THE ROLES OF PROSTATE PROGENITOR CELLS AND SURVIVIN IN INFLAMMATION-INDUCED PROSTATE EPITHELIAL HYPERPLASIA

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Dedication

This thesis is dedicated to my parents Ming Wang and Xiaomei Tan. Thank you for giving me the best education and support. To my wife Liting Deng, thank you for encouraging and supporting my study in all aspects.

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THE ROLES OF PROSTATE PROGENITOR CELLS AND SURVIVIN IN INFLAMMATION-INDUCED PROSTATE EPITHELIAL HYPERPLASIA

Prostate inflammation is a common health concern as an important risk factor for prostate cancer and Benign Prostatic Hyperplasia (BPH). Inflammation induces epithelial apoptosis and epithelial hyperplasia, suggesting that inflammation promotes the tissue repair and regeneration process. Progenitor cells are critical in maintaining epithelial homeostasis in adult tissues. However, the roles of prostate progenitor cells, especially during prostate inflammation, are understudied. I proposed that prostate epithelial progenitor cells (PEPCs) are involved in inflammation-induced epithelial hyperplasia, and are driven by regulation from inflammatory pathways.

Here, we showed that sphere formation ability of prostate epithelial cells is increased by inflammation. We identified that a population of prostate progenitor cells, named prostate epithelial progenitor cells, were expanded by inflammation under the regulation of IL-1/insulin-like growth factor 1 (IGF-1) signaling pathway, a previously identified critical regulation pathway of inflammation-induced

epithelial hyperplasia. The expansion of PEPCs also correlated with the intensity of inflammation.

We then identified that survivin was upregulated in prostate epithelial cells by inflammation and was mainly co-localized with proliferation markers in prostate epithelial cells. This upregulation depended on IL-1/IGF-1 signaling. *In vivo* treatment with the survivin inhibitor LQZ-7F reduced both survivin expression and proliferation in prostate epithelial cells during inflammation. Using our label-retaining strategy, we compared the survivin expression pattern in two prostate regeneration models. We discovered that different populations of progenitor cells may be involved in different regeneration processes. We identified that survivin was expressed in a specific population of reactivated cells that respond to the inflammatory environment and was independent of the known slow-cycling stem cells found in the prostate epithelium. In summary, I have identified that PEPCs are involved in epithelial hyperplasia and are dependent on survivin signaling. My work defines how survivin serves as a key regulator of epithelial hyperplasia in an inflammatory environment.

Travis Jerde, Ph.D., Chair

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List of Abbreviations

Abbreviation	Full name
ABP	acute bacterial prostatitis
AEC1	alveolar type I cell
AEC2	alveolar type II epithelial cell
AIP	asymptomatic inflammatory prostatitis
AP	anterior lobe or anterior prostate
AR	androgen receptor
BOO	bladder outlet obstruction
BPH	benign prostatic hyperplasia
BrdU	bromodeoxyuridine
CBP	chronic bacterial prostatitis
CK	cytokeratin
COX-2	cyclooxygenase-2
CP/CPPS	chronic prostatitis/chronic pelvic pain syndromes
CRPC	castration resistant prostate cancer
CXCL	chemokine (C-X-C motif) ligand
CZ	central zone
DASC	distal airway stem cell

DHT	dihydrotestosterone
DLP	dorsal-lateral lobe or dorsal lateral prostate
E	estrogen
EdU	5-ethynyl-2'-deoxyuridine
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ERα	estrogen receptor α
ERβ	estrogen receptor β
ES	embryonic stem
FGF	fibroblast growth factor
GFP	green fluorescent protein
H&E	hematoxylin and eosin
HGPIN	High-Grade Prostatic Intraepithelial Neoplasia
HIF-1	hypoxia-inducible factor-1
HSC	hematopoietic stem cell
IF	Immunofluorescence staining
IFN-γ	Interferon γ
IGF-1	insulin-like growth factor 1
IGF-1R	IGF-1 receptor

IKK	I kappa B kinase
IL	interleukin
IL-1RA	IL-1 receptor antagonist
IPSS	international prostate symptom score
KO	knock out
LESC	limbal epithelial stem cell
LHRH	luteinizing hormone-releasing hormone
LUTS	lower urinary tract symptoms
MCP-1	monocyte chemoattractant protein-1
MDSC	myeloid-derived suppressor cell
MIP	macrophage inflammatory proteins
MMP	matrix metalloproteinases
MSC	mesenchymal stem cell
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAID	nonsteroidal anti-inflammatory drug
NSC	neural stem cell
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2

PIN	prostatic intraepithelial neoplasia
PSA	prostate-specific antigen
PSMA	prostate specific membrane antigen
Pten	phosphatase and tensin homolog
PZ	peripheral zone
RFP	red fluorescent protein
ROS	reactive oxygen species
SFM	serum free medium
SP	side population
STAT3	signal transducer and activator of transcription 3
T	testosterone
TAC	transit amplifying cells
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinase
TLR	Toll-like Receptor
TNF-α	tumor necrosis factor-α
TWEAK	TNF-related weak inducer of apoptosis
TZ	transitional zone
VP	ventral lobe or ventral prostate

Chapter 1 Introduction

A. Prostate structures

The prostate is an exocrine organ and an accessory gland of the male reproductive system that is only found in mammals. It secretes the prostate fluid that constitutes about 30% of total volume of the semen. The prostate fluid contains proteins and minerals (i.e.: zinc) that maintain the viability of sperm cells. The prostate is also one of the major organs that produce dihydrotestosterone (DHT) in humans due to its high expression level of 5α-reductase. 5α-reductase transforms testosterone (T) to Dihydrotestosterone (DHT). DHT has 3-5 times more activity on androgen receptor (AR) compared to T. The transformation from T to DHT increases the AR signaling in the prostate. In humans, the prostate is a walnut-size organ that lies under the bladder and surrounds the urethra. Anatomically, the human prostate can be divided into 4 zones. The peripheral zone (PZ) is the largest part of the prostate which constitutes about 70% of the total prostate mass. Most prostate cancers (70-80%) develop from the peripheral zone (Fig 1.1). The transitional zone (TZ) is in the upper part of the prostate and is inside of the PZ. It is the smallest part of the prostate which is around 10% of the total prostate. Benign prostatic hyperplasia (BPH) usually originates from the TZ in aged male (Fig 1.1). Overgrowth of the TZ will increase the pressure on urethra and bladder which

may block the urinary tract leading to lower urinary tract symptoms (LUTs). The central zone (CZ) surrounds the TZ in the middle of the prostate. is the CZ composes approximately 20% of the total prostate mass. It has ductal structures which connect to the prostate and the seminal vesicle (Fig 1.1). There is also a non-glandular anterior fibromuscular stroma region in the prostate. The stromal nourishes prostate epithelium and regulates its functions and growth. These cells play an in dispensable role in the development of BPH and prostate cancer. The anatomy structure of the prostate differs in different animals. Human and canine prostates have compact structures as an integrity, while the mouse and rat prostates consist of different lobes [1]. Mouse prostate consists of 4 separate lobes. The ventral lobe (VP) is attached to the urethra under the bladder and partially wrapped the urethra (Fig 1.1). The dorsal lobe (DP) lies at the base of the seminal vesicle. The lateral lobe (LP) is between and on the outer side of the VP and DP (Fig 1.1). The dorsal and lateral lobes are always referred as Dorsal-lateral lobes (DLP) (Fig 1.1). The anterior lobe (AP), also known as the coagulating glands, is attached to the seminal vesicle (Fig 1.1). Based on limited developmental studies and mRNA comparisons, it has been determined that the PZ in human is homologous to DLP in the mouse. The CZ shares similarities with the AP in the mouse. The TZ in humans, from where the BPH develops, does not have a counterpart in the mouse [1].

These differences in anatomy may result in different pathologies. For example, dogs can spontaneously develop BPH-like symptoms which do not occur in rodents. Dogs also have spontaneous prostate cancer and metastasis to bone, similar to human prostate cancer [1]. These are major disadvantages that limit the application of mouse/rat as animal models for prostate research.

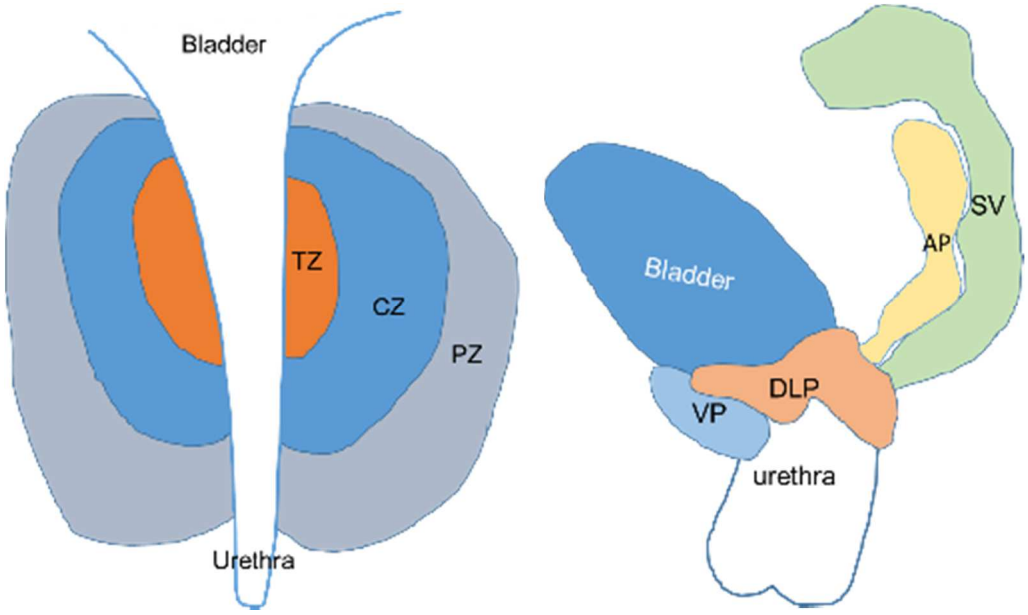


Fig 1.1 Anatomy structure of human and mouse prostate

Left: human prostate; right: Mouse prostate TZ: transitional zone; CZ: central zone; PZ: peripheral zone; VP: ventral prostate; AP: anterior prostate; DLP: dorsal-lateral prostate; SV: seminal vesicle.

There are 4 different major types of cells in the prostate: basal cells, luminal cells, neuroendocrine cells and stromal cells. The basal cells and luminal cells

are the two major types of epithelial cells in the prostate. The basal cells lie along with the basement membrane in a discontinuous pattern. They are characterized by cytokeratin (CK)-14 and P63 positive staining. The progenitor cells of prostate epithelium are considered to be located in the basal cell compartment since basal cells can differentiate into other types of epithelial cells such as luminal cells. The luminal cells are terminally differentiated epithelial cells. They are columnar-shaped with the secretion function. Luminal cells are characterized by CK8/18 and NKx3.1 positive staining. The luminal cells are androgen receptor (AR) positive cells and are considered as the origin of prostate cancer. The neuroendocrine cells are a rare population in the prostate. They are positive for chromogranin A staining. They are associated with certain types of prostate cancers and their numbers in the prostate cancer have prognosis significance. However, their functions are still unclear. The stromal cells are the major cell type in the prostate stromal compartment. They support and regulate the growth of prostate epithelium through epithelial-stromal interaction. The stromal cells play important roles in the development of BPH and prostate cancers. The cancer associated fibroblast in the stromal compartment is necessary for prostate epithelial cell transformation and thus for cancer initiation. Other cell types, such as endothelial cells, smooth muscle cells, residence macrophages, can also be

found in the prostate. They are crucial for physiological and pathological situations in the prostate.

B. Prostate Inflammation

3 major types of prostate diseases are usually found in human: prostate inflammation, BPH, and prostate cancer. Clinically, prostate inflammation is usually under-diagnosed since it is commonly asymptotic and patients will not aware the initiation of prostate inflammation unless it causes any symptoms like LUTs. Most prostate inflammation cases are diagnosed when a biopsy is conducted for another purpose such as BPH and prostate cancer, which means prostate inflammation is already in its chronic phase in these cases. Clinically, prostate inflammation is classified into four categories: 1. acute bacterial prostatitis (ABP), is caused by bacteria or virus infection and is an uncommon type of prostate inflammation which may count for 5% or fewer prostatitis cases; 2. chronic bacterial prostatitis (CBP), CBP is also microbial-caused inflammation, but it cannot be effectively treated; 3. chronic prostatitis/chronic pelvic pain syndromes (CP/CPPS), is the most common type of prostate inflammation that contributes to more than 90% of total prostate inflammation. It is identified as non-microbial related, but its mechanisms can be multi-factorial and are still

unknown; 4. asymptomatic inflammatory prostatitis (AIP), it is asymptomatic, but diagnosed by immune cells in expressed prostatic fluid.

Prostate inflammation is a common health problem in the American society. About 1.8% of males who make up approximately 2 million individuals in the US, report to have CP/CPPS symptoms [2] which include frequent urinating, difficulty urinating, lower urinary tract pain and etc. This indicates that there is an even larger population that may be affected by prostate inflammation. Little is known about how and when prostate inflammation is initiated and developed in human. Unlike BPH and prostate cancer, both of which are majorly age-related diseases, puberty chronic prostatitis can be found in human [3]. Numerous risk factors have been identified for prostate inflammation, including bacterial infection, prostate calculus, urinary tract sphincter over contraction, abnormal immune activity, and psychological problems [4]. However, the mechanisms underlying that how do these factors contribute to prostate inflammation in human are still poorly understood.

Although the prostate inflammation is not life-threatening, it is considered as an important risk factor for both BPH and prostate cancer. The prostate inflammation is commonly found in BPH and prostate cancer specimens and it

can promote the progression of these diseases [5]. Delongchamps and associates analyzed autopsy specimens obtained from 93 men with histological evidence of BPH and found that chronic inflammation was found in 50% of prostates with cancer [6]. These suggest prostate inflammation as an important pathological factor of BPH and prostate cancers.

a. Prostate inflammation and BPH

BPH is diagnosed by increased proliferating of prostate epithelial cells (histologically) and enlarged prostate. BPH is often progressed and is associated with LUTS and bladder outlet obstruction (BOO) in patients. BPH is a tightly age-related disease. The incidence of BPH increases from 42% in males ages between 51 to 60, to 90% in males ages between 81 to 90 [7]. This suggests an almost inevitable fate of developing BPH in aged males.

The first line anti-BPH drugs fall into two categories: the 5α-reductase inhibitors and the alpha-blockers. The 5α-reductase inhibitors, such as dutasteride or finasteride, block the production of DHT by inhibiting T transformation and thus reduce the activation of AR in the prostate. These drugs impede the enlargement of prostate since prostate epithelial cells are highly androgen signaling dependent. However, 5α-reductase inhibitors have fewer

effects on stromal cells since they are not androgen dependent. The overgrowth of stromal cells also contributes to the development of BPH. The alpha-blockers, such as terazosin, block the α-androgenic receptor to relax the smooth muscle cell contraction in prostate and bladder. The alpha-blockers target symptoms but not the cell proliferation. They relieve BPH symptoms but have no effect on inhibiting prostate enlargement. Thus, the alpha-blockers alone is not ideal for long-term BPH control. Chronic inflammation is associated with higher International Prostate Symptom Score (IPSS) and larger prostate volume in the REduction by DUtasteride of prostate Cancer Events (REDUCE) study [8]. This suggests that inflammation may drive BPH development. Thus, anti-inflammatory drug treatments are expected to be potential therapies for BPH. Anti-inflammatory strategies have been shown effective in treating BPH, at least on relieving symptoms in patients. Celecoxib can effectively reduce refractory nocturia in BPH patients [9]. Patients treated with permixon have lower inflammation intensity, evaluated by lymphocytes B infiltration, TNF-α, and IL-1 beta level in the prostate. Permixon also reduces their IPSS, suggesting alleviations of BPH symptoms by anti-inflammatory treatment [10]. The combination of nonsteroidal anti-inflammatory drugs (NSAID) and alpha-blockers can further alleviate the symptoms of BPH/LUTS. Patients treated with doxazosin in combine with tenoxicam have better improvements of their IPSS comparing to

single treatment [11]. A combination of doxazosin with celecoxib also showed similar improvements in BPH patients [12]. However, the effects of anti-inflammatory treatment also raise controversy. Clinical trials show that daily use of aspirin or ibuprofen cannot reduce BPH/LUTS risks in human [13]. A recent study using the cohorts from the Prostate Cancer Prevention Trial shows that the application of NSAID increases BPH risks [14]. This suggests that a complex network of mechanisms underlies the association between inflammation and BPH progression. The effects of inflammation on BPH initiation and progression may depend on different regulation pathways.

The mechanisms of how inflammation contributes to BPH development are not fully understood. The prostate inflammation creates a reactive inflammatory environment and disrupts hormone balance, both of which may increase prostate epithelial cell proliferation and thus increase the risk of BPH. In humans, the decline in T : estrogen (E) ratio in aged males is considered as the main factor which contributes to BPH progression [15]. This partly explains the age-related incidence of BPH. AR is found in both epithelial and stromal compartments in BPH specimens [16]. The luminal epithelial cells in the prostate are androgen dependent while the stromal cells are usually castration-resistant, suggesting that the epithelial cells are the primary targets of androgen signaling. In humans, the

BPH regions contain 2.2 times more DHT compared to the non-diseased regions in human and the BPH stromal regions have 2-3 times more 5α-reductase activity compared to non-diseased regions [17]. This suggests that despite the lower ratio of circulating T/E in aged males, the elevation of local hormone level disturbs the homeostasis of hormone regulation and contributes to BPH progression. Interestingly, the mechanisms underlie androgen regulation in the prostate are more complicated than we expected. Studies in rabbit prostate show that testosterone treatment can reverse the high-fat diet induced prostate inflammation and BPH, suggesting that in some cases testosterone may have a protective effect against prostate inflammation [18]. E2 treatment in castrated rats induces inflammatory markers such as TNF-a, cyclooxygenase-2 (COX-2) and macrophage inflammatory proteins-1α (MIP-1α), while co-treatment with androgen can reduce these inflammatory marker expressions in a dose-dependent manner [19]. Consistent with the T/E ratio change-induced BPH in human, combination treatments of T and E in castrated animals lead to prostate inflammation and BPH [20]. This is used as a major animal model for BPH and prostate inflammation study. Thus, the mechanisms underlying how androgen contributes to the initiation and progression of BPH remain controversial.

Inflammation increases the metabolism of estrogen in prostate and shifts the expression pattern of estrogen receptors (ER) [21]. Both types of estrogen receptors, ERα and ERβ, have been found in the prostate. ERα is primarily expressed in stromal cells in prostate and its expression level varies according to the progression of BPH [22]. Thus, its main function in the prostate may be through regulating stromal cells and the stromal-epithelium interaction. Selectively knocking out (KO) ERα in the prostate stromal cell results in less stromal branching during prostate development and increased stromal cell apoptosis, while ERα KO in epithelial cells does not affect prostate development [23]. ERβ expression in rodents differs from that in human. ERβ is primarily expressed in luminal cells in rodents, while its expression level varies a lot in human specimens in both epithelial and stromal compartments [24]. Knocking out ERβ in mice induces epithelial hyperplasia, blockade of epithelial differentiation and increase inflammation in the prostate [25]. ERβ has the anti-proliferative function in prostate epithelial cells. Loss of ERβ is correlated with malignant lesions in the prostate [26]. Activation of ERβ inhibits NF-κB signaling through regulating hypoxia-inducible factor-1 (HIF-1). Loss of ERβ is inversely correlated with IKKβ/p65 activity in both ERβ KO mice and human prostate cancer specimens [27]. This further suggests ERβ's anti-proliferative and tumor suppressive functions. In addition, the racial disparity of BPH incidence is also

postulated to be related to estrogen signaling. Phytoestrogens uptake from daily diet is thought to be a protective factor against BPH in Asian populations.

Other factors, such as inflammatory factors, cytokines, and chemokines, also affect epithelial cells proliferation during inflammation. High-fat diet induces epithelial hyperplasia through activation of signal transducer and activator of transcription 3 (STAT3) and NF-κB signaling to increase pro-inflammatory cytokines and their regulated gene products [28]. An aging associated up-regulation and elevated secretion of CXC-type chemokines (CXCL1, CXCL2, CXCL5, CXCL6, CXCL12) and interleukins (IL-11, IL-33) are observed in prostate stromal compartment [29], suggesting an increased inflammation intensity along with the progression of BPH. In addition, urine levels of CXCL-8, CXCL-10, and IL-1RA are associated with prostate size [30]. Serological analysis indicates that prostate size and plasma prostate-specific antigen (PSA) levels are positively correlated with IL-8 and monocyte chemoattractant protein-1 (MCP-1) concentrations [31]. Thus, inflammation is associated with prostate growth.

In summary, inflammation is a potential therapeutic target for treating BPH. Prostate inflammation is associated with BPH. It affects BPH progression through

regulating hormone signaling and inflammatory factors related signaling pathways. However, the mechanisms involved are only partly understood.

b. Prostate inflammation and prostate cancer

Prostate cancer is a common heath issue of males in the U.S. It has the highest estimated annual new cases (around 180,000) which were 21% of total new cancers cases among all cancer types in males in 2016. It caused around 26,120 deaths in 2016, which is the second leading causes of cancer death (8% of total cancer deaths in males, Cancer fact 2016). Most prostate cancers (70-80%) originate from the PZ in human prostate. However, the initiation of the tumor is still unclear. Many factors have been correlated to the cancer initiation, such as gene, race, diet, life style, and environment [32]. The prostate cancer develops from prostatic intraepithelial neoplasia to androgen-dependent invasive cancer and then to castration-resistant prostate cancer (CRPC) which is partly driven by the androgen deprivation therapy. Clinically, the most important marker used to surveillance prostate cancer risk is the PSA level. A circulating PSA level larger than 4ng/ml suggests a high risk of prostate cancer. In prostate cancer patients, PSA level is also associated with the malignancy of cancer. Both surgical and pharmacological methods can be applied in treating prostate cancer. Inhibition of androgen signaling is the primary strategy of treating prostate

cancer. The androgen level in the prostate can be reduced through surgical castration (by removing the testis) or chemical castration (i.e., Luteinizing hormone-releasing hormone (LHRH) treatment). Another strategy is to inhibit the production of androgen. CYP17 inhibitors such as Abiraterone can be applied to further reduce androgen level in addition to castration strategy. AR inhibitor is the third strategy to reduce AR signaling in prostate cancer. AR inhibitors such as Enzalutamide can prevent T from binding to AR and inhibit AR nuclear translocation and thus to inhibit AR signaling. Other methods, such as radiation therapy, chemotherapy, and cryosurgery, are also involved in clinical prostate cancer treatments.

Prostate inflammation is among the top risk factors for prostate cancer. It is usually defined by the presence of infiltrating lymphocytes in prostate tissues. It increases the risk of cancer initiation in the prostate. In animals, inflammation increases prostate cancer initiation when prostate epithelial cells lose the tumor suppressor phosphatase and tensin homolog (Pten) [33]. In human, chronic inflammation in the benign prostate is correlated with the following occurrence of high-grade prostate cancer [34]. In men without suspicion of prostate cancer, intra-prostatic inflammation is associated with higher PSA level, which suggesting a higher risk of prostate cancer [35]. This indicates inflammation as a contributor

to cancer initiation. The correlation between inflammation and cancer initiation makes anti-inflammatory drugs the potential cancer prevention candidates. However, the outcome of applying these drugs in prevention prostate cancer is not very encouraging. Whether applying NSAID in patients can decrease PSA levels or not is still controversial [36]. On the other hand, it has been shown that NSAID slightly increases the risk of prostate cancer in patients [37]. It is also arguing that regular aspirin use cannot alter the circulating inflammatory factors, such as IL-1, CCL13, CCL17, soluble vascular endothelial growth factor receptor 2 and soluble tumor necrosis factor receptor 1, in many tissues including prostate [38]. Failure of NSAID in preventing prostate cancer may partly because of lower COX-2 expression in tumors compared to benign tissues [39], it may also due to the complexity nature of inflammation. It has to be noticed that the correlation between inflammation and prostate cancer initiation is not one-directional. Data derived from the REDUCE study shows that base line chronic inflammation is correlated with smaller prostate volume in prostate cancer patients [40]. Baseline prostate atrophy, which suggesting the presence of inflammation, is negatively correlated with the prostate cancer risks [41]. Moreover, a racial analysis shows that Asian people who have the highest incidence of acute prostate inflammation have the lowest prostate cancer incidence, while American African population has the lowest acute prostate inflammation risk with the top risk of prostate

cancer [42]. This suggests that the patterns of inflammation, i.e.: lymphocytes infiltration profiles, are also determinants of prostate cancer initiation. Several recent studies show that a large neutrophil-lymphocyte ratio indicates a poor prognosis and shorter survival in metastatic castration-resistant prostate cancer patients [43, 44]. The T cell-dominant immune reaction in acute inflammation may explain why acute inflammation may have prostate cancer preventive effects. However, the mechanism underlying is largely unknown.

Inflammation creates a microenvironment which facilitates cancer progression by breaking the hormone balance and increasing cytokines and inflammatory factors release. Testosterone treatment reduces cytokine release and immune cell infiltration in high-fat diet-induced prostatitis [18]. This suggests that loss of testosterone level may facilitate inflammation, and thus promotes the androgen independent cancer imitating cell growth. On the other hand, estrogen induces pro-inflammatory cytokine release in rat prostate inflammation model [45]. In combination, this suggests that the increased estrogen signaling and decreased androgen signaling in aged male promotes inflammation and the tumorigenic environment. Prostate inflammation increases cell survival factors, such as IGFs, hedgehogs, IL-6, fibroblast growth factors (FGFs), and transforming growth factors (TGFs), in mouse prostate [46]. Cytokines released by macrophage can

promote loss of AR signaling and accumulation of reactive oxygen species (ROS), and thus promotes cancer initiation [47]. Expression of inflammation-related factors, such as TGF-β, IL-6, gp-130, etc. is correlated with the progression of prostate cancer in human [48]. IL-6 and IL-1 drive the PSA-prostate specific membrane antigen (PSMA) positive cell clone expansion in prostate cancer [49]. Up-regulated IL-17 (A, E and F), as well as increased infiltration of inflammatory cells, are observed in BPH and prostate cancer specimens compared to those in the non-diseased specimens [50]. Sex steroid hormones cause inflammation by affecting the expression of inflammatory factors through DNA methylation regulation in prostate cancer cells [51]. Targeting shared risk factors of prostate inflammation and prostate cancer may improve the inflammatory microenvironment and thus the benefit prostate cancer patients. High-fat diet is known as a risk factor for both prostate cancer and prostate inflammation. Reducing fat in the diet can decrease expression of inflammatory cytokines and angiogenetic factors in prostate cancer patients thus attenuate the proliferative environment [52].

Cytokines and inflammatory factors modulate cancer cell proliferation, migration, and apoptosis. Activation of the IL-6 receptor by prostaglandin E2 (PGE-2) increases cell proliferation in PIN lesion [53]. IL-6 also increases

prostate cancer cell proliferation [54]. Age-related increase of IL-17 released by T cells can activate inflammatory signaling in cultured prostate cancer cell lines and promote their proliferations [55]. Inflammatory cytokine profile in the immune cell conditional medium can affect PC-3 cell proliferation [56]. Overexpression of PGE2 in prostate cancer cell lines promotes their proliferations and tumor formations in nude mice [57]. Inflammatory factors regulate cancer cell invasiveness through regulating cell migration. TNF-α increases prostate cancer cell migration through inhibiting β-catenin/E-cadherin association [58]. Activation of ERα signaling in cancer-associated fibroblast reduces CCL5 and IL-6 expression and thus partly reduces prostate cancer cell invasion *in vitro* [59]. Infiltration of monocyte into cancer region is correlated with poor prognosis in CRPC patients [60]. Co-culture of monocyte with PC3 cells increases the cell invasion [61]. MCP-1 recruits monocyte to prostate epithelium during inflammation. Its expression level is associated with prostate cancer pathological stages. Overexpression of MCP-1 in PC-3 cells increases cell proliferation and invasion [62]. Monocyte increases prostate cancer cell line invasion through CCL-2 dependent NF-κB signaling activation [63]. Cancer cell survival is another target of inflammatory factors. Matrix metalloproteinases-26 (MMP-26) exhibits anti-inflammatory effects in prostate cancers. Expression of MMP-26 is correlated with cell apoptosis in high-grade prostatic intraepithelial neoplasia (HGPIN) and

prostate cancer specimens in human [64]. COX-2 overexpression in prostate cancers induces Bcl-2 and VEGF expression, and thus reduces cancer cell apoptosis in prostate cancer specimens [65]. Pten-loss induced IL-8 overexpression can further activate the HIF-1a and NF-κB signaling to maintain cell surviving in prostate cancer [66]. On the other hand, inflammation may also increase cancer cell apoptosis by modulating cell responses to apoptotic-ligands. Inflammatory cytokines such as TNF-α/IFN-γ can modulate the cells response to TNF-related weak inducer of apoptosis (Tweak) and thus induce cell apoptosis in PC3 cells [67]. The apoptotic aspect of inflammation in prostate cancer may sustain a noxious but tumorigenic microenvironment which increases cell transformation and cancer progression.

Moreover, cytokines released during inflammation can modulate cancer progression in the prostate. Circulating cytokines including MIC1, IL-1RA, IL-1β, IL-4, IL-6, IL-12, and IFN-γ are correlated with docetaxel resistance in PC-3 cells and with cancer progression in CRPC patients [68]. In addition, hormone unbalance can aggravate the prostate cancer by aberrantly inducing expression of various cytokines [51, 54]. IL-17 induces epithelial-mesenchymal transition (EMT) to facilitate prostate cancer progression through the activation of MMP-7 [69]. Modification of some key regulators in the prostate can promote both

inflammation and cancer progression. This further supports that inflammation may accelerate cancer initiation or progression in the prostate. Recently, it is shown that overexpression of the prostate-specific G-protein coupled receptor in mouse increases inflammation and low-grade PIN in Pten (+/+) mice and accelerates cancer initiation of Pten null mice [70]. Loss of tissue inhibitor of metalloproteinase 3 (TIMP3) in prostate cancer increase tumor growth, cell invasion, and angiogenesis, as well as inflammatory cell infiltration [70]

Inflammation induces hypoxia in prostate tissue. Increase level of HIF-1α is observed in LPS or urine reflux induced prostate inflammation models. This suggests a hypoxic environment is induced by inflammation [71, 72]. On the other hand, hypoxia can activate the inflammasomes in cultured prostate epithelial cell lines, suggesting that hypoxia can further increase inflammation intensity in prostate cells[73]. ERβ has anti-proliferative effects and can protect tissue from inflammation induced tissue damage. Loss of ERβ in prostate cancer cells increases the HIF-1α level [27], this may provide a correlation between hypoxia and inflammation induced hormone imbalance. Hypoxia facilitates the initiation and progression of prostate cancer. HIF-1α mediated the EMT in prostate cancer stem cells [74], which may promote the malignancy of prostate cancer. Hypoxia can increase AR expression and activate its transcriptional

activity [75], which serves as a possible mechanism of CRPC. Hypoxia also affects cell invasion, cell proliferation, and cell apoptosis in prostate cancer cells [76-78], all of which may facilitate the progression of prostate cancer. The interactions between immune cells and prostate cancer cells lead to immune surveillance escaping of cancer cells. The cancer initiation and progression processes require an immune suppression to help the cancer cells evading immune surveillance. Myeloid-derived suppressor cells (MDSCs) are a major immune suppressive cell population derived from the myeloid lineage. They are highly active in infectious diseases and cancers. MDSCs inhibit CD8+ T-cell proliferation and IFN-γ production during acute prostate inflammation [79]. Prostate tumors express low levels of chemokines (CCL5, CXCL9, CXCL10) which recruit cytotoxic immunes cells but are abundant of MDSCs attractive chemokines such as CCL2, CCL22, and CXCL12 [80]. Circulating MDSC and regulatory T cells are enriched in prostate cancer patients compared to those in healthy people [81]. Chronic inflammation in prostate expands the Gr-1+/CD11b+ MDSCs and thus leads to immune suppression and facilitates prostate cancer initiation [82] .MDSCs are enriched in prostate cancer tissues in Pten null mice [83]. Activation of the mTOR pathway in these cells can increase prostate cancer cell proliferation and metastasis [84]. Elimination of MDSCs inhibits prostate cancer progression and increases survival in mouse models [83]. In addition,

MDSCs can also serve as osteoclast progenitors which may promote prostate cancer bone metastasis [85]. Prostate cancer cells can alter the immune responses to increase their survivals. Loss of HLA class I in prostate reduces cytotoxic T cell recognition [86]. Expression of Toll-like Receptor (TLR) 7 is reduced in prostate cancer cells, while its activation can reduce cell proliferation, suggesting an altered immune response in prostate cancer cells [87]. Moreover, the clinical prostate cancer treatments such as androgen ablation also affect immune cells in the prostate. T cell infiltrations in both benign glands and tumors are found in the prostate specimens after androgen ablation [88].

In summary, inflammation promotes prostate cancer initiation and progression through creating a tumorigenic microenvironment. The inflammatory factors released during inflammation may affect cancer cells proliferation, survival and their interaction with immune cells. The inflammation induced tumorigenic environment is harmful to non-cancer cells, but cancer cells take advantages in this environment through evading immune surveillance and utilizing this immune active but proliferative milieu.

C. Prostate epithelial stem cells

a. Stem cells

Stem cells are undifferentiated cells which can self-renewal or differentiate into other types of cells. Stem cells determine the fates of themselves through different division patterns: symmetric and asymmetric division. A stem cell can give rise to identical daughter cells through symmetric division. The 2 daughter cells can be stem cells which are identical to the parental cell, or can be differentiated into progenitor or more differentiated cells. A stem cell can maintain its population through asymmetric division which is critical for its self-renewal trait. In asymmetric division, stem cells can generate two daughter cells, one is identical to the parental cells, while the other is a differentiated cells. The stem daughter cells will maintain the population of parental stem cells, while the differentiated cell will enter further cell cycle or differentiation to become terminallyly differentiated cells (functional cells). Stem cells can give rise to progenitor cells, which are more specialized cells compared to stem cells and with a limited potency of proliferation and differentiation. Progenitor cells can only differentiate into specific types of tissue cells. Progenitor cells can compensate tissue loss through differentiating into functional terminally differentiated cells in the tissue they reside. Since progenitor cells do not express self-renewal capacity, they need higher level stem cells to replenish their population. There

are generally 2 categories of stem cells, the embryonic stem (ES) cells and the adult stem cells. ES cells are highly pluripotent cells. They reside in the inner layer of the blastocyst and possess potentials to differentiate into all kinds of cells during embryonic development due to their undetermined differentiation fates. The ES cell has unlimited proliferation ability and can be directed to differentiate into any cells of the body. These make ES cell a potential resource of the human organs for therapeutic purpose. ES cells from mouse and human have been successfully obtained and cultured. The mouse ES cells have been used for genetic manipulation to produce transgenic mice, while the use of human ES cells is still limited by both technique and ethical reasons. The adult stem cell is the other major category of stem cells. They reside in the adult tissues and their major function is maintaining tissue homeostasis. In most cases, their differentiation fates are limited to the tissue which they reside. Adult stem cells can only differentiate into certain types of intermediate or terminally differentiated cells depend on the tissue they reside in. They are also capable for self-renewal through asymmetric division. Adult stem cells are very rare populations in their organs, for example, their population is only 0.01% in bone marrow [89] and is about 0.2% in prostate epithelium. The characteristics of adult stem cells, such as their distributions, differentiation patterns, self-renewal, and responses to stimuli, vary from organ to organ. These highly organ-specific traits enable adult

stem cells to adapt their environments and to exert their functions under most situations. Experimentally, adult stem cells are characterized by their self-renewal ability, clonogenic ability and differentiation ability.

There are several sub-categories of adult stem cells based on their organ localization and functions, including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), neural stem cells (NSCs), and epithelial stem cells. HSCs are derived from bone marrow and are the origin of red blood cells, white cells, and platelets. Most blood cells have relatively short lifespans and need to be replenished every day to maintain the transportation, repair and immune functions. This makes HSCs a highly dynamic population of stem cells. Around 8-10% long-term HSCs enters cell cycle every day in a mouse to give rise to other blood cells [89]. This suggests HSCs may get replenish from a higher level of precursor stem cells or from self-renewal. MSCs are bone marrow-derived stromal stem cells. They distribute in lots of tissues. MSCs can give rise to a variety of different differentiated cells, such as osteoblast, cartilage cells, adipocytes and stromal cells. MSCs play a key function in maintaining the homeostasis of connective tissues. Nerve cells were thought to be unable to divide and cannot be repaired until the discovery of neural stem cells. Different neuron stem cells have been identified [90, 91]. The neural stem cells can give

rise to the 3 major lineages of nerve cells: neuron, astrocytes, and oligodendrocytes. Neural stem cells are high active during embryonic development, while their functions in adult tissue are still not fully understood. Epithelial tissues have protective, absorptive and secretive functions. Their functions depend on the integrity of epithelium. Some of the epithelial tissues, such as skin tissues, require a fast turn-over rate to maintain their functions. Epithelial stem cells are the custodians of adult tissue homeostasis. They play crucial roles in tissue repair and regeneration. Epithelial stem cells have been identified in multiple organs and their pivotal involvements in maintaining epithelium homeostasis have been shown in organs such as lung, intestine, kidney, prostate, testis, etc. for the fulfillment of their functions [92]. Local epithelial stem cells are capable of giving rise to other epithelial cells by undergoing terminally differentiation [92-94]. This differentiation is crucial for maintaining the structure of intact epithelium. Thus, a local stem cell pool may play important roles in maintaining the functional organization of the epithelium and in responding to noxious conditions.

b. Inflammation and epithelial stem cells

Inflammation is a well-known protective mechanism against exogenous pathogens or endogenous noxious substance. It is identified in epithelium by

infiltration of immune cells. On the other hand, inflammation is also a major event which causes tissue damage to tissues including epithelium. Cell-cell interactions between epithelial cells and local immune cells (i.e. macrophages) recruit circulating leukocytes and lead to the secretion of a variety of inflammatory factors in tissue. The changes in this inflammatory factors profile modulate the behavior of epithelial stem cells and drive tissue repair and regeneration. Emerging studies have been focused on the interactions between inflammation and stem cells. The associations between inflammation and stem cell ablation or overproduction suggest the mechanisms of inflammation-induced epithelial damage.

The definition of prostate epithelial stem cells is still controversial. Several different panels of stem markers have been applied to isolate prostate epithelial stem cells. These stem cells have been shown to be capable of sphere formation and tissue reconstruction, but their abilities of differentiate to functional epithelial cells or tissues need to be further verified. Prostate epithelial stem cells cannot be defined by one or two stem cell markers, or be defined by a specific location in tissues. Thus, it is difficult to describe or compare different populations of prostate stem cells from different models or cell harvest methods. This disadvantage makes it impossible to directly and precisely tracing or knocking

out a specific population of epithelial stem cells in the prostate. Thus, direct evidence of prostate epithelial stem cells' functions *in vivo* is missing. The consequences of aberrant differentiation or ablation of epithelial stem cells in the prostate are still unclear. This limitation may be overcome by using complicated strategies involve multi-strains of transgenic mice. However, it requires further investigation. On the other hand, the complexity of stem cell populations in the prostate is also a major obstacle in prostate research. Both basal and luminal compartments of the prostate have their own stem cell population to maintain their structure independently. Epithelial differentiation across compartments can happen under pathological conditions. This suggests a trans-differentiation between different lineages of epithelial stem cells happens, or there is intermediate type of stem cells which has traits of both compartments and can differentiate to both basal and luminal cells in prostate epithelium. The tans-differentiation leads to more overlap between different stem cell populations and more difficulties to study a stem cell population independently.

Although the function of prostate epithelial stem cell in tissue regeneration remains unclear, the functions of epithelial stem cells in other tissues, such as cornea, lung epithelium, and intestine epithelium, in both physiological and pathological conditions, especially in the inflammatory environment, have been

well established. The studies in these tissues may shed light on the prostate field by introducing the mechanism of stem cells' roles in maintaining epithelial homeostasis and cell hierarchy during inflammation. .

Stem cells are necessary for maintaining the homeostasis in many epithelial tissues. Deficiency of stem cells may lead to tissue damage and inflammation in epithelial tissues. Limbal epithelium stem cells (LESCs) LESCs reside in the basal layer of the limbus of the cornea [95, 96]. Removing corneal epithelial stem cell via keratectomy or chemical injury introduces chronic eye inflammation[97]. [98]. In humans, 21% of specimens from chemically-induced LESCD patients were shown to have severe inflammation [99]. The loss of airway stem cells causes severe disease and potentiates inflammation in lung. Ganciclovir (GCV)-mediated ablation of Clara cell secretory protein positive cells in the mouse lung injury model reduced both Clara cells and AEC2s in different compartments of the airway [100]. The reductions of these cells shift leukocyte profile from macrophage to neutrophil/lymphocytes and increase protein leakage from the vasculature. This indicates a potentiated inflammation in lung after chemical injury. Genetic ablation of Clara cells in mouse induces peribronchiolar fibrosis, a disease associated with chronic inflammation in the lung [101]. Small intestine epithelial stem cells locate in the +4 position of intestinal crypt and can give rise

to other terminally differentiated secretory cells such as Paneth cells and neuroendocrine cells [102]. Knocking out of adenosine deaminase acting on RNA 1 in intestine leads to loss of Lgr5+ intestine stem cells and inflammation [103].

Inflammation induces stem cell proliferation and differentiation in epithelial tissues. In human colonic mucosal samples, crypt epithelial stem cells express TLR-2, TLR-4, and TLR-5, suggesting that epithelial stem cells can directly respond to bacterial infection [104]. Trichinella spiralis infection in the small intestine increases the Paneth cell number and crypt proliferation, suggesting an activation of stem cells and a shift of differentiation to secretory cells [105]. Crypt stem cell expansion is also observed in radiation-induced colitis or spontaneous ileitis in mice [106, 107]. Tissue damage can also activate epithelial stem cells in a tissue repair purpose. Intestinal quiescent cells can recapitulate stem cell states after intestine injury [102]. Ectopic activation of STAT5 in intestine epithelial caused crypt expansion, alleviating radiation-induced mucositis [108].

A recent study focused on CK5+/P63+ distal airway stem cells (DASCs) showed that DASCs population is induced to expand in number following influenza-induced inflammation [109], while ablation of DASCs leads to the formation of pre-fibrotic lesions.

In summary, the studies in corneal, intestine and lung epithelial stem cells indicate that epithelial stem cells maintain the homeostasis of epithelial tissues. Loss of epithelial stem cells disrupts the structure and integrity of epithelium, and finally leads to inflammation (Fig 1.2 A). The epithelial stem cell population is regulated by inflammation in these tissues. An expansion of epithelial stem cells may suggest the activation of tissue repair-regeneration process during inflammation (Fig 1.2 B). Thus, we expected that the stem cell in the prostate epithelial compartment may possess similar functions and responses to inflammation as stem cells in other tissues do.

c. Prostate epithelial stem cells

In the prostate, the expression pattern of cytokeratin markers in different prostate cells suggests the existence of a differentiation pathway for maintaining the homeostasis of the prostate epithelium [110]. A Hoechst 33342 expulsive side population (SP) of prostate epithelial cells can be isolated from BPH or prostate cancer patients. SP cells can form spheroid and ductal structures in a 3D culture system. They express stem cell markers such as CD133 [111, 112]. In mice, putative prostate epithelial stem cells characterized by slow-cycling and high proliferation potential reside in the proximal region and can recapitulate glandular structures [113]. Within this region, c-kit positive epithelial stem cells are

identified. These progenitors form prostatic structures in renal transplantation [114]. Additionally, prostate cells expressing markers that correspond to undifferentiated stem cells in other tissues have been described [115]. Lineage restrictive stem cells in prostate reside in the basal cell layer. Moreover, the luminal layer also serves as a potential stem cell reservoir. Resident luminal cells have been identified as progenitors to produce terminally differentiated (luminal) cells.

Homeostasis of prostate epithelium also depends on stem cells. Knocking out Dicer in mouse prostate results in decreased stemness of Sca-1+/CD49f+/Lin- (linage markers: a cock tail of markers to label all hematopoietic cells) prostate epithelial stem cells and increased prostate atrophy [116]. An increase in the proportion of transit amplifying cells (TACs) is identified in hormone-induced BPH in dogs [117], indicating that TACs or stem cells which produce TACs are proliferating during inflammation. However, terminally differentiation of these stem cells is shown to be blocked by unknown pathways during prostate diseases such as BPH. This leads to the accumulation of TACs and cancer-initiating cells [118]. Sca-1+/CD49f+/Lin- prostate epithelial stem cells expanded after inflammation in an autoimmune mouse prostate inflammation model [119]. Taken together, these data suggest that inflammation induces an aberrant proliferative response in epithelial stem cells and block their normal

differentiation. This leads to an accumulation of intermediate type cells which may contribute to epithelial hyperplasia.

d. Intersection of inflammation and epithelial stem cell

Inflammation induces the release of pro-inflammatory cytokines such as IL-1α, IL-1β, IL-6 and TNF-α, and anti-inflammatory cytokines, such as IL-10 and TGF-β in tissues. This factors can affect epithelial stem cell proliferation and differentiation to regulate the tissue regeneration induced by inflammation..

In vitro treatment of intestinal stem cells with IL-1β results in increased expression of stem cell markers and promotes their sphere forming abilities [120]. Activation of IL-6/stat3 signaling pathway promotes airway basal stem cell to ciliated cell differentiation, while IL-6 knocking out in mice results in fewer ciliated cells and the regeneration of more secretory cells, suggesting that IL-6 signaling directs the differentiation of basal stem cells [121]. A transcriptional profiling comparison between LESCs and terminally differentiated corneal epithelial cells showed that inflammatory cytokines including IL-1α, IL-1β, IL-6 and IL-8 are the top hits of differentially expressed regulators of stemness, differentiation and angiogenesis pathways [122]. IFN-γ deprives hair follicle stem

cell niches by inducing bulge immune privilege collapse and this causes Lichen planopilaris [123].

Activation of development pathways is a feature of inflammation in many tissues. The epithelial stem cells can be regulated by this signaling pathways during inflammation and thus participate in tissue repair and regeneration. Wnt/β-catenin pathway is a developmental pathway which is activated during inflammation. Wnt/β-catenin pathway plays important role in regulating epithelial stem cells. β-catenin is necessary for hair follicle stem cell proliferation. Epithelial deletion of β-catenin in hair follicles results in an inflammatory response in skin [124]. In the small intestine, the role of Wnt/β-catenin pathway is more complicated. PI-3 kinase induced β-catenin activation increases intestinal stem cell proliferation in a mouse colitis model [125]. The overexpression of Kaiso, a transcriptional factor that can negatively regulate β-catenin mRNA level, induces both crypt stem cells expansion and inflammation in the small intestine [126]. Suz12 is a key regulator of stem cell differentiation in intestinal stem cells. Suz12 KO in intestinal stem cells increases IL-1β induced activation of NF-κB and C/EBPβ pathways [127]. IGF-1 is another developmental regulator which is crucial for prostate development. IGF-1 plays important roles in regulating tissue repair and regeneration through affecting stem cell populations. IGF-1 affects

corneal epithelium wound healing process through promoting LESCs proliferation and migration [128]. IGF-1 promotes tissue regeneration after irradiation through activating intestine stem cell proliferation [129]. IGF-1 also plays crucial roles in renal epithelium regeneration through regulating stem cell behaviors [130].

e. Therapies on inflammation and epithelial stem cells

Chronic inflammation leads to long-lasting damage to epithelium, which can eventually override the regeneration ability of stem cells or distort their differentiation directions. Inflammation creates a proliferative microenvironment through releasing inflammatory factors and cytokines. These factors may directly or indirectly act on stem cells, which make them attractive therapeutic targets. Manipulation of these factors may alleviate the damage to stem cells caused by inflammation and contribute to the reconstitution of stem cell populations.

IL-1 is one of the earliest pro-inflammatory signals and can activate the release of other inflammatory cytokines such as IL-6 and IL-8. Targeting IL-1 receptor using an antagonist peptide reduces the production of IL-6 and IL-8 in LESCs and also reduces inflammation caused by LESCs transplantation [131]. Conditioned medium contains IL-1 receptor antagonist (IL-1RA) from mesenchymal stem cells restores cell permeability of alveolar epithelial cell after

hypoxia injury [132]. The addition of IL-1RA in *ex vivo* LESCs cultures reduces LESCs apoptosis [133]. Anti-inflammatory cytokines IL-10 and TGF-β protect the colon from LPS-induced crypt cell loss and epithelial damage [134]. Chronic intake of anti-inflammatory drug aspirin also increases crypt cell expansion in non-inflamed rat duodenum [135]. Taken together, this suggests that stem cell expansion is not only a consequence of inflammation but also a protective mechanism against inflammation.

Epithelial stem cell transplantation is another strategy of reconstructing local stem cell populations. LESCs transplantation is a well-developed therapy to treat LESCD. LESCs transplantation in humans is first described by Pellegrini and his colleagues in the autologous transplant of LESCs cultured from the healthy eye to the damaged eye of a patient [136]. The corneal epithelium is re-constructed after the transplantation. Reduced corneal vascularization and inflammation are observed in both animal experiments and clinical cases [137, 138]. Interestingly, transplantation of oral mucosal epithelial cells also improves LESCD symptoms and reduces inflammation intensity [139], suggesting that anti-inflammatory effects of a stem cell population are not necessarily organ specific, but may depend on the presence of the intact epithelial structure. Trans-differentiation between lineages also contributes to the reconstruction of stem cell populations.

This further supports stem cell transplantation as a plausible way to restore epithelium structure and to alleviate inflammation.

Although anti-inflammatory effects of other stem cells such as mesenchymal stem cell have been well illustrated, epithelial stem cell therapy for treating inflammation is yet to be demonstrated in organs such as lung and intestine. Successes in animal experiments have shed light on manipulation of inflammation using epithelial stem cells. Human amniotic epithelial cells transplantation reduces inflammatory IL-6 release in bleomycin-induced lung injury model in mouse [140]. Transplantation of intra-colonic stem cells ameliorates colon inflammation in IL-10 knockout-induced colitis mouse model [141].

Epithelial stem cells are essential for maintaining epithelium homeostasis and are crucial for the repair/regeneration of damaged tissues. Maintenance of the epithelial stem cell population is indispensable for fulfillment of normal tissue functions. Inflammation is a key component of various human diseases and inflammatory events such as cytokine release and immune cell recruitment can result in damage to the epithelium. Inflammation-induced tissue damage can either activate stem cell proliferation and differentiation to compensate for the

epithelial cell loss or directly destroy stem cell populations which may lead to epithelium breakdown and the worsening of inflammation. Stem cell proliferation and differentiation can be distorted under inflammatory conditions. These results in failure to regenerate functional epithelial cells and the accumulation of abnormal epithelial cells culminate in hyperplasia. By the same token, genetic or exogenous loss of stem cells results in abnormal growth of epithelium and destruction of epithelium structures, followed by inflammatory symptoms (i.e. ulceration). Restriction of inflammation or reconstruction of epithelium presents a plausible and attractive meaning of recovering both epithelium structures and tissue functions. Stem cell therapy and anti-inflammatory therapy represents potential ways to clinically manipulate inflammation-induced epithelium dysfunction.

f. Roles of stromal cells and neuroendocrine cells in prostate inflammation

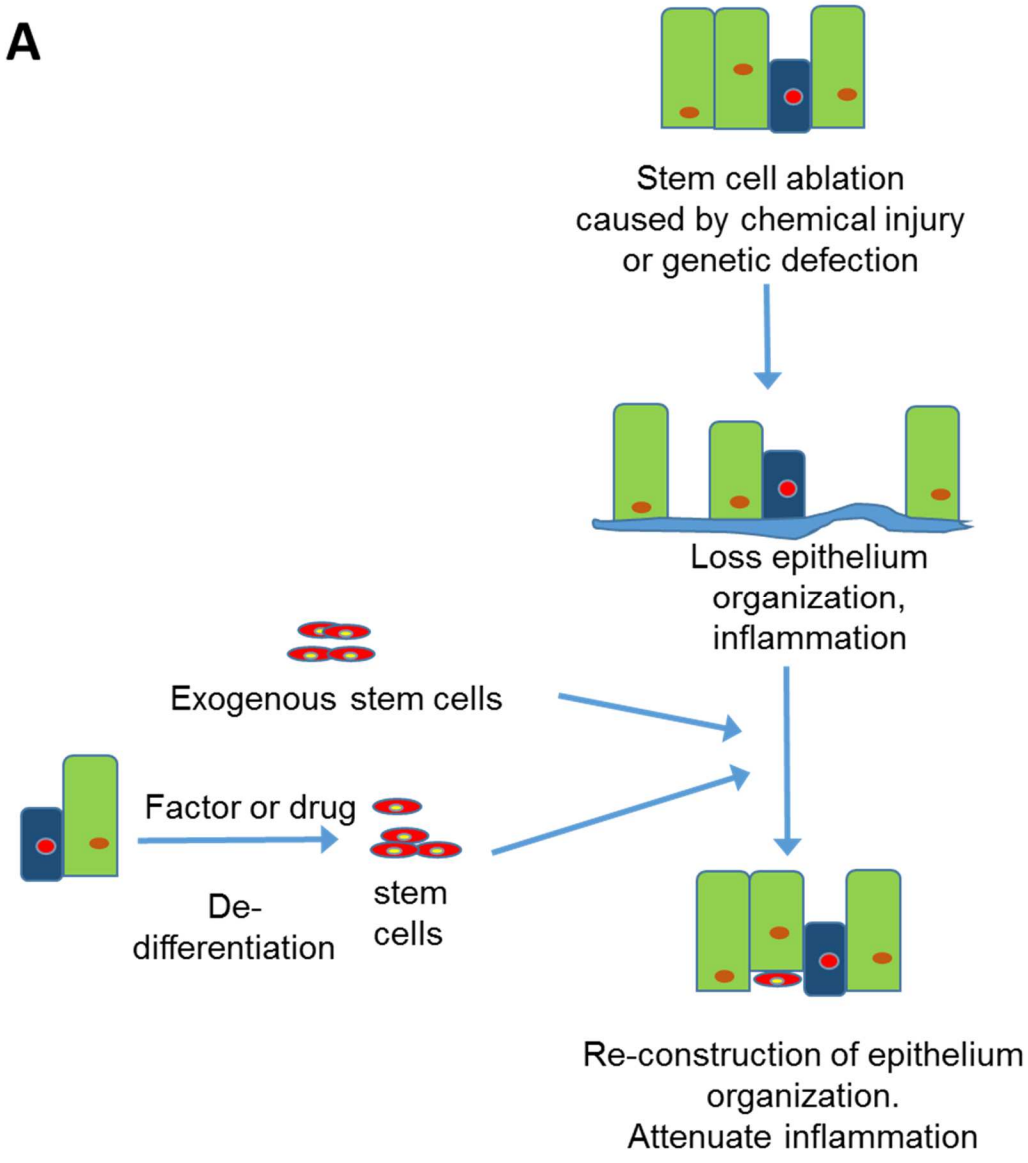
Prostate stromal cells and neuroendocrine cells also participate in the pathological process of prostate inflammation. The overgrowth of prostate stromal cells is a key feature of BPH. The epithelium to the stromal ratio of prostate decreases in BPH specimens compared to non-diseased prostates. This suggests a stronger proliferation in the stromal compartment compared to it is in the epithelial compartment. In bacteria induced acute inflammation in the

prostate, the stromal cells experience smooth muscle hypertrophy and then a phenotype transition to secretory phenotype [142]. Inflammatory factors released by epithelial cells can promote stromal cell proliferation through interaction with mast cell in pathogen-induced prostate inflammation [143]. Epithelial cells released IL-1 increases the production of CXCL-1, CXCL-2, CXCL-3, and IL-8 in prostate [144]. Stromal cells can also produce inflammatory factors such as CXCL8 and CCL2 to recruit immune cells which may facilitate the progression of prostate inflammation [145]. Loss of smooth muscle type stromal cells may increase the invasiveness of prostate cancer cells under chronic inflammation condition [146]. Smooth muscle cell-specific knocking out of AR in prostate stromal cells increases inflammation and epithelial hyperplasia in the prostate, suggesting a key function of stromal cells in maintaining prostate hormone balance [147]. Neuroendocrine cells produce factors which regulate the growth of the prostate. Neuroendocrine cell marker chromogranin A and neuron-specific enolase expression is increased in chronic inflamed rat prostate, suggesting an increase in neuroendocrine cells differentiation induced by inflammation [148]. In prostate cancer cells, inflammatory factors can increase neuroendocrine differentiation [149]. Inflammation increases the neuroendocrine phenotype which indicates a stronger malignancy and worse prognosis in prostate cancer patients [150]. Thus, inflammation has strong effects on stromal cells and

neuroendocrine cells. Their interactions with epithelial cells play important role in the pathological process of prostate inflammation.

D. Summary

Prostate inflammation is a common health issue and is tightly correlated with BPH and prostate cancer. It promotes the initiation and progression of BPH and prostate cancer through a variety of signaling pathways such as cytokines, growth factors, and hormone related pathways. The integrity of prostate epithelium is maintained by prostate epithelial stem cells. Inflammation affects epithelial stem cell population in many tissues including prostate. Loss of stem cells in epithelial tissues leads to epithelial dysfunction and may induce inflammation. Inflammation manipulates stem cell populations to activate tissue repair and regeneration. Thus, restoration of epithelial stem cell population may contribute to the reconstruction of epithelium structures and the recovery of epithelium functions.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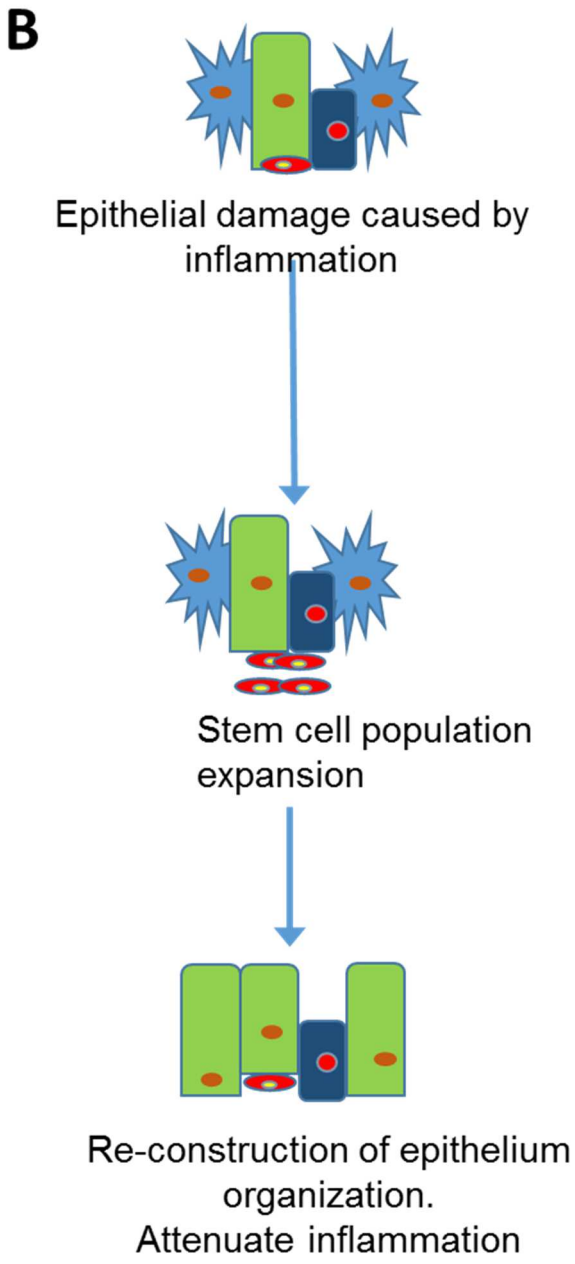


Fig 1.2 Stem cell-dependent tissue repair during inflammation

A: exogenous stem cel- dependent tissue repair. B: local stem cell expansion induced tissue repair

Chapter 2 Material and Methods

A. Methods

a. Mouse prostate inflammation model

1. Bacteria preparation

E.coli strain 1677 was cultured in 25ml LB medium at 37 ℃ overnight. The medium contains bacteria was collected and centrifuged at 1500g for 10min. The bacteria pellet was collected and washed with sterile PBS twice and was re-suspended in 10ml sterile PBS. The OD600 was measured to determine the concentration of *E.coli* and the *E.coli* suspension is diluted to 1 X 10^6/ml (OD600=0.118) in sterile PBS.

2. Bacteria instillation

C57BL/6 or IL-1-R1(-/-) mice (Charles River Ltd.) at 8-12 weeks were deprived from water for 2h, then were anesthetized using isoflurane. *E.coli* strain 1677 solution (1 X 10^6/ml in sterile PBS) is instilled through sterile catheter into the prostate as previous described (100μl/mice) at day 0 [151], PBS is used as vehicle. Mice were sacrificed after instillation based on the experiment design. 5-bromo-2'-deoxyuridine (BrdU) is intraperitoneal (I.P.) injected into animal (10mM, 200μl/mouse) 2 hours before sacrifice to label all proliferating cells. Prostates were collected within 30min after animal sacrifice for following experiments.

b. Tissue dissection

Mouse prostate was collected within 30min after the animal is sacrificed. The fat tissue around prostate was removed and the prostate was dissected to separate different lobes using stereomicroscope in cold sterile PBS with antibiotics. All lobes (including AP, DLP, and VP) were collected separately for following experiments.

c. Tissue fixation and embedding

The tissue for immunofluorescence analysis was fixed in 10% formalin in PBS for 72hs, and then the tissue was washed with distilled water twice to remove excessive formalin. Then the tissue was dehydrated by treating with 50% ethanol, 70% ethanol, 2 X 95% ethanol, and 2 X 100% ethanol, 30min/step. The tissue was then treated with 100% xylene twice, 30min/step, followed by two 50% xylene+50% paraffin treatments, 30min/step. The 50% xylene+50% paraffin treatment was replaced with 100% paraffin and the tissue was incubated at 59℃ in oven for 30min. Then the paraffin was refreshed and the tissue was incubating at 59℃ overnight. The tissue was embedded in paraffin in the following day and was microtomed into 5μm slice for immunofluorescence (IF) or hematoxylin and eosin stain (H&E) staining.

d. Immunofluorescence staining

Paraffin embedded slides were heated in oven at 59℃ for 2h to remove paraffin. The slides were washed with xylene 3 times, 5min each, followed by 3 times methanol wash, 5min each. The slides were then washed with distilled water for 5min. For antigen retrieval, the slides were treated with boiled citric buffer for 20min then were cooled down in distilled water for 5 min. The tissue was circled with hydrophobic pen and washed with PBST for 15min. The slides were blocked with blocking buffer for 2 hours at RT. The tissue was treated with primary antibodies diluted in blocking buffer at 4℃ overnight, then was washed with PBST for 15min to remove excessive primary antibody. The tissue was treated with fluorophore labelled secondary antibody at RT for 1hr. For nuclear staining, the tissue was treated with Hoechst 33342 in PBST at RT for 10min, followed by 2 PBS washes and 3 distilled water washes, 5min each. Mounting medium was used to mount the slides and the slides were kept in 4℃ for short term and in -20℃ in for long term stock.

e. Primary cells preparation

The prostate tissue from the tissue dissection step was collected and washed with PBS contains antibiotics 3 times, 5 min each, in centrifuge tube. The tissue was kept in collagenase working solution (500µl/prostate in 1.5ml centrifuge

tube). The tissue was chopped into small pieces (1 mm^3 cubes) in collagenase working solution and was incubated in 37℃ water bath for 30min. The supernatant was collected into a centrifuge tube with 10ml growth medium on ice. The tissue was digested in 500μl fresh collagenase working solution for a total of 3 times. All the supernatant was collected and the remaining chunks were washed with PBS for 3 times and were digested in 500μl 0.25% trypsin in 37℃ water bath for 30min. The supernatant was collected into the centrifuge tube with growth medium on ice. The tissue was then digested in 500μl 1% trypsin in 37C water bath for 30min. All the supernatant collected from each digestion was combined in the centrifuge tube with growth medium on ice. The supernatant was vortexed and was filtered using 40μm cell mesh. The supernatant was then centrifuged at 500g for 10min. The cell pellet was collected for following experiments.

f. Flow cytometry

The primary cell pellet was re-suspended in pre-chilled stain wash solution and was washed twice with the stain wash solution. For each wash, the cells were centrifuged at 500g for 10min. The cell pellet from each prostate was re-suspended in 200μl stain wash solution on ice. The cell numbers were counted using hemocytometer. Primary antibodies of surface antigens were added into to

the cell suspension. For every 1 million cells, 2μl of each antibody was added. The cells were incubated with antibodies on ice for 30min, and then were washed with stain wash solution. The cells were fixed with BD fixation & permeabilization buffer at 4℃ for 25min, followed by 2 washed with stain wash solution. The primary antibodies of intracellular antigens were added into the cell suspension. For every 1 million cells, 2μl of each antibody was added. The cells were incubated with antibodies on ice for 30min, and then were washed with stain wash solution and were re-suspended in stain wash solution to a concentration less than 1 X 10^6 cells/ml. The cells were kept on ice before flow cytometry analysis.

g. Prosta-sphere formation assay

Primary prostate cells collected from the “primary cell preparation” step were incubated in growth medium at 37℃ overnight to attach the stromal cells. The supernatant with all unattached epithelial cells was collected and was centrifuged at 500g for 10min. The cell pellet was washed twice with cold PBS, and was re-suspended in sphere formation medium. The cell numbers were counted using hemocytometer, and the cell concentration was adjusted to 10000 cells/ml. The cell suspension was added into ultra-low attachment systems, for 60mm dishes, 3ml cell suspension (30000 cells in total)/dish was added, for 96 wells plate,

100μl (1000 cells)/well was added. The cells were cultured at 37℃ for 14-21days. The sphere number and the sphere size were quantified as readout. When using E6 or E7 cells, started with the PBS wash and re-suspended the cell in sphere growth medium. When treating with IGF-1 or other reagents, added the reagents with the cell suspension and renewed the treatment every 7 days. For GFP and RFP labelled sphere formation, GFP and RFP labeled cells were added in a 1:1 ratio in all experiments.

To measure the size and number of spheres, 9 pictures were taken each well for the spheres grown in 96 wells plate. The sphere sized and single cell size was measured using Adobe Photoshop. For each sphere or single cell, two mutually perpendicular diameters were measured and the average diameter was used as the diameter of the sphere or the single cell, respectively. The diameter of spheres was represented as equal to the number of cells. To calculate this, the diameter of spheres was divided by the average diameter of 20 single cells, the result is used as the sphere diameter. To quantify the sphere number, only the sphere with a diameter larger than 3 cells was counted. For spheres cultured in dishes, the spheres were centrifuged under 500g for 10min. The pellet was re-suspended in 100μl sphere medium in 96 wells plate and then the sphere size and number were measured as described.

h. Sphere fixation and processing

The prosta-spheres were collected and were centrifuged at 300g for 5min. The supernatant was gently removed and the cell pellet was washed with 500μl cold PBS. The spheres were fixed with 95% ethanol at RT for 30min, and then were collected by centrifuging at 500g for 10min. 10μl pre-heated histogel was added into the sphere and was mixed well. The histogel pellet was kept at 4℃ to be solidified. The histogel pellet was processed according to the “tissue fixation and embedding steps”, started with the 50% ethanol treatment step.

i. Cell culture

Prostate epithelial cells lines E6 and E7 were cultured in complete growth medium at 37℃, 5% CO_2 condition. 0.25% trypsin was used to digest the cells and is neutralized with complete growth medium. Cells were passaged every 3 days.

j. Cell proliferation assay

Prostate epithelial cell lines E6 and E7 were seeded in a 96-wells plate at a concentration of 5000 cells/well. Cells were serum-starved for 24h using serum free medium (SFM), followed by vehicle (5% BSA in PBS), IGF-1 (100ng/ml, Life technology Ltd.) or LQZ-7F (Curtesy from Dr. Jian-ting Zhang) treatment (100μl

medium/well). The treatment medium was refreshed every day. The cells were fixed in methanol for 15min in RT and were dried in chemical hood for 1 hour.

k. Methylene blue assay

Methanol fixed cell were stained with methylene blue working solution for 2 hours, followed by 2 distilled water washes. The plate was dried in RT for 2 hours. The cells were then dissolved in 100µl 5% HCl in distilled water and OD630 was measured using plate reader (H2 Bio). 100µl 5% HCl in distilled water in blank wells is used as background. The OD630 value of each well subtracted the OD630 background value was used as result. The final results were normalized as the percentage of control groups.

l. Immune blotting

1. Cell treatment

E6 and E7 cells were seeded into 6 wells plate at a concentration of 3 X 10^5 cells/well and incubate at 37℃ overnight. The cells were then serum starved using SFM for 24 hours. The cells were treated with IGF-1 or other inhibitors depended on experiment designs. After treatment, the cells were washed with cold PBS and were lysis by adding 100µl/well lysis buffer. The plate was kept on ice for 10min and the cells were scratched with cell scraper. The cell lysate was

transported into Eppendorf tube and kept on ice for 30min. The cell lysate was then centrifuged at 1500g for 10min to remove the cell debris. The supernatant was collected as protein sample for BCA test.

2. BCA test

Serial dilutions of BSA standard solution (concentration: 0.0625mg/ml, 0.125mg/ml, 0.25mg/ml. 0.5mg/ml, and 1mg/ml) were prepared using BSA standard and lysis buffer for BCA test. In 96 wells plate, 35µl of standard solution or protein samples was added into each well, 2 replicates/sample. The BCA reagent A and B were mixed in a 1:50 ratio to make BCA reaction buffer. 70µl BCA reaction buffer was added into each well on samples in 96 wells plate. The plate was incubated at 37℃ for 30min, OD570 was read on plate reader. The protein concentration was calculated using standard curve.

3. Gel running

The protein samples were prepared using 2X loading buffer and lysis buffer. The protein concentration was adjusted to make the loading amount of protein is the same between samples. The samples were loaded on 4%-20% gradient gel. The gel was ran under 90V for 1.5h. Bio-rad dual color precision ladder was used as protein marker.

4. Transmembrane and blocking

The gel was washed with distilled water for 5 min. The gel and the thick blotting paper was soaked in transfer buffer for 10min. Activate The PVDF membrane was activated in methanol for 2 min and was balanced in transfer buffer for 20min. The gel was transmembraned at 10V, 400mA for 30min. The PVDF membrane was collected and blocked in blocking buffer for 2 hours at RT.

5. Antigen probing and developing

The primary antibody was diluted in blocking buffer or other buffers depends on antibodies instructions. The membrane was incubated with primary antibody at 4℃ overnight. The membrane was washed with PBST for 6 times, 10min each. The secondary antibodies were diluted in blocking buffer. The membrane was incubated with secondary antibody at RT for 2 hours. The membrane was then washed with PBST for 4 times, 10min each, followed by 2 PBS washes, 10min each. The membrane was developed using Thermo fisher Femto or Pico developing buffer and was exposed on X-ray film in dark room. The film was scanned on computer and was analyzed using Adobe photoshop software.

6. Stripping and re-probe

After developing, the membrane was washed with PBS for 2 times, 10min each. The membrane was stripped using stripping buffer for 15min, then washed with PBST for 5 time, 5min each. Then the membrane was blocked in blocking buffer for probing other antigens.

m. SiRNA transfection

E6 and E7 cells were seeded into 6 wells plate at a concentration of 2 X 10^5 cells/well. Fitc conjugated control siRNA or survivin siRNA were transfected using Hiperfectene reagent following the instruction manual at a final siRNA concentration of 100nm. Cells were cultured overnight and then were subjected to followed experiments. Transfection efficiency was confirmed by observing green fluorescent in control group using fluorescent microscopy and further confirmed by western blot.

n. Lentiviral shRNA transfection

1. Antibiotic concentration titration

E6 or E7 cells were cultured in complete growth medium at a confluency between 40% to 60%. Antibiotics (puromycin or hygromycin) were added in

culture medium in a concentration gradient manner. The best concentration for selection was determined by the antibiotic concentration which killed 100% non-transfected cells after 3 days treatment.

2. Lentiviral transfection

For cultured E6 and E7 cells, cells were seeded at a concentration of 2 X 10^5 cells/well in 6 wells-plate, 24 hours before transfection. Lentiviral particles at 1 X 10^6 TFU were mixed with complete growth medium containing 5μg/ml probrene in 1:100 ratio (10μl lentiviral particles/ml in medium) and were incubated at RT for 5min. 2ml of medium contains lentiviral particles was added on E6 or E7 cells in each well of the 6 wells plate. The plate was incubated at 37℃ overnight. The cells were washed with PBS for 5 times and the medium was replaced with fresh complete growth medium. 48 hours after transfection, the cells were passaged into T25 flasks. Antibiotics were added into medium at the concentration determined by the antibiotics titration step after the cell attached to T25 flasks. The medium in the flasks was refreshed every day. Non-transfected cells were seeded in T25 flasks as control. The transfected cells were collected 3 days after transfection. The inhibition efficiency was determined using fluorescent microcopy or western blot. The cells collected were used as a pool for following experiments.

For primary cell collected from prostate, the cells were re-suspended in complete growth medium at a concentration of 1 X 10^5 cells/ml. Lentiviral particles of 1 X 10^6 TFU were mixed with the cell suspension in a 1:100 ratio (10μl/ml). The primary cells were incubated with the lentiviral particles at 37℃ overnight in a 6 well-plate. The cell suspension is collected after lentiviral infection and is washed with PBS for 3 times. The cells are centrifuged at 500g for 10min and then re-suspend sphere growth medium for prosta-sphere growth experiment.

o. Renal transplantation of PEPCs

PEPCs from inflamed mice were sorted by flow cytometry into low-attachment 96-well plates containing transplant medium (50 μl/well, DMEM supplied with 20 ng/ml EGF, 10 ng/ml FGF, 1% HEPES, and 1% antibiotics) and was cultured for 3 days. 1, 10, 100 or 1000 PEPCs were mixed with 8,000 urogenital mesenchymal cells, respectively in transplant medium (50μl/well) with 4 μg/ml cold Matrigel. The mixture was solidified at 37℃ and culture with 100μl transplant medium. The formed Matrigel plugs were then implanted surgically under the renal capsules of nude mice (CD-1 background). One plug was inserted into each kidney. The mice were sacrificed on day 60 after

transplantation. Kidneys were harvested and fixed in 4% paraformaldehyde at 4°C for 72 h followed by tissue processing and dissection.

p. LQZ-treatment

LQZ-7F was dissolved in 10% DMSO in PBS at 25mg/ml. Mice were injected with LQZ-7F (100µl/mouse, I.P.) 1 day prior to bacterial instillation and then 2 days after instillation. The prostates were collected for follow analysis.

q. Dual-labelling retaining assay in inflammation model

Pregnant CD1 mice were injected with BrdU at embryonic day 16 (E16) through I.P injection. After birth, the male litters from the BrdU labelled pregnant CD-1 mice were collected and were injected with Edu when they were 8 weeks old. EdU was inject (I.P.) 1 dose per day for 1 week. The EdU injection was chased 1day before bacteria instillation. The animals were inflamed and the prostates were collected for immunofluorescence analysis base on experiment design.

r. Dual-labelling retaining assay in prostate re-growth model

Pregnant CD1 mice were injected with BrdU at embryonic day 16 (E16) through I.P injection. After birth, the male litters from the BrdU labelled pregnant

CD-1 mice were collected and were castrated by removing their testis at the age of 8 weeks. The mice were kept for 2 weeks to let the prostate atrophy. After 2 weeks, the mice were subcutaneously implanted with 2 vehicle or testosterone capsules, respectively. 6 hours after testosterone capsule implantation, the mice were injected with EdU. The EdU was loaded 1 dose/day until day 2 after testosterone treatment. The mice were sacrificed at day3 or day 14 after testosterone implantation. The prostates were collected for immunofluorescence analysis.

B. Materials

a. Cell culture

E6 and E7 cells were kept by our lab. Cell culture medium, antibiotics, HEPES buffer, trypsin, and additives were from Hyclone; flasks, culture dishes, and plates were from Corning or BD pharmaceutics; Ultra-low attachment culture plates and dishes were from Corning; Fetal Bovine serum was from Atlanta Biologics.

b. Immune blotting

4-20% gradient SDS-page and western apparatus were from Bio-rad. BCA buffer, developing buffer Femto and Pico, phophotase and protease inhibitor, and BSA were from Fisher Scientific.

c. Animal models

IL-1R1 KO mice and *E.coli* 1677 were from our lab. C57BL/6 mice and GFP transgenic mice were from Jackson Lab, nude mice and CD-1 mice were from Charles River, dTomato transgenic mice were a gift from Dr. Karren Pollock; Matrigel was form BD pharmaceutics; BrdU and EdU were from Fisher Scientifics.

d. Immunofluorescence

Normal Donkey Serum, Hoechst 33342, histogel, and mounting medium were from Fisher Scientific.

Anti-survivin antibody was from Cell signaling; anti-CK5 antibody was from BioLegend; anti-CK8, Anti-BrdU antibodies were from Novus biologicals; anti- b-actin antibody was from Sigma Aldrich; anti-CK14 antibody was from Abcam; all the conjugated antibodies and Isotype controls for flow cytometry were from BD

pharmaceutics and Bioss; all the fluorescent labelled secondary antibodies were from Life technology; all the HRP-conjugated secondary antibodies were from Fisher Scientific; EdU detection kit was from Fisher Scientific.

e. SiRNA and Lentiviral vectors

Survivin siRNA was from Cellsignaling; Allstar neg scramble siRNA was from Qiagen; control and shSuvivin lentiviral vector was from Santa Cruz biologics; Survivin overexpression lentiviral vector and control vector were from Cyagen & Vectorbuilder.

f. Facilities

Flow-cytometry sorter: BD FacsAria; analyzer: BD LSR4; Fluorescent Microscope: Leica DMI6000B.

g. Other materials

All the chemical reagents and lab consumptions were from Fisher Scientific.

C. Statistical analysis

All statistical works were done using GraphPad Prism 5. The data were analyzed using student t-test, One-way ANOVA, Two-way ANOVA or Chi-square

test. For cell-based assay, the n-number of each experiment represented the number of independent test. For each independent test, the result was calculated by the average of 3-6 replicates. For animal experiments, the n-number of each experiment represented the number of animals used in each group.

Chapter 3 Inflammation-induced PEPCs expansion in mouse prostate

A. Introduction

Prostate inflammation is a common health issue all around the world. It is correlated with 2 major types of prostate diseases: prostate cancer and BPH. Prostate inflammation is frequently found in biopsies from prostate cancer patients [5]. Emerging evidence implicate inflammation of the prostate with cancer initiation in this gland[152].Prostate inflammation is considered as one of the most important risk factors for prostate cancer [153, 154]. Prostate inflammation triggers cancer initiation by creating a cancer favorable environment. Inflammatory mediators induced by prostate inflammation including IL-6, VEGF, and IL-10 are correlated with prostate cancer [155, 156]. Bacterial infection, prostate calculus, urinary tract sphincter over contraction, abnormal immune activity, and mental disorders have been shown as risk factors of prostate inflammation [4]. However, the mechanisms underlying the initiation and development of prostate inflammation, and how inflammation promotes the cell apoptosis and survival in BPH and prostate cancer have not been determined yet.

BPH is characterized by enlargement of the prostate. It includes the proliferation of both epithelial and stromal cells [157]. Prostate inflammation is

frequently found in human BPH specimens, suggesting a tight correlation between them. Inflammatory factors, such as IL-6, IL-1α/β, and IL-8, are thought to drive epithelial proliferation in prostate and contribute to the pathological progression of BPH. The REDUCE study shows that prostate inflammation is correlated with prostate volume. This establishes a correlation between prostate histology and the symptomology of prostate diseases [158]. However, the mechanisms underlying prostate inflammation and BPH are still far away from being understood.

Dr. Jerde previously reported that the activation of developing pathway IL-1/IGF-1 signaling pathway is necessary for epithelial hyperplasia induced by inflammation. This pathway shared by tissue development and inflammation suggests a potential role of stem cells during inflammation. Although prostate stem cells have been studied for decades, their responses to inflammation and their roles in epithelial hyperplasia have never been shown.

Thus, we hypothesized that inflammation activates prostate progenitor cells under the regulation of IL-1/IGF-1 signaling and leads to epithelial hyperplasia. We focused on a specific population of progenitor cells, PEPCs, which is defined by CD133+/Sca-1+/CD44+/CD117+/Lin- and have been shown to be capable of

forming the prostatic structure *in vivo*. We investigated the PEPCs' behavior in an inflammatory condition.

B. Results

a. Inflammation increases prostate epithelial cell sphere formation

Inflammation induced cell apoptosis and cell proliferation suggest that prostate stem cells or progenitor cells may be involved in this tissue regeneration process. To test this hypothesis, I evaluated the sphere formation ability of primary prostate epithelial cells from 3 days-inflamed or the non-inflamed control mice using prosta-sphere formation assay.

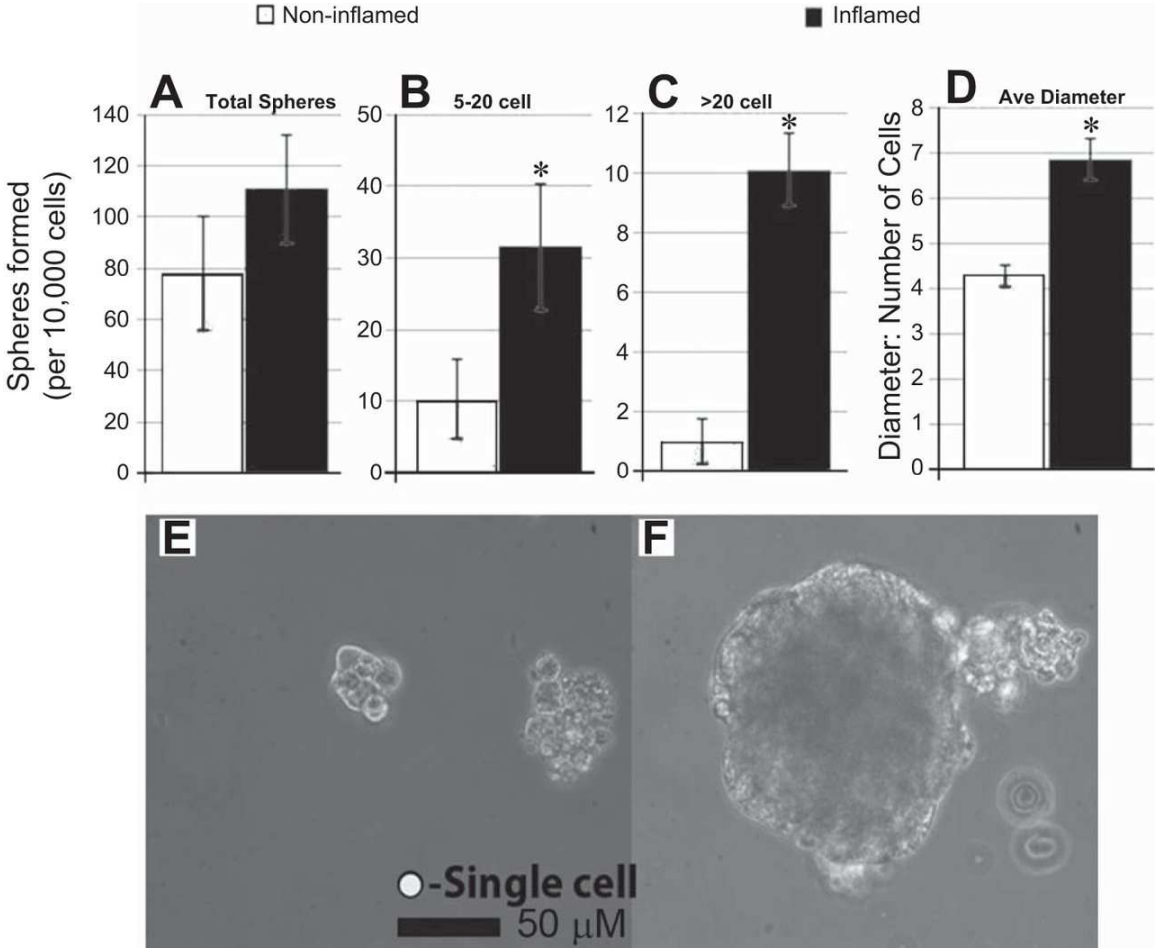


Fig 3.1 Inflammation increased prostate epithelial cells sphere formation

A: Average sphere numbers formed per 10000 cells. B, C: average numbers of medium (5-20 cells in diameter, B) and large (>20 cells in diameter, C) spheres formed per 10000 cells, only sphere with a diameter larger than 3 cells were quantified. D: average diameter of the spheres formed per 10000 cells. E, F: example pictures of spheres formed by cells from non-inflamed (E) and inflamed (F) prostate. * $P<0.05$, t-test, n=5, bar graphs were shown as mean±STDEV. Wang et al. 2015 [159].

Isolated epithelial cells from 3 days-inflamed and the non-inflamed control mice were grown in anchorage-independent conditions for 21 days at a density of 10000 cells per ml in ultra-low attachment dishes. The total volume of medium in each dish was 3ml. After growth, the numbers of cell mass meeting the criteria for counting as a “sphere” (>3 cells diameter) were counted by microscopy, and their individual size (as measured by the number of cells in diameter in a 2-dimentional plane, calculated by the diameter of spheres divided by the average diameter of a single cell) was determined. Although inflammation did not significantly increase the total number of spheres formed by the primary prostate epithelial cells (Fig 3.1 A), the average number of medium (diameter 5-20 cells) sphere formed per 10000 cells was increased 3-fold from 10±5.50 spheres to 31 ±8.82 spheres (Fig 3.1 B). The average number of large (diameter >20 cells) sphere formed per 10000 cells increased 10 folds from 1±0.74 sphere to 10± 1.21 spheres(Fig 3.1 C) There was no significant difference in average sphere diameter between the two groups, probably because of the large amount small spheres which diminished the difference. These data demonstrate that total sphere number was not increased in cells cultured from inflamed prostates, but suggests that epithelial cells cultured from inflamed prostates have the capacity to form larger spheres than those from control prostates. Thus, sphere formation ability is increased in the inflamed prostate, which may suggest a stronger

proliferation in this anchorage-independent cells or a larger sphere forming cell population, or both, induced by inflammation.

b. Increased cell-cell adhesion in inflamed prostate

Sphere formation assay was firstly used to evaluate the anchorage-independent proliferation of neuronal progenitor cells, and then, was used in other fields. It has been identified that cell proliferation contributes to the sphere size, while the number of spheres majorly depends on the number of sphere-forming cells. However, it has also been studied that the sphere formation assay is a highly dynamic process in which the spheres can move and communicate, and attach to each other. In this case, the increase of the medium and large spheres we observed may be due to a higher cell proliferation rate and/or increase in the cell-cell attachment. To address this question, We isolated cells from control or inflamed prostates from GFP and dtTomato-RFP transgenic mice and co-cultured both under anchorage-independent conditions mixing 7,500 cells (5000/ml, 1.5ml) from GFP-expressing mice with 7,500 cells (5000/ml, 1.5ml) from dtTomato-RFP-expressing mice) for 7 days (Fig 3.2 A-D). If the cell-cell attachment does not contribute to the formation of large or huge spheres, then I should expect all the spheres are mono-colored. On the other hand, if cell-cell attachment contributes to the formation of large or huge spheres, then most

medium or large spheres should be dual-colored since the larger sphere may come from several small spheres. The larger the sphere is, the higher incidence it may fuse with another sphere of different color.

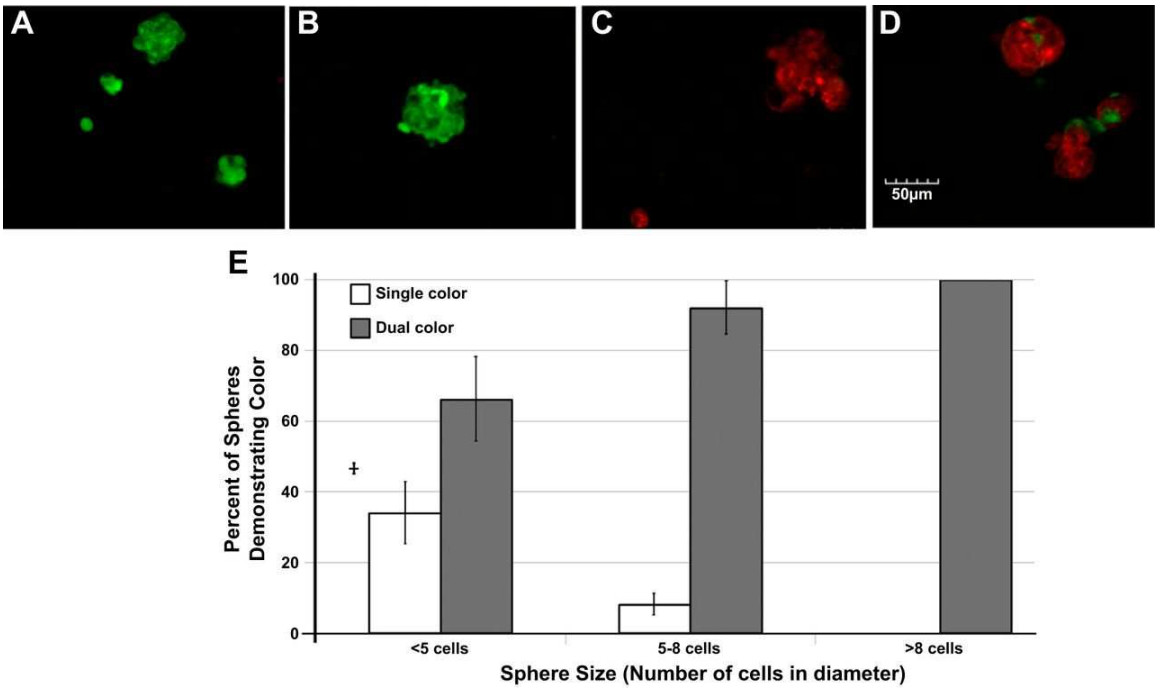


Fig 3.2 Cell-cell adhesion contributes to medium and large sphere formation

A: spheres formed by cells from GFP transgenic mice. B, C: spheres formed by cells from inflamed GFP and RFP transgenic mice, respectively, D: spheres formed by cells from RFP and GFP mice mixed in a 1:1 ratio. E: color analysis of spheres of different sizes. Bar graphs were shown as mean±STDEV. Wang et al. 2015 [159].

Fluorescent images from these spheres showed that the majority of the cultured spheres contained cells from both mice, indicating that epithelial cells cultured in sphere culture medium were aggregating. In particular, all spheres that grew to 8 cells in diameter or larger had both GFP and RFP components, indicating that the larger spheres were made up of multiple potential sphere-forming cells (Figure 3.2 E). On the other hand, the percentage of mono-colored spheres was higher in small sphere group (diameter < 5 cells). This result suggested that cell-cell attachment contributes to sphere formation. It was possible that inflammation increases cell adhesion signaling (i.e. E-cadherin, integrin, and fibronectin) and promotes cell attaching to each other to form larger spheres. In addition, we also confirmed the single cell diameter and the total cell numbers recovered from the spheres. There was no difference of cell diameter between spheres from non-inflamed or inflamed groups (Fig 3.3 A). This indicated that the difference in the size of spheres was only determined by the cell numbers, but not the single cell size in these spheres. This was further confirmed by the total cell number recovered from spheres. More cells could be recovered from the inflamed group than from the non-inflamed group (Fig 3.3 B). This suggested either a higher proliferation rate or a lower apoptosis rate in inflamed groups.

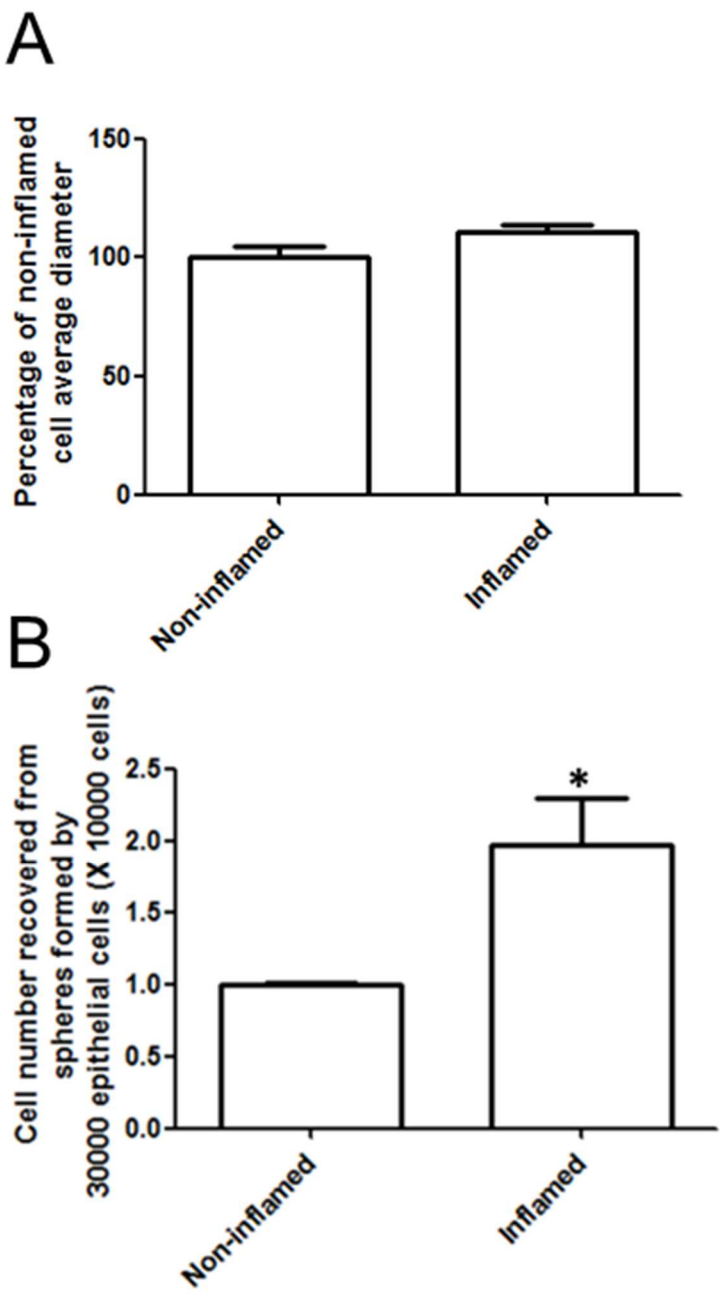


Fig 3.3 Validation of prosta-sphere formation assay.

A: percentage of average cell diameters of cells from inflamed mouse prostate to those of cells from non-inflamed mouse prostate. B: numbers of cell recovered from spheres formed by 30000

cells from non-inflamed or inflamed animals after 21 days' culture. *: $P<0.05$, t-test, n=3. Bar graphs were shown as mean±SEM

c. PEPCs exhibit strong sphere formation ability

Stem cells are capable of forming spheres in anchorage-independent culture. One possible explanation for the increased sphere formation ability in inflamed prostate was the expansion of stem cell populations. Thus, I analyzed the stem cells in our prostate inflammation model. Unlike those in other epithelial tissues such as intestine epithelium, the epithelial stem cells in the prostate cannot be identified by a single marker. In addition, the definition and characterization of prostate epithelial stem cells remain controversial. Several different sets of markers have been applied in prostate epithelial stem cells analysis, each of which may reflect a different population of stem cells. Here I analyzed a specific population of sphere-forming cells: the prostate epithelial progenitor cells (PEPCs), which is defined by CD133+, c-kit+, Sca-1+, CD44+, and Lin-. PEPCs have been shown to be capable of forming a prostatic structure in renal transplantation and are so far the most potent prostate stem cells that have been identified. To investigate the behavior of PEPCs during prostate inflammation, I first identified the sphere formation ability of PEPCs using different numbers of cells in prosta-sphere formation assay (Table 1) and compared the sphere formation ability of PEPCs with it of the non-stem prostate cells identified as Lin-/Sca-1-/c-kit-/CD133-/CD44- population.

Table 1 Prosta-sphere formation assay using PEPCs and non-stem prostate cells after 11 days in culture

Cell type	Starting cell number/well (cells/well)	Wells contain spheres / total wells	Percentage of wells that contain spheres.
PEPCs	1	0/96	0%
PEPCs	10	14/48	29%
PEPCs	100	19/20	95%
non-stem prostate cells	500	0/96	0%
Combination	1 PEPC + 499 non-stem prostate cells	0/48	0%

As shown in table 1, after 11 days in culture, PEPCs could form spheres in a cell density as low as 10 cells/100ul medium/well in 96 wells plates. The chance to detect sphere went up to 95% of wells when the cell concentration was increased to 100 cells/100µl medium/well. On the other hand, the non-stem prostate cells failed to form spheres even when the cell concentration reached up to 500 cells/100µl medium/well. This suggested that PEPCs have stronger

sphere formation ability compared to the non-stem population of prostate cells. In addition, it was noticed that single PEPC could not from sphere during long-term culture (up to 40 days). This suggested that PEPCs need to create a proliferative environment to promote their sphere formation, probably by releasing growth factors or cytokines. In this case, a single cell needed much longer time to accumulate enough growth factors for sphere formation. There is another possibility that there are multi sub-populations of PEPCs which are complementary to each other to facilitate sphere growth, while a single PEPC can never meet another cell from other populations. Moreover, the failure of forming spheres in the combination group (1 PEPC+ 499 non-stem prostate cells/well) suggested that the sphere formation depends more on the number of “sphere forming seeds” (PEPCs) than on the total number of cells. In addition, it also suggested that the non-stem prostate cells cannot provide the environment required by PEPCs for sphere formation.

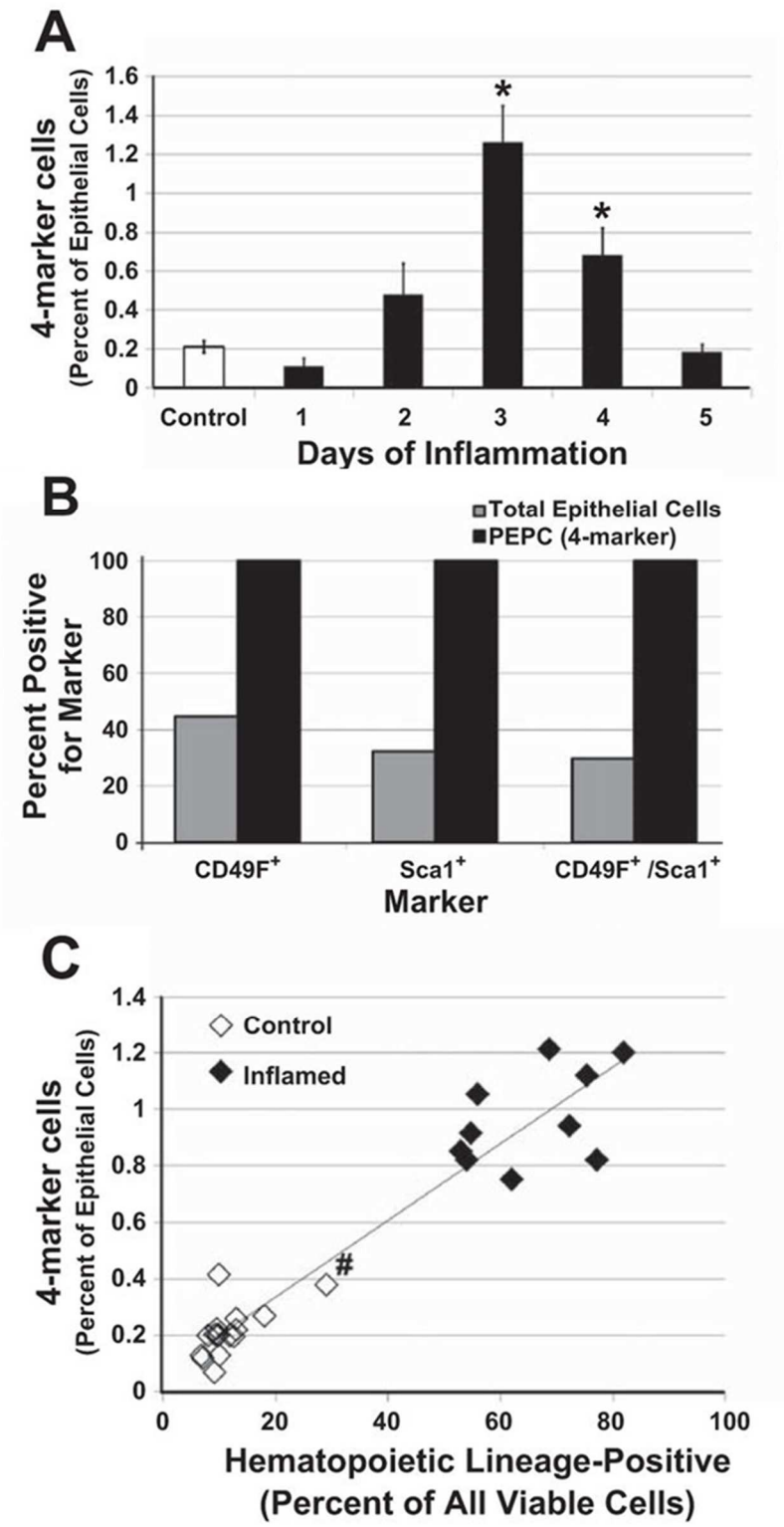
d. Inflammation induces PEPCs expansion in prostate

We then analyzed the PEPCs population change during inflammation in our mouse prostate inflammation model using multi-colored flow cytometry. We isolated epithelial cells from non-inflamed and inflamed mouse prostates each day during the 5-days inflammation induction process and analyzed them by flow

cytometry for PEPCs which were negative for Lin markers and positive for the critical 4-marker panel (Figure 3.4 A). The percentage of PEPCs was significantly increased from 0.2%±0.13 on day 0 to a peak of 1.2%+0.55 on days 3 post induction of inflammation and then dropped to base level on day 5 after inflammation. This time course change of PEPCs during inflammation suggested a highly regulated process which expands PEPCs during inflammation. I then compared our PEPCs with other published prostate epithelial stem cell markers including CD49f. We found that 100% of PEPCs were also CD49F positive, while CD49F and Sca-1 double positive cells consist 29% of the total epithelial population (Fig 3.4 B). This demonstrated that the Sca-1+/CD49f+ stem cell population includes the PEPCs and other populations.

To further establish the correlation between PEPCs populations and inflammation, we analyzed the correlation between PEPCs population and the intensity of inflammation. In our previous study, we used H&E staining to score the inflammation intensity. In PEPCs analysis, we use the whole prostate for flow cytometry. Thus, I investigated the percentage of Lin positive cells, which means bone-marrow derived cells (majorly immune cells) as a surrogate of histology analysis.

Preliminary data indicates that more than 60% total cell population are Lin positive fraction on histological analysis on inflamed specimens while Lin positive cells are only 20% in non-inflamed specimens. Thus, we set up this criterion for the quality of flow cytometry and found that the PEPCs population was correlated with Lin positive cell percentage, with a Pearson correlation coefficient of 0.957 (Fig 3.4C) Lin positive population peak also occurred on day 3, the same as PEPCs population peaks. Thus, this demonstrated that PEPCs population is correlated with inflammation intensity. To further investigate the effect of inflammation on PEPCs' sphere formation ability, we isolated PEPCs and Lin- cells (represent the total prostate cell population) from non-inflamed and inflamed animals and applied them in prosta-sphere formation assays. The sphere formation ability of PEPCs was much higher than that of Lin- cells, demonstrated by the higher number of the spheres formed (Fig 3.4D). However, there was no difference of PEPCs from non-inflamed or inflamed groups in terms of the sphere numbers. The size distribution of spheres was not different between inflamed and non-inflamed groups (Fig 3.4E). This suggested that inflammation does not change the sphere formation ability of PEPCs, instead, inflammation induces expansion of PEPCs populations which give rise to more spheres comparing to the non-inflamed group.


A
4-marker cells
(Percent of Epithelial Cells)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
Control
1
2
3
4
5
Days of Inflammation
B
Total Epithelial Cells
PEPC (4-marker)
Percent Positive for Marker
100
80
60
40
20
0
CD49F+
Sca1+
CD49F+ /Sca1+
Marker
C
4-marker cells
(Percent of Epithelial Cells)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Inflamed
#
0
20
40
60
80
100
Hematopoietic Lineage-Positive
(Percent of All Viable Cells)

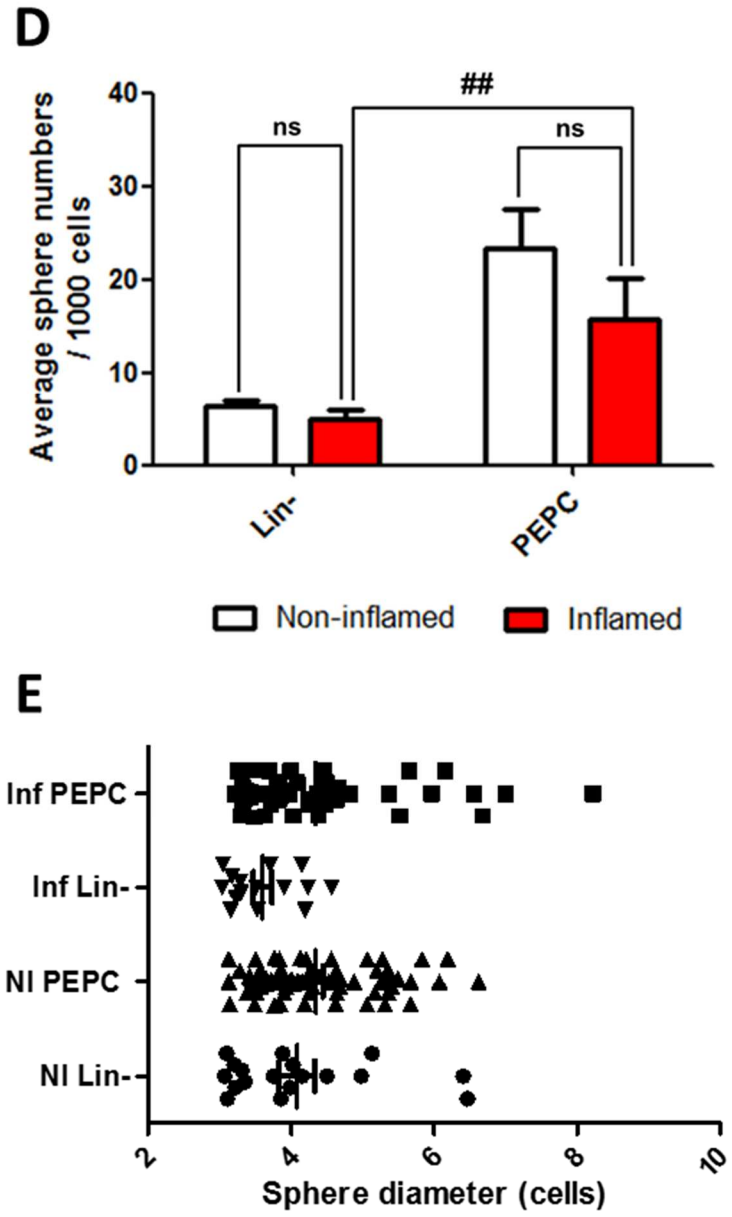


Fig 3.4 Inflammation increased PEPCs population in prostate without affecting their sphere formation ability

A: time course analysis of PEPCs in mouse prostate inflammation model *: P<0.05, One-way ANOVA; B: CD49F/Sca-1 expression in PEPCs and total epithelial cells. C: correlation of PEPCs population and inflammation intensity. D: PEPCs' sphere formation ability compare to Lin-

population, ## p<0.01 PEPCs group vs. Lin- groups， n=3, Two-way ANOVA, ns: no significance.); bar graphs were shown as mean±SEM. E: sphere diameter distribution. Each dot represented the diameter of a single sphere. Wang et al. 2015 [159].

e. PEPCs forms prostatic structure in renal transplantation

Although PEPCs has been reported to form a prostatic structure in renal transplantation, this has never been confirmed and needs to be further investigated. We isolated 1, 10 or 100 PEPCs by flow sorting, kept them in anchorage-independent conditions for 3 days, combined it in medium with Matrigel with 8000 urogenital mesenchymal cells obtained from the embryos of dtTomato-RFP-expressing mice and implanted the resulting pellet under the renal capsule of nude mice for 60 days. The transplants were harvested, fixed and stained after 60 days (Fig 3.5). We transplanted 10 total animals in this method using 1, 10, or 100 PEPCs and compared them to non-4-marker cells (epithelial cells that do not express all four markers). 7 of the 10 implants grew prostatic structures as shown in Fig 3.5, while no implants from the 4-marker negative population were able to grow prostatic structures. We also detected the RFP and the mature mouse prostate epithelial cell marker probasin expression in this transplantation formed by PEPCs. As shown in Fig 3.6 D, the stromal parts are RFP positive (Green channel, RFP was stained using anti-RFP antibody and Alexa-488 labeled secondary antibody), indicating that they were from the urogenital mesenchymal cells of dtTomato-RFP-expressing mice. The epithelial parts were RFP negative and were probasin positive (Red channel), suggesting that the epithelial parts were from PEPCs and were functional prostate epithelium

proved by probasin expression. Thus, we confirm that PEPCs are capable of forming prostatic structure *in vivo*.

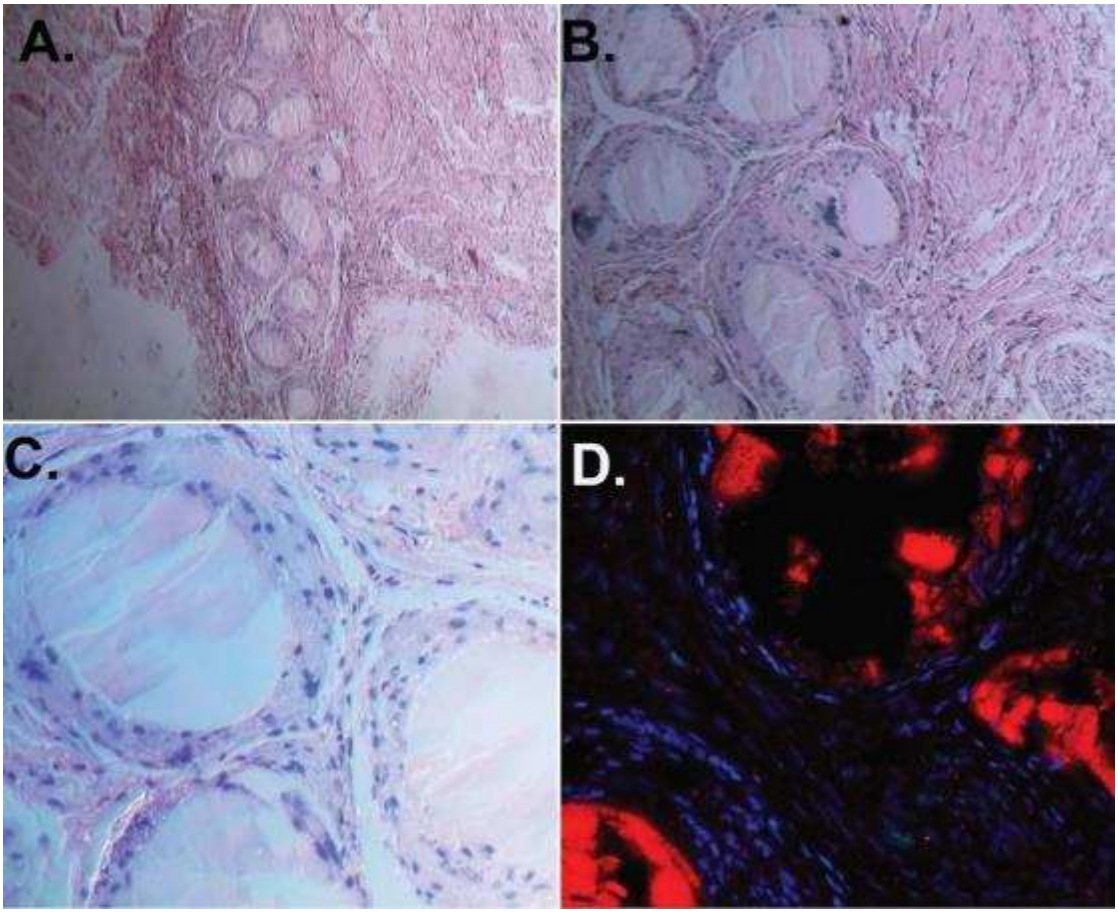


Fig 3.5 PEPCs formed a prostatic structure in renal transplantation in nude mice.

A-C: H&E staining of prostatic structure in renal transplantation under different magnification. D: RFP (Green, Alexa-488 labeled secondary antibody) and probasin (Red) expression in renal transplantation. The epithelial layer is probasin positive and RFP negative, indicating that it is from PEPCs, but not from the urogenital mesenchymal cells. Wang et al. 2015 [159].

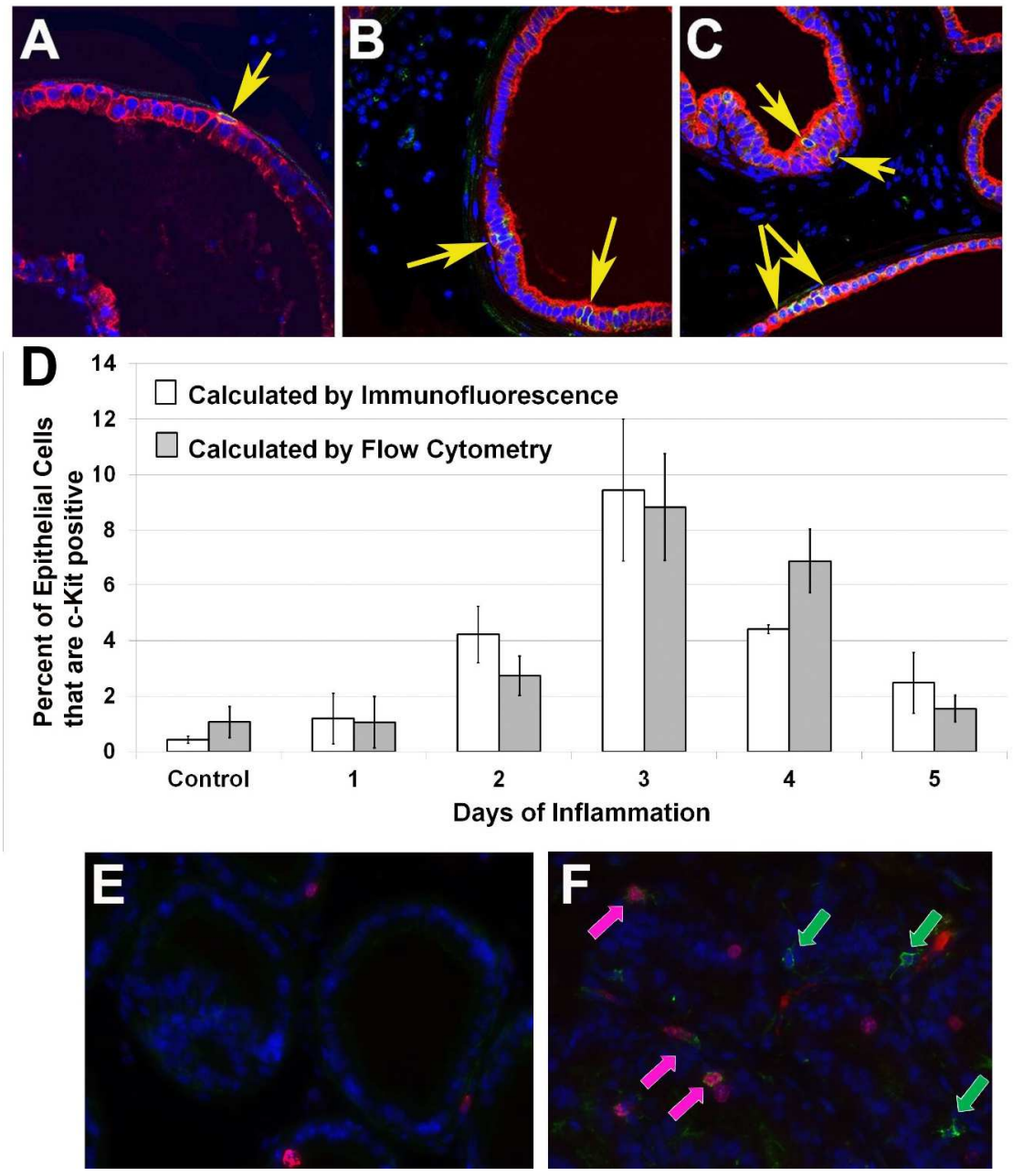


Fig 3.6 C-kit expanded from basal compartment to luminal compartment during inflammation

A-C: c-kit (Green) expression pattern on day 0 (A), day 2 (B) and day 3, yellow arrow refers to c-kit positive cells (C) during prostate inflammation (Red: Pan-Ck). D: c-kit positive cells populations calculated by flow cytometry and immunofluorescence were close. E, F: co-staining of c-kit

(Green, showed by the green arrow) and BrdU (Red, showed by the red arrow) in non-inflamed

(E) and Inflamed prostate (F). Bar graphs were shown as mean±STDEV. Wang et al. 2015 [159].

f. The basal-luminal population shift in c-kit positive cells

Among the four stem cell markers we used to define PEPCs, c-kit (CD117) is the most selective marker which was expressed in 1.6% (±0.6%) of isolated cells in non-inflamed prostates, while the percentage of the other 3 markers were much higher (Sca-1: 32% (±8.6%), CD44: 40% (±12.2%), and CD133: 23% (±7.8%)). The localization of c-kit positive cells may reflect the stem cell behavior in prostate inflammation. The c-kit positive cells were a very rare population localized in the basal layer of prostate epithelial cells in non-inflamed prostate (day 0) (Fig 3.6A)/ At day 2 and day 3 after inflammation, the c-kit positive epithelial appeared in the luminal compartment (Fig 3.6 B, C). The time course expression pattern of c-kit fitted for that of PEPCs during prostate inflammation (Fig 3.6D). By co-staining of c-kit with BrdU, we showed the mitotic c-kit positive cells in the inflamed prostate. This suggested a dynamic population expansion of c-kit positive cells from basal to the luminal compartment through cell proliferation (Fig 3.6 E, F).

g. PEPCs expansion depends on IL-1 signaling

Dr. Jerde's previous study identified that IL-1/IGF-1 signaling is necessary for inflammation induced epithelial hyperplasia. We then investigated whether the inflammation-induced expansion of PEPCs is mechanistically similar to reactive

hyperplasia. We applied IL-1 receptor 1 (IL-1R1) KO mice in our mouse prostate inflammation model and analyzed the c-kit expression pattern and PEPCs population during prostate inflammation. IL-1R1 KO mice have lower c-kit positive expression comparing to the WT mice at day 3 after inflammation (Fig 3.7A). The inflammation induced PEPCs expansion was also reduced in IL-1R1 KO mice compared to that in the WT mice group (Fig 3.7B). Thus, this result demonstrated that PEPCs expansion is at least partly depends on IL-1 signaling and it shares the regulation pathways with inflammation-induced epithelial hyperplasia. It suggests a potential correlation between progenitor cells and epithelial hyperplasia.

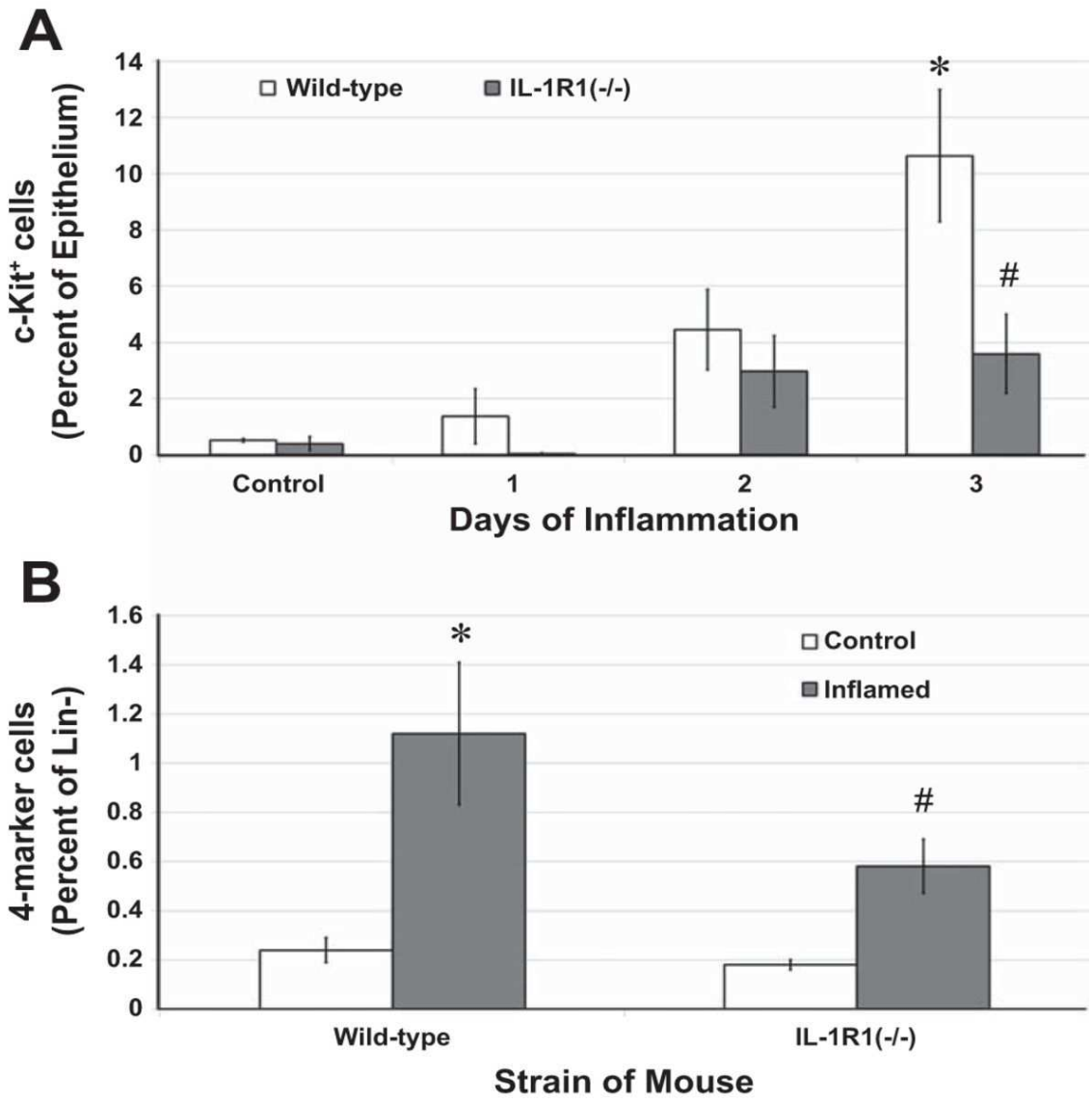


Fig 3.7 PEPCs expansion is IL-1 dependent in prostate inflammation

A: time course analysis of epithelial c-kit expression in mouse prostate inflammation model.*: P<0.05 versus control group. #: P<0.05 versus WT, One-way ANOVA, n=3; B: PEPCs population analysis in IL-1R1 KO mice. *: P<0.05 vs the control group. #: P<0.05 versus WT inflamed, One-way ANOVA, n=5-8. Bar graphs were shown as mean±STDEV. Wang et al. 2015 [159].

C. Discussion

Our data indicated that the activation of prostate epithelial stem cells during inflammation. Both the increased sphere formation ability and the expansion of PEPCs population confirm these findings. Inflammation increased the percentage of PEPCs by 6 folds, from 0.2%± 0.13 of the isolated prostatic epithelial cells to 1.2%±0.55 at day 3 after inflammation. This increased percentage dropped back to basal level at day 5 after inflammation, suggesting that PEPCs are under highly dynamic regulation during inflammation. Unfortunately, we were lacking the techniques to trace PEPCs *in vivo* to reveal their fates during inflammation; we speculated that the decrease of PEPCs population after day 3 is driven by the differentiation of PEPCs. During the differentiation of PEPCs, they will gradually lose the 4 stem cell markers and differentiate into lower-level progenitor cells or terminally differentiated cells. The progenies of PEPCs will not be recognized as PEPCs on flow cytometry since they no longer keep the 4 stem cell markers. On the other hand, at inflammation day 5, prostate still kept a basal level of PEPCs which may be used as a backup to response to further stimulus. This further implied that PEPCs may have self-renewal ability to maintain its population. Our finding of c-kit positive cells shifted from basal to luminal compartment supported this differentiation hypothesis. It suggested that c-kit positive cells or PEPCs may be involved in both basal and luminal regeneration, a basal to luminal trans-

differentiation may occur during inflammation, as also recognized in other studies.

The application of sphere formation assay on the prostate is so far one of the best experiments to evaluate cell proliferation in an anchorage-dependent condition of prostate epithelial cells since anchorage-independent growth is a critical feature of stem-like cells [113, 160-162]. Our results did not show an increase in total sphere numbers formed by cells from inflamed animals. Considering the assumption that a sphere is derived from a single sphere forming cell, this result was contradicted to what we found about the PEPCs population during inflammation and PEPCs' stronger sphere formation ability comparing to non-PEPCs populations. Moreover, the increase of medium and large spheres also suggested an increased sphere formation in prostate epithelial cells from inflamed animals. Thus, we introduced the dual-color sphere formation strategy and cultured spheres in a 1:1 ratio of epithelial cells from GFP-expressing mice and dtTomato-expressing mice. The results clearly showed that the incidence of dual-colored spheres increased along with the size of spheres. These results lead us to the conclusion that more cell-cell aggregation happens when forming large spheres. We found an equal number of spheres between non-inflamed and inflamed groups but larger spheres in the inflamed group. This

observation indicated a higher total cell numbers in the inflamed groups which were also confirmed by the cell number recovered from each group (Fig 3.3B). When considering that there were more “sphere seeds” (PEPCs, or other sphere-forming cells) in inflamed groups, we postulated that inflammation increases cell-cell adhesion. The mechanisms underlying how inflammation induces cell-cell adhesion is still unknown. However, changes on cell-adhesive molecule expressions, as well as circulating cell adhesive molecules such as ICAM, have been identified in prostate cancer specimens [163-165]. Cell adhesive molecules can regulate cell behavior through many signaling pathways such as TGF-β, β-catenin, and integrin signaling. These may directly, or indirectly affect the integrity of prostate epithelium, modify the stem cell milieu and regulate progenitor cell behaviors. These may finally contribute to the proliferative microenvironment induced by inflammation.

The repetitive cycle of androgen ablation and re-addition can induce prostate atrophy and regeneration, which suggests the existence of androgen-independent prostate progenitor cells [166]. Several stem cell markers, including CD133, CD44, Sca-1, CD117 and CD49f, have been used to identify prostate stem cells. Gao et al. identified that the c-kit stem cell factor (SCF) receptor is the most important marker for prostate epithelial stem cells since only c-kit positive

cells can form the prostatic structure in renal transplantation [114]. C-kit positive cells are capable of generating secretive luminal cells as demonstrated by probasin expression, in addition, these cells were also capable of proliferation as shown in prosta-sphere formation assay. We transplanted 10 animals with PEPCs and 7 of them grown to prostate-like structures while the non-PEPCs cell failed to do so. Thus, the defined panel of PEPCs, in particularly including c-kit in the panel, is more specific to locate the functional stem cells in the prostate which is responsible for inflammation-induced tissue regeneration.

The definition of prostate stem cells remains controversial. Other surface markers have been used for isolation of stem cell, most notably the Sca-1/CD49f panel [119]. This panel produces significantly enrichment on prostate graft growth and sphere formation comparing to non-stem prostate epithelial cells. To compare our PEPCs with this panel, we also analyzed the expression pattern of CD49f in our PEPCs since Sca-1 was already on our panel. Given that CD49f+/Sca-1+ cells were 10-20% of total epithelial cell populations which is much more than expected population of progenitor or stem cells, we postulated that this population may represent for a medium to low level of lineage progenitor cells that have very limited differentiation potential. The result showed that all the

PEPCs were CD49f, suggesting PEPCs are included in the CD49f panel and PEPCs panel is a more strict definition of progenitor cells in the prostate.

The roles of PEPCs in prostate diseases have never been reported. However, the roles of prostate stem cells in prostate cancer and BPH are attracting more interests. The correlation between inflammation and cancer initiation in prostate has been shown recently [167] and chronic inflammation increased prostate intraepithelial neoplasia (PIN) in our WT mouse inflammation models. The differentiation from basal to luminal cells in Pten null mice indicates the involvement of stem or progenitor cells in cancer initiation. Lots of studies have indicated the existence of prostate cancer stem cell in cancer tissues, circulating blood, and cultured cancer cell lines, such as PC3, DU145 and LNCaP cells [160, 168, 169]. These cancer stem cells are capable for recapitulating other cancer cell populations under the selection of chemotherapy drugs or other treatments. However, little is known about the origin of cancer stem cells. The expansion of PEPCs during inflammation suggested that PEPCs are responsive to the inflammatory environment and may be involved in later events such as loss of tumor suppressor and cell transformation.

The trans-differentiation from basal to luminal cells is critical for the homeostasis of epithelium and for maintaining the epithelial structure and functions during inflammation. This trans-differentiation rarely happens in physiological conditions. The trans-differentiation also significantly accelerates the formation of PIN lesion in prostate. Our results demonstrated the role of c-kit positive cells for basal-luminal phenotype transition during inflammation, which may provide an insight of the cells which are responsible for this trans-differentiation and for neoplasia initiation.

BPH is a slow progressive enlargement of the prostate gland and is age-related in human. Most of BPH derive from the transitional zone. Little is known about the biological processes of BPH development since the cause of BPH is unknown. Our results demonstrated that PEPCs population expands during the inflammation-induced epithelial hyperplasia, together with increased cell proliferation in mouse prostate. The progenies of PEPCs may reside in the epithelial compartment and differentiated to luminal, basal or TAC cells, and finally give rise to a clone of highly proliferative cells. On the other hand, chronic inflammation is the major type of human prostate inflammation. This indicates that inflammation may persist in both animals and human which results in an inflammatory environment. This environment may continuously expand PEPCs or

similar progenitor or stem cells populations to increase the aberrant proliferation of epithelial cells in the prostate. Thus, the expansion of PEPCs at the very beginning of inflammation can result in long-term effects of epithelial hyperplasia. This is also confirmed by the severe epithelial hyperplasia observed in chronic inflamed animals. It is reasonable for us to postulate that PEPCs expansion may also contribute to the development of BPH, the mechanism underlying which may also involve stem or progenitor cells [170, 171]. However, to confirm this hypothesis, a more accurate lineage tracing study needs to be performed.

Jerde et al. previous demonstrated that development pathways, such as IL-1/IGF-1 signaling, are activated during inflammation. This makes inflammation a development-like process of tissue regeneration. Thus, we investigated whether PEPCs expansion is also the consequence of development signaling activation. Our results in IL-1R1 KO mice showed a 50% reduction of the PEPCs expansion during inflammation. This was not the consequence of lower inflammation intensity since we set the 60% Lin positive cells criteria that assured the inflammation intensity was comparable between WT mice and IL-1R1 KO mice. In addition, IL-1R1 KO mouse is shown to have effective inflammation through neutrophils, lymphocytes, and macrophages [151] .Thus, this reduction in PEPCs expansion may depend on the stromal-epithelial interaction-based IGF-1 release.

Admittedly, this reduction was not 100% abolishment since there may be other signaling mediators, such as IL-6, sonic-hedgehog, PDGF and TGF-β, which may also be activated by inflammation and are known to participate in stem or progenitor cell regulations. Thus, further studies need to be performed to figure out other pathways that regulate prostate progenitor cells during inflammation.

Chapter 4 IGF induces prostate epithelial cell proliferation through survivin

A. Introduction

A direct correlation between inflammation and epithelial hyperplasia has been established using animal prostate inflammation models [33, 167]. Elevation of both cell apoptosis and cell proliferation have been identified in prostate inflammation models [142, 172], suggesting a tissue regeneration process taking place to compensate the cell loss induced by inflammation. Infiltration of immune cells such as neutrophil and macrophage introduces the pro-inflammatory cytokines, apoptosis factors and growth factors. These inflammatory factors may coordinately regulate the apoptosis-proliferation process. Aberrant regulation of this regeneration process shifts the hierarchy of prostate epithelial cells, results in the disruption of prostate epithelium and increases prostate cancer risks [152]. However, the mechanism underlying inflammation-promoted epithelial cell proliferation and the mediators regulating epithelial hyperplasia in the prostate is largely unknown.

Survivin is a dual-functioning protein that has both anti-apoptosis and cell-cycle regulation functions. It can bind to other IAP family member to inhibit caspase activation. It is also a necessary component of the chromosomal passenger complex (CPC) that can bind to microtubule to facilitate the formation

of CPC and cleavage furrow [173]. Loss of survivin in mitosis may result in mitosis catastrophe [174]. Survivin is primarily known as a cancer marker due to its overexpression in most human cancers including prostate cancer [175]. Its expression level is associated with drug resistance and malignancy of human cancers [176]. However, in non-cancerous tissues, the functions of survivin are understudied, partly due to the absence of its expression in many tissues including prostate. In adult tissue, survivin is highly expressed in high-turnover rate tissues such as vascular endothelium, colonic epithelium, and activated lymphocytes [177]. This suggests a cell proliferative role of survivin in these tissues. We previously identified an increase of survivin expression level in inflamed mouse prostate, human BPH, and prostate cancer specimens [46]. This suggests a potential correlation between survivin expression and inflammation induced prostate epithelial cell proliferation. However, the role of survivin in inflammation induced prostate epithelial hyperplasia and its regulation pathways have never been studied.

IGF-1 is a growth factor that is structurally similar to insulin. It binds to the IGF-1 receptor (IGF-1R) and leads to IGF-1R phosphorylation to activate downstream signaling pathways such as AKT and mTOR. IGF-1 exhibits cell proliferative and protective effects [178] in various cell types including skeletal

myoblasts [179], fibroblast [180], and lens epithelial cell [181]. IGF-1 is a key regulator of prostate embryonic and puberty development. The IGF-1 level is correlated to prostate size [182]. Loss of IGF-1 may lead to defective prostate development [183], while IGF-1 receptor knockout mice are embryonic lethal [184].

Emerging studies have suggested a more complicated role of IGF-1 in inflammation. Increased IGF-1 signaling may protect tissue from inflammation-induced tissue damage in the retina [185]. Inhibition of IGF-1 signaling in macrophage accelerates atherosclerosis [186], while that in myeloid reduces skin inflammation [187]. Previous studies show that IGF-1 level is increased during prostate inflammation and it is necessary for inflammation reduced epithelial hyperplasia, but its downstream factors are still unclear [151, 188]. Thus, in this chapter, we proposed that survivin serves as a key mediator of epithelial cell proliferation in an inflammatory environment and is regulated by IGF-1 in prostate inflammation. To investigate our hypothesis, we evaluated survivin expression pattern in mouse prostate inflammation model and human specimens. We also analyzed the role of survivin in mediating prostate epithelial cells proliferation both *in vivo* and *in vitro*. In addition, we examined the roles of IL-1 and IGF-1 ,

which are key regulators of epithelium hyperplasia, on survivin expression regulation using specific inhibitors and transgenic mouse models.

B. Results

a. Survivin up-regulation in prostate epithelial cells

Our lab previously identified an up-regulation of survivin expression induced by inflammation. Thus, we first investigated the survivin expression pattern in our mouse inflammation model. We harvested the prostates from time-course inflamed animals and used immunofluorescence to probe survivin-positive epithelial cells. Survivin staining was rare in non-inflamed animals (Fig 4.1 A, B). It confirmed what has been reported before. The percentage of survivin positive epithelial cells gradually increased during the 5 days timespan of inflammation and reached a peak of more than 40% at day 5 post inflammation (Fig 4.1 A). By co-staining of survivin and epithelial cell marker Pan-CK, we showed that survivin was expressed primarily in the epithelial compartment but can also be detected in stromal cells (Fig 4.1 B, C).

Cell proliferation is a key feature of epithelial hyperplasia. Survivin is known for its cell proliferative function by participating in CPC assembles, and lacking survivin may result in mitotic catastrophe. This makes survivin an interesting

candidate of regulating epithelial cells proliferation during inflammation. To test the correlation between survivin up-regulation and cell proliferation, we analyzed the proliferation pattern of epithelial cells based on BrdU incorporation. We injected BrdU 2 hours before animals were sacrificed to label all the cells which were synthesizing DNA (in S phase) at the very moment. As we expected, BrdU labeling in the epithelial compartment was increased from 1% in the non-inflamed group to 12% in the inflamed groups (Fig 4.2 A, B). Interestingly, co-staining of BrdU and survivin demonstrated that most survivin positive cells were also BrdU positive in the inflamed group (Fig 4.2 A, B). This correlation was weaker in the non-inflamed group, probably because of the rare distribution of survivin positive cells (Fig 4.2 C). These results suggest that as a necessary factor of mitosis, survivin expression may be driven by cell proliferation during inflammation.

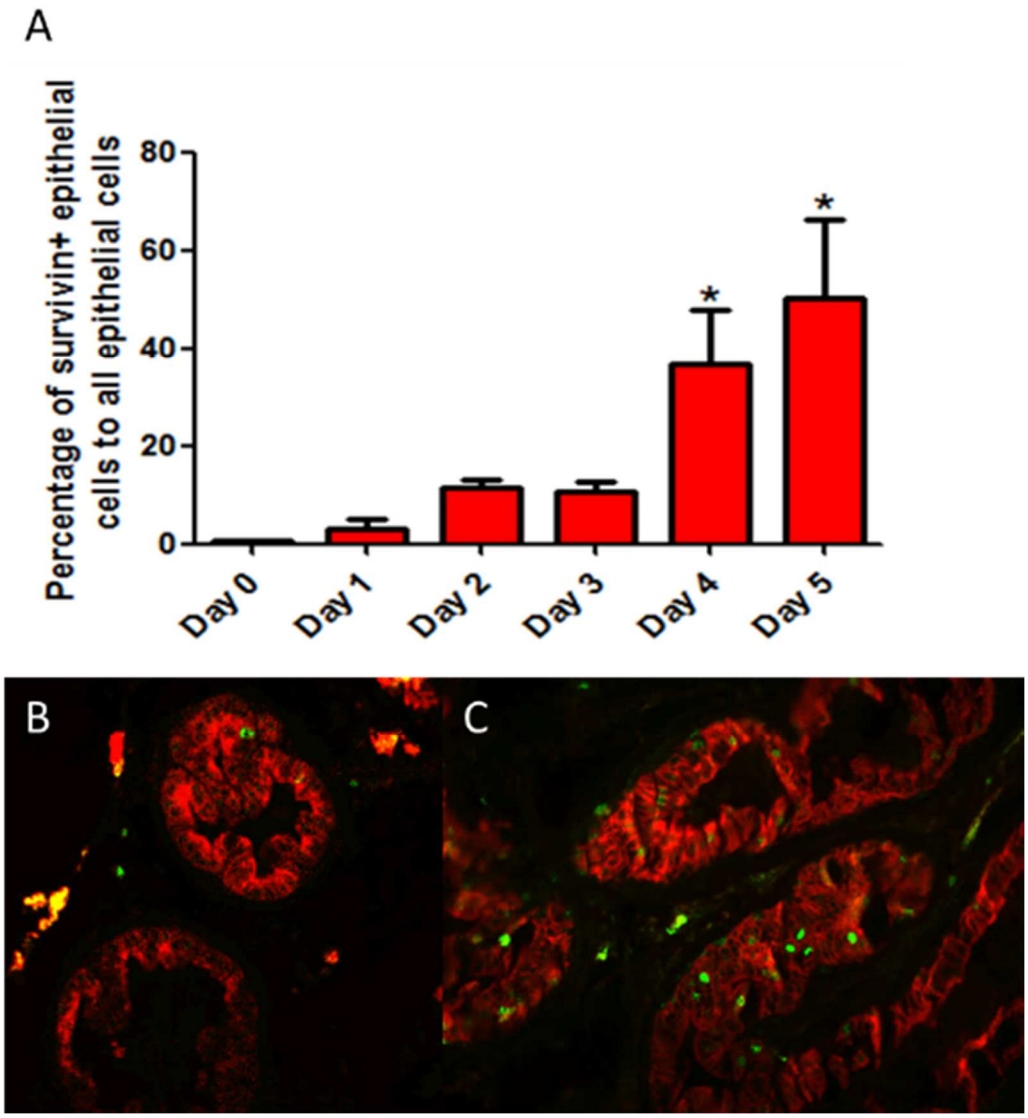
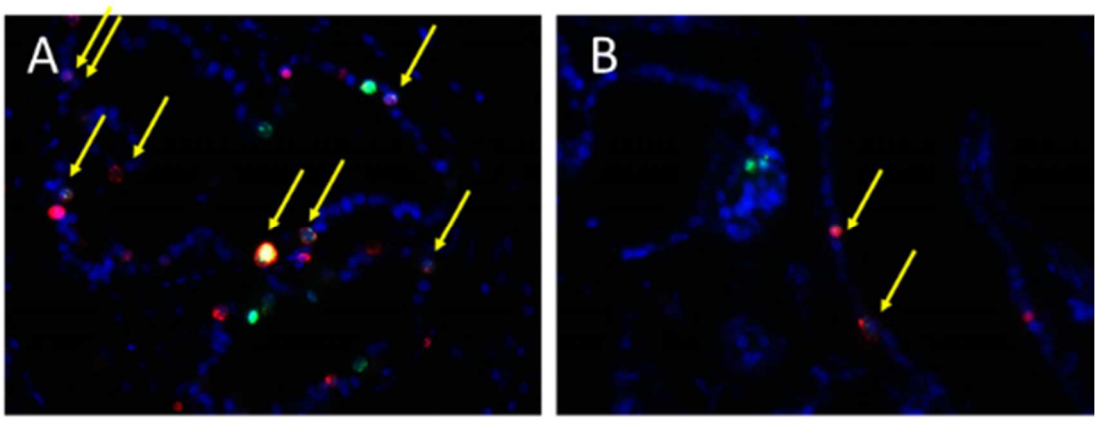
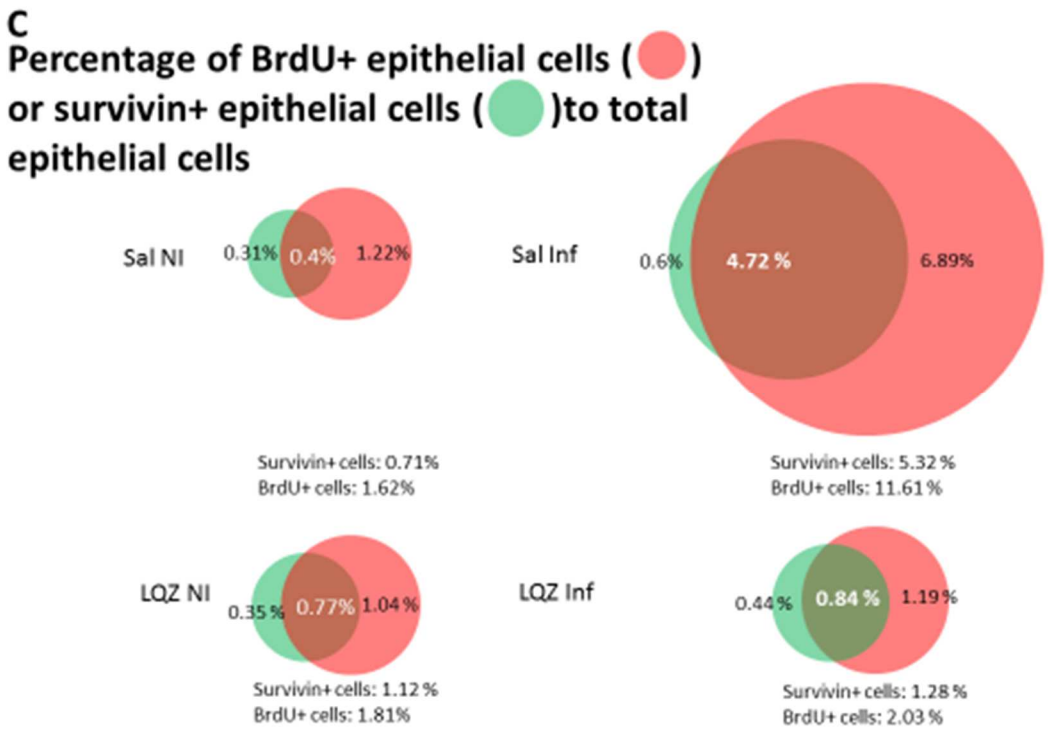


Fig 4.1 Increased survivin expression in epithelial cells during prostate inflammation

A: quantification of the percentage of survivin positive epithelial cells in total epithelial cells. *: P<0.05 One-way ANOVA, n=3, B,C: IF staining of survivin (Green) and epithelial cell marker Pan-CK (Red) in 0 day (B) and 3 days (C) post bacteria instillation. Bar graphs were shown as mean ±SEM.


A
B


C
Percentage of BrdU+ epithelial cells ( )
or survivin+ epithelial cells ( )to total
epithelial cells
Sal NI
0.31%
0.4%
1.22%
Survivin+ cells: 0.71%
BrdU+ cells: 1.62%
Sal Inf
0.6%
4.72 %
6.89%
Survivin+ cells: 5.32 %
BrdU+ cells: 11.61 %
LQZ NI
0.35 %
0.77%
1.04 %
Survivin+ cells: 1.12 %
BrdU+ cells: 1.81%
LQZ Inf
0.44 %
0.84 %
1.19 %
Survivin+ cells: 1.28 %
BrdU+ cells: 2.03 %

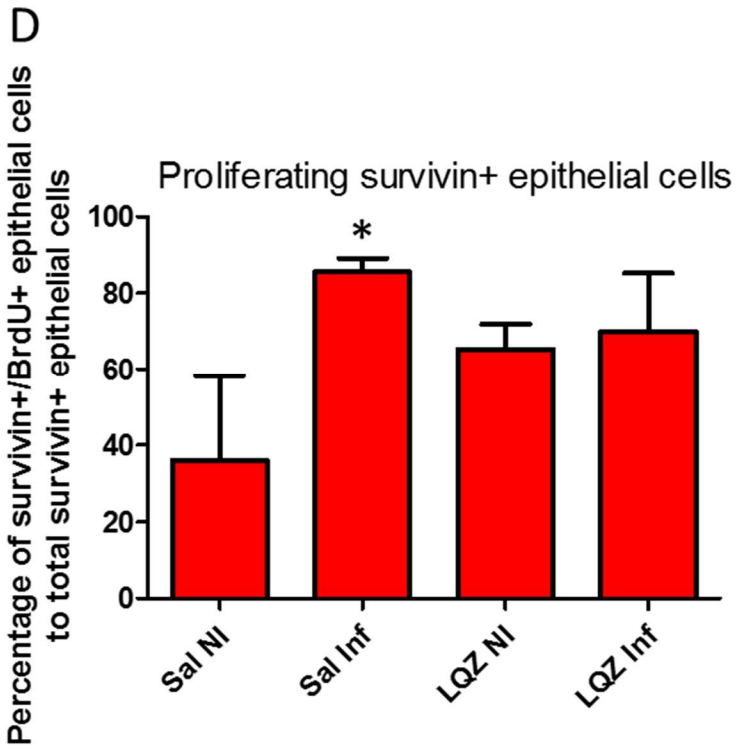


Fig 4.2 Survivin positive epithelial cells were proliferating during inflammation

A, B: survivin (Green) and BrdU(Red) co-staining in Veh inflamed (A) and LQZ-7F inflamed (B) mice at 3 days post-bacterial instillation; yellow arrows showed the dual positive cells. C: distribution of survivin and BrdU in epithelial populations. The overlap of green and red circles represented the cells that were survivin+/BrdU+. D: the percentages of proliferating survivin+ epithelial cells (survivin+/BrdU+) to total survivin+ epithelial cells. *:$P<0.05$, One-way ANOVA, n=3-6, bar graphs were shown as mean±SEM.

b. Inhibition of survivin reduces cell proliferation during inflammation

To further confirm the correlation between survivin and cell proliferation during prostate inflammation, we inhibited survivin expression in our mouse inflammation model. Survivin is necessary for cell cycle. The well-established survivin inhibitor YM155 is a transcriptional inhibitor. It inhibits survivin expression by inhibiting the Sp-1 site dependent transcription. This makes it a dirty drug since it will affect all the genes related to the Sp-1 site. Our collaborator Dr. Jian-ting Zhang developed a novel survivin inhibitor LQZ-7F. It increases survivin protein degradation through inhibiting its dimerization with other XIAPs and thus increased its proteasome-dependent degradation [189]. We pretreated C57BL/6 mice with this drug 1 day before the induction of inflammation and dosed the mice at day 1 after inflammation. The tissues were collected 2 or 3 days after bacteria instillation and were subjected to immunofluorescence analysis. Our results showed that LQZ-7F treatment significantly decreased the percentage of survivin positive prostate epithelial cells to total epithelial cells in inflamed animals (Fig 4.3A). BrdU incorporation was also reduced by 50% in LQZ-7F treated inflamed animals (Fig 4.3B). Since survivin is known essential for immune cell maturation in adult animals, globally survivin inhibition by LQZ-7F may affect immune cells and alter the inflammation patterns. To exclude this possibility, we evaluated CD45 positive cells populations in LQZ-7F treated inflamed animals.

There was no significant difference in the CD45 positive population in the prostate in inflamed groups between vehicle and LQZ-7F treated animals (Fig 4.3 C, D). This suggested that the reduction of survivin expression and BrdU incorporation was not due to the change in inflammation intensity. Thus we demonstrated that survivin inhibition leads to the reduction of proliferation in prostate epithelial during inflammation.

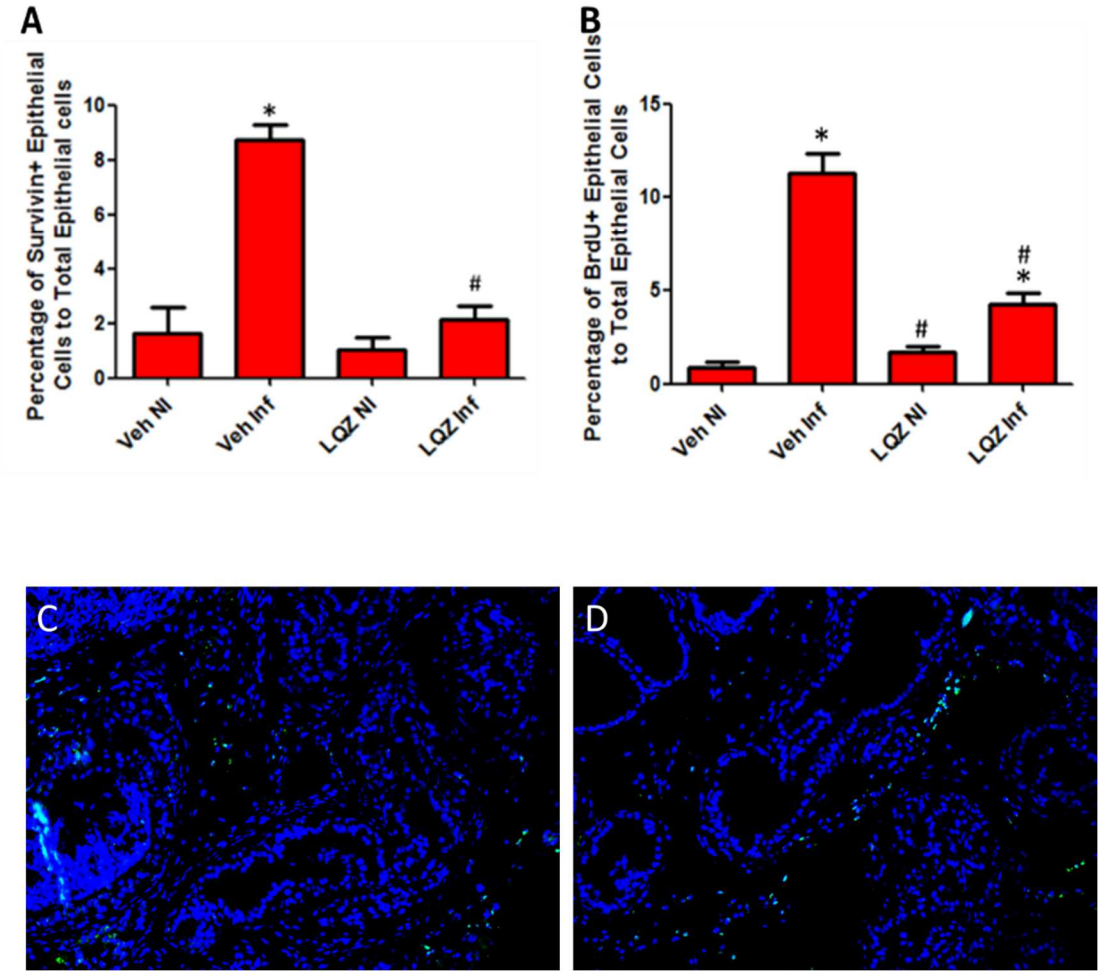


Fig 4.3 LQZ-7F treatment reduced survivin expression and cell proliferation in mouse prostate inflammation model without affecting inflammation intensity

A, B: quantification of the percentage of survivin positive epithelial cells (A) and BrdU positive epithelial cells (B) in total epithelial cells. * $P<0.05$ vs vehicle non-inflamed, #: $P<0.05$ vs vehicle inflamed, One-way ANOVA, n=5-8, bar graphs were shown as mean±SEM; C, D: IF staining of CD45 (Green) in Vehicle Inflamed group (C) and LQZ-7F inflamed group (D).

c. IL-1/IGF-1 dependent survivin up-regulation in prostate

We previous demonstrated that IL-1/IGF-1 signaling regulated lots of events during inflammation including PEPCs expansion and epithelial hyperplasia. We then postulate that survivin expression is also under the regulation of IL-1/IGF-1 signaling since it is tightly correlated with epithelial cell proliferation. IL-1R1 KO mice and IGF-1 receptor inhibitor Picropodophyllin (PPP) were introduced to establish the correlation between survivin and IL-1/IGF-1. We treated the animal with PPP, and then induce inflammation in treated animals and IL-1R1 KO mice. We analyzed survivin expression in prostate epithelial cells through co-staining of survivin and Pan-CK using IF. PPP treatment reduced the percentage of survivin positive epithelial cells in inflamed animals, while survivin positive epithelial cells population in IL-1R1 KO mice cannot be expanded by inflammation (Fig 4.4). Our results demonstrated that survivin expression in prostate epithelial cells depends on IL-1/IGF-1 signaling. Since PEPCs, epithelial hyperplasia and survivin expression are all under the same regulation pathway, we postulate that the up-regulation of survivin during inflammation may play important roles in regulating the behavior of prostate stem cells such as PEPCs.

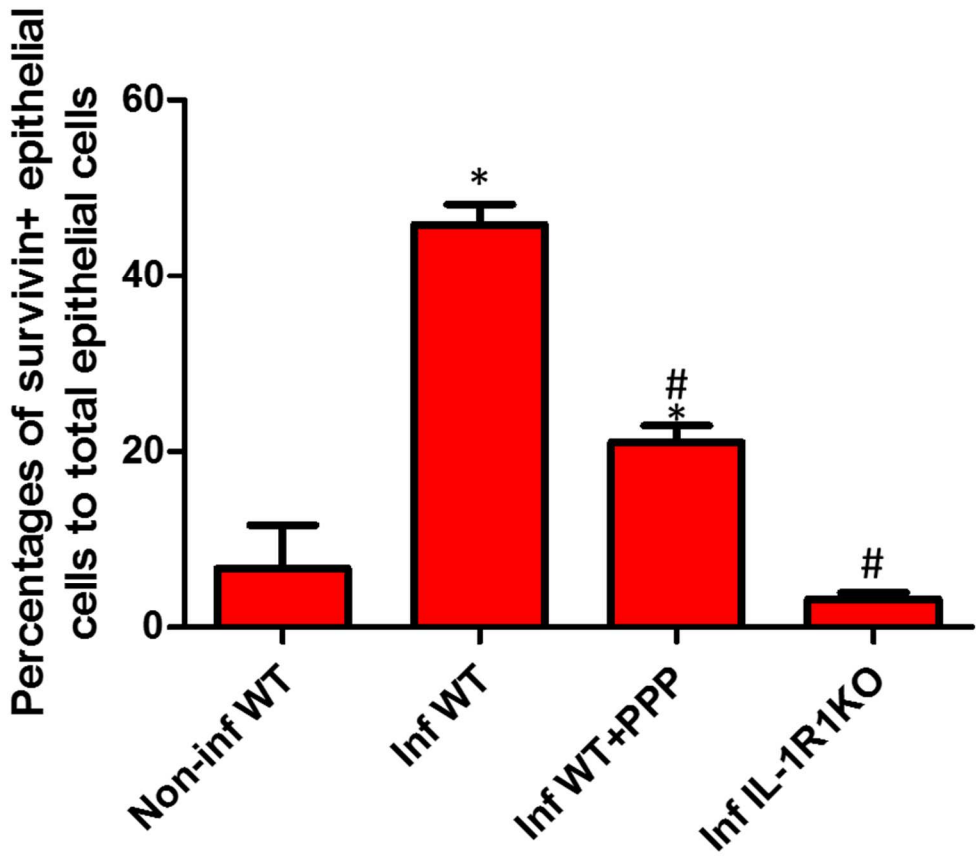


Fig 4.4 Inhibition of IL-1/.IGF-1 signaling reduced the percentage of survivin positive epithelial cells to total epithelial cells in inflamed mouse prostate

The percentage of survivin positive epithelial of total epithelial cells was increased by inflammation and can be inhibited by PPP treatment or in IL-1R1 KO mice. *: $P<0.05$ vs WT+vehicle, #: $P<0.05$ vs WT+Inf, One-way ANOVA, n=3, bar graphs were shown as mean $\pm$ SEM.

d. IGF-1 induces survivin expression in E6 and E7 cells

As a mediator of inflammation, IGF-1 is under the regulation of IL-1 signaling and is released by stromal cells to activate epithelial cell proliferation during prostate inflammation. IGF-1 is known for its cell metabolism and proliferative effects. Since we have shown that survivin's proliferative effects during inflammation, we postulate that IGF-1 increases survivin expression in prostate epithelial cells. To test our hypothesis, we treated prostate non-cancerous epithelial cell lines E6 and E7 with IGF-1 in a time course manner and probe IGF-1 expression using western blot. In both E6 and E7 lines, IGF-1 induced survivin expression in 0.5h. This survivin up-regulation last for 3-6 hours, and survivin level dropped at 24 hours (Fig 4.5). This suggests that IGF-1 regulates survivin expression in prostate epithelial cell lines. There may be feedback loops which maintain survivin expression levels since IGF-1 induced survivin up-regulation is attenuated at 24 hours. Survivin is known as a fast turnover protein with a half-life around 30min in prostate epithelial cells. Cyclin-B1/CDK-1 complex can phosphorylate survivin to increase its stability at G2/M checkpoint. This phosphorylation is essential for survivin's mitotic function. Thus, IGF-1 induced survivin expression may also correlate with IGF-1's well known proliferative aspect.

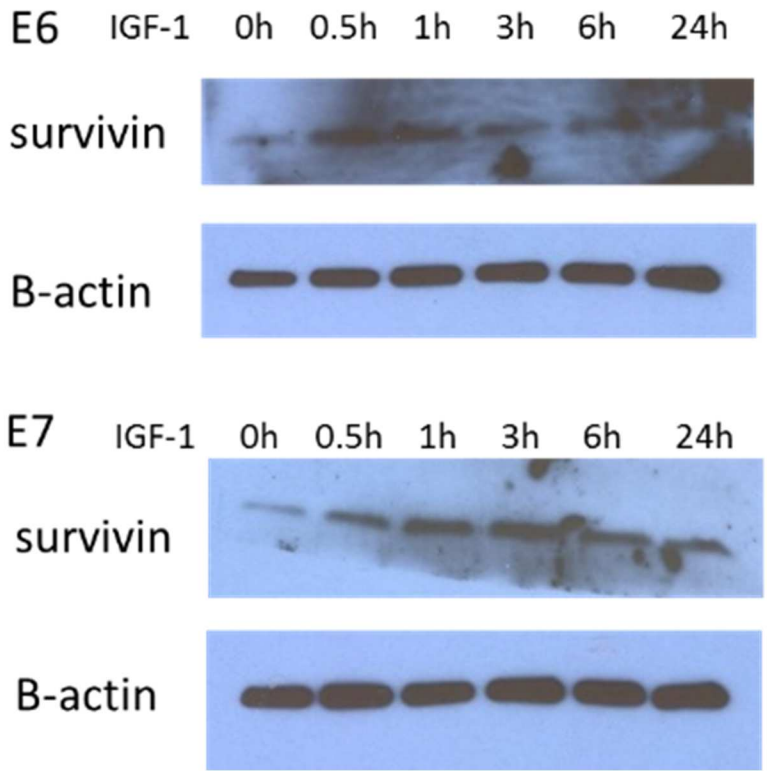


Fig 4.5 IGF-1 induced survivin expression in E6 and E7 cells

Time course treatment of IGF-1 in E6 and E7 cells, survivin and b-actin were probed by western blot.

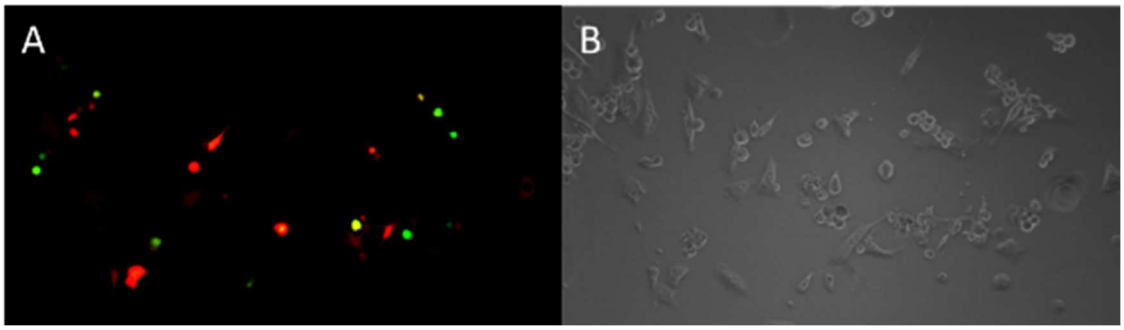


Fig 4.6 Survivin is indispensable for prostate epithelial cells

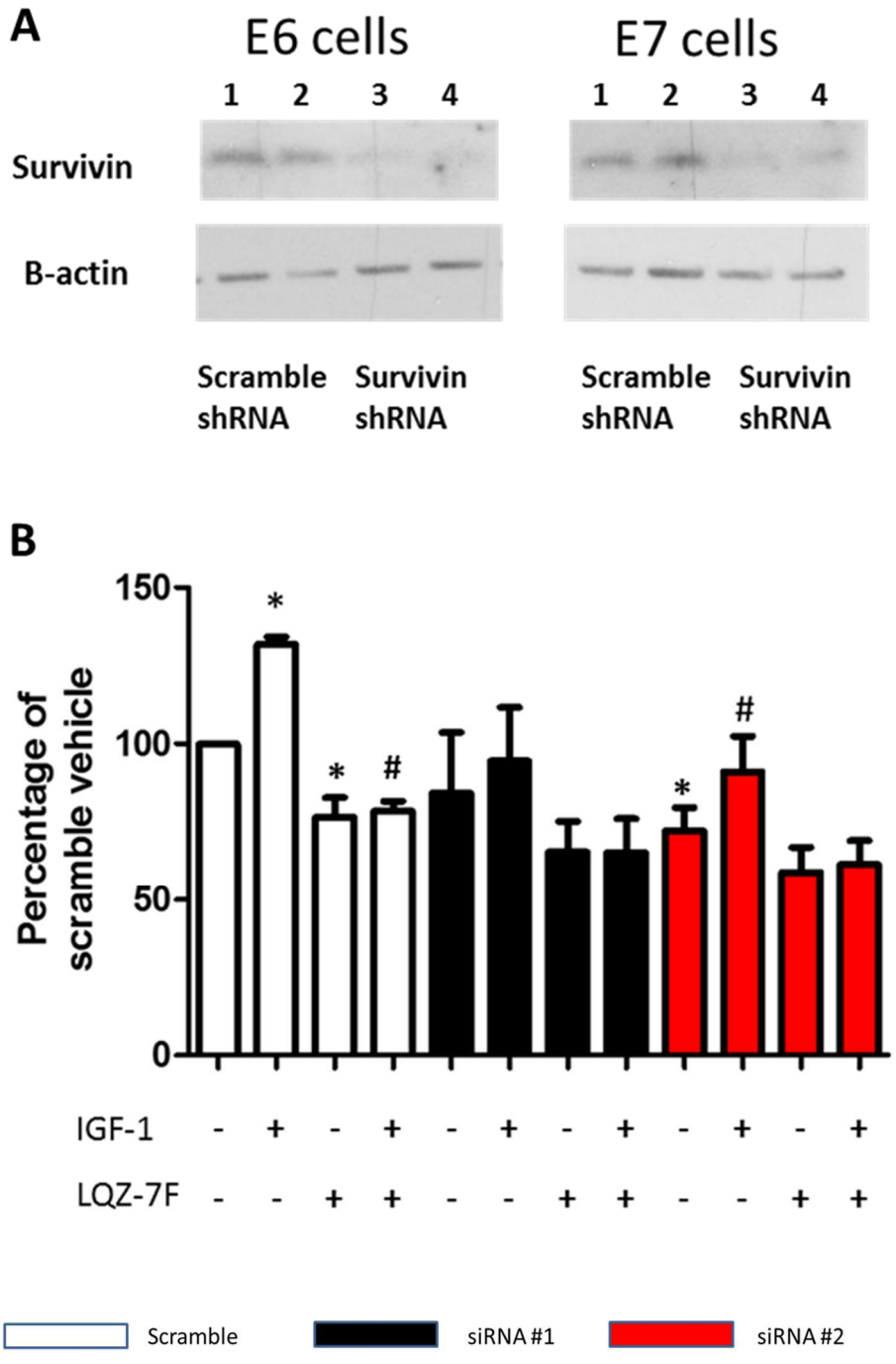
PC3 cells transfected with survivin CRISPR/CAS9 plasmid (GFP labeled, green, panel A) and survivin HDR (RFP labeled, red, panel A) plasmids. All the cells transfected with the survivin

CRISPR/CAS9 plasmid (GFP) were round cells and could not attach to the culture flask. A: fluorescent. B: bright field.

e. IGF-1 induces cell proliferation is survivin-dependent *in vitro*

Thus, we then analyzed IGF-1 induced cell proliferation to test whether it is survivin-dependent. We first tried to completely knock out survivin in cells lines using CRISPR/Cas9 technique. However, we failed to obtain any positive clones. Fluorescent microscopy showed that all the cells in which survivin had been successfully knocked out (GFP+) were failed to grow in culture (Fig 4.6). This suggested that survivin is essential for cell proliferation since survivin deficiency may cause a mitotic catastrophe as previously reported.

Thus, we adopted RNAi technique and pharmacological inhibitor LQZ-7F to partially knock down survivin expression in E6 and E7 cells. E6 and E7 cell were transfected with 2 survivin siRNA of different target sequences, respectively, and then were subjected to IGF-1 or LQZ-7F treatment for 5 days. We evaluated the cell proliferation using methylene blue assay and verified the survivin knockdown using western blot.


A
E6 cells
E7 cells
1 2 3 4
1 2 3 4
Survivin
B-actin
Scramble shRNA
Survivin shRNA
Scramble shRNA
Survivin shRNA
B
Percentage of scramble vehicle
150
100
50
0
*
*
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IGF-1 - + - + - + - + - + - +
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Scramble
siRNA #1
siRNA #2

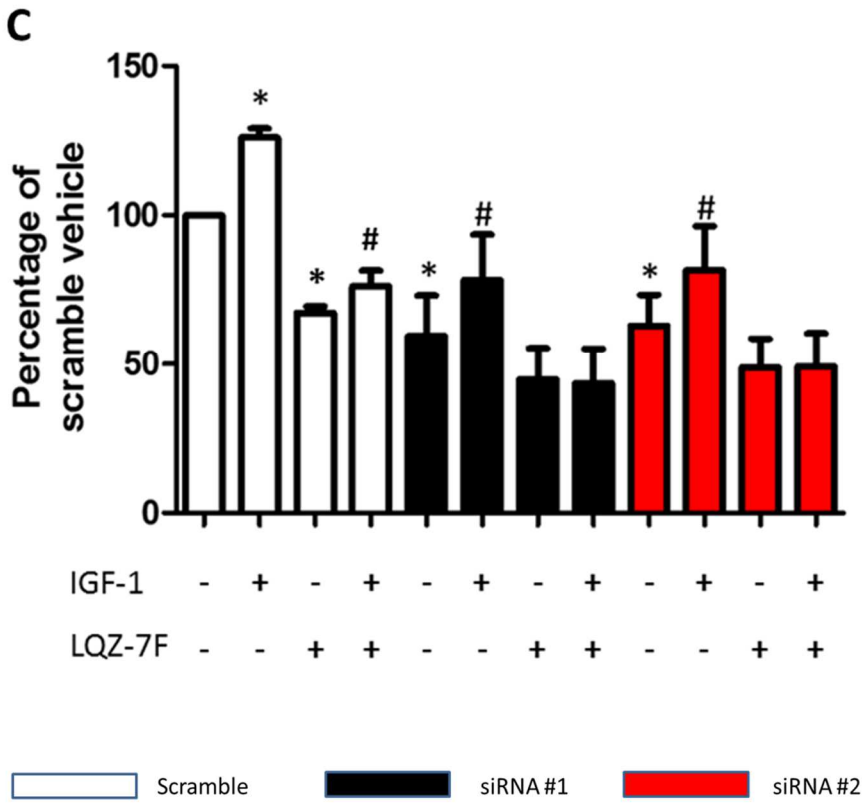


Fig 4.7 Survivin knockdown attenuated IGF-1 induced cell proliferation in prostate epithelia cells lines

A: western blot showed effective knockdown of survivin in E6 and E7 cells, Lane 1-2: scramble siRNA. B,C: methylene blue assay showed proliferation of cell treated for 5 days by IGF-1, LQZ-7F or survivin siRNA in E6 (B) and E7 (C) cells. *: P<0.05 vs IGF-1(-)/LQZ-7F(-)/Scramble group. #: P<0.05 vs IGF-1(+)/LQZ-7F(-)/Scramble group. One-way ANOVA, n=6, bar graphs were shown as mean±SEM.

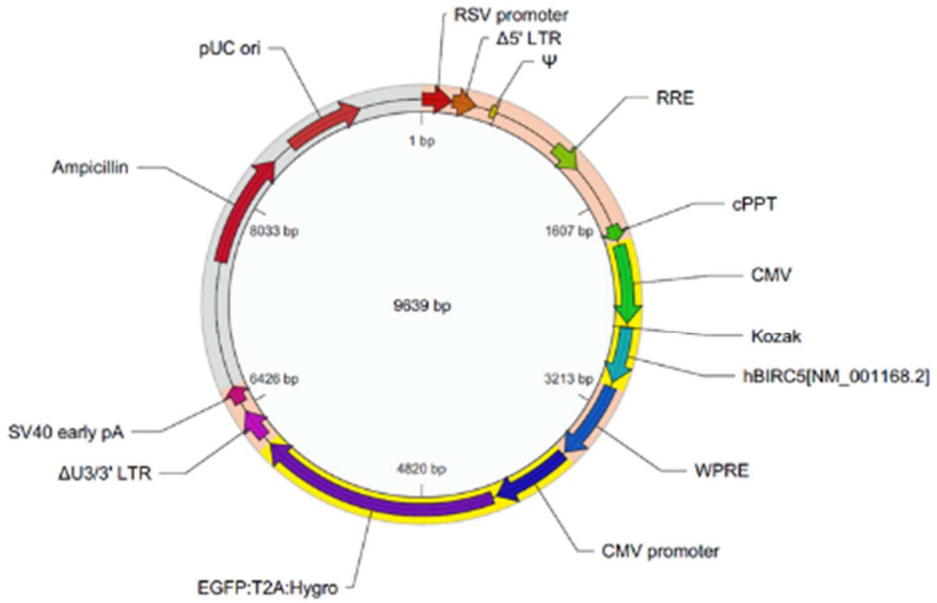
Survivn siRNAs shown effective knock down in both cells line (Fig 4.7 A). 5 days' treatment of IGF-1 induced a 30-50% increase in cell numbers in both lines transfected with scramble siRNA. LQZ-7F treatment reduced cell numbers in both vehicle and IGF-1 treated groups, and it totally abolished the IGF-1 induced cell proliferation. SiRNA also attenuated IGF-1's effect on cell proliferation. Co-treatment survivin siRNA and LQZ-7F further reduced the cell number in all groups and abolished IGF-1 effects (Fig 4.7 B, C). These results demonstrated that IGF-1 induced cell proliferation requires the presence of enough amount of survivin. Lack of survivin inhibits cell proliferation both with and without the presence of IGF-1. This result supplemented our *in vivo* data that IGF-1 induced epithelial cell proliferation by inducing survivin expression. It also confirmed what we found in LQZ-7F experiment that inflammation-induced epithelial proliferation in prostate depends on survivin expression.

f. Survivin overexpression does not affect cell proliferation

Survivin is known as a dual-functioning protein with both proliferative and anti-apoptotic functions. Its major role in cells may depend on its amount and localization. Due to its role as a part of the CPC complex, survivin may serve as a necessary part of mitosis, but not a driver. To test the effect of survivin overexpression in prostate epithelial cells, we overexpressed survivin using the

lentiviral vector: pLV[Exp]-EGFP:T2A:Hygro-CMV>hBIRC5[NM_001168.2]. This vector overexpresses human survivin under the control of CMV promoter which helped us to avoid most known transcriptional level regulation of survivin expression (Fig 4.8 A). We also use m-Cherry overexpression lentiviral vectors as a control. E6 and E7 cell were transfected with the survivin or the m-Cherry overexpression control lentiviral vectors and then were subjected to antibiotic selection. The positive cells after selection were cultured for 3 weeks and were treated with IGF-1 in a time course manner. We first verified the overexpression of survivin in E6 and E7 cell lines using fluorescent microscopy. A dramatic increase of survivn expression can be observed in both E6 and E7 lines. Survivin is weakly expressed in E6 and E7 cells in the nucleus. The overexpression of survivin showed both nucleus and cytoplasm localization (Fig 4.8 B). It was noticed that survivin is majorly nuclei localized during mitosis which can be easily characterized by the distribution of cell nuclei in cultured cells. In other phases of cell cycle, survivin can be found in the cytoplasm.

A


RSV promoter
Δ5' LTR
Ψ
pUC ori
RRE
1 bp
Ampicillin
cPPT
8033 bp
1607 bp
CMV
9639 bp
Kozak
hBIRC5[NM_001168.2]
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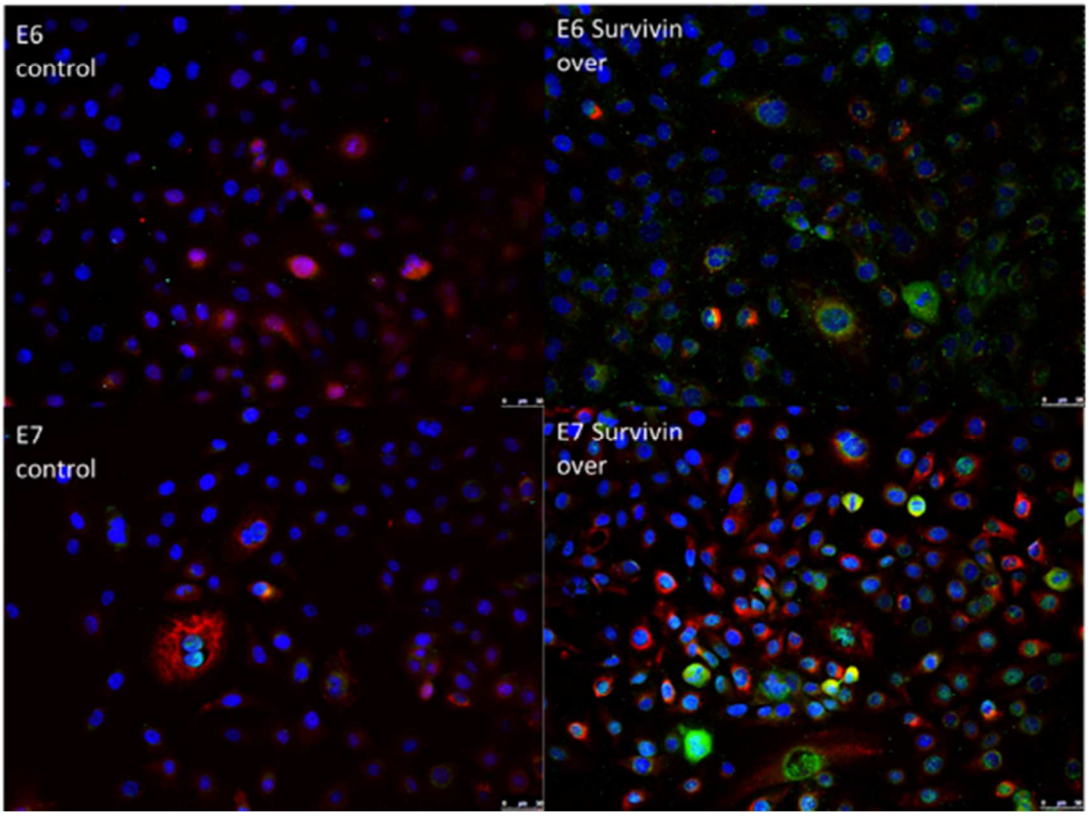
3213 bp
SV40 early pA
WPRE
ΔU3/3' LTR
4820 bp
CMV promoter
EGFP:T2A:Hygro

B


E6
control
E6 Survivin
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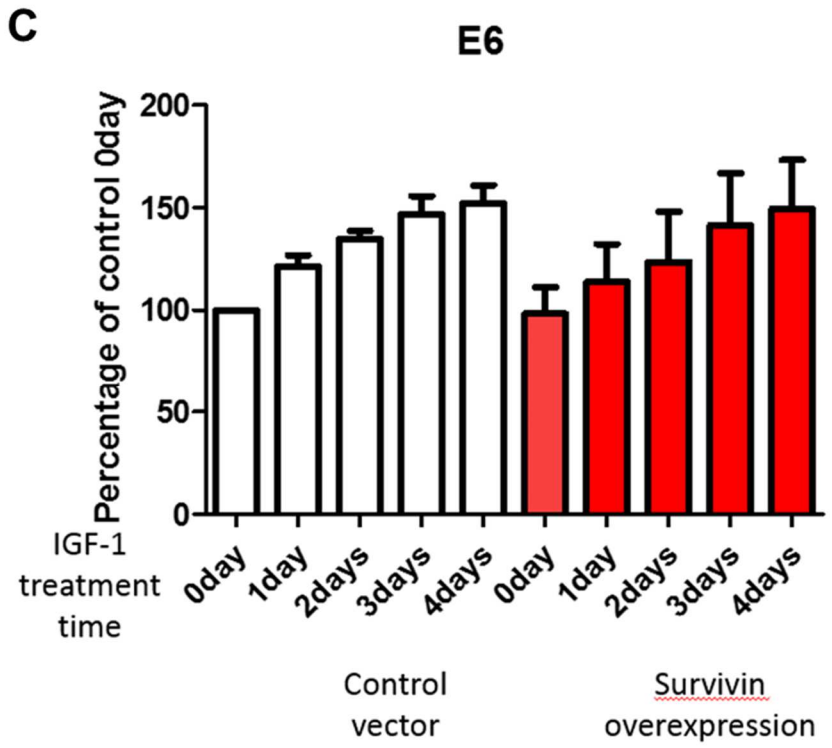
C
E6
Percentage of control 0day
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150
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50
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IGF-1 treatment time
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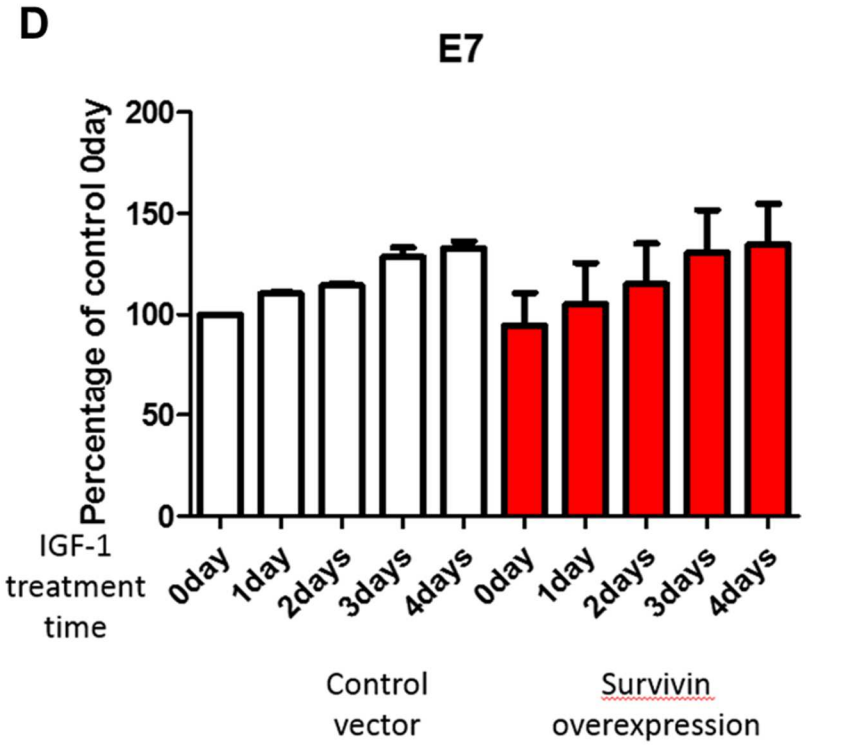
Control vector
Survivin overexpression


D
E7
Percentage of control 0day
200
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IGF-1 treatment time
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3days
4days
Control vector
Survivin overexpression

Fig 4.8 Survivin overexpression does not affect cell proliferation induced by IGF-1

A: plasmid map of survivin overexpression lentiviral vector; B: IF staining showed survivin overexpression in E6 and E7 cells. C, D: IGF-1 induced cell proliferation in E6 and E7 survivin overexpression cell lines, no statistical significance was reached between groups. Two-way-ANOVA, n=3, bar graphs were shown as mean±SEM.

Methylene blue assay demonstrated that IGF-1 induced proliferation in both control vector and survivin overexpression vector-transfected cell lines. There was no statistical difference between control and survivin overexpression groups in neither the response to IGF-1 nor the baseline of proliferation. These results lead us to the conclusion that only minimum amount of survivin is required for cell proliferation. An excessive amount of survivin cannot drive cell proliferation.

g. Survivin expression in chronic inflamed human prostates.

We last analyzed Non-diseased and BPH human prostate specimen to confirmed what we found in the mouse model. Interestingly, the survivin expression in human samples was not correlated with the proliferation level as demonstrated by ki-67 staining. Much more survivin+ cells comparing to ki-67 positive cells were found in the human samples. The geographic distribution of survivin+ cells suggested that survivin expressed in epithelial compartment in human specimens. This may because the sample we have were from aged men and they were already in chronic inflamed condition. Thus, this supported our idea that survivin may have different regulation and functions other than proliferative aspects in chronic inflammation.

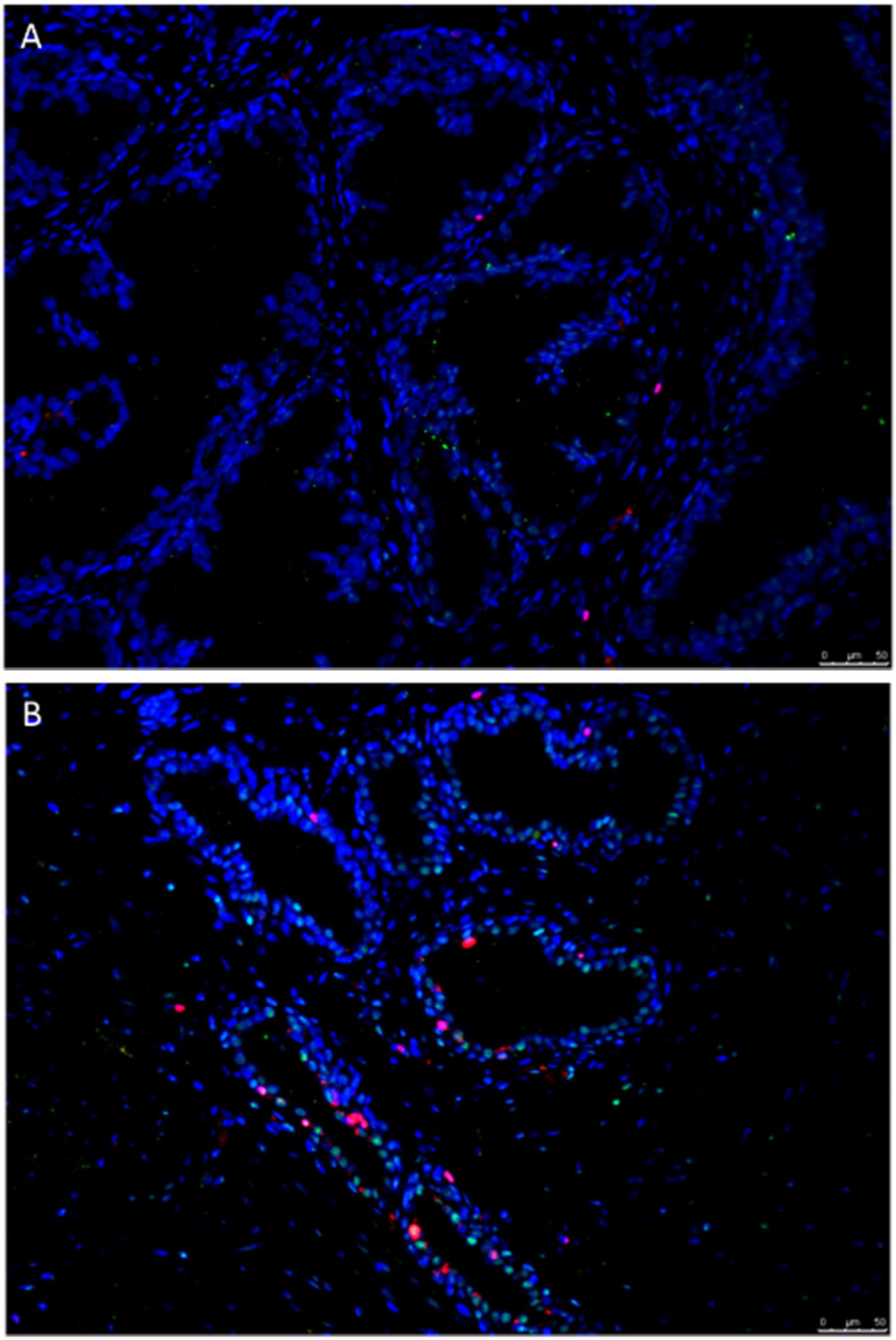


Fig 4.9 Survivin positive cells were not proliferating in human prostate specimens

IF staining of survivin and Ki-67 in human specimens. Survivin (Green) and Ki-67 (Red) was shown in non-diseased (A) or BPH (B) samples.

C. Discussion

Numerous risk factors of prostate inflammation have been identified, such as depression, smoking, high-fat diet, etc. but the mechanisms underlying these risk factors are still unknown. An increased incidence of prostate inflammation is found along with increasing age of human, suggesting the persistence of chronic inflammation in the prostate. Excessive cell proliferation is a key feature of inflammation, but mechanism driven cell proliferation during inflammation is still controversial.

Survivin is an essential component of mitosis. It participates in the assembly of CPC and cleavage furrow. It begins to be expressed at S phase and is fast degraded after mitosis [190-192]. In addition, survivin is also an important regulator during tissue development, especially in apoptosis-dependent tissue development such as limb and thymus. Survivin KO mice is embryonic lethal. These make survivin an interesting regulator during tissue repair and regeneration. In prostate epithelial cells and other cells, survivin's half-life is about 30-60min [190], suggesting a constant expression of survivin requires sustained activation of survivin mRNA transcription or a modulation of survivin degradation. By loading BrdU to animal 2h before sacrifice, we discovered a major overlap of survivin and BrdU staining in prostate epithelial cells of inflamed

animals, suggesting a strong proliferative trait in survivin positive cells. However, whether it is proliferation that drives survivin expression or survivin expression is an event independent of proliferation is debatable. BrdU incorporation only occurs in S phase of cell cycle, while survivin protein stability is increased by cyclin-B1-CDC2 complex at G2/M phase and is fast degraded after telophase of mitosis [190, 191]. Given that, a survivin+/BrdU+ epithelial cell was in its S phase when we loaded the BrdU. After 2 hours, it was in its M phase with survivin expression at the time we fixed the tissue. If so, this double-positive cell went from S phase to M phase within 2 hours, while taking into considering that G2 phase is usually 30% of the whole cell cycle which may last 18-24h, this may be an impossible assumption. Thus, it is likely that survivin was already expressed at the time BrdU is incorporated, and it may serve not only a component of mitosis but also a license of cell proliferation. Our apoptosis analysis showed no difference in cell apoptosis upon to survivin inhibition, suggesting anti-apoptosis is not the primary function of survivin in the inflamed prostate. However, more experimental evidence need to be provided before any conclusion can be made.

Inflammation has been identified as one of the top risk factors of cancer initiation. Known as a cancer marker, survivin induced by inflammation in prostate may suggest a potential correlation between inflammation and prostate

cancer initiation. Although expression level may varies, survivin expression level was much higher in prostate cancer cell lines such as PC3, LNCaP and Du145 than that in non-cancerous cell lines E6 and E7. It has been shown that survivin is required by cancer cell since survivin knockdown may cause growth inhibition and apoptosis both *in vitro* and *in vivo* in cancer cell lines or cancer tissues [193-195]. In addition, survivin overexpression can be used as a diagnostic cancer marker and is associated with poor prognosis outcome in some human cancers [196-198] .

There are also a few studies showing that survivin overexpression is necessary for cancer initiation [199] or can increase cancer initiation risk [200]. However, the mechanism underlying how survivin is involved in cancer initiation remains unclear. In our inflammation model, survivin was induced along with the increase of cell proliferation and was co-localized with proliferation markers at the beginning stage of acute inflammation (0-3 days after inflammation began). This suggests that survivin is induced in demand of proliferation in prostate epithelial cells. In a later stage of acute inflammation (5-7 days after inflammation began), the proliferation level in prostate dropped down while survivin expression level was maintained in a plateau phase. More important, at day 5-7 after inflammation, survivin positive epithelial cells were less proliferative since

survivin and BrdU overlap was reduced. This suggested that an inflammatory environment forces survivin expression and prevents it from cell cycle-regulated degradation. Several inflammatory factors or growth factors, such as IL-1β, IGF-1, PDGF, EGF, TGF-β, etc. [201-203] has been identified as regulators of survivin expression in different tissues. However, the regulation pathway of survivin during prostate inflammation is still unknown. We demonstrated that inflammation induced survivin expression and epithelial hyperplasia shared the IL-1/IGF-1 pathways, and both can be inhibited by the IGF-1 receptor inhibitor, IL-1R1 deficiency or the survivin inhibitor.

In chronic inflammation animals, we observed a comparably high level of survivin but low proliferation rate, suggesting that survivin's function in chronic inflammation was largely independent of proliferation. We found a high expression level of survivin in human samples in both non-diseased and BPH group comparing to that in mice, while the ki-67 level in those groups varied. Meanwhile, we observed inflammation in both groups. Since it is impossible to know when the inflammation begins in prostates of these patients, we postulate that they are chronic inflamed due to their age and known epidemiology studies. Thus, the human results also fitted for our hypothesis that survivin in chronic inflammation may have functions other proliferation. In addition, overexpression

of survivin in cancer specimens was associated with proliferation, suggesting other mechanisms may be involved in the re-start of cell proliferation in survivin overexpressed cancer cells.

In summary, we showed that IGF-1 induced survivin expression in inflamed prostate epithelial cells is necessary for inflammation-induced cell proliferation and epithelial hyperplasia. Most survivin positive epithelial cells were proliferating at 3 days post inflammation. The persistence expression of survivin in the chronic inflamed prostate may be associated with prostate cancer initiation, but the mechanisms involved require further study.

Chapter 5 Role of survivin in prostate progenitor cells

A. Introduction

Epithelial stem cells are crucial for maintaining the integrity of epithelium and epithelial hierarchy through producing new terminally differentiated epithelial cells. Deficiency of epithelial stem cells may cause epithelium disruption and inflammation. Prostate epithelial stem cells have been identified in both basal and luminal compartment to maintain the tissue homeostasis in physiological condition. During pathological condition such as inflammation, a basal to luminal differentiation may serves as a major route to maintain epithelium structure. Several markers, including Epcam, CD44, CD49f, CD133, CK5/14, ABCG2, Sca-1, CD117 and SCF [204-206], have been introduced to characterize prostate stem cells. Other strategies, for example, Hoechst 33342 exclusion and BrdU labelling retaining are also applied based on the slow-cycling and DNA dye exclusion characteristics of prostate stem cells. Although the prostate stem cells have been shown to be capable for proliferation and differentiation in *in vitro* and *ex vivo* studies, their behaviors and regulation pathways *in vivo* are largely unknown.

Survivin is a dual-functioning protein which can bind to XIAP to inhibit smac/diablo activation and thus inhibits caspase cleavage and apoptosis. It can

also bind to Aurora B and microtubule to regulate mitosis. Survivin has been shown as an indispensable part for mitosis since survivin knocking out leads to mitosis catastrophe. Limited facts are known about the role of survivin in regulating stem cells behaviors. Survivin is majorly studied in tumor stem cells since it is a cancer marker in human. In non-tumor tissues, survivin may serve as a stem cell keeper that helps to maintain the undifferentiated stage of stem cells. Knocking out survivin in endothelial cells results in embryonic lethal. Diffuse hemorrhages are observed at E9.5 together with heart failure and endothelial cells abnormality, suggesting dysfunctions of endothelial progenitor cells differentiation and cardiomyocyte development [207] . Survivin is partially co-localized with stem cell marker CK15 in the canine hair follicle, suggesting its important role in maintaining hair follicle stem cells [208]. β-catenin/TCF4 regulated survivin expression is necessary for maintaining corneal epithelial stem cells in wound healing model [209, 210]. In skins, survivin expression maintains the viability of epidermal stem cells [211]. It is expressed in a subpopulation of keratinocyte in the basal layer of human skins [212]. In rat testis, survivin can be regulated by SCF [213]. This implies that survivin expression may correlate with c-kit, a known stem cell marker and the receptor of SCF, in testis. Thus, survivin may play important roles in spermatogenesis and maturation. In the prostate, survivin was not expressed in non-diseased conditions but is overexpressed in

BPH and prostate cancer specimens [214]. Survivin is also identified in neuroendocrine cells, a highly plastic population, which may help them evading from apoptosis and contributing to cancer initiation [215]. In summary, survivin expression in stem cells may strengthen their anti-apoptotic and proliferation abilities to maintain their population and makes them a potential target of cancer initiation.

The role of survivin in prostate epithelial cells during inflammation is understudied; here we propose that survivin induced by inflammation in prostate epithelial cells is expressed in a specific population of epithelial cells. This population can rapidly response to noxious stimuluses such as inflammatory factors to maintain the homeostasis of prostate epithelium. In addition, survivin can affect prostate epithelial stem cell behaviors during inflammation.

B. Results

a. Survivin affects epithelial cells phenotype transition in vitro

We previous demonstrated that survivin shares the same regulation pathway with epithelial cell proliferation, epithelial hyperplasia, and PEPCs expansion during inflammation in the prostate. Survivin is known as a key regulator during apoptosis directed tissue development. The outburst of survivin during inflammation under the regulation of a development signaling pathway suggested a potential role of survivin in stem cell regulation. To test this hypothesis, we first analyzed the expression of survivin in sphere culture. We cultured survivin positive E6 and E7 cells in anchorage-independent condition for 14days to form spheres. The spheres formed were collected and were subjected to immunofluorescence analysis. To test the potential phenotype transition during sphere formation, we stained adherent-cultured E6 and E7 cell for CK5 and CK8 expression. Both E6 and E7 cells were dominantly basal phenotypes since they are CK5 strongly positive and CK8 weakly positive (Fig 5.1A, B). After anchorage-independent culture, the sphere formed by E6 or E7 cells were dominantly CK8 positive with weak CK5 expression in the outer layer of spheres (Fig 5.1E,F), suggesting a basal to luminal phenotype transition during sphere culture. Interestingly, although E6 and E7 cells were both survivin positive cells, we failed to detect survivin expression in spheres (Fig 5.1G). Since survivin is

tightly correlated with cell proliferation, we would not be able to detect survivin if the spheres formed by E6 and E7 cells were derived from cell aggregation but not cell proliferation. To test this possibility, we introduced labeling retaining strategy in sphere culturing. The E6 and E7 cells were cultured with 3.1ug/ml BrdU for 30days to label all cells with BrdU. The BrdU labeled cells were then applied in sphere formation assay in the BrdU-free medium. The BrdU in proliferating cells will be diluted by cell dividing while non-proliferative cells will have stronger BrdU staining. Our results showed different staining intensity of BrdU in a single sphere (Fig 3.1 G), suggesting different proliferation rates in cells formed a sphere. Thus, the loss of survivin during sphere formation was likely to be an event independent of proliferation.

b. Phenotype analysis of survivin positive epithelial cell *in vivo*

Our *in vitro* data suggested survivin may participate in epithelial cells phenotypes regulation. Thus, we then analyzed the phenotype of survivin positive epithelial cells in our mouse prostate inflammation model by staining survivin with basal cell marker CK5 or P63, or with luminal cell marker CK8. In the inflamed prostate, survivin was expressed in both basal cells (Fig 5.2 A, B) and luminal cells (Fig 5.2 C). This indicated that both luminal and basal compartments can response to inflammation through cell proliferation. The

percentage of basal survivin positive epithelial cells to total survivin positive epithelial cells was elevated by 2 folds in 3 days' inflamed group comparing to non-inflamed control (Fig 5.2 D), suggesting a luminal to basal shift of survivin expression during inflammation.

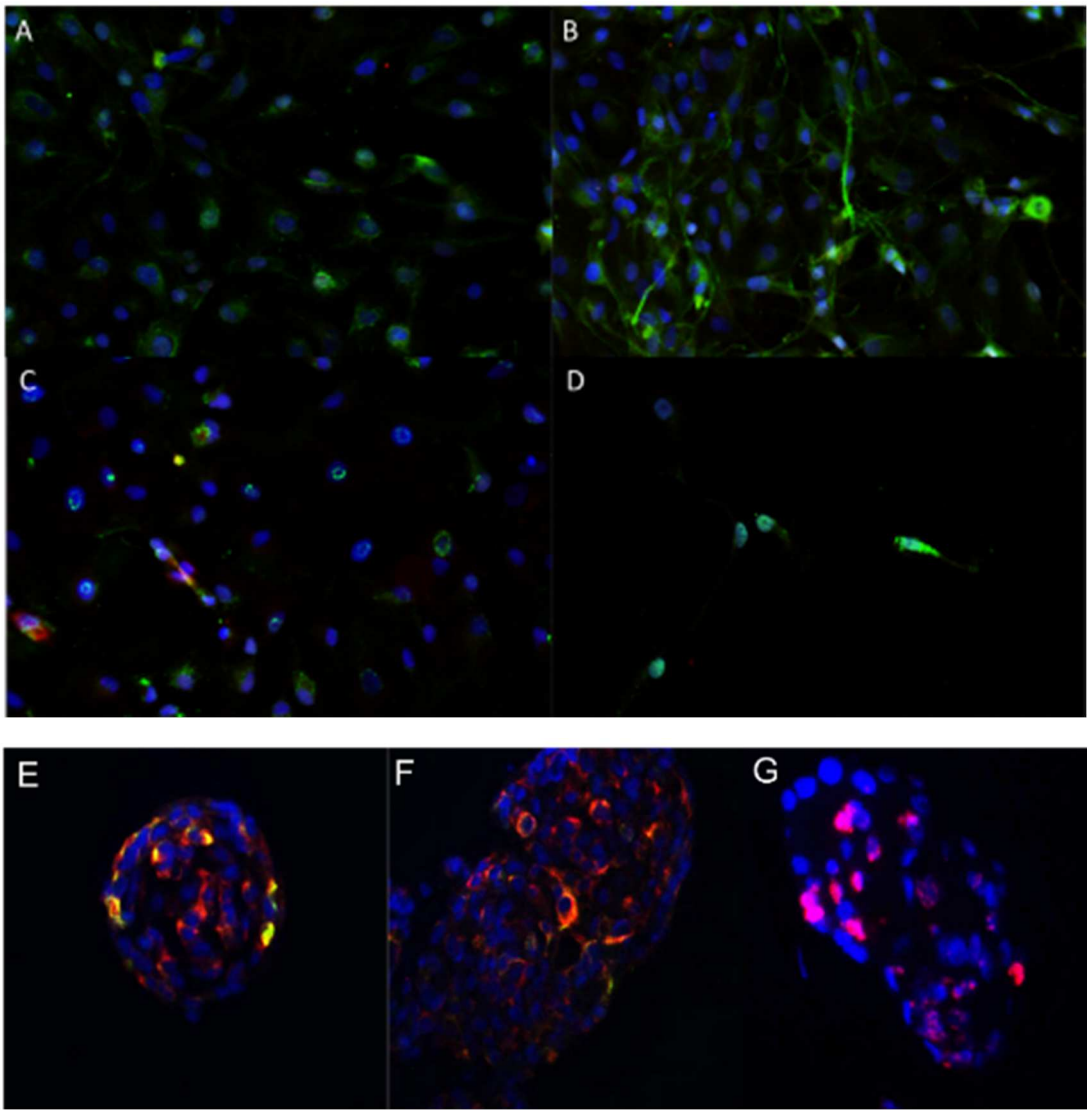


Fig 5.1 Changes of survivin and cytokeratin expression in prostate epithelial cell lines during sphere formation

IF staining showed cytokeratin and survivin expression in E6 and E7 cells. A, B: E6 (A) and E7 (B) cells are CK5 (Green) strongly positive /CK8 (Red) weakly positive in adherent culturing. C, D: E6 (C) and E7 (D) cells are survivin positive (Green) /CK8 (Red) weakly positive in adherent culturing. Nucleus was stained using Hoechst 33342 (Blue); E, F: spheres formed by E6 (E) and E7 (F) cells are CK8 (Red) strongly positive. CK8 (Green) only expressed in certain cells. G:

Sphere formed by E7 cells are survivin (Green) negative. Nucleus was stained using Hoechst 33342 (Blue). Label-retaining assay using BrdU (Red, in fig G) shows different proliferation rate of cells in spheres (G).

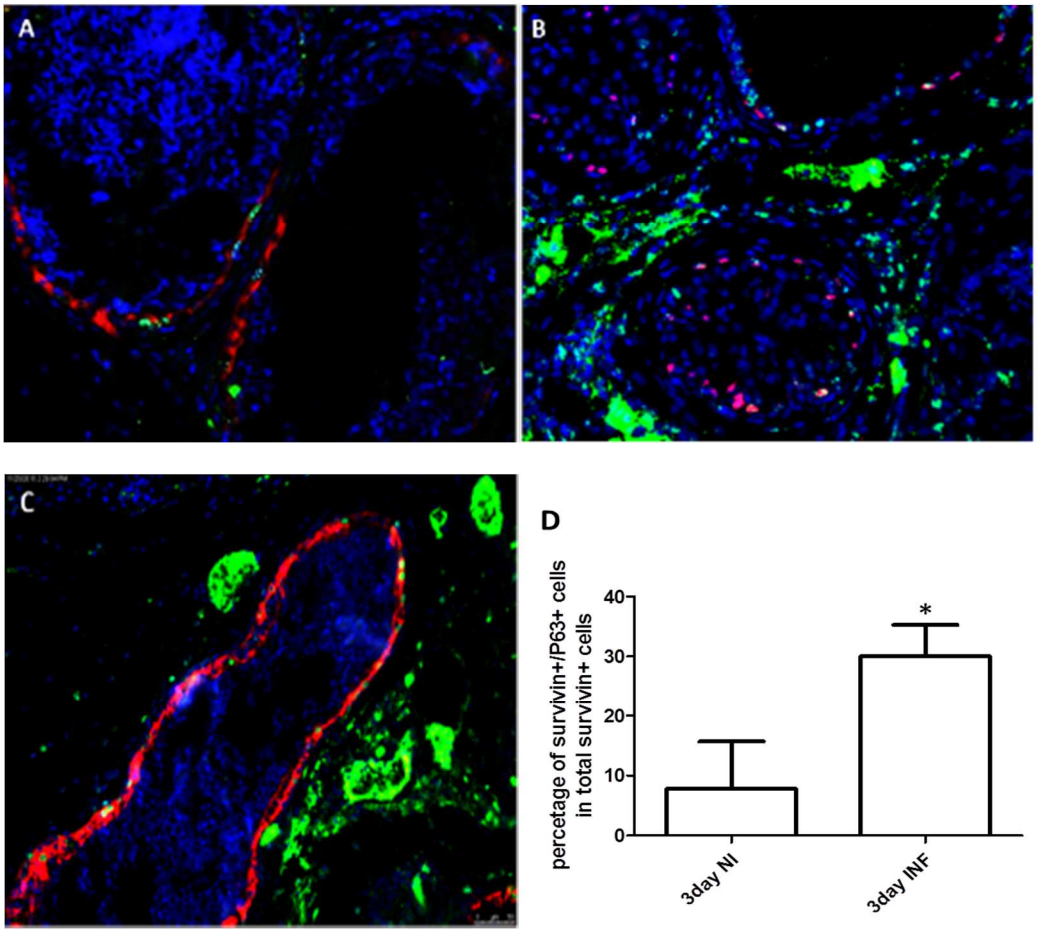
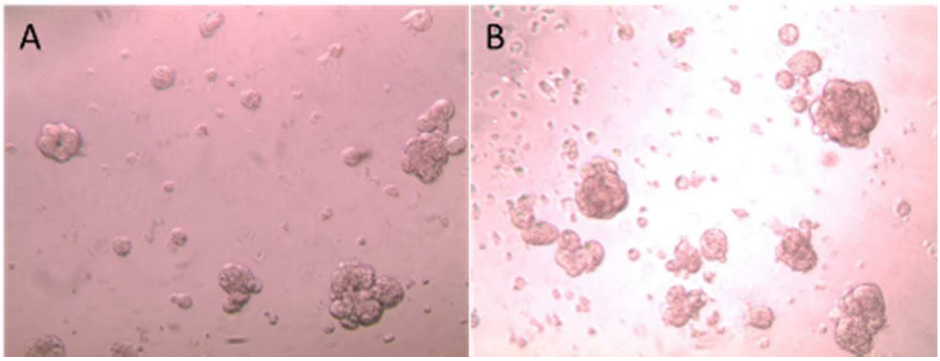
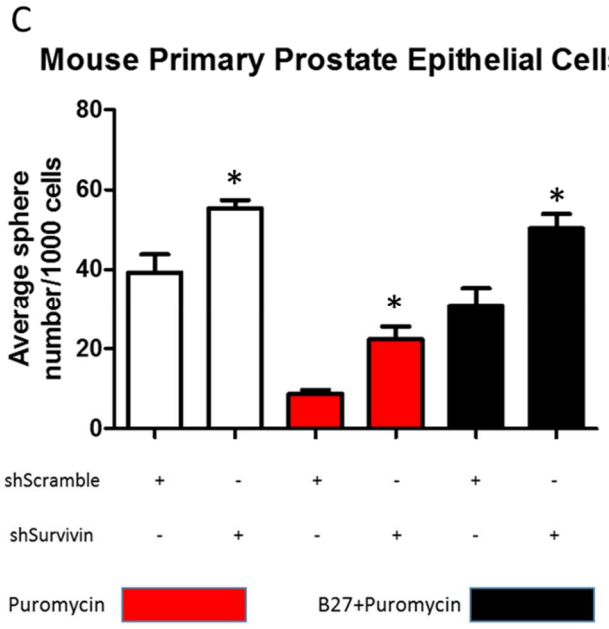


Fig 5.2 Phenotype analysis of survivin positive epithelial cells in prostate inflammation model

A: co-staining of survivin (Green) and basal cell marker CK14 (Red); B: co-staining of survivin (Green) and basal cell marker P63 (Red); C: co-staining of survivin (Green) with luminal marker CK8 (Red); D: percentage of survivin positive basal cells of total survivin positive cells. *: $p<0.05$, student t-test, n=3-6, bar graphs were shown as mean±SEM.


A
B


C
Mouse Primary Prostate Epithelial Cells
Average sphere number/1000 cells
80
60
40
20
0
*
*
*
shScramble + - + - + -
shSurvivin - + - + - +
Puromycin
B27+Puromycin

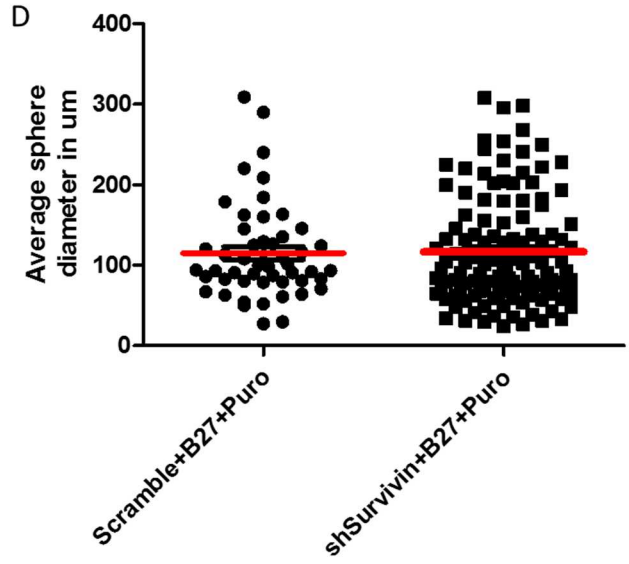

D
Average sphere diameter in um
400
300
200
100
0
Scramble+B27+Puro
shSurvivin+B27+Puro

Fig 5.3 Survivin knockdown increases sphere formation ability in primary cultured prostate epithelial cells

A, B: sphere formation from primary epithelial cells transfected with control (A) or survivin shRNA (B); C: quantification of sphere number in control shRNA and survivin shRNA groups, *: $p<0.05$ vs the shScramble sample in each group, respectively, t-test, n=4-6, bar graphs were shown as mean±SEM; D: quantification of sphere size, each dot represents the diameter of a single spheres, no static significance was reached, t-test.. Red line indicates the median sphere size.

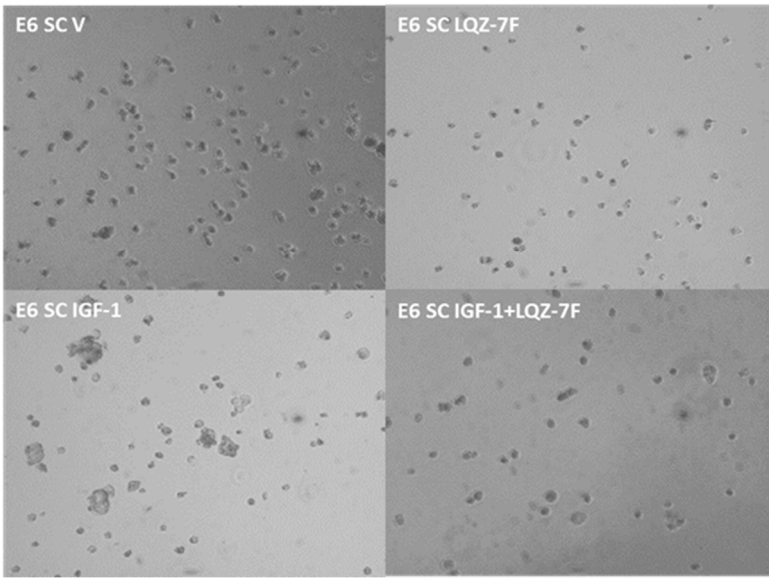
c. Survivin inhibition increases sphere formation

To further evaluate the correlation between survivin and stem cells, we knocked down survivin using lentiviral vectors based survivin shRNA in E6, E7, and primary cultured prostate epithelial cells and tested their sphere formation abilities. We transfected the primary cells with lentiviral particles for 24 hours and then applied these cells in sphere formation assay with/without puromycin or nutrition supplement B27 treatment. Transfection of survivin shRNA increased the number of spheres formed by primary epithelial cells with/without the presence of B27 supplement or antibiotic puromycin selection, suggesting this effect is not nutrition dependent or cause by transfection efficiency (Fig 5.3 A-C). Using sphere size analysis, we did not find any difference in the sphere size distribution (Fig 5.3 D), suggesting that survivin knockdown may not affect cell-aggregation. In E6 and E7 cells, we transfected the cells with lentiviral particles and cultured them in medium with puromycin for more than 30days. The positive clone were collected and passaged as a pool. The cells were then used for sphere formation assay combined with IGF-1 or LQZ-7F (1um) treatment. Similar results were obtained as those in primary cells. IGF-1 treatment significantly increased the sphere formation in E6 and E7 cells. Survivin shRNA increased the baseline sphere formation in non-IGF-1 treated groups. IGF-1 treatment in survivin shRNA groups still increased sphere formation, while the fold change of

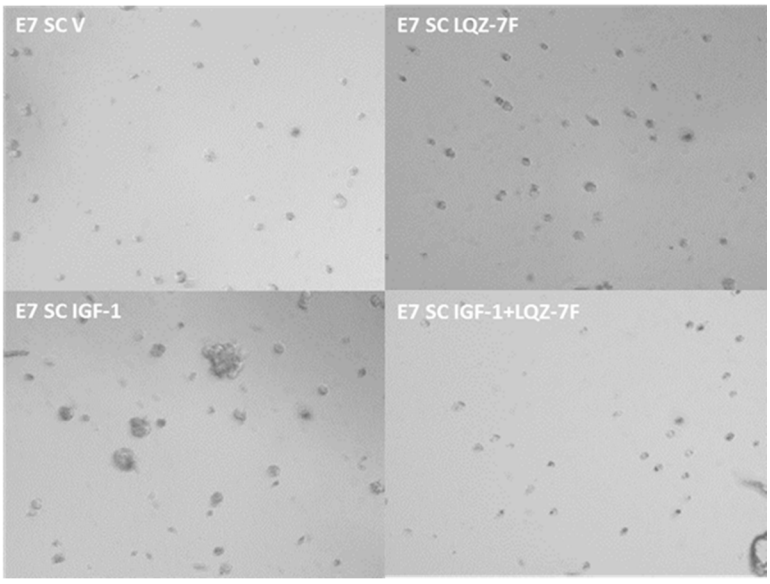
sphere in survivin shRNA group was reduced compared to that in control shRNA group (Fig 5.4).

Little is known about the role of survivin in prostate stem cell differentiation. Survivin is mostly known as a positive regulator of stem cell or is co-localized with stem cell markers in some tissues. Overexpression of survivin has been shown to be associated with the expression of stem cell markers such as c-kit. Survivin is critical in bone marrow cell differentiation, such as T cell maturation. However, in the prostate, our results showed that survivin was a negative regulator of anchorage-independent cell growth in prostate epithelial cells. This suggested that survivin may have inhibitive effects on prostate epithelial stem or progenitor cells. Our survivin staining in spheres also supported this since survivin was negative in spheres.

A


E6 SC V
E6 SC LQZ-7F
E6 SC IGF-1
E6 SC IGF-1+LQZ-7F

B


E7 SC V
E7 SC LQZ-7F
E7 SC IGF-1
E7 SC IGF-1+LQZ-7F

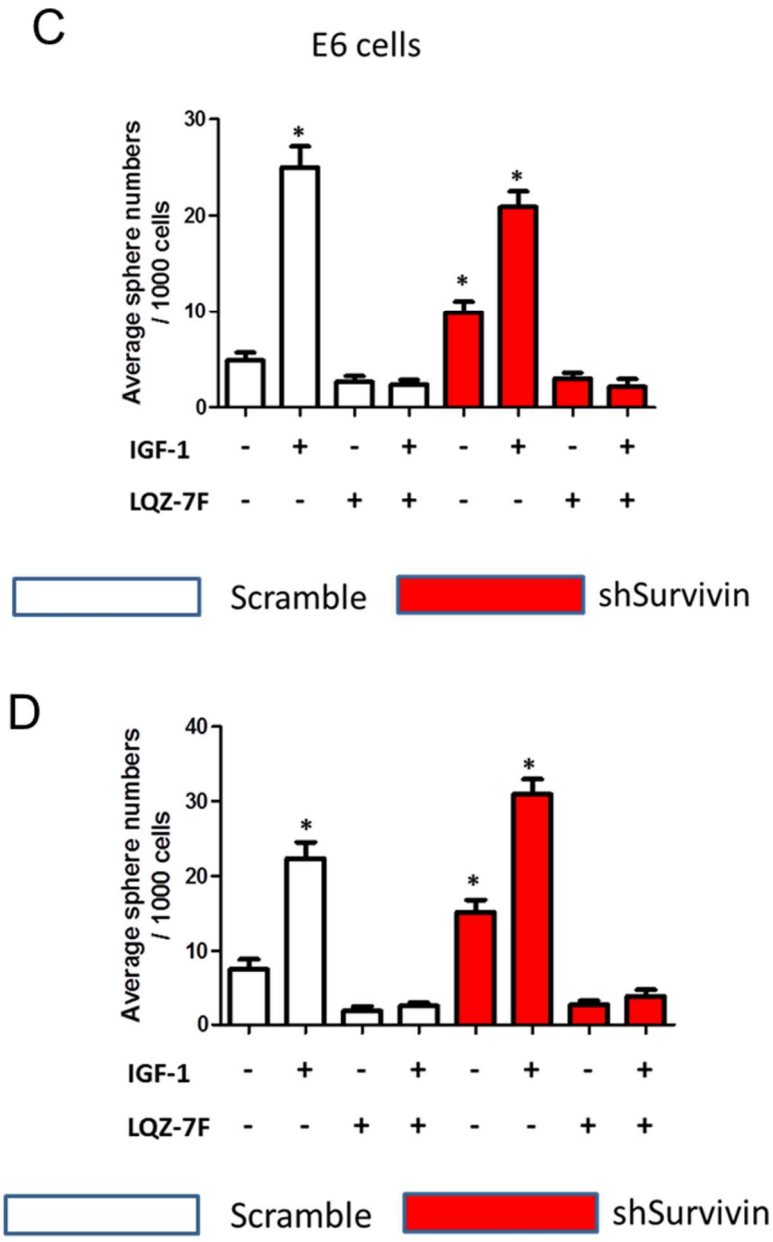


Fig 5.4 Survivin knockdown increased sphere formation in E6 and E7 cells

A, B: sphere formed by E6 (A) or E7 (B) cells treated with scramble shRNA, IGF-1 or LQZ-7F;

C,D: quantification of sphere numbers formed by E6 or E7 cells transfected with scramble or survivin shRNA, *: $P<0.05$ vs Scramble/IGF-1(-)/LQZ-7F(-) group, One-way ANOVA, n=5, bar graphs were shown as mean±SEM.

d. Dual labeling retaining assay in prostate inflammation model

The PEPCs expansion observed during inflammation suggested the participation of stem or progenitor cells in prostate epithelial hyperplasia. Unfortunately, we cannot directly trace PEPCs differentiation *in vivo* during inflammation since the multi-markers tracing techniques are not available. To further evaluate the role of progenitor cells and the role of survivin in stem cell regulation *in vivo*, we adopted an alternative dual-labelling lineage tracing strategy in our mouse inflammation model.

Pregnant CD1 mice were injected with BrdU at embryonic day 16 (E16) when the prostate was in its first development peak that the ducts begin branching. All the proliferating cells at E16 were labeled by BrdU. The cells continue proliferating after E16 was not detected as BrdU-positive cells since the post-puberty development diluted the BrdU incorporated, while the cells only proliferate limited times since embryonic development maintained the BrdU until the adulthood and can be detected by immunofluorescence staining. The BrdU positive cells in the epithelial compartment were considered as stem cells since their slow-cycling traits and their self-maintaining during adulthood. The maintaining of epithelial homeostasis requires the elimination of defective epithelial cells and the production of new epithelial cells. This process happens

even in a non-diseased condition. The mechanisms involved in the maintaining of prostate epithelial homeostasis are only partly understood. Both basal and luminal compartments may have independent stem or progenitor cells populations to maintain their homeostasis in physiological conditions. However, whether these progenitor cells participate in the response to bacteria induced inflammation is unknown. Thus, we introduced a second DNA incorporation reagent 5-ethynyl-2′-deoxyuridine (EdU) to label all the progenitor cells which maintain the epithelial homeostasis. Although EdU has a similar structure as BrdU and they are both thymidine analogs, EdU can be detected using chemical-based assays with no cross-reactions with BrdU. We collected the male litters from the BrdU labeled pregnant CD-1 mice and injected them with EdU when they were 8 weeks old. We loaded 1 dose of Edu every day for 1 week to increase the chance of labeling stem or progenitor cells since the proliferation level in the prostate in physiological condition is low. The EdU injection was chased 1day before the bacteria instillation. The animals were inflamed and the prostates were collected for immunofluorescence analysis. Using this strategy, the prostate epithelial cells fall into 3 categories: 1. BrdU-positive epithelial cells, these cells remain slow-cycling state since embryonic stage and are considered as stem cells in the prostate; 2. EdU positive cells, these cells that are responsible for maintaining the homeostasis of the prostate in physiological

condition and their progenies are also EdU positive cells; 3, ki-67 positive cells, the cells proliferating during inflammation are ki-67 positive (Fig 5.5).

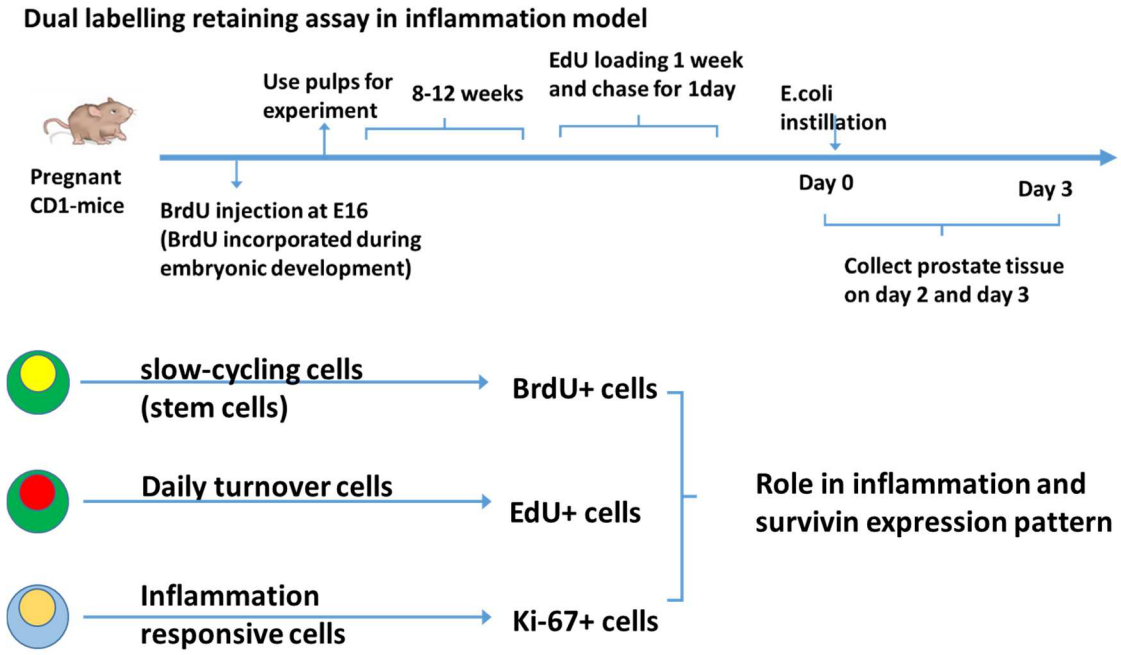


Fig 5.5 Dual labelling retaining strategy in prostate inflammation model

e. BrdU positive cells do not proliferate during inflammation.

Proliferation is the featured response to inflammation; we first examined the proliferation profile in BrdU positive cells. BrdU positive slow cycling cells were considered as stem cells in the prostate, surprisingly, we failed to detect proliferation in BrdU positive cells using ki-67 staining in animals 2 days or 3 days post-inflammation (Fig 5.6 A, B). This suggested that the slow-cycling stem cells did not proliferate during the inflammation, thus their contribution to epithelial hyperplasia may be minimum. In addition, co-staining of BrdU with CK5 shown that most BrdU positive cells were not CK5 positive (Fig 5.6 C). The slow-cycling cell did not reside in the basal compartment where the putative progenitor cells were supposed to be.

f. BrdU positive slow cycling cells are survivin negative

We then evaluated the survivin expression pattern in BrdU positive populations. Immunofluorescent staining showed that most slow-cycling BrdU positive cells in mouse prostate were also survivin negative in both non-inflamed and inflamed group. We only find a few double positive cells which may be caused by unspecific staining (Fig 5.7 A-C). In addition, the number of BrdU positive cells was not difference between non-inflamed and inflamed group (Fig 5.7 C). Since we already identified that survivin positive cell are also proliferating

during inflammation, it is also confirmed our previously finding that BrdU positive cells were neither survivin positive nor proliferative during inflammation. The results further supported that the BrdU positive slow-cycling cells did not response to inflammation.

g. EdU positive cells are survivin negative during inflammation

Edu labeled cells in prostate had proliferated at least once during the one week injection of EdU before the inflammation was induced. It is also possible that these Edu positive cells can further proliferate during inflammation to produce new epithelial cells. We found only 5 Edu+/Survivin+ cells in a total of 50 fields from 10 inflamed animals, this indicated that survivin was not expressed in Edu positive cell 2 or 3 days after inflammation (Fig 5.8 A-C). In combine, these results suggested that the survivin positive cells during inflammation are a cell population that response to inflammation and is independent of other putative progenitor cells.

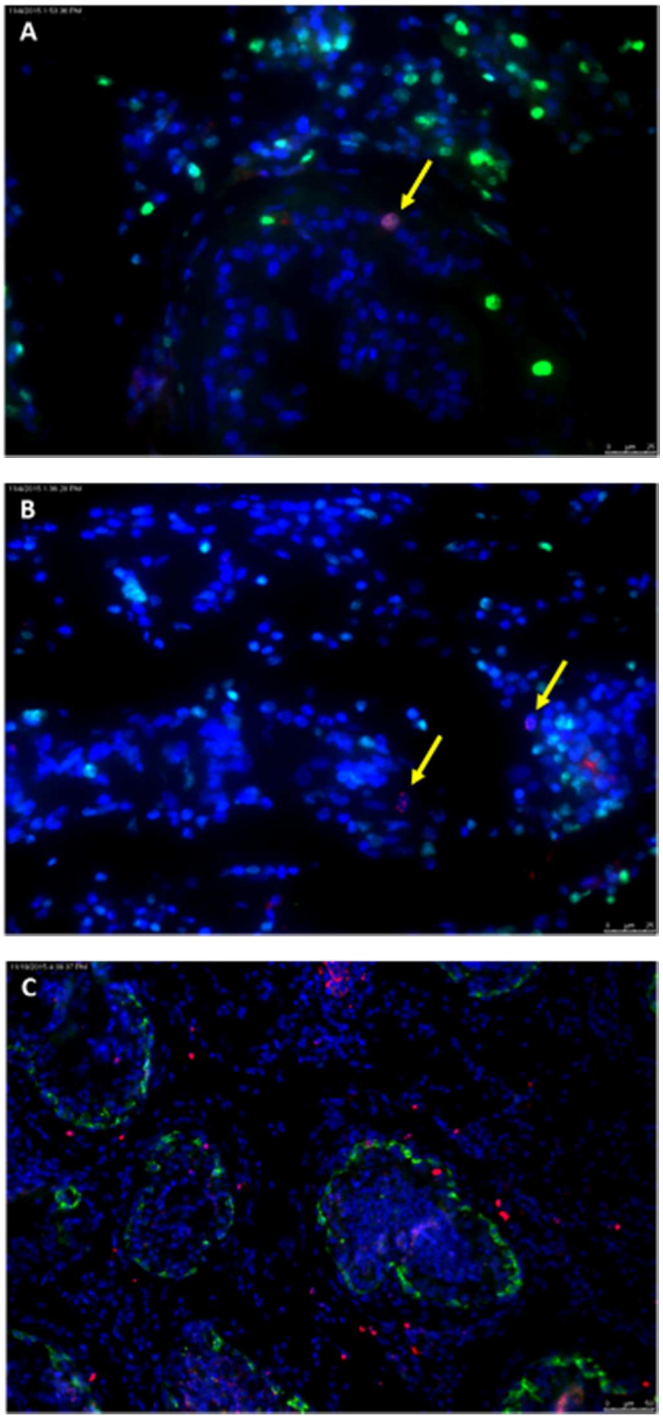
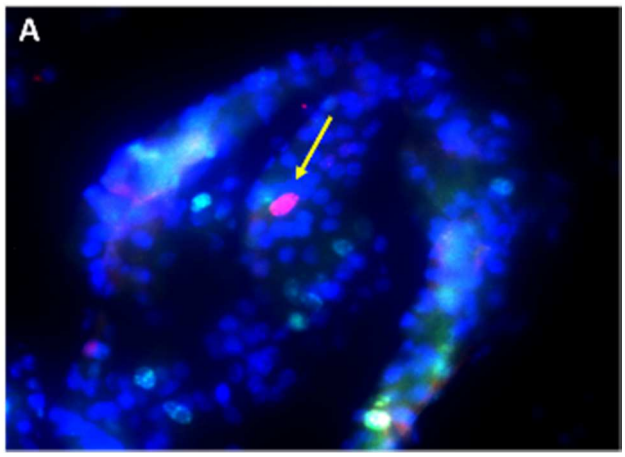
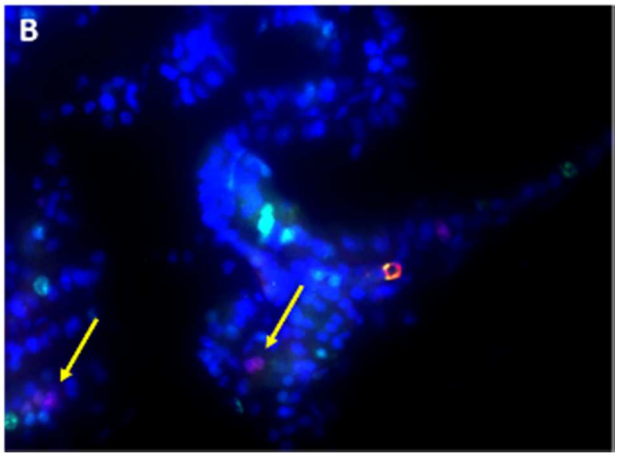


Fig 5.6 BrdU positive slow-cycling stem cells did not proliferate during inflammation

A, B: co-staining of BrdU (Red) and ki-67 (Green) in 2 days inflamed (A) and 3 days inflamed (B) animals; yellow arrows showed the BrdU positive cells, BrdU positive epithelial cells were ki-67

negative during prostate inflammation; C: BrdU (Red) and CK5 (Green) co-staining in 3 days inflamed animal.


A


B

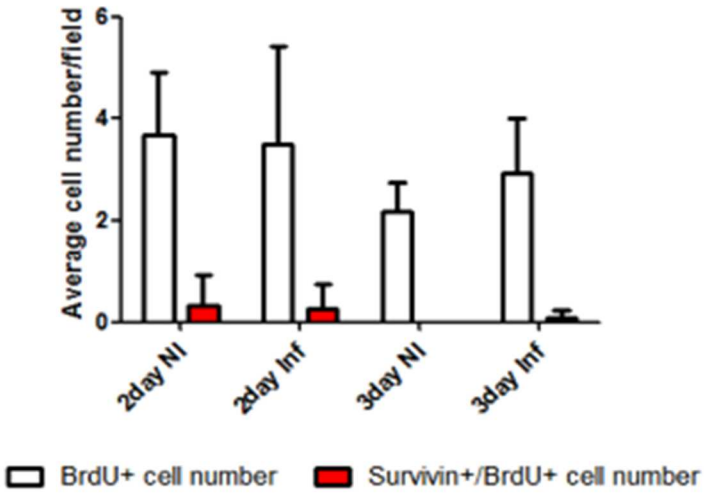

Average cell number/field
6
4
2
0
2day NI
2day Inf
3day NI
3day Inf
BrdU+ cell number
Survivin+/BrdU+ cell number

Fig 5.7 BrdU positive cells were survivin negative cells in mouse prostate

A, B: BrdU (Red) and survivin (Green) co-staining in 2 days inflamed (A) and 3 days inflamed (B) prostates; C: quantification of BrdU positive epithelial cells, and survivin+/BrdU+ epithelial cells. Data is presented as the number of cells/field, No-statistical significance was reached between groups, n=3-5, bar graphs were shown as mean±SEM.

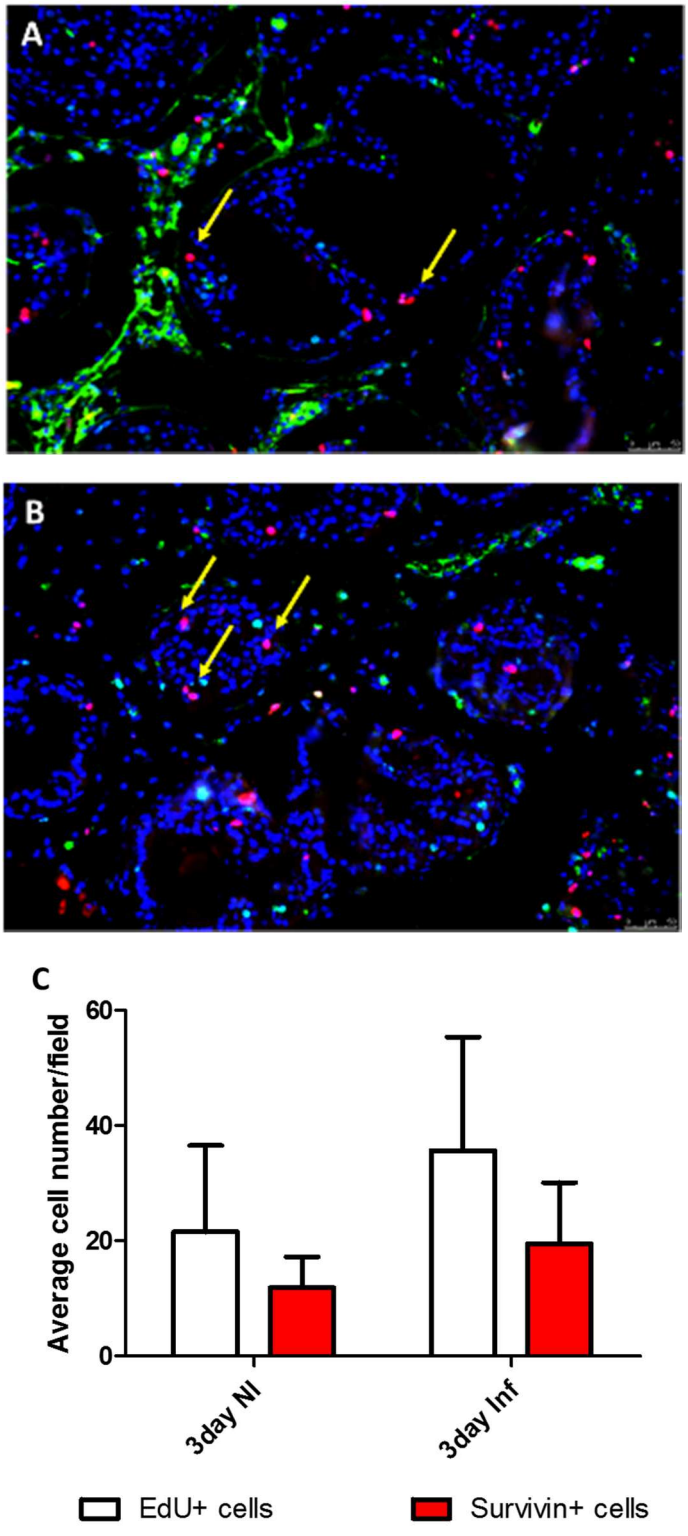

A
B
C
Average cell number/field
60
40
20
0
3day NI
3day Inf
EdU+ cells
Survivin+ cells

Fig 5.8 Edu positive cells did not express survivin during inflammation

A, B: EdU (Red) and survivin (Green) co-staining in 3 days non-inflamed (A) and 3 days inflamed (B) prostates; C: quantification of EdU positive epithelial cells, and survivin positive epithelial cells, data was presented as average number of cells per field. No dual positive epithelial cell were found in the epithelial compartment, no statistical significance was reached, Two-way ANOVA, n=3-7, bar graphs were shown as mean±SEM.

h. Dual labeling retaining assay in prostate re-growth model

Our results suggested that the slow-cycling BrdU positive cells remain "dormant" during bacteria-induced inflammation. The tissue regeneration pathways are activated to repair the damage caused by pathogens. This leads to our postulation that the slow-cycling stem cells are not activated because the damage caused by inflammation is still tolerable by the tissue. Other low-level stem cells or progenitor cells may be capable of the tissue repair tasks. We showed that survivn can regulate cell stemness and it is exclusively expressed in a population of epithelial cells which respond to inflammation. Thus, we then decided to investigate whether survivin also play important roles in other more intense tissue regeneration models. The prostate is a hormone-dependent organ, the maintenance of prostate epithelial structures are highly androgen-dependent. Ablation of androgen leads to the disruption of prostate epithelial structures and apoptosis of luminal cells which is androgen dependent. The prostate loses most epithelial cells and finally atrophy after long-term androgen ablation. Only the androgen independent epithelial cells may survive after androgen ablation.

Re-introduction of androgen in the prostate after castration induces re-constitution of epithelial structures and recapitulation of luminal cells. The tissue damage induced by androgen ablation is more intense than that induced by

inflammation since the prostate loses most luminal cells. This androgen-dependent tissue re-growth involves proliferation and differentiation of prostate progenitor cells; we investigated the expression pattern of survivin in this model. We adopted a modified dual-labelling tracing strategy for this purpose.

Pregnant CD-1 mice were labeled with BrdU at E16 as described previously. The male litters were castrated when 8 weeks old. 14days after castration, the litters were subjected to androgen treatment. Androgen capsules were implanted subcutaneously, and the capsules with 70% ethanol in PBS were used as vehicle control. EdU were loaded to the animal 6 hours after capsules were implanted and 1 dose/day until day 2 post-instillation. The animals were sacrificed 24 hours after the day 2 injection of EdU, or at 14 days post-instillation. The prostates were collected and were subjected to immunofluorescence analysis. The BrdU positive cells are slow-cycling cells that survive during castration period. The EdU positive cells are the cells response to androgen treatment and proliferate during the 3 days or 14 days re-growth time. The cells which are proliferating when the animals are sacrificed will be positive for proliferation marker ki-67 (Fig 5.9)

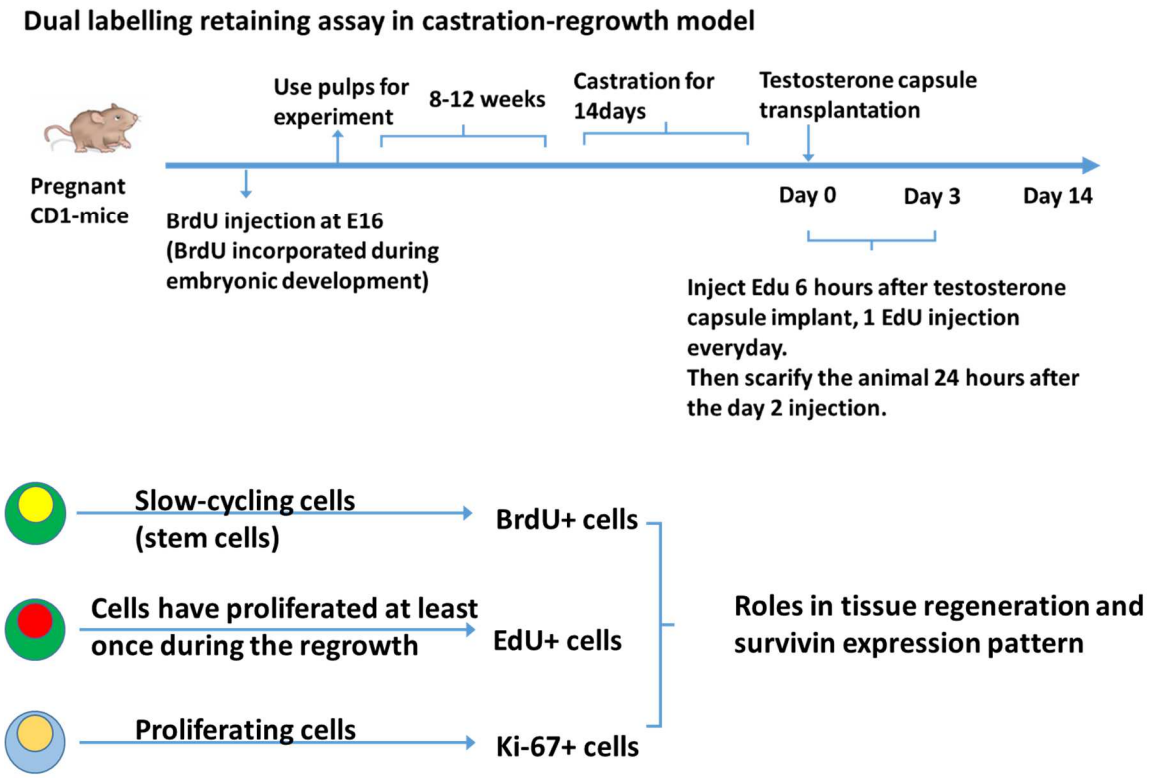


Fig 5.9 Dual labelling retaining assay in castration-re-growth model

i. BrdU positive slow-cycling cell proliferates in re-growth model

We first evaluated the survivin expression in BrdU positive slow-cycling cells in this androgen-re-growth model by co-staining of BrdU and survivin. The percentage of BrdU positive epithelial cells of total epithelial cells was increased by 4 fold in the testosterone group comparing to the vehicle group. The percentage of survivin positive cells of total epithelial cells was also increased by 6 folds in testosterone group (Fig 5.10 A-D). Although in a very rare case, we can find BrdU+/survivin+ cells only in 1 out of the 4 animals in the inflamed group, the BrdU and survivin were mutually exclusively presented in epithelial cells in both groups. Increased percentage of BrdU positive cells suggested that the slow-cycling stem cells were proliferated during the re-growth process. This also supported what we hypothesized previously that slow-cycling stem cells will be activated when the tissue damage excesses the repair threshold of other progenitor cells. The increase of survivin positive cells indicated that survivin was also involved in testosterone induced tissue regeneration.

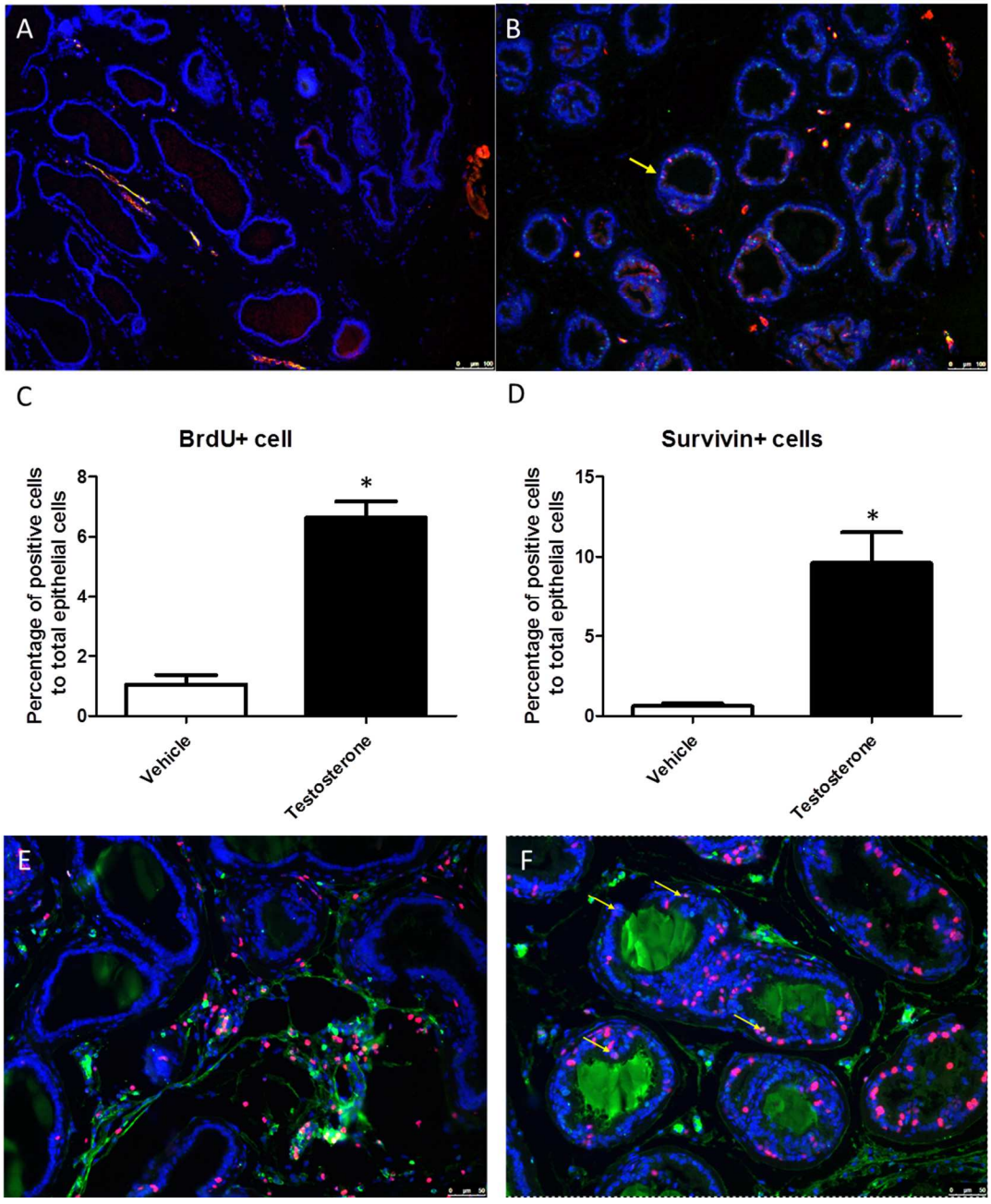


Fig 5.10 Increase of BrdU positive cells and survivin positive cells during testosterone induced tissue regeneration 3 days post testosterone capsule implantation

A, B: BrdU (Red) and survivin (Green) co-staining in 3 days vehicle (A) and 3 dayd testosterone (B) treated prostates, yellow arrow showed double-positive cells.; C, D: the percentage of BrdU positive epithelial cells (C), and survivin positive epithelial cells (D) to total epithelial cells, *:

P<0.05, t-test, n=3-4, bar graphs were shown as mean±SEM; E, F: BrdU (Red) and EdU (Green) co-staining in 3 days vehicle (A) and 3 days testosterone (B) treated prostates, yellow arrow showed double-positive cells.

To further support the idea that BrdU positive slow cycling cells were proliferative during re-growth phase, we co-stained BrdU and EdU in our model. BrdU+/EdU+ cells were located in the testosterone group (Fig 5.10 F), suggesting that BrdU positive cells had at least proliferated once during the 3 days testosterone treatment. We then evaluated the phenotype of BrdU positive cells in this re-growth model. Co-staining of BrdU and CK5 showed that BrdU positive cells were CK5 negative in vehicle group, while a few BrdU positive cells (0.4% of total epithelial cells) were positive for CK5 in testosterone group. There was no difference in CK5 positive cells percentage between groups (Fig 5.11 A-D)

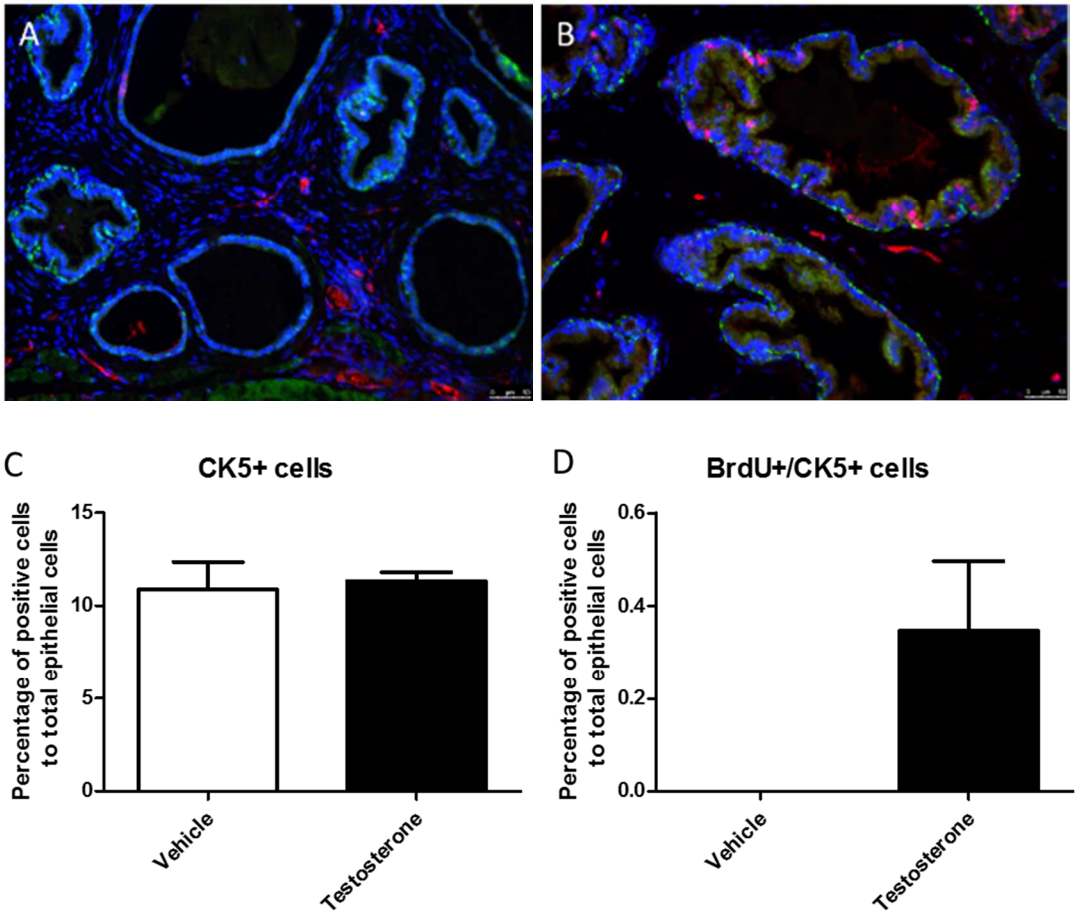


Fig 5.11 BrdU/CK5 co-staining in testosterone re-growth model

A, B: BrdU (Red) and CK5 (Green) co-staining in 3 days vehicle (A) and 3 days testosterone (B) treated prostates; C, D: quantification of the percentage of CK5 positive (C) and CK5+/BrdU+ cells (D) to total epithelial cells. n=3-4, no statistical significance was reached in C. No statistical work was performed in D since the number of positive cells in vehicle group is 0; bar graphs were shown as mean±SEM.

j. EdU positive cell proliferates limited times during re-growth

The next question for us to answer is the proliferation pattern of epithelial cells during the testosterone-induced tissue re-growth event. There are two possible proliferation patterns that the epithelial cells may exhibit. One is that some epithelial stem cells produce secondary progenitor cells. The secondary progenitor cells will produce terminally differentiated epithelial cells. The other possibility is that only a small population of stem cells continues producing new terminally differentiated cells. In the first case, we will detect ki-67 positive expression in most Edu positive cells since they continue proliferating, otherwise, the second case may be the dominate proliferation pattern during the re-growth phase. Co-staining of EdU and ki-67 in prostate 3days after testosterone treatment showed that both EdU positive epithelial cell and ki-67 positive cells were increased in testosterone group (Fig 5.12 A-D). Even in the vehicle group, around 4% of total epithelial cells were EdU positive, suggested that the castration created a highly dynamic microenvironment which promotes epithelial proliferation.

EdU+/ki-67+ cells were slightly increased in testosterone group. However, percentages in both groups were only 0.4-0.8 percent, much lower than a random distribution expectation (tested by chi-square, table 2). This suggests

that EdU positive cells were prone to be ki-67 negative in both groups, which supported our postulation about the second proliferation pattern of epithelial cells.

k. EdU positive cell are majorly survivn negative

Around 15% to 20% epithelial cells in testosterone groups were either survivin positive or EdU positive, however, less than 1% of total epithelial cells were positive for both. This suggested that at the time point that we sacrificed the animal, survivin was only expressed in those cells which had never proliferated during the 3 days' re-growth phase (Fig 5.13 A, B, D). In addition, we showed that survivin is negative in some highly proliferative regions in the vehicle group (Fig 5.13, C), suggesting that survivin expression in vehicle group was independent of cell proliferation.

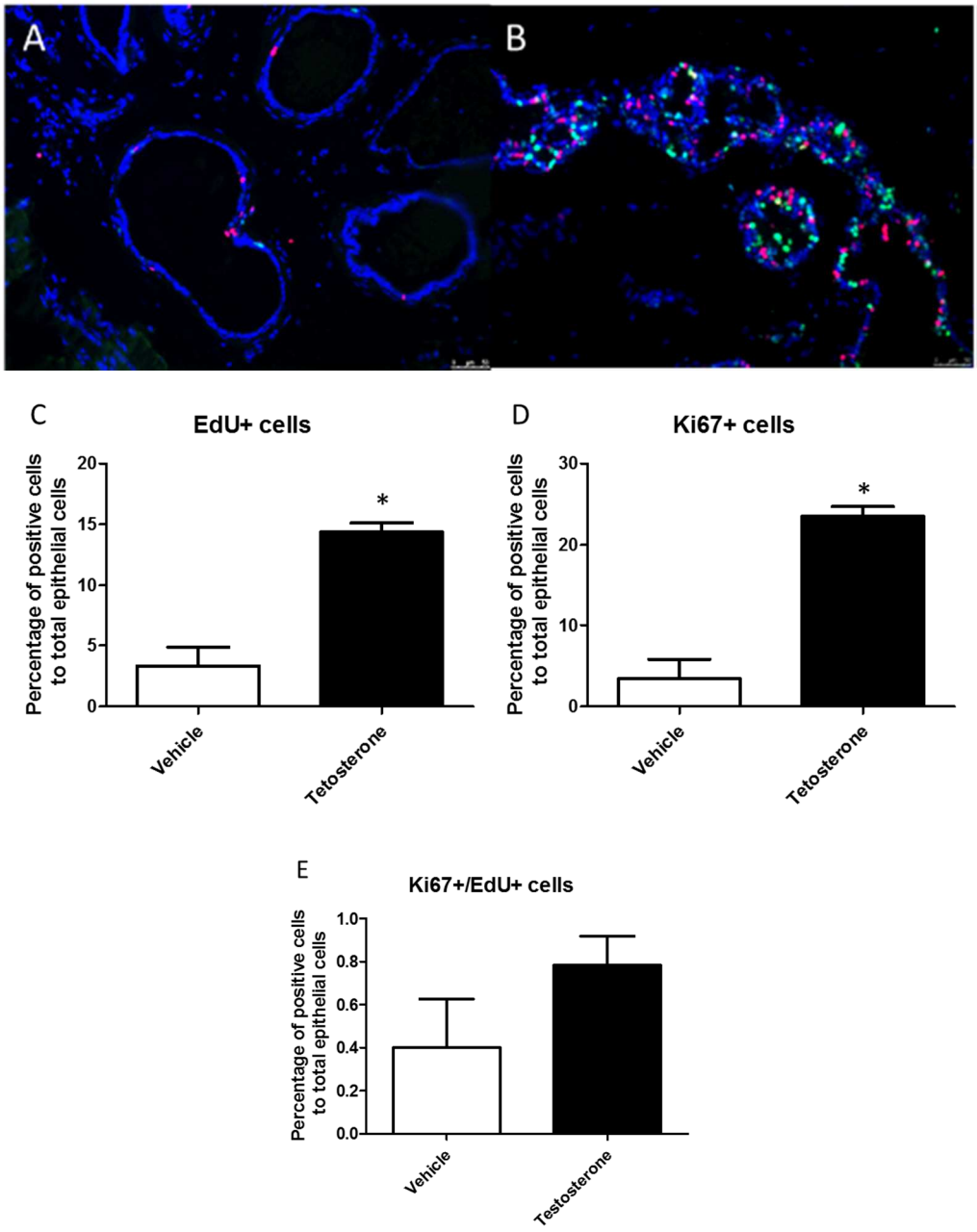


Fig 5.12 EdU and ki-67 staining in the prostate-re-growth model

A, B: EdU (Red) and ki-67 (Green) co-staining in 3day vehicle (A) and 3 day testosterone (B) treated prostates; C-E: quantification of the percentage of EdU positive cells (C), ki-67 positive cells(D) and double-positive cells (E) to total epithelial cells, *:$p<0.05$, t-test, n=3-4, bar graphs were shown as mean±SEM.

I. Time course analysis of survivin expression in re-growth model

Testosterone-induced regrowth reaches a plateau phase and prostates size stops increasing after 14days of testosterone treatment. We evaluated the survivin expression pattern at day 14 of testosterone treatment. The percentage of survivin positive cells and ki-67 positive cells decreased in day 14 testosterone group comparing to those in day 3 testosterone groups. The BrdU positive cell percentage showed no difference at day 14 compare to that at day 3. (Fig 5.14 A-G) This suggests that the epithelial proliferation was reduced and survivin expression was consistent with proliferation pattern in re-growth phase. BrdU positive slow-cycling cells also stopped proliferating after day 14 since their BrdU was not diluted and was still detectable, suggested that the slow cycling cell were important for re-growth, but not the maintenance of epithelial structure after re-growth phase.

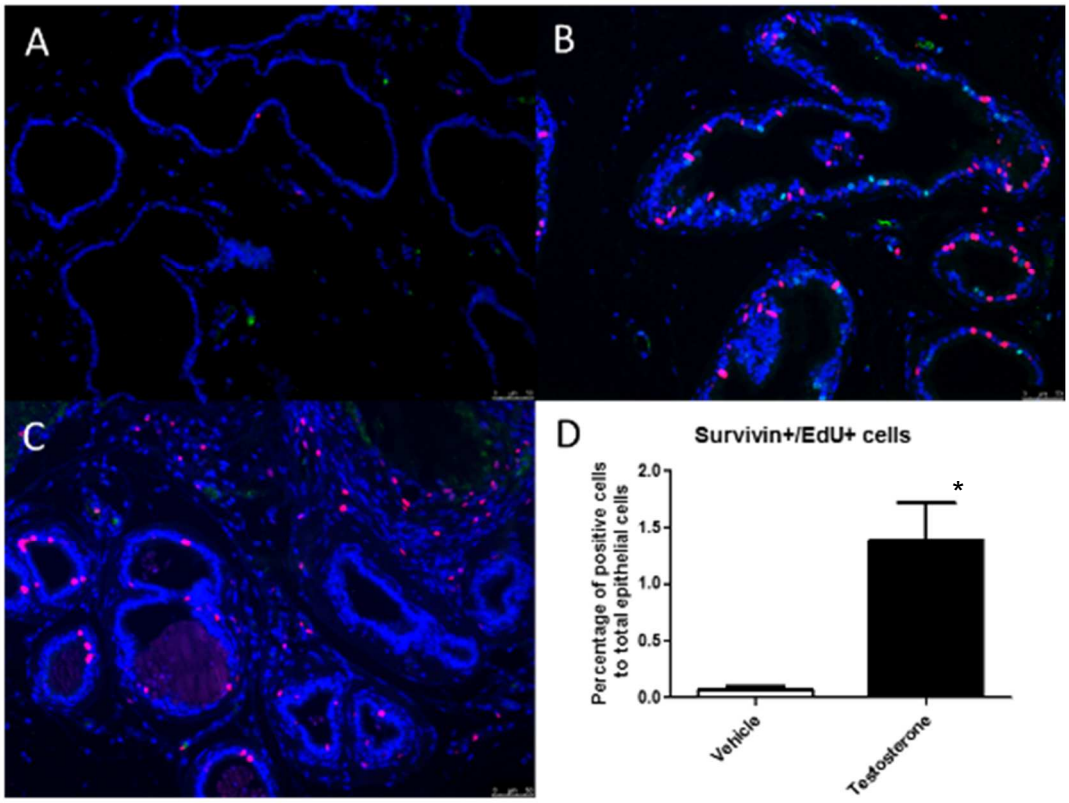


Fig 5.13 Co-staining of EdU and Survivin in re-growth model

A, B: Co-staining of survivin (Green) and EdU (red) in vehicle group (A) and testosterone group (B); C: survivin (Green) is negative in highly proliferative regions in vehicle group; D: percentage of survivin+/Edu+ cells in vehicle and Testosterone group, *: $P<0.05$, t-test, n=3-4, bar graphs were shown as mean±SEM.

Chi-square Vehicle

Chi-square, df	28.50, 1
P value	< 0.0001
P value summary	***
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

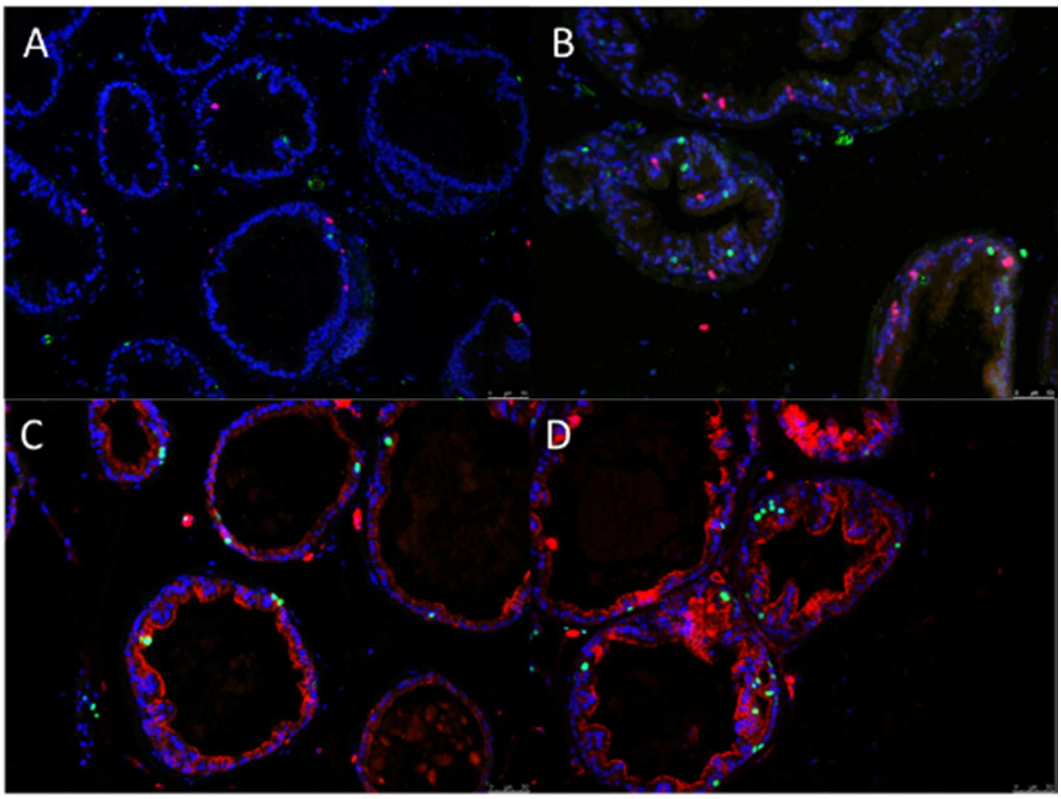
Data analyzed	Ki-67-	Ki-67+	Total
BrdU-	3481	101	3582
BrdU+	103	14	117
Total	3584	115	3699

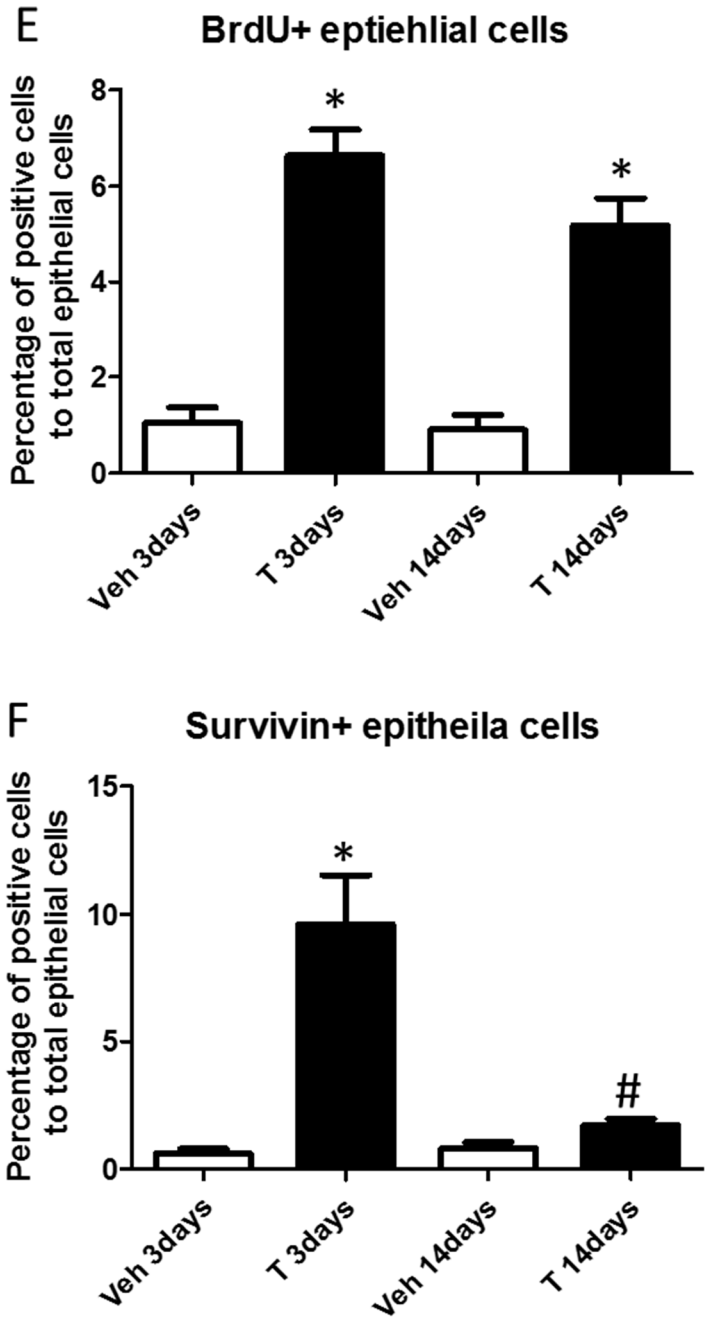
Chi-square Testosterone

Chi-square, df	186.5, 1
P value	< 0.0001
P value summary	***
One- or two-sided	Two-sided
Statistically significant? (alpha<0.05)	Yes

Data analyzed	Ki-67-	Ki-67+	Total
BrdU-	3878	1393	5271
BrdU+	835	47	882
Total	4713	1440	6153

Table 2 Chi-square test of the distribution of Ki-67positive cells and EdU positive cells in prostate regrowth model


A
B
C
D


E
BrdU+ eptiehlial cells
Percentage of positive cells to total epithelial cells
0
2
4
6
8
*
*
Veh 3days
T 3days
Veh 14days
T 14days
F
Survivin+ epitheila cells
Percentage of positive cells to total epithelial cells
0
5
10
15
*
#
Veh 3days
T 3days
Veh 14days
T 14days

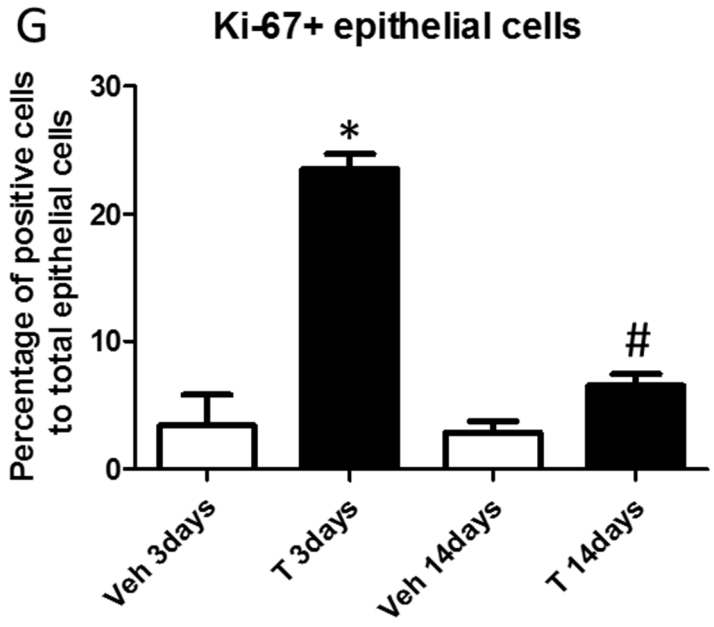


Fig 5.14 Survivin, ki-67 and BrdU staining pattern at 14days after testosterone treatment

A, B: survivin (Green) and Brdu (Red) staining in the vehicle (A) and the testosterone (B) groups after 14 days' tissue regrowth. C, D: ki-67 (Green) and Pan-CK (Red) in the vehicle (C) and the testosterone (D) groups after 14 days' tissue regrowth. E-G: quantification of the percentages of Brdu positive (E), survivin positive (F) and ki-67 positive (G) epithelial cells to total epithelial cells in tissue regrowth model, *: $p<0.05$ vs veh 3days, #:$p<0.05$ vs T 3days, One-way ANOVA, n=3, bar graphs were shown as mean±SEM.

C. Discussion

Inflammation is a common protective mechanism against exogenous pathogens or endogenous noxious substances. The cell-cell interaction between tissue cells and local immune cells (i.e. macrophages) recruits leukocytes and leads to secretion of a variety of cytokines into the tissue. Inflammation induces both tissue damage and tissue regeneration. The growth factors and cytokines released during inflammation create a proliferative microenvironment which promotes tissue regeneration by increasing epithelial cell proliferation and reconstitution of epithelial structures. The roles of epithelial stem cells in maintaining epithelial homeostasis have been shown in many tissues such as Lung, cornea and intestine epithelium [95-97, 102]. Prostate epithelial stem cells are also indispensable to maintain the epithelial structure. Loss of prostate epithelial stem cells induces tissue atrophy. For example, knocking out Dicer in mouse prostate results in decreased Sca-1+/CD49f+/Lin- prostate epithelial stem cells and prostate atrophy [116]. However, the role of prostate epithelial stem cells under inflammation condition is understudied.

Embryonic injection of BrdU labels some slow-cycling epithelial cells in the adult mouse prostates. These slow-cycling cells are considered as important stem cells which maintain the epithelial homeostasis [113]. Our study on this

slow-cycling cells showed that these cells were not activated during the acute phase of bacteria-induced inflammation since they were neither ki-67 positive nor survivin positive. On the other hand, the slow-cycling cells proliferated during the testosterone induced tissue regeneration. These results lead to our postulation that the tissue may utilize different populations of stem cells in response to different situations. The slow-cycling cells are considered as high-level stem cells which play important roles during tissue development. Thus, testosterone induced tissue regeneration may require the participation of these cells since castration ablates most luminal cells and probably also other low-level progenitor cells. In the inflammation condition, the majority of prostate epithelium remains intact despite being infiltrated by neutrophils or T cells. Thus, a lower level stem cells, in our case probably the PEPCs, can rapidly response to the stimulation from inflammation and start tissue regeneration process. Our analysis on BrdU positive cells at 14 days after testosterone treatment in the prostate re-growth model also supports this hypothesis. We found comparable percentages of BrdU positive cells in day 3 and day 14 samples in testosterone group. The BrdU labeling may last 6 passages in proliferating cells. Thus, the slow-cycling cells experience less than 6 cell cycles from embryonic day 16 to the end of testosterone treatment, otherwise, their BrdU level would be too low to be detected. Thus, it is reasonable for us to postulate that these slow-cycling cells

experience limited cell cycles and stopped proliferating at sometime between day 3 and day 14 of testosterone treatment. This suggests that the slow-cycling cells are responsible for the beginning of tissue regeneration since we observed a 4-folds increase of BrdU positive cells in testosterone treated group. BrdU positive cells ceased proliferation because a tissue balance was reached, or other progenitors, for example, their progenies, took over their tasks to further repair the prostate through day 14. However, we need a more delicate lineage tracing strategy to test this hypothesis.

The role of survivin in stemness regulation remains unclear. Most studies on survivin and stem cells focused on their tumor-related aspects. Survivin is thought to be a positive regulator of stemness since it is expressed in tumor stem cells [216-218]. A recent study about survivin in Lgr5 positive intestine stem cells shows that survivin is necessary for the maintenance of intestine epithelial stem cells [218]. These effects may be through maintaining a functional mitosis to support stem cell self-renewal and tissue homeostasis. The proliferative aspect of survivin is crucial not only to stem cells but also to all proliferative cells. Most terminally differentiated epithelial cells lose their proliferation traits while stem and progenitor cells still keep them. This makes survivin more important in stem

cell regulation. However, this is not enough to explicit all the roles of survivin in prostate epithelial cells.

Our results showed that survivin knocking down increased sphere formation in prostate epithelial cell lines and primary cultured prostate epithelial cells. This effect seems not related to P53 functions since E6 and E7 cells were P53 null and PRb null, respectively, and they had the similar response to survivin knocking out. On the other hand, we also showed that survivin knocking down reduces cell proliferation in E6 and E7 cells. This seems contradicted to the results in sphere formation assay since sphere formation assay evaluates the anchorage-independent proliferation. We postulated that survivin is necessary for differentiated cell proliferation in prostate epithelium. The sphere forming cells, which may be the stem/progenitor cells in prostate, do not require survivin for cell proliferation. This hypothesis was supported by our results. The spheres formed by E6 and E7 cells were survivin negative. This may suggest that survivin is not required by sphere formation. In addition, our labeling retaining assays indicated that the BrdU+ slow-cycling cells were survivin negative. This further supported our idea that survivin is not necessary for stem cell proliferation, or at least a very low level of survivin is enough for stem cell proliferation since our did not reach a 100% knocking down of survivin in either cell lines. Another possible

explanation is survivin may affect phenotype transition in prostate epithelial cells. The spheres formed by E6 and E7 cell were survivin negative. It is possible that the cells form spheres were already dormant at the time we harvested them and thus they did not require survivin for their proliferation. However, this is unlikely since our lab previously showed BrdU incorporation during sphere formation which suggested the sphere-forming cells were proliferative. It is also possible that sphere formation requires reduced expression level of survivin to promote the phenotype transition of sphere-forming cells. The E6 and E7 cells experienced phenotype transition from CK5 dominant type to CK8 dominant or in another word, a basal phenotype to luminal phenotype. The stratified spheres formed by E6 and E7 cells expressed CK5 in the outer layer and CK8 in the inner layer, suggesting a differentiation occurred and E6/E7 cells were directed to different sub-populations. This suggested a potential role of survivin in regulating prostate epithelial cell proliferation since loss of survivin and the phenotype transition occurred simultaneously during sphere formation.

In vivo analysis of survivin expression in epithelial cells also showed that during inflammation the survivin positive cells shifted from the luminal to the basal population. One potential mechanism underlying inflammation-induced epithelial hyperplasia is that the normal differentiation of epithelial cells is

disrupted and lots of epithelial cells are trapped in the intermediate type between basal and luminal cells. This failure of generating functional terminally differentiated epithelial cells further activates the epithelial proliferation that finally leads to hyperplasia. We observed an increase of survivin expression in response to inflammation. Thus this suggested that the overexpression of survivin in epithelial cells may block the cell differentiation, disrupt the cell hierarchy and contribute to the epithelial hyperplasia.

By dual labeling retaining strategy, we were able to separate the stem or progenitor cells based on their functions. Our results showed that the EdU positive cells in inflammation model were not survivin positive. This leads to an interesting conclusion that the cells responsible for replacing apoptotic cells in physiological condition do not respond to inflammation. Otherwise, these cells would intake EdU during the week before inflammation and performed a robust proliferation during the inflammation; we would have a higher chance to detect the survivin expression in the EdU positive cells since the EdU may be retained in cells for 5-6 passages. On the other hand, another possibility must be mentioned. Asymmetric division is a trait of stem cells and it enables stem cells to self-renewal when producing a daughter cell. If the last division of those EdU positive cells were a symmetric division which means the mother cell lose its

progenitor trait and become 2 identical terminally differentiated daughter cells, those cells will retain the EdU forever and never enter cell cycle again. Nevertheless, this still supports our idea that different populations of stem or progenitor cells may have different functions in maintaining tissue homeostasis. In combined with the BrdU experiment results, our study drove to a conclusion that survivin is expressed in a specific population of cells which do not proliferate during physiological condition but is responsive to inflammation.

Our regrowth model added more information to this conclusion. The survivin expression in re-growth model was majorly in BrdU- and EdU- cells. This further supported our idea about a tissue regeneration-specific population which is survivin positive during re-growth phase. A few EdU positive cells also expressed survivin or ki-67, suggesting that these cells can replicate more than once to produce new epithelial cells during the re-growth phase. In the vehicle treated group, there were some loci which were abundant of EdU positive cells, suggesting a high proliferation level, but were survivin negative. This demonstrated that survivin expression may be independent of proliferation in androgen ablated animals. In addition, survivin was significantly upregulated by testosterone. The intersection between these 2 models is tissue regeneration.

This makes survivin an interesting regulator of tissue regeneration in prostate epithelium.

At day 14 of testosterone treatment, both survivin expression and cell proliferation dropped to baseline in the re-growth model. This is different from what we observed in the inflammation model in which survivin persisted after the acute phase of inflammation. We also found a strong survivn expression with a low level of proliferation in human samples which may represent a chronic phase of inflammation. Thus, survivn expression fits for the level of proliferation in the acute phase of inflammation and the entire re-growth phase. Survivin expression deviates from the proliferation level during the chronic phase of inflammation, suggesting different regulation mechanisms of survivin expression may take charges in this two models. Moreover, the regrowth model is driven by testosterone alone, while the inflammation model involves live bacteria, various cytokines, and inflammatory factors. This difference may also cause the different regulations of survivin and stem cell behavior. At last, we cannot ignore the fact that inflammation also happened in the regrowth model since hormone imbalance also causes inflammation in the prostate. Thus, there may be overlap between these two models that shapes the regulation of survivin more like inflammation-driven pathways.

In summary, we found survivin expression in a specific population of cells which is responsible for tissue regeneration, but not in putative slow-cycling stem cells or in EdU positive daily turn-over cells. Survivin may regulate cell stemness by blocking their differentiation.

Chapter 6 Conclusion and future direction

A. Conclusion

Here I demonstrated that inflammation increased the stemness of prostate epithelial cells. A population of epithelial stem cells named PEPCs was expanded during inflammation. This expansion was IL-1/IGF-1 dependent and was correlated with inflammation intensity. PEPCs possessed higher stemness comparing to non-PEPCs cells, demonstrated by stronger sphere formation ability and forming a prostatic structure in renal transplantation. A shift of c-kit expression from basal to luminal compartment happened during inflammation, indicating a differentiation of stem cells to maintain the tissue homeostasis in the prostate.

The dual-functioning protein survivin was up-regulated in prostate epithelial cells during inflammation. Survivin expression was associated with cell proliferation in the acute phase of inflammation. Survivin persisted during the chronic phase of inflammation while the proliferation level in epithelial cells went down. This also happened in human BPH samples. Survivin expression during inflammation shared the same regulation pathway IL-1/IGF-1 signaling with PEPCs expansion and epithelial hyperplasia. IGF-1 induced survivin expression in prostate epithelial cells. Knocking down survivin using RNAi technique or

pharmacological inhibitor LQZ-7F reduced cell proliferation in E6 and E7 cells, and reduced IGF-1 induced cell proliferation *in vitro*. LQZ-7F treatment *in vivo* reduced survivin expression and cell proliferation in prostate epithelial cells during inflammation without affecting inflammation intensity. Overexpression of survivin in E6 and E7 cells did not affect cell proliferation induced by IGF-1.

BrdU positive slow-cycling cells did not respond to inflammation but proliferated during testosterone-induced prostate re-growth. Survivin was expressed in a specific population of cells that responses to tissue regeneration. Survivin may regulate cell stemness by blocking cell differentiation (Fig 6.1).

Emerging evidence support that prostate inflammation contributes to the initiations and progressions of BPH and prostate cancer. However, the mechanisms underlying the pathological process of prostate inflammation in human are still largely unknown. The clinical treatments to BPH and prostate cancers are focused on the prostate cells. Most of the treatments, such as 5α-reductase inhibitors, androgen receptor inhibitors, and chemotherapy, inhibit cell proliferation or induce apoptosis in prostate epithelial cells without involving the inflammatory aspects. It is probably because of the complexity of inflammatory environment which makes it hard to locate the targets that drive epithelial cells

proliferation. Many inflammatory factors, such IL-6, TGF-β, VEGF, IL-1, IGF-1, etc. can promote epithelial proliferation in BPH or cancerous prostate tissues. Inhibition of one or several of these factors may induce compensation effects from other inflammatory factors and increases the risks of side-effects caused by drug-drug interaction. In addition, several recent clinical trials of using general anti-inflammatory drugs such as NSAID for BPH or prostate cancer preventive purposes did not present encourage results.

Most of the inflammatory signaling pathways converge at the prostate epithelial cells. Epithelial cells experience fate decision which determines their survival or apoptosis during inflammation. This makes this fate decision a potential therapeutic target for treating prostate diseases. However, the mechanism underlying this fate decision is unknown. Prostate epithelial cells are continuously replenished by epithelial stem cells under physiological condition. Under pathological conditions such as inflammation, the epithelial stem cells maintain the epithelial cell population through cell proliferation and differentiation. Little is known about the epithelial stem cell populations which restore the epithelial structures during inflammation. We are the first to demonstrate the expansion of progenitor cells population in prostate inflammation. This suggested a critical role of PEPCs in tissue repair/regeneration. This also provided epithelial

stem cells as a potential therapeutic target for inhibiting epithelial hyperplasia. Epithelial hyperplasia is not evenly distributed all over the prostate in BPH samples, instead, there are focal points of hyperplasia in the prostate of which the adjacent regions remain healthy. Comparing to inhibition the growth of the most epithelial populations through androgen ablation, targeting the differentiation of prostate epithelial stem cells such as PEPCs may be a more specific strategy with fewer side effects on luminal cells in the health regions. Inhibition of epithelial stem cells proliferation and differentiation will remove the cell source of epithelial hyperplasia in pathological regions which have high cell turnover rate, but will have minimum effects on the healthy regions since they are less dependent on these stem cells. We showed that the PEPCs expansion can be manipulated by inhibiting inflammatory/development singling pathways. This attempt of targeting stem cells in prostate epithelial hyperplasia demonstrated that it is a practical way to manipulate epithelial stem cell behaviors during inflammation. Admittedly, our results need to be further verified in human. It is impossible to isolate PEPCs from human prostate since the stem cell marker Sca-1 is mouse exclusive. Substitute markers, for example, CD166, need to be applied in isolating human prostate epithelial stem cells. , which may bring the question that we can never have the stem cell population from human as same as the PEPCs. Another major obstacle of studying prostate epithelial stem cells

and inflammation in human is the availability of non-diseased samples. Although prostate inflammation is age-related in human, little is known about its cause and initiation. Thus, it is almost impossible to obtain human samples with no inflammation. Thus, a tissue culture based *ex vivo* system is required to verify our results in human. Despite these drawbacks, our results in PEPCs expansion and its regulation not only provided a possible mechanism to explain inflammation-induced epithelial hyperplasia but also introduced a potential therapeutic target of prostate diseases.

The chronological order of inflammation and BPH or prostate cancer is probably a “the chicken or the egg dilemma” in human since BPH and prostate cancer specimens without inflammation can be identified despite their small percentages. However, it is more likely that inflammation occurs earlier than BPH or prostate cancer in human since inflammation can be detected in puberty while the other two diseases are typically age-related. In experimental animal models, the inflammation is an earlier event comparing to the following epithelial hyperplasia. Our results about PEPCs expansion, epithelial hyperplasia, and survivin expression were focused on the acute phase (the first 3-7 days) of inflammation in the prostate. We showed that the inflammation-induced epithelial hyperplasia can be inhibited in the acute phase. This could partly explain the

failure of anti-inflammatory treatment in BPH and prostate cancer patients. Chronic inflammation is always accompanied with BPH or prostate cancer. This means the BPH or prostate cancer patients have been inflamed for years. The long-term immersion into the inflammatory environment may change the gene expression profile in prostate epithelial cells, such as reduced expression level of cox-2 and elevated expression of IL-6, and thus leads to resistance to anti-inflammatory drugs. A new epithelium homeostasis may also be established since lots of undifferentiated cells are trapped in the epithelium. They may modify the proliferative milieu in the prostate to reach a new balance of cell proliferation and cell death, otherwise, an infinite tissue growth should be observed in chronic inflamed prostates. Thus, this re-constructed epithelium homeostasis may show different reactions to therapeutic treatments. Our results suggested a benefit of the early manipulation of inflammation before a new epithelial homeostasis is established. Inhibition of inflammation in an early stage may stop the epithelium destruction and prevent the distorted differentiation of stem cells. Thus the normal epithelial cell hierarchy may be preserved and the tissue damage will be minimized. Clinically surveillance of early inflammation may require new systems to test inflammation in the prostate with minimal interventions in prostate tissue since physical trauma used for biopsy can also result in inflammation.

Our results of survivin upregulation during inflammation set up a correlation between inflammation and cancer initiation. Survivin is rarely expressed in non-diseased prostate and is overexpressed in prostate cancer. We showed that survivin expression persisted after the acute phase of inflammation. Its expression also deviated from cell proliferation during the chronic phase, suggesting its functions other than cell proliferation in chronic inflamed prostates. Inhibition of cancer initiation is important for cancer prevention. Epithelial cells with survivin overexpression are suspicious candidates of cancer initiating cells. Moreover, our labeling retaining assay suggested that survivin-expressing cells are the population which responds to inflammation. Inhibition of survivin reduces epithelial proliferation. The survivin expression pattern is also confirmed in human samples. This leads to our postulation that cancer initiating cells may originate from this survivin positive population since they take advantage of the inflammatory environment and are highly proliferative while the survivin negative populations are not. Thus, survivn is a marker for the epithelial population with potential cancer initiating cells. Tracing the survivin expression pattern after inflammation may benefit cancer prevention in the prostate.

B. Future Direction

Limited by the multi-marker tracing techniques *in vivo*, we did not directly demonstrate the fates of PEPCs and their progenies *in situ* in the animal model. Although we demonstrated the differentiation potential of PEPCs by renal transplantation, the progenies of PEPCs and their roles during inflammation-induced epithelial hyperplasia remain a question. An orthotropic injection of labeled (i.e. GFP) PEPCs into mouse prostate may be a solution. The exogenous PEPCs can be traced and the phenotype of their progenies can be analyzed *in situ*. This will tell us the differentiation of PEPCs in situ and the types of epithelial cells produced by PEPCs. An alternative way is to knock out PEPCs in inflammation condition. An inducible c-kit promoter driven suicide gene (i.e.TK) is an available choice since the highest stringency of c-kit among the 4 stem cell markers. However, it is inevitable that we would remove other progenitor populations since c-kit is not specific to PEPCs. In addition, the inflammation caused by physical trauma needs to be taken into consideration. These studies will provide a deeper insight of the roles of epithelial progenitor cells in inflammation and later events such as cancer initiation.

Another question needs to be answered is the role of survivin in prostate progenitor cells. A cell-specific knockdown or overexpression of survivin need to

be performed in PEPCs, or other stem/progenitor populations to further investigate survivin's functions. A virus based survivin knockdown strategy can be applied in animal since the specificities and side effects of pharmacological inhibitors can be avoided. Using cell-specific promoter driven RNAi technique of survivin can knock down survivin in a small population of target cells. This will help us to link the function of survivin to a specific population of cells and to evaluate the importance of survivin.

Survivin is known as a dual-functioning protein and a tumor marker in most human cancers. We hypothesize that the overexpression of survivin persists in some cells after the inflammation. This is supported by our results in human specimens and chronic inflamed animals. We showed that survivin inhibition decreases cell proliferation while overexpression of survivin has no effect. Thus, the overexpression of survivin during inflammation may not drive cell proliferation, but unlock a restriction of low survivin amount to facilitate epithelial cells proliferation. This entitles the cells a license for further proliferation. Thus, it is reasonable to postulate that survivn overexpression may increase the risk of cell transformation and cancer initiation in the prostate. Emerging evidence about the roles of survivin in cancer stem cells makes survivin a potential marker of cell transformation. Thus, we need to investigate whether survivin overexpression will

increase cell migration or invasiveness. Whether survivin overexpression or inhibition may change the incidence of cancer initiation in genetically modified animals under inflammation condition (i.e. Probasin promoter driven Pten null mice) also need to be studied. Moreover, *in vivo* labeling of survivin expression cells (i.e.: survivin-GFP fusion proteins) will enable us to trace and analyze the phenotype of survivin positive cells. This will tell us whether survivin overexpression is correlated with cancer initiating cells.

During my graduate study in Dr. Travis Jerde's lab, I am involved in the prostate epithelial stem cell and inflammation research project. This research experience developed my interests in the regulation of prostate epithelial cells under pathological conditions. I enjoyed my work in epithelial stem cell research which helps me to establish a deep understanding of epithelial stem cells and the microenvironment that facilitates tissue repair and regeneration. I also established a more comprehensive understanding about inflammation and its effects on the prostate. Under the rigorous training from Dr. Travis Jerde, I established a skeptical attitude of doing research and a strict way to interpret my data. This will benefit me in my future research. In addition to my knowledge background, I have great chances to learn lots of new experimental techniques,

especially in animal surgery fields, all of which are necessary for pursuing my future positions.

I enjoy my experience of doing research in the field I am interested in. This precious experience will help me in the future on my way to science.

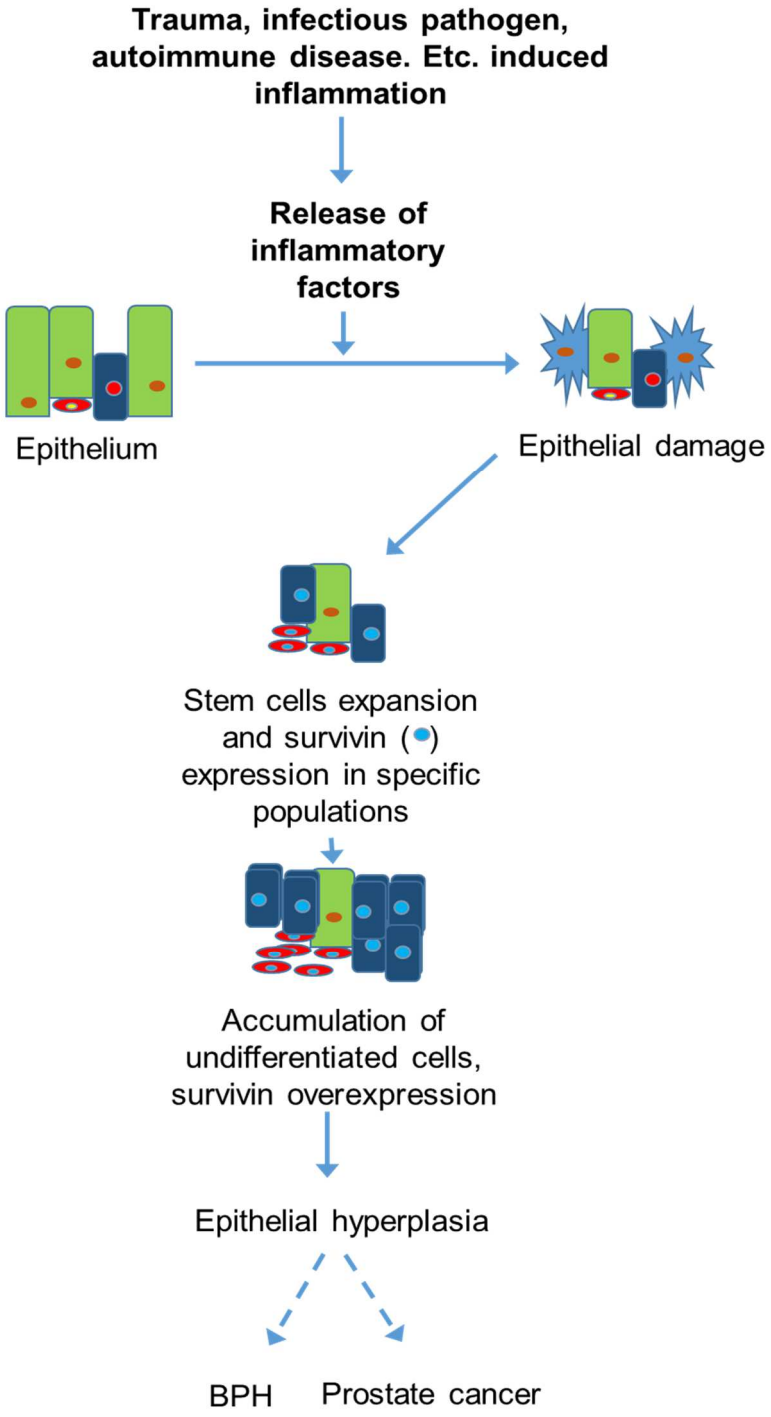


Fig 6.1 Survivin and epithelial stem cells in tissue repair and regeneration in response to prostate inflammation

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2016. **17**(1).

CURRICULUM VITAE

Liang Wang

Education

Ph.D. in Pharmacology Feb 2017

Indiana University, Indianapolis, IN GPA: 3.88

M.S. in Biochemistry Jul 2010

Nankai University, Tianjin, China GPA: 3.7

B.S. in Biology Jul 2006

China Agricultural University, Beijing, China GPA: 3.7

Research Experience

Ph.D. Student, Advisor: Dr. Travis Jerde, Department of Pharmacology & Toxicology, Indiana University School of Medicine, Indianapolis, IN, 2011-2017

- Discovered a pathological mechanism of prostate inflammation by demonstrating IL-1/IGF-1 mediate prostate progenitor cells expansion in response to inflammation.
- Identified the role of survivin in regulating epithelial cell differentiation and proliferation as mechanism of inflammation induced epithelial hyperplasia.
- Designed and optimized in vitro/in vivo assays to study survivin's function and the cell origin of hyperplasia in prostate inflammatory diseases.

- Performed data analysis, generated multiple manuscripts, and presented research discoveries at international conferences.

M.S. Student, Advisor: Dr. Ju Zhang, Department of Biochemistry, Nankai University, Tianjin, China 2007-2010

- Established an adenoviral reporter system in primary human prostate stromal cells to screen anti-Benign Prostatic Hyperplasia (BPH) drugs.
- Screened phytoestrogens for anti-BPH effects using cell-based assays and animal models. Identified 2 candidates with better therapeutic effects compared to clinical used medicines

Work Experience

Policy Researcher, Institute of Science Technology Information (a public policy study institute), Tianjin, China 2010-2011

- Performed public policy analysis for biomedical industry development.
- Drafted the medical device chapters of the planning for biomedical industry development in Tianjin, China.
- Performed patent analysis for biomedical market investigation and policy research.
- Organized the Plant Drug Forum as a team leader at the 2011 Tianjin Bio-economy International Conference.

Publications

Peer-Reviewed Articles

1. **Wang L**, Zoetemelk M, Chitteti BR, Ratliff TL, Myers JD, Srour EF, Broxmeyer H, & Jerde TJ (2015). Expansion of prostate epithelial progenitor cells after inflammation of the mouse prostate. *Am J Physiol Renal Physiol*, 308(12), F1421-1430.
2. Wang HH, **Wang L**, Jerde TJ, Chan BD, Savran CA, Burcham GN, Crist S, & Ratliff TL (2015). Characterization of autoimmune inflammation induced prostate stem cell expansion. *Prostate*, 75(14), 1620-1631.
3. Wang T, Green LA, Gupta SK, Kim C, **Wang L**, Almodovar S, Flores SC, Prudovsky IA, Jolicoeur P, Liu Z, & Clauss M (2014). Transfer of intracellular HIV Nef to endothelium causes endothelial dysfunction. *PLoS One*, 9(3), e91063.
4. Peng Y, Shi J, Du X, **Wang L**, Klocker H, Mo L, Mo Z, & Zhang J (2013). Prostaglandin E2 induces stromal cell-derived factor-1 expression in prostate stromal cells by activating protein kinase A and transcription factor Sp1. *Int J Biochem Cell Biol*, 45(3), 521-530.
5. Green LA, Petrusca D, Rajashekhar G, Gianaris T, Schweitzer KS, **Wang L**, Justice MJ, Petrache I, & Clauss M (2012). Cigarette smoke-induced CXCR3

receptor up-regulation mediates endothelial apoptosis. *Am J Respir Cell Mol Biol*, 47(6), 807-814.

6. Zhang Z, **Wang L**, Mei M, Zhu Y, Du X, Lee C, Park I, Zhang J, & Shi J (2010).Both nongenomic and genomic effects are involved in estradiol's enhancing the phenotype of smooth muscle cells in cultured prostate stromal cells. *Prostate*, 70(3), 317-332.

Primary Research Articles (in preparation)

1. **Wang L**, Zoetemelk M, JT Zhang, Ratliff TL, Srour EF, Broxmeyer H, Jerde TJ. Regulatory pathways of inflammation induced epithelial hyperplasia in prostate.
2. **Wang L**, Jerde TJ. Identification of the cell origin of inflammation-induced prostate epithelial hyperplasia.

Invited Oral Presentation

Wang L (2015). Inhibition of survivin in prostate epithelium reduce inflammation induced prostate epithelium hyperplasia. *John P. Donohue Urological Research Symposium*, Indianapolis, IN, Feb 13th, 2015. * Recognized by the Presentation Award.

Professional Skills

Cell Biology Skills

Cell Culture (Primary Cell/Cell Line); Gene Transfection (Plasmid/RNAi/Virus); Cell Functional Assays (Proliferation/Apoptosis/Reporter Assays); Multi-color Flow Cytometry; Spheroid and 3D Culture.

Histology Skills

Tissue Processing; Fluorescent Microscopy; Immunohistochemistry/Immunofluorescence; Paraffin/Cryo Tissue Microