foxa1 but do not express Foxa2. This led us to hypothesize that Foxa2 expression is a key factor in the NE differentiation of prostate tumors. In order to test this hypothesis we decided to knock out the expression of Foxa2 in mouse model for prostate neuroendocrine tumors. We used the TRAMP mice for these studies. Expressing both SV-40 large and small T-antigen specifically in the prostatic luminal epithelial cells using the prostate specific probasin gene promoter generated TRAMP mice. These mice were initially reported to form adenocarcinomas of the prostate but a consensus pathology

report for the MMHCC on mouse models of prostate cancer describes TRAMP prostate tumors as poorly differentiated small cell carcinomas (NE cancer) that express NE markers and show NE metastasis (Shappell et al, 2004). Preliminary results show that the TRAMP neuroendocrine PIN lesions as well as poorly differentiated NE tumors express Foxa2, Synaptophysin (marker for neuroendocrine cells) and lose androgen receptor expression. In order to generate TRAMP mice with a knockout of FoxA2 gene, a two step breeding plan was followed as shown in Figure 4-1. The final goal was to breed two floxed alleles and one copy of Nkx3.1-Cre into the TRAMP mice, generating TRAMP/FoxA2$^{loxP/loxP}$ as shown in Figure 4-1(A). Simultaneously two floxed alleles of FoxA2 were bred into the Nkx3.1-Cre mice (Figure 4-1B) generating Nkx3.1-Cre$^{+/-}$/FoxA2$^{loxP/loxP}$. In the final breeding TRAMP/FoxA2$^{loxP/loxP}$ animals were bred with Nkx3.1-Cre$^{+/-}$/FoxA2$^{loxP/loxP}$ as shown in Figure 4-1(C) to generate TRAMP mice with a knock out of Foxa2(TRAMP/Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$) along with other age matched controls. Nkx3.1 is an epithelial cell specific gene that is detected as early as E14 in the developing mouse prostate. Thus it targets Cre expression in a prostate specific manner and at a very early age. The Nkx3.1-Cre mouse is a knock-in mice thus only one copy of Nkx3.1-Cre was bred in to avoid complete loss of Nkx.3.1 expression. Using Nkx3.1-Cre to knock out Foxa2 expression caused loss of Foxa2 expression during early prostate budding. Immunohistochemical analysis was done to confirm that the loss of Foxa2 during early prostate development did not affect the architecture, histology or the outcome of the experiment. Parallel to this, histological analysis was done to confirm that the loss of one copy of Nkx3.1 did not affect the outcome of the experiment. The loss of Foxa2 expression was confirmed by immunohistochemical analysis. In the

TRAMP/Nkx3.1-Cre/Foxa2$^{loxP/loxP}$ mice, NE prostate tumors still developed but with the loss of Foxa2 the pro-neuronal gene mASH-1 (mouse achaete scute homolog-1) was expressed. In wild type TRAMP NE tumors, Foxa2 but not mASH-1 is expressed. NE tumors from both the wild type TRAMP and Foxa2 knocked out TRAMP express synaptophysin and T-antigen and both show loss of androgen receptor. These results indicate that either Foxa2 or mASH-1 can serve as an early marker for NE differentiation and suggest that either factor alone contributes to NED in the prostate tumors. mASH-1 expression has been associated with the neuroendocrine phenotype. hASH-1 (human achaete-scute homolog-1) expression is tightly linked to the NE phenotype in lung cancer. hASH-1 expression can be seen in virtually all classic SCLC lines as well as typical and atypical bronchial carcinoid lines, and in many NSCLC lines with NE features (Khoor et al, 2004). Studies utilizing antisense oligonucleotides directed at hASH-1 suggest that depletion of this factor reduces NE markers in cultured SCLC cells (Borges et al, 1997). In addition to this, overexpression of hASH1 in airway epithelial cells of transgenic mice, in concert with SV40 Large T antigen, is sufficient to induce aggressive lung tumors with a NSCLC-NE phenotype (Linnoila et al, 2000). In this study, in the absence of T-antigen, extensive airway epithelial proliferation but no NE trans-differentiation was observed. Bigenic mice, expressing both hASH-1 and T-antigen exhibit widespread hyperplasia of NE-reactive epithelial cells and adenocarcinomas with NE features (Linnoila et al, 2000). hASH-1 expression appears to be an important feature of medullary thyroid carcinoma, paraganglioma and carcinoid tumors of the foregut (lung, thymus and pancreas) and midgut (duodenum, jejunum and ileum). Recently, Gordon and colleagues have demonstrated exceptionally high-level expression of mash1

in a mouse model of prostatic NE carcinoma. This mouse model expresses SV40 Large T-antigen in prostate neuroendocrine cells using cryptdin2 gene promoter (Hu et al, 2002).

Results

Foxa1 expression in the E19 UGS is not affected by knocking out of Foxa2

Foxa1 and Foxa2 are closely related members of the forkhead family of transcription factors. As mentioned earlier Foxa1 is expressed early during prostate budding and is expressed in the adult prostate luminal epithelial cells. On the contrary Foxa2 is expressed only at the tips of growing prostatic buds from E18-E21. Experiments designed to knock-out Foxa2 in the buds using the Nkx3.1-Cre mice show no difference in Foxa1 expression. Figure 4-2 shows the expression pattern of Foxa1 in the epithelial cells of the wild type C57Bl6 background (A). The floxing of Foxa2 alleles (Figure 4-2B) and the expression of Cre (Figure 4-2C) has no effect on the Foxa1 expression. The loss of Foxa2 in Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$ does not alter the expression of Foxa1 in the E19 UGS (Figure 4-2(D).

Foxa2 expression is effectively knocked out in early prostatic budding in Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$

Foxa2 is expressed at E19 through E21 at the tips of the prostatic buds and Nkx3.1 starts expressing around E14. The loss of Foxa2 expression in the Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$ mice was confirmed using immunohistochemistry. Figure 4-3(A) shows the expression of Foxa2 in the C57Bl6 wild type mouse UGS. Panel B of Figure 4-3

confirms that floxing of alleles has no affect on the expression of Foxa2. Expression of Cre or loss of one copy of Nkx3.1 has no affect on the expression of Foxa2 (Figure 4-3C). Figure 4-3(D) shows that Nkx3.1-$Cre^{+/-}$/$Foxa2^{loxP/loxP}$ does not express Foxa2 at the tips of growing prostatic buds in the embryonic UGS compared to the wild type mice or Nkx3.1-$Cre^{+/-}$ mice .

Floxing of Foxa2 alleles has no affect on the prostate histology

The loss of foxa2 expression in the UGS of the Nkx3.1-$Cre^{+/-}$/$Foxa2^{loxP/loxP}$ was confirmed by immunohistochemistry. The Foxa2 knocked out animals along with the age matched controls were sacrificed at 8 weeks of age to check for any histological changes or for any obvious changes in the architecture of prostate lobes**.** Haemotoxylin and Eosin staining did not show any significant histological changes at 8 weeks of age. At one year of age 5 animals of each genotype were sacrificed and analyzed for any histological changes. Figure 4-4 shows the histological features of the anterior prostate (A), the dorsal prostate (B), lateral prostate (C) and ventral prostate (D) of a one year old Nkx3.1-Cre^{wt}/$Foxa2^{loxP/loxP}$ mouse. The results show that in this case floxing of Foxa2 alleles has no apparent affect on the architecture or histology of any of the prostate lobes.

Haploinsufficiency of Nkx3.1 does not alter the prostate tissue architecture

Loss of one copy of Nkx3.1 does alter the architecture of the prostate lobes. Figure 4-5 shows the Haemotoxylin and Eosin staining on the prostatic lobes of a one year old Nkx3.1-Cre+/- mouse. Panel A of Figure 4-5 shows the architecture of the anterior lobe which does not seem to be affected by the loss of one allele of Nkx3.1. The dorsal prostate (B) and the lateral prostate (C) of the one year old Nkx3.1 haploinsufficient mice shows a very thin layer of epithelial cells. It appears as if the cells

are sloughing off the basement membrane and falling into the lumen. The ventral prostate (D) shows pilling up of luminal epithelial cells and hyperplasia. This could be a result of haploinsuffiency of Nkx3.1 gene, referred to as a tumor suppressor in prostate cancer.

One year old Foxa2 knockout mice have reduced number of basal cells in the lateral prostate compared to age matched control mice

Figure 4-6 shows Haematoxylin and Eosin staining on the anterior prostate (A), dorsal prostate (B), lateral prostate (LP) and ventral prostate (VP) of the one year old Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$ (Foxa2 knock out mice) shows that there is no obvious architectural abnormality in the Foxa2 knock out mice. The dorsal prostate and the lateral prostate show the same sloughing off of the cells into the lumen and the lateral prostate has areas of hyperplasia as seen in the Nkx3.1-Cre +/- mice in Figure 4-5. One year old Foxa2 knockout mice and the age matched control mouse prostate lobes were analyzed for the expression of androgen receptor, Foxa1 (prostate epithelial cell specific transcription factor) and p63 (basal cell marker). Foxa1 and androgen receptor expression was uniform for both the knock out animals and the age matched controls of other genotypes (results not shown). p63 expression on the other hand was not identical in all the genotypes with the lateral prostate of the one year old Foxa2 knockout mice having lower number of basal cells (Figure 4-7 E and F) compared to the lateral prostate of Nkx3.1-Cre+/- mice (Figure 4-7 A and B) and Foxa2$^{loxP/loxP}$ mice (Figure 4-7 C and D).

The TRAMP neuroendocrine tumors are different from the 12T-10 neuroendocrine prostate tumors

Chapter II of this study has discussed the molecular mechanism that lead to NE differentiation in the neuroendocrine prostate tumors of the 12T-10 mouse model. The

NE PIN lesions as well as the advanced NE tumors of the 12T-10 model express both Foxa2 and mash-1 followed by neurogenin-3 and Nkx2.2 (genes involved in pancreatic endocrine cell differentiation). The TRAMP neuroendocrine tumors do express synaptophysin (Figure 4-8 A), a marker for NED. They show a loss of androgen receptor (Figure 4-8 B), a feature associated with neuroendocrine differentiation of prostate tumors. TRAMP neuroendocrine tumors also express Foxa2 (Figure 4-8 C). The expression of Foxa2 in TRAMP tumors is seen in the neuroendocrine PIN lesions and advanced NE tumors but as these tumors advance to very poorly differentiated sheets of tumors cells, Foxa2 expression seems to be lost. This could be either due to the selection of a cell population that does not need Foxa2 expression or because these cells have gone far from the starting cell population and do not any more require Foxa2 expression. The advanced undifferentiated TRAMP neuroendocrine tumors do not express mash-1 (Figure 4-8 D). The expression of Foxa2 and mash-1 was confirmed on eight different TRAMP tumor samples. Thus, these results suggest that although both the 12T-10 NE prostate cancer model and the TRAMP model develop NE prostate tumors, the pathways that lead to the phenotype or the genes that mark the NE tumors are different in the two models. Since Foxa2 was expressed early in both models, it suggested to us that Foxa2 was a key player in the TRAMP tumors and thus we decided to knock out Foxa2 to see if its absence would block the formation of NE tumors.

Floxing of Foxa2 alleles in TRAMP mice have no affect on gene expression

Neuroendocrine tumors from TRAMP/Foxa2$^{loxP/loxP}$ mice were analyzed for the expression of Foxa2 and mash-1 to confirm that the floxing of alleles did not result in loss of Foxa2 or expression of mash-1 in the TRAMP tumors. Figure 4-9 (A) shows that

these tumors express synaptophysin. These tumors do not express androgen receptor (Figure 4-9B). Foxa2 is expressed (Figure 4-9C) in the NE tumors from TRAMP/Foxa2$^{loxP/loxP}$ mice and mash-1 is not expressed in these tumors (Figure 4-9 D). This confirms that the floxing of Foxa2 alleles has no effect on the gene expression of Foxa2 and mash-1 and these tumors behave the same as the wild type TRAMP tumors.

Loss of Foxa2 in TRAMP tumors switches on mash-1 expression

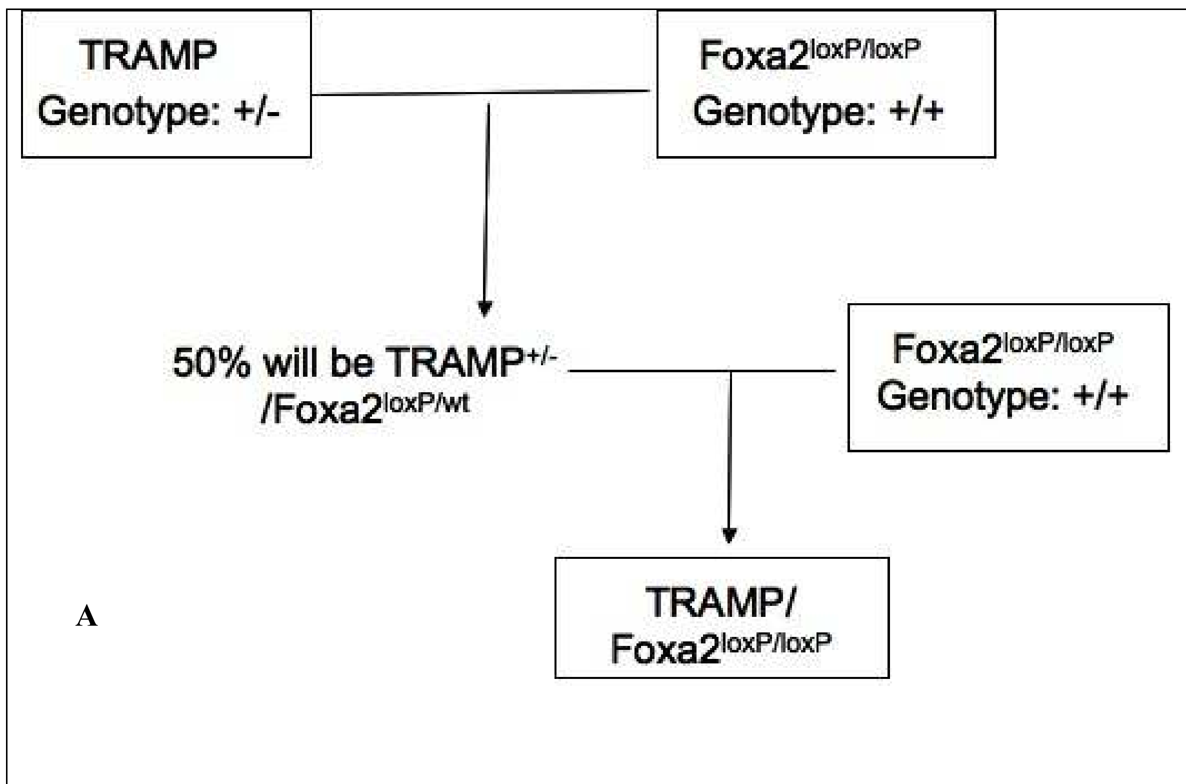
This study now focused on understanding the role of Foxa2 in the development of neuroendocrine prostate tumors in the TRAMP model. To do so, a knockout of Foxa2 in the TRAMP line was made. We used the Nkx3.1 driven Cre recombinase to knock out Foxa2 as shown by the immunohistochemical analysis (Figure 4-10 C), where Foxa2 expression is lost. These tumors still expressed synaptophysin (Figure 4-10 A), marker for NE tumors. This suggests that the foxa2 knocked out TRAMP mice still develop NE prostate tumors even in the absence of Foxa2. Also these tumors demonstrated the same loss of AR (Figure 4-10 B) as seen in a typical NE tumor. The most interesting observation was that the Foxa2 knock out TRAMP neuroendocrine tumors expressed mash-1 (Figure 4-10 D) in large pockets of cells, which was absent in the wild type TRAMP tumors. These results are based on a study of 8 mice where 7 of the Foxa2 knockout TRAMP mice showed expression of mash-1. This suggests that in the absence of Foxa2, mash-1 may be switched on and it may now play a key role in NED of the TRAMP tumor. Figure 4-11 is representative of the results of a poorly differentiated NE tumor arising from the DP of a Foxa2 knock out TRAMP tumor. This tumor shows expression of synaptophysin (Figure 4-11A), loss of AR expression (Figure 4-11 (B)), no Foxa2 expression due to the knock out (Figure 4-11C) and mash-1 expression is switched

on (Figure 4-11 D). These knock out studies suggest that the NE tumors in TRAMP either express Foxa2 or mash-1, at the same time there are some cells within the TRAMP NE tumors where we did not detect the expression of either Foxa2 or mash-1. This may mean that a NE cell population can develop without either Foxa2 or mash-1 or that expression occurred at an earlier stage but that once the tumor acquired NED, continued expression of these transcription factors (Foxa2 or mash-1) was not required to maintain NED. The knock out of Foxa2 in TRAMP was generated using the Nkx3.1-Cre mice. These mice lack one functional Nkx3.1 gene. Therefore, as a control, one year old Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$ mice prostate lobes were immunostained for Foxa2 and mash-1. The results confirm that the loss of one copy of the Nkx3.1 gene does not result in the expression of mash-1 (results not shown).

Over expression of mash-1 in NeoTag1 cells results in increase in NSE and ChrA expression

NeoTag1 is one of the three cell lines derived from bigenic mice that express both the Large T-antigen and the neomycin resistance gene, both specifically targeted to prostatic epithelium by using the probasin promoter (Wang et al, 2006). These cells were found to be androgen responsive and can generate PIN lesions and adenocarcinoma when grafted with UGM (Wang et al, 2006). In order to determine whether mash-1 can cause NED, we overexpressed mash-1 in the NeoTag1 cells and monitored the expression of NSE (Neuron Specific Enolase) and Chromogranin A, markers for neuroendocrine differentiation. Figure 4-12 (A) is a western blot confirming the over expression of mash-1 in NeoTag1 cells infected with mash-1 lentiviral expression vector compared with cells infected with empty vector. NE-10 tissue was used as a positive control. Figure 4-12 (B)

shows that there is a very low level of NSE in the empty vector control NeoTag1 cells and that the addition of mash-1 greatly increased the NSE expression. More ChrA then NSE expression is detected in the empty vector control NeoTag1 cells but the addition of mash-1 still increased the over all levels of ChrA (Figure 4-12 C). These results indicated that the over expression of mash-1 in NeoTag1 cells was sufficient to cause NED *in vitro*. This supports the idea that the emergence of mash-1 expression in TRAMP after the knock out of Foxa2 is sufficient to result in NED.



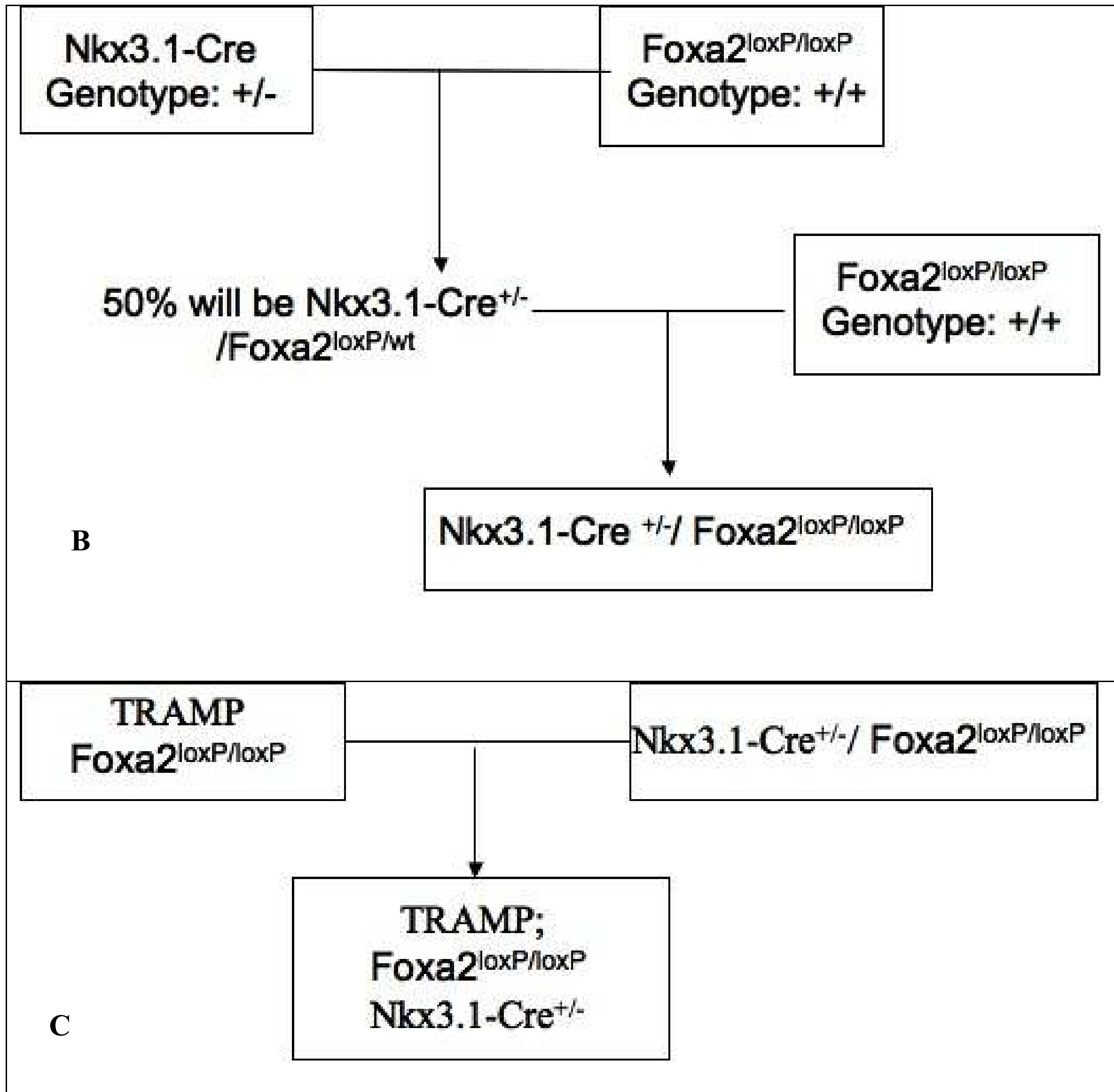


Figure 4-1: Flow charts showing the breeding scheme used to generate TRAMP mice with a knock out of Foxa2 gene.

The first step was to breed in two alleles of Foxa2 into the TRAMP (A) and Nkx3.1-Cre (B) background. The final step was to breed the TRAMP mice with floxed Foxa2 alleles into the Nkx3.1-Cre background(C)

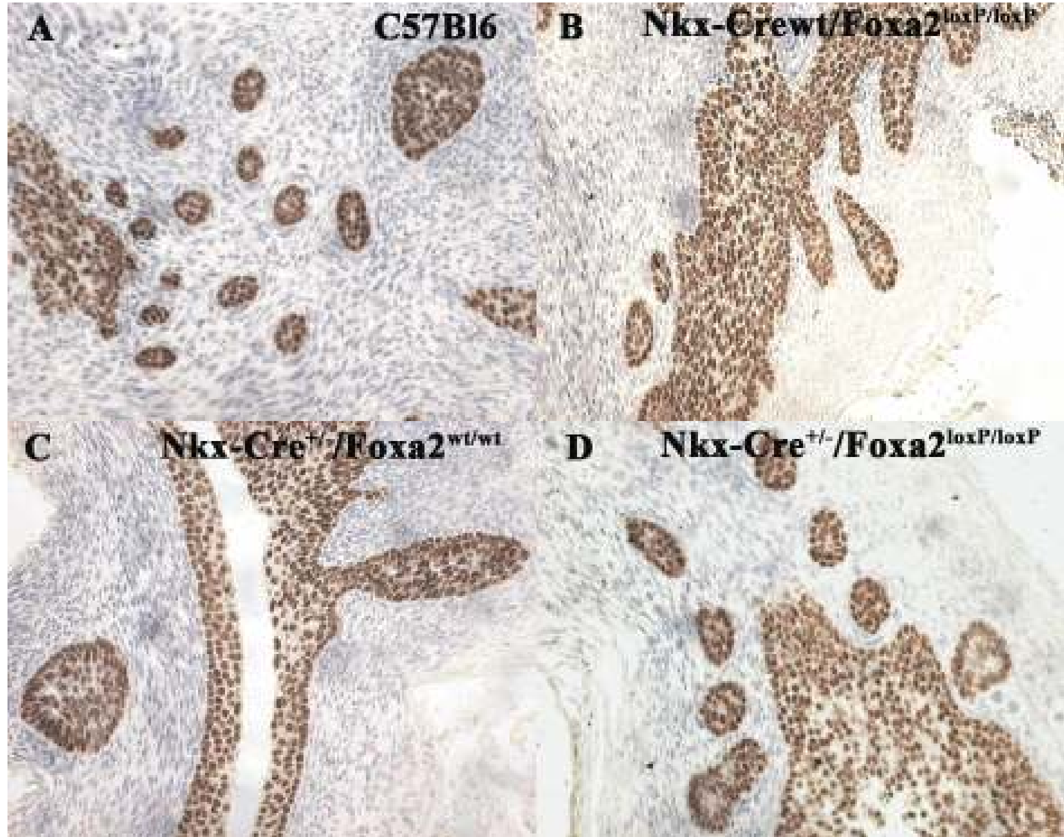


Figure 4-2: **Foxa1 expression in E19 UGS**

Immunohistochemistry showing expression of Foxa1 in the E19 UGS of wild type C57Bl6 mice embryo (A), Nkx3.1-Cre$^{wt/wt}$/Foxa2$^{loxP/loxP}$ (B), Nkx3.1-Cre$^{+/-}$/Foxa2wt (C) and Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$(D). Foxa1 expression is not altered in any of the genotypes compared to the C57Bl6 wt E19 UGS.

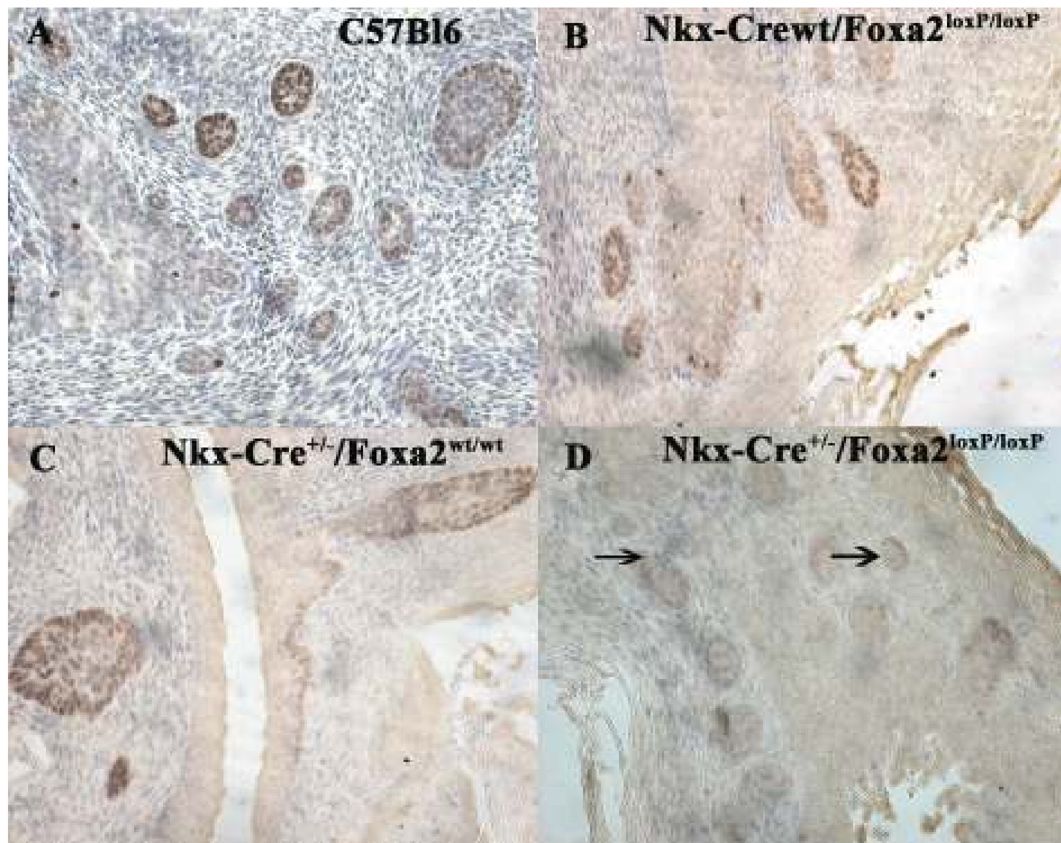


Figure 4-3: Foxa2 expression in E19 UGS

Immunohistochemistry showing expression of Foxa2 in E19 UGS. Foxa2 expression is seen at the tips of growing prostatic buds in E19 UGS of C57Bl6 wt type mice (A), Nkx3.1-Cre$^{wt/wt}$/ Foxa2$^{loxP/loxP}$ UGS (B), Nkx3.1-Cre$^{+/-}$/Foxa2wt (C). The arrows in panel D point to the tips of the prostatic buds where Foxa2 expression is knocked out in E19 UGS from Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$ mice (D).

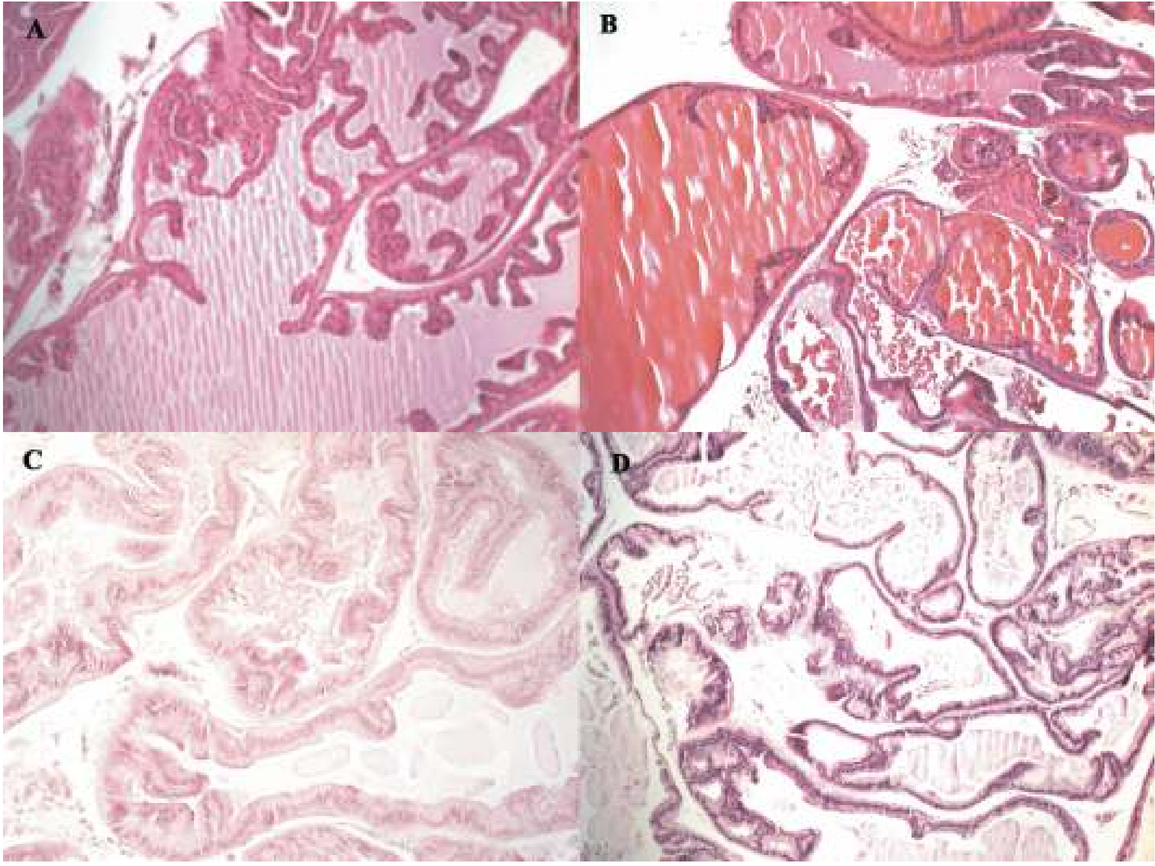


Figure 4-4: Histology of 1year old Foxa2 floxed animals

Haematoxylin and Eosin staining showing the histological features of anterior prostate (A), dorsal prostate (B), lateral prostate (C) and ventral prostate (D) of a one year old Foxa2$^{loxP/loxP}$

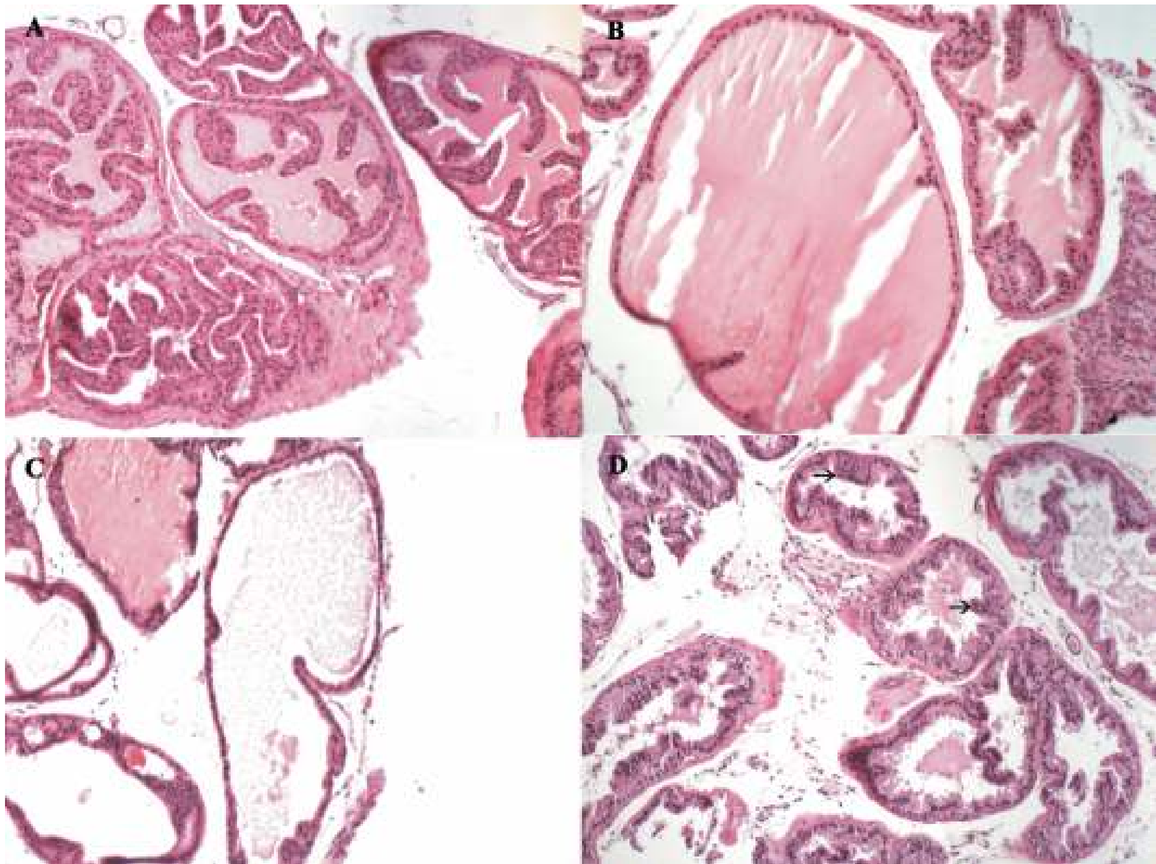


Figure 4-5: Histology of prostate from one year old Nkx3.1-Cre heterozygous animal

Haematoxylin and Eosin staining showing histological features of different lobes of a one-year-old Nkx3.1-Cre+/- mice. The architecture of the AP looks like a normal prostate (A). The DP (B) and the LP (C) show thinned lining of the ducts with the cells sloughing off into the lumen. The VP (D) shows pilling up of the cells or hyperplasia.

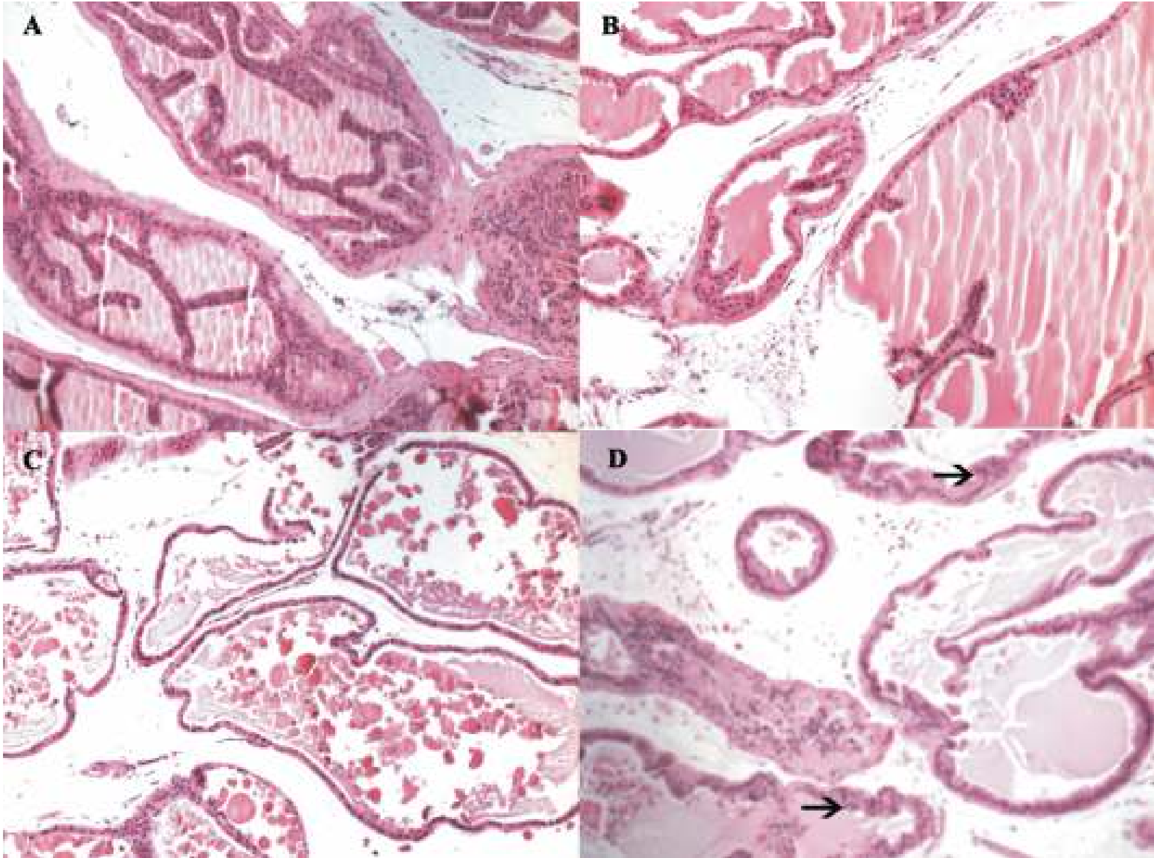


Figure 4-6: **Histology of prostate from one year old Foxa2 knockout animal**

Haematoxylin and Eosin staining showing the histological features of the anterior prostate (A), dorsal prostate (B), lateral prostate (LP) and ventral prostate (VP) of the one year old Nkx3.1-$Cre^{+/-}$/$Foxa2^{loxP/loxP}$ (Foxa2 knock out mice).

20 X 40 X

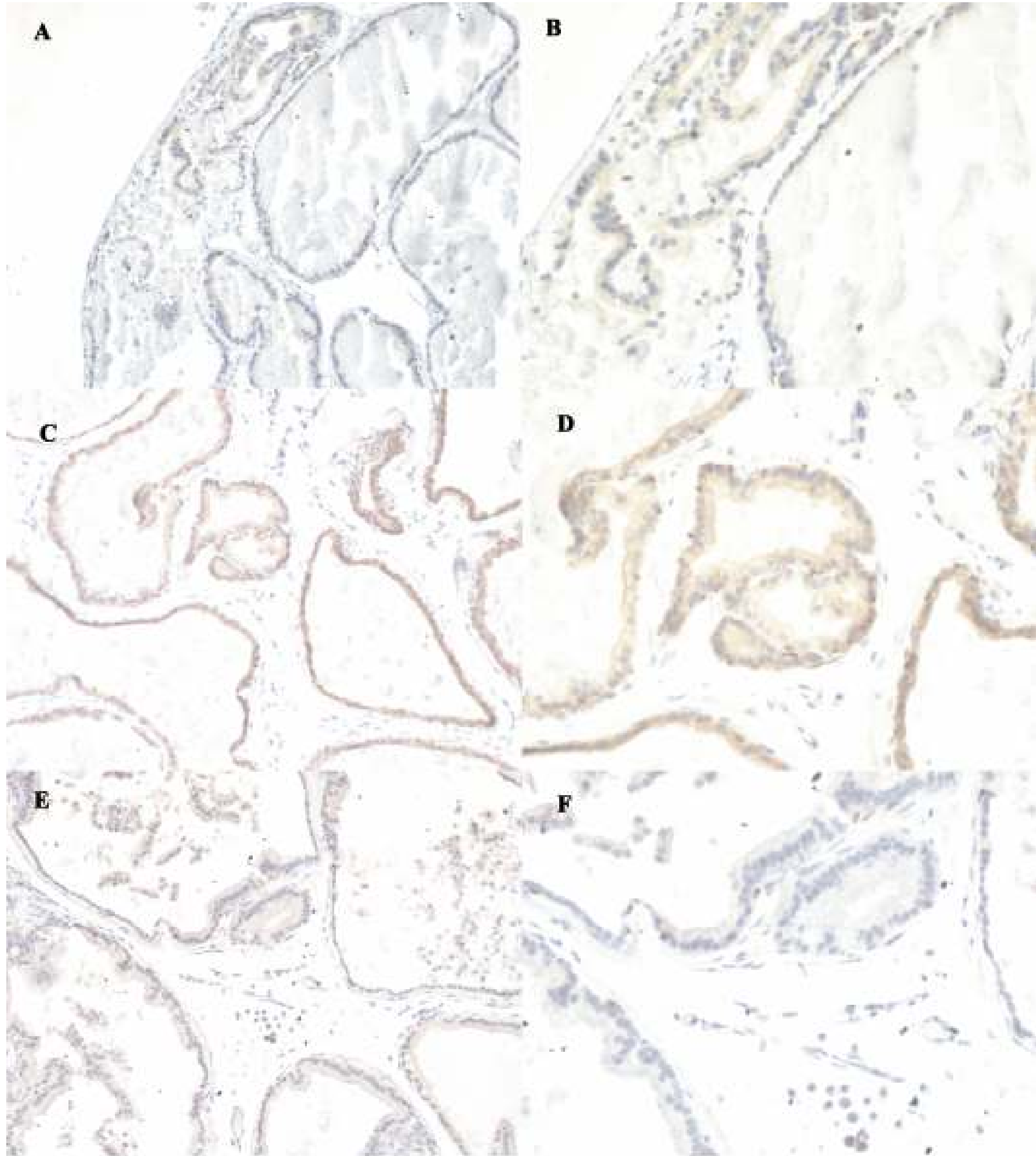


Figure 4-7: **p63 expression on lateral prostates from one year old animals**

Immunohistochemistry showing expression of p63, a basal cell marker in the lateral prostate of one-year-old Nkx3.1-Cre$^{+/-}$ mice (A) and (B); one year old Foxa2$^{loxP/loxP}$ mice (C) and (D); one year old Nkx3.1-Cre$^{+/-}$/Foxa2$^{loxP/loxP}$ (E) and (F).

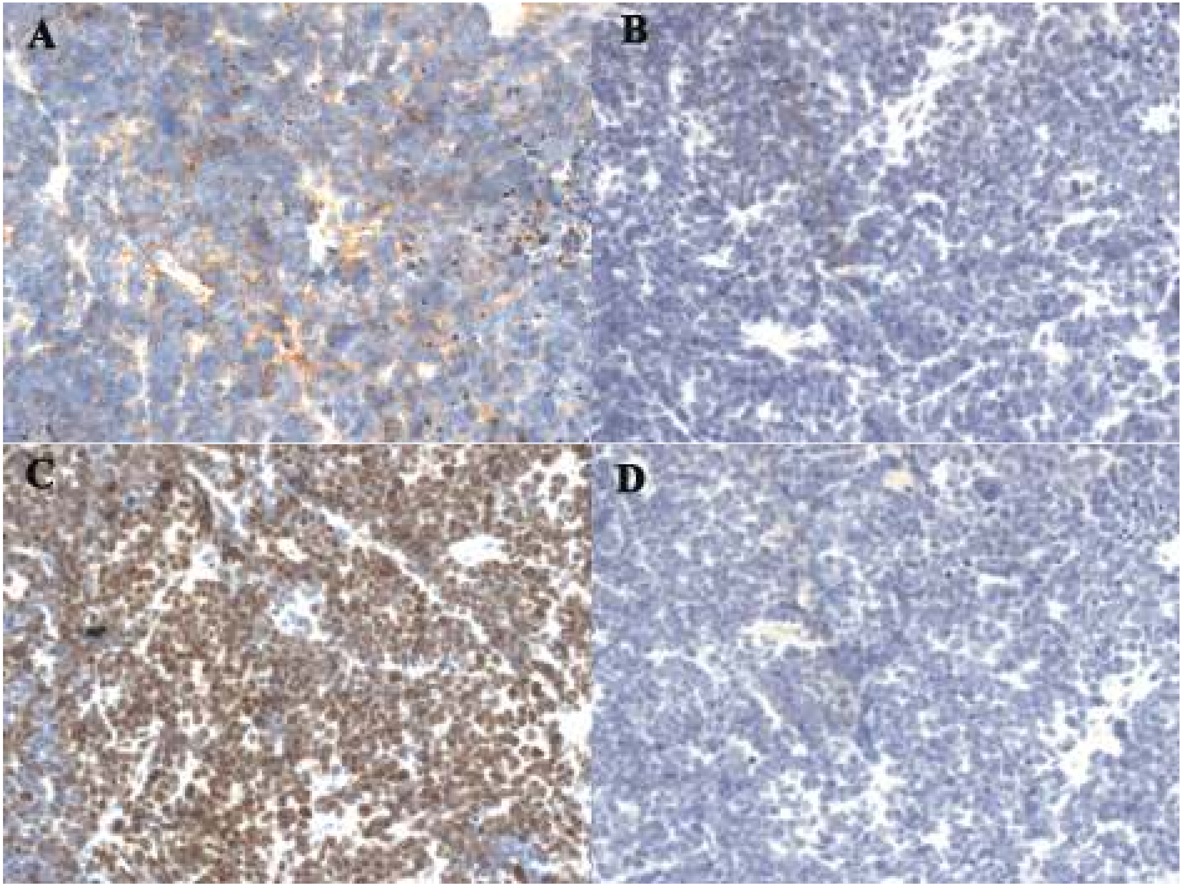


Figure 4-8: TRAMP NE prostate tumors

Serial sections of a TRAMP neuroendocrine tumor showing expression of synaptophysin (A), androgen receptor (B), Foxa2 (C), and mash-1 (D) by immunohistochemical analysis**.**

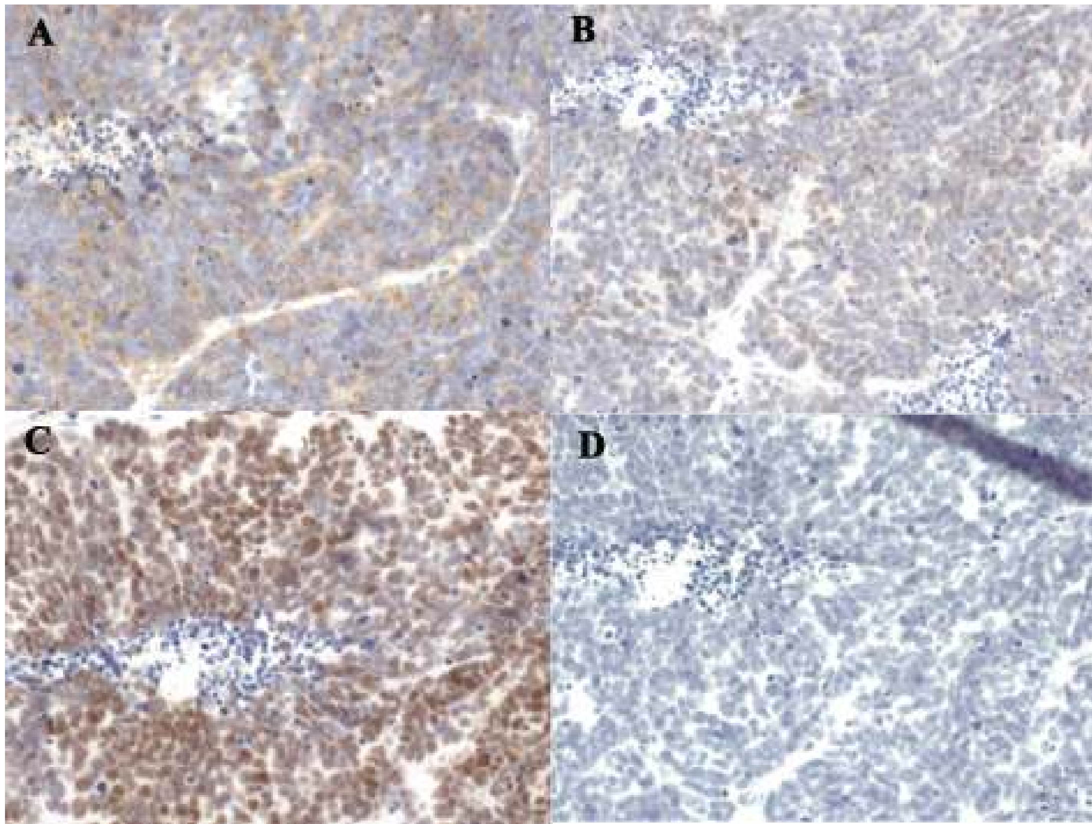


Figure 4-9: **NE tumor from TRAMP/ Foxa2$^{loxP/loxP}$ mouse**

Immunohistochemical analysis on serial sections of a neuroendocrine prostate tumor from a TRAMP/Foxa2$^{loxP/loxP}$ mouse. This tumor expresses synaptophysin (A), does not express AR (B), Foxa2 is expressed (C) and mash-1 is not expressed in these tumors (D).

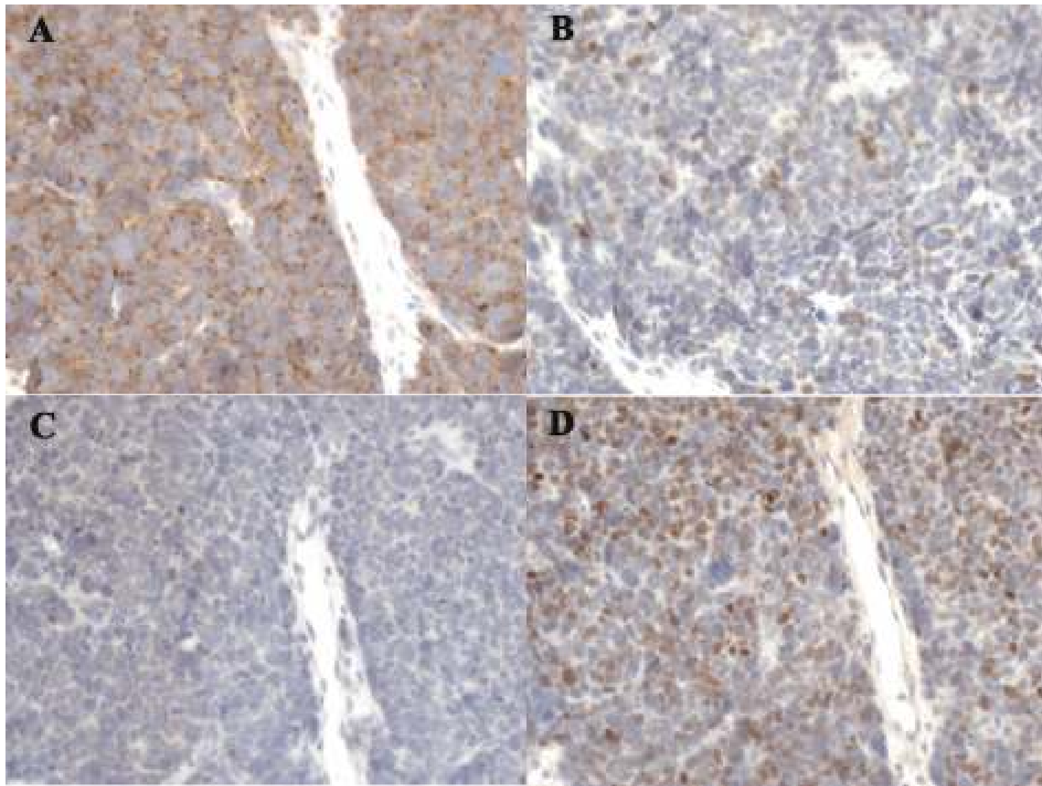


Figure 4-10: NE tumor from Foxa2 knock out TRAMP prostate

Immunohistochemistry showing expression of synaptophysin (A), androgen receptor (B), Foxa2 (C) and mash-1 (D) in the neuroendocrine prostate tumor from a TRAMP/Foxa2$^{loxP/loxP}$/Nkx3.1-Cre$^{+/-}$ mice (foxa2 knock out in TRAMP). This tumor is negative for Foxa2 and shows expression of mash-1.

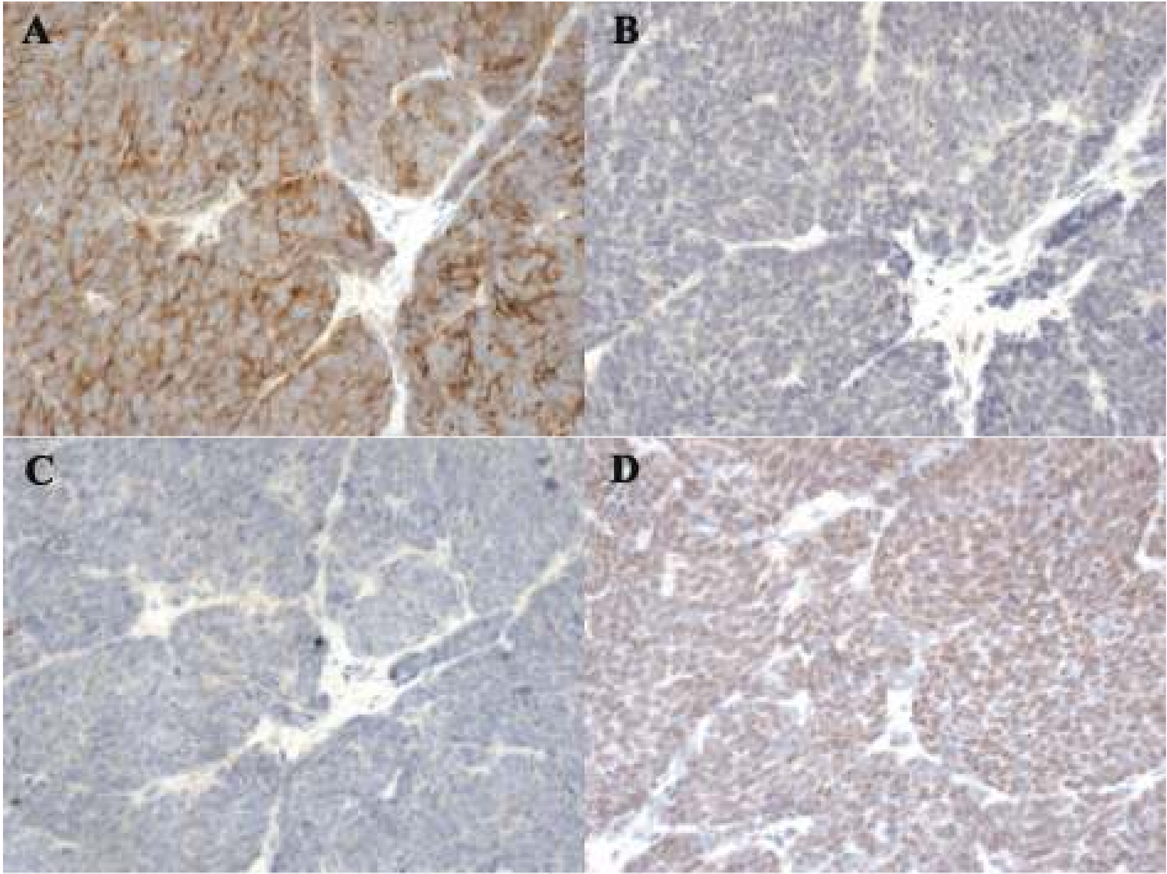
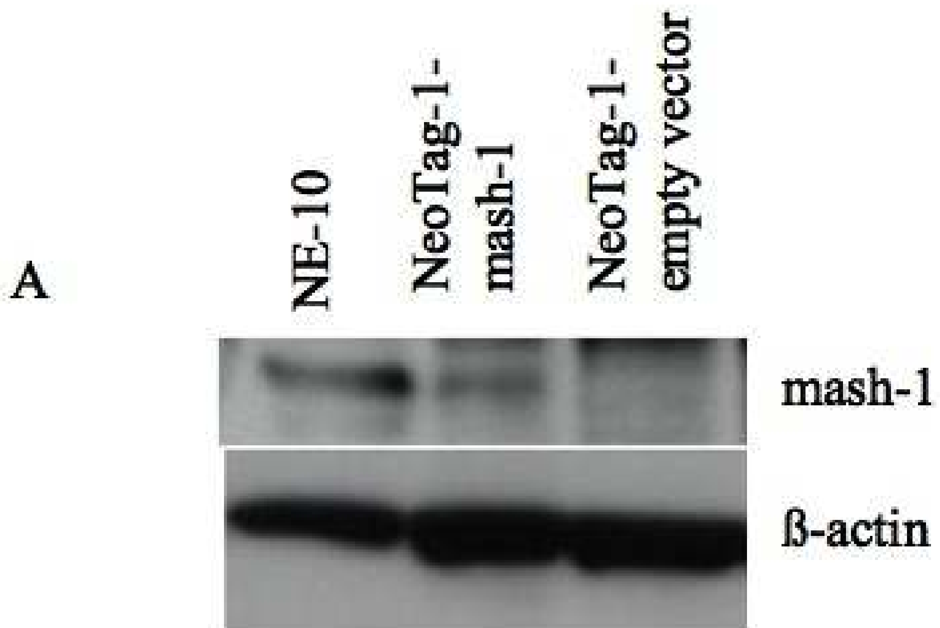


Figure 4-11: NE tumor from another Foxa2 knock out TRAMP prostate

A poorly differentiated NE prostate tumor arising in the DP of a Foxa2 knock out in TRAMP mice showing expression of Synaptophysin (A), androgen receptor loss (B), no Foxa2 (C) and extensive mash-1 expression(D).


A
NE-10
NeoTag-1-
mash-1
NeoTag-1-
empty vector
mash-1
ß-actin

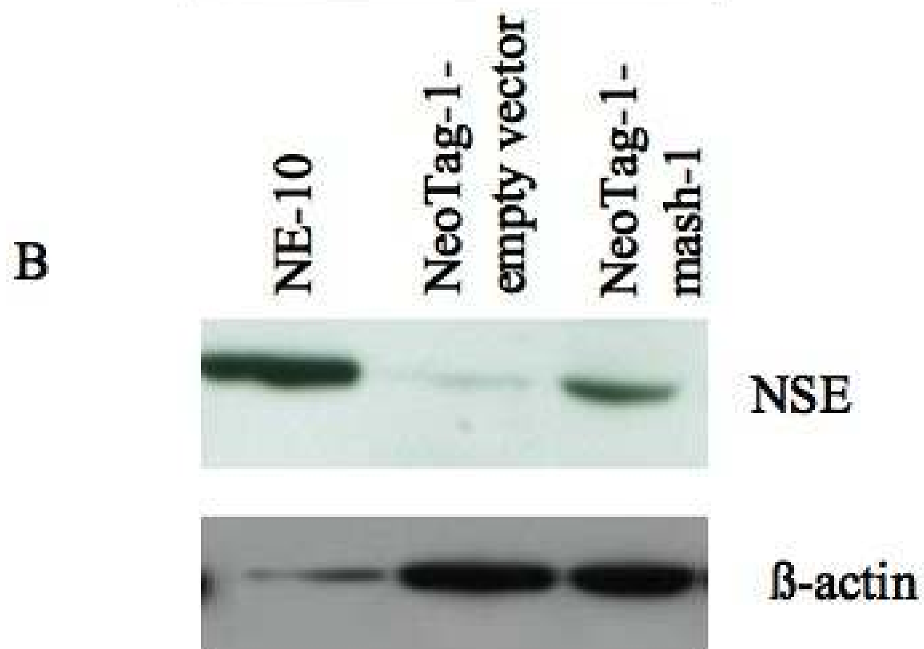

B
NE-10
NeoTag-1-
empty vector
NeoTag-1-
mash-1
NSE
ß-actin

Figure 4-12 continued

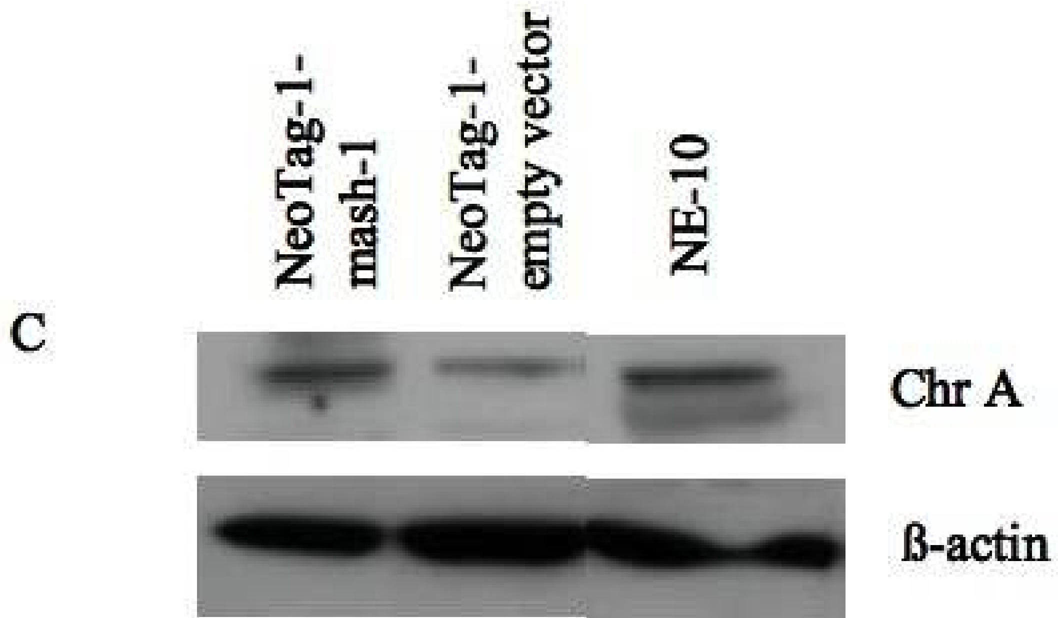


Figure 4-12: Western blot analysis on mash-1 overexpressing NeoTag-1cells

(A): Western blot showing expression of mash-1 in NeoTag1 cells infected with mash-1 lentiviral vector. NE-10 acts as a positive control.

(B): Western blot showing and increase in NSE expression after infecting cells with mash-1 expression vector.

(C): Western blot showing an increase in ChrA expression after mash-1 over expression in Neo-tag-1 cells.

CHAPTER V

DISCUSSION

The main focus of this study was to understand the mechanism that controls neuroendocrine differentiation in prostatic adenocarcinomas and/or leads to primary neuroendocrine prostate cancers that are neuroendocrine tumors (small cell carcinoma). Primary neuroendocrine cancer of the prostate is rare but if a patient presents with this tumor, the prognosis is poor. Neuroendocrine differentiation of prostatic adenocarcinoma is commonly reported but the prognostic impact of NE differentiation on prostate cancer is conflicting, as will be discussed later.

To study the mechanism of NED, we have used two different mouse models that develop neuroendocrine prostate tumors. Our studies show that the progression of tumor and the gene expression profile is different in the two models. The 12T-10 model was developed by expressing only the SV-40 large T-antigen in prostatic epithelial cells by using the prostate specific probasin promoter (Masumori et al, 2001). TRAMP, the other NE prostate cancer mouse model was generated by expressing both the SV-40 large and small t-antigen in prostatic epithelial cells using the probasin promoter (Gingrich, 1996).

By definition, neuroendocrine refers to the communication between the nervous and the endocrine system resulting in the release of secretions. Others and our group have documented that prostatic NE secretions have growth promoting effects on prostatic cancer cells (Jin et al, 2004; Kohsuke, 2006). The embryonic origin of neuroendocrine cells in the prostate (ectodermal or endodermal) remains unclear. If, during prostatic

development, the NE cells arise from the neural crest, they should have ectodermal features. However, if NE cells are endodermal in origin, then they may share a common progenitor cell with the basal and luminal epithelial cells of the prostate. Although the origin of the NE cells has not been a pivotal question, it nevertheless is fundamental to understanding the plasticity of prostatic cancer during tumor progression. In rare cases, human prostate tumors are NE cancers (Okada, 1996; Ketata, 2006) and frequently human prostatic adenocarcinomas undergo neuroendocrine differentiation, both conditions associated with poor prognosis (Turbat-Herrera, 1998; Yashi et al, 2006). In contrast, a report by McWilliam indicated that the presence of NE cells have no clinical or prognostic significance (McWilliam, 1997). The variable prognostic significance of NE differentiation in different studies could be attributed to methodological differences in determining NE differentiation, variances in the interpretation of the results, as the methods used were semi-quantitative; and differences in the cohorts of patients studied.

However, numerous studies have shown that the paracrine effects of NED can cause adjacent androgen dependent cells to transform into androgen insensitive cells. Most of the studies have discussed the effects of NED on prostate cancer but there has been very little work done on the mechanisms that can lead to NE differentiation. In the 12T-10 study, we have shown that prostate neuroendocrine differentiation follows the same molecular pathway involved in endocrine differentiation of pancreatic ß-cells, which are endodermal in orgin. Further, we report that normal prostatic NE cells as well as NE prostatic cancers express Foxa1 and Foxa2 (Mirosevich, 2005; Mirosevich, 2006), two forkhead proteins frequently associated with endodermally derived tissues (Ang et al, 1993).

Figure 5-1A shows the sequence of transcription factors that are expressed in pancreatic endocrine precursor cells. First, endodermal transcription factors, HNF6, Foxa2 and HNF1 are expressed. These differentiating endocrine cells express Notch ligands (delta and serrate) on the surface and activate Notch signaling in adjacent cells, this prevents neighboring cells from differentiating into the same cell type. Notch signaling mediates its response through activation of Hes genes (Jan, 1993). Hes genes act as transcriptional repressors and Hes1 inhibits the expression of pro-endocrine gene, Ngn3(Apelqvist, 1999; Jensen, 2000) (figure 5-1A). Previous studies have shown that activation of the Notch-1 receptor leads to activation of a Hes-1 promoter construct as well as activation of the endogenous Hes-1 gene (Jarriault et al, 1998). Further recent work using antisense oligos against Notch-1 in cell culture and overexpression of Notch-1 in transgenic mice indicates that Notch-1 regulates Hes-1 expression (Shan et al, 2006). Hes-1 is known to bind to the N box–related sequence of the mash-1 promoter and repress mash-1 transcription (Chen et al, 1997). Animals lacking Ngn3 fail to develop endocrine cells (Gradwohl et al, 2000) and ectopic expression of Ngn3 causes premature differentiation of pancreas into endocrine cells (Apelqvist, 1999; Schwitzgebel et al, 2000). Once Ngn3 is activated in the progenitor cell, that cell is destined to become an endocrine cell. The Ngn3 promoter contains inhibitory Hes1 binding sites proximal to the TATA box (Lee et al, 2001). In addition to Hes-1, the Ngn3 promoter contains binding sites for HNF1, Foxa2 and HNF6 (Jacquemin et al, 2000; Lee et al, 2001). Figure 5-1A shows that Nkx2.2 is a downstream target of Foxa2 and Ngn3 which interact synergistically to recruit transcriptional activators to Nkx2.2 gene promoter (Watada et

al, 2003). Nkx2.2 expression in the pancreas marks the final development of the mature insulin secreting ß-cells.

Figure 5-1B demonstrates that the transcription factors involved in pancreatic endocrine differentiation are sequentially expressed in prostate NE cancer cells. Foxa2, an endodermal transcription factor is expressed early in the TRAMP and 12T-10 PIN lesions. In the HGPIN lesions Foxa2 and synaptophysin are all expressed, but only occasional HGPIN samples express mash-1. However, once NE cancer appears, the cells routinely express mash-1. Similarly, hASH-1 has been reported to be highly expressed in human medullary thyroid cancer and small cell lung carcinoma (Ball, 1993; Ito, 2003; Jiang et al, 2003).

As the high grade neuroendocrine PIN lesions grow to an advanced and undifferentiated NE cancer, there is loss of Hes-1 expression. Thus, hes1 mediated inhibition of Ngn3 is removed and these NE cells express the pro-endocrine gene, Ngn3. As during pancreatic development (Watada et al, 2003), Ngn3 and Foxa2 can recruit other transcription factors to the Nkx2.2 promoter permitting expression of Nkx2.2 in some of the advanced NE tumors. Figure 5-1B shows that the NE lung metastases express Ngn3 and Foxa2 but never express Nkx2.2 while liver metastases express Foxa2 and Nkx2.2 but they do not express Ngn3. Therefore, expression of Nkx2.2 in NE tumors is not absolutely dependent upon the expression of both Ngn3 and Foxa2. Further, the difference in Ngn3 and Nkx2.2 expression between different metastatic sites indicates that a unique pattern of gene expression exists in NE cells that either allows their growth at a given site and/or is controlled by the tissue microenvironment at the site of metastases. Foxa2 can bind to the distal and proximal Ngn3 promoter and activate gene

expression, Hes-1 on the contrary specifically inhibits the Ngn3 promoter (Lee et al, 2001). This observation can be explained through the Foxa2 positive early PIN lesions in 12T-10 prostates, which still express Hes-1 that prevents the expression of Ngn3. In the 12T-10 transgenic and NE-10 allograft model of NE prostatic cancer, we can confirm the loss of Notch signaling by the loss of Hes-1 shown by RT-PCR. Similarly, the loss of Notch signaling has been reported in lung NE cancers (Collins, 2004), neuroblastomas (Axelson, 2004), a childhood tumor originating from cells of developing sympathetic nervous system as well as in the neuroendocrine phenotype in gastrointestinal carcinoids (Nakakura et al, 2005). These results suggest that the genes involved in pancreatic endocrine differentiation are expressed in the same sequential manner during NED of prostatic cells. This study presents markers for molecular mechanism that can either be used to detect early stage NE tumors or to target therapy to a subset of NE prostate tumors.

The NE tumor progression in TRAMP mice is very different from the 12T-10 mouse model, with the TRAMP developing very aggressive and large NE tumors between 24 to 36 weeks of age unlike 12T-10 that develop LGPIN, followed by HGPIN after 30 weeks of age and NE cancer after one year. TRAMP NE tumors show loss or decrease of androgen receptor with the progression of NE tumors from a more differentiated to poorly differentiated form and the tumors do express synaptophysin. The TRAMP NE tumors unlike the 12T-10 NE tumors do not generally express mash-1. Immunohistochemical studies have detected only a few faintly positive mash-1 cells in the TRAMP tumors but they are always Foxa2 positive. This led us to hypothesize that Foxa2 might be a critical component in NED of prostate tumors. In this study we used the

TRAMP model to better understand the role of Foxa2 in NE differentiation of the prostate tumors. We have generated mice with a conditional knock out of Foxa2 in the TRAMP NE tumors by crossing the TRAMP foxa2 floxed mice with Nkx3.1Cre+/- mice. Loss of foxa2 in the TRAMP mice prostates did not prevent the formation of neuroendocrine tumors. The Foxa2 knock out TRAMP mice still develops NE tumors but many of the NE cells now express mash-1 after the loss of Foxa2. Not all the tumor cells express mash-1 after the loss of Foxa2, suggesting that the TRAMP NE tumor cells are of at least three different kinds based on these results: 1) NE cells that require Foxa2 expression; 2) NE cells that lose Foxa2 but now express mash-1; and 3) NE cells that lose Foxa2 due to the knock out but still do not express mash-1. It is possible that in the third case, the early tumors do express mash-1 but as they progress to very high grade NE cancers they no longer express mash-1. Regardless, this suggests that the TRAMP NE tumors are very heterogeneous and may contain three different types of NE cells, each having a distinct gene expression profile yet all expressing synaptophysin.

Unlike 12T-10 tumors, the TRAMP neuroendocrine prostate tumors do not show a complete loss of androgen receptor, instead most of the TRAMP NE cells express low levels of androgen receptor. The expression of androgen receptor in the Foxa2 knock out TRAMP PIN or NE cancer looks similar to TRAMP alone. There does not seem to be a co-relation between the expression of either Foxa2 or mash-1 to the expression level of AR in the respective cells. This suggests that AR receptor status in the NE cells is independent of the levels of Foxa2 or mash-1. Overall, this study suggests that Foxa2 and mash-1 can be used as markers during NED of PIN and in a subset of neuroendocrine prostate tumors. Further, transfection of mash-1 into the mouse prostatic NeoTag1 cell

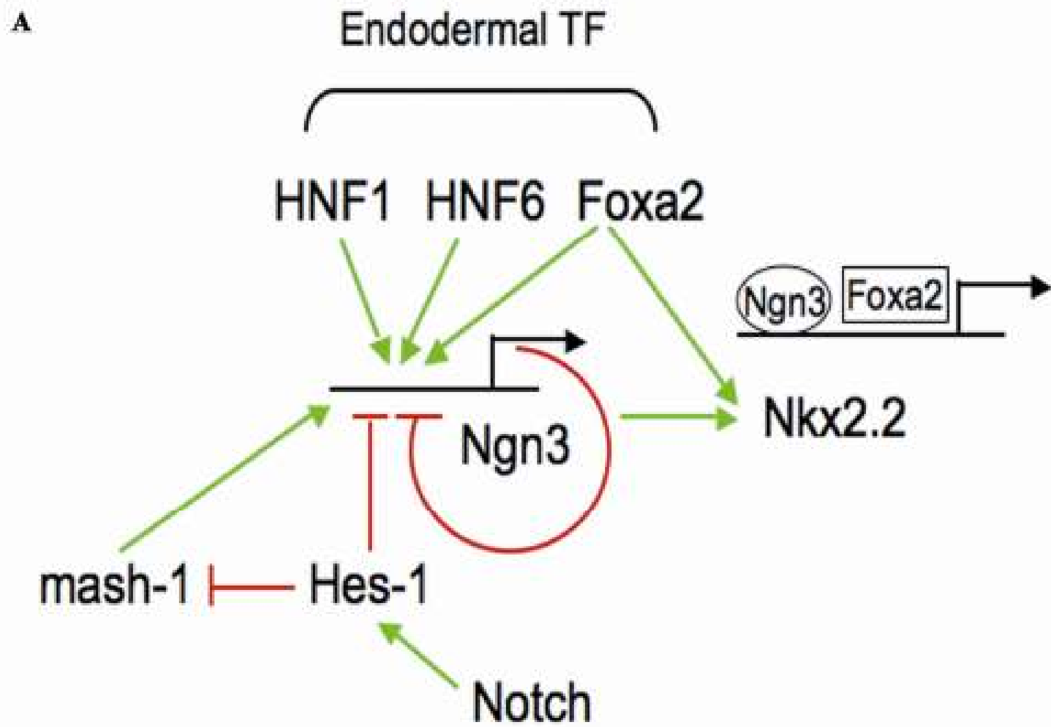
line (Figure 4-12) demonstrates that in Large T-antigen immortalized cells mash-1 is able to initiate expression of NE markers. Taken together, the expression of mash-1 in Foxa2 knock out TRAMP mice and the ability of mash-1 to NED NeoTag1 suggest that a Foxa2 independent pathway that now utilizes mash-1 can result in NED of the TRAMP tumor. At the same time there appears to be at least one more pathway that can cause neuroendocrine differentiation in the TRAMP tumors since some NE tumors are negative for both Foxa2 and mash-1. Further, unlike the 12T-10 neuroendocrine tumors, the TRAMP tumors do not express either Ngn3 or Nkx2.2 as seen by RT-PCR.

Although both 12T-10 and the TRAMP mice develop NE prostate tumors, there is a difference between the rate of progression of these tumors and the gene expression profile. The two mouse models use different versions the probasin gene promoter to target the transgenes specifically to the prostate epithelial cells. The 12T-10 uses the LBP promoter that is 10.8 kb in size while TRAMP uses the small -426 PB promoter. The site of integration may affect the expression of the transgene as well as alter gene expression near the site of integration. The transgene in the 12T-10 line is located on the X-chromosome but there is no information on the transgene location in the TRAMP model. Another major difference is that the 12T-10 model expresses only the large T-antigen while the TRAMP mice express both large T-antigen and small t-antigen. The difference in the NE tumors between these two mice models could be due to the expression of small t-antigen in the TRAMP models.

This study has unraveled a few important facts about the neuroendocrine differentiation of prostate tumors. The studies on the 12T-10 neuroendocrine mouse line have identified key transcriptions factors involved in NED. Foxa2, mash-1, Ngn3 and

Nkx2.2 could be used at the very least as markers for NED. In addition to this, the difference in the expression of Ngn3 and Nkx2.2 in the lung and liver metastasis such that Ngn3 is expressed only by the lung NE metastasis and Nkx2.2 is expressed only by the liver metastasis, suggests a difference between the tumor cells migrating to these different organ sites. The expression of these two genes could be used as a marker for site specific metastasis. The other possibility could be that there is no distinction between the cells that migrate to the liver or the lung but once the tumor cells are at the metasatic site, the microenviornment controls gene expression of the tumor cells.

The Foxa2 knock out studies performed on the TRAMP tumor model has been very informative. It suggests that the NE tumors in at least the TRAMP mice model can develop without the expression of Foxa2. After the loss of Foxa2 in these tumors, mash-1 is switched on. There are some Foxa2 knockout TRAMP NE tumors that do not express mash-1. This suggests that there are at least three different pathways to NED, one that requires foxa2, a second that needs mash-1 expression and the third that can do without either Foxa2 or mash-1. Overall, this study has demonstrated interesting characteristics about neuroendocrine differentiation in prostate tumors.



B

The start of NE differentiation

	LGPIN	HGPIN	NE PCa
Syn	+/-	+	+
AR	+/-	-	-
Foxa2	+	+	+
Mash-1	+/-	+	+
Ngn3			+
Nkx2.2			+

Metastasis

Liver
Nkx2.2 + (33%)

Lung
Ngn3 + (100%)

Figure 5-1: Diagramatic representation of sequential gene expression in prostate tumors

(A) Diagrammatic representation of gene expression in pancreatic endocrine cell differentiation. Endodermal factors are the initial ones to be expressed. Notch signaling prevents cells from differentiating into endocrine cells and maintains normal cell differentiation. Notch signaling mediates its response through Hes-1 (hairy/enhancer of split) that represses pro-endocrine gene, neurogenin3 (Ngn3). Hes-1 also down regulates mASH-1. Ngn3 and Foxa2 interact synergistically to recruit transcriptional activators to Nkx2.2 promoter. Nkx2.2 is expressed in pancreatic ß-cells.
(B) Diagrammatic representation of sequential gene expression with progression of neuroendocrine prostate cancer from LGPIN (low-grade prostatic intraepithelial neoplasia) to HGPIN (high grade PIN) to poorly differentiated NE cancer followed by lung and liver metastases.

Future Direction

Determine the role of mash-1 in NED: The knock out of Foxa2 in TRAMP mice results in expression of mash-1 in the NE prostate tumors. Over expression of mash-1 in NeoTag-1 cells results in increase of NSE (Neuron Specific Enolase) and ChrA expression. This suggests that mash-1 could cause NED. This can further be confirmed by growing the mash-1 overexpressing cells subcutaneously in the nude mice. This would confirm the importance of mash-1 in neuroendocrine differentiation of prostate tumors.

Determine the importance of Ngn3 and Nkx2.2 in organ specific metastasis: The NE prostate tumors of the 12-T10 mice express Ngn3 but rarely express Nkx2.2 in a few scattered cells. The NE liver metastasis express Nkx2.2 but do not express Ngn3 and lung NE metastasis express Ngn3 but not Nkx2.2. To elucidate whether these two genes are markers for site specific metastasis or if the expression of these genes in the primary tumors governs the site of metastasis, we should implant Ngn3 and nkx2.2 overexpressing cells into the mouse prostate and watch the site of metastasis of each cell type. This will determine the importance of Ngn3 and Nkx2.2 in NE metastasis.

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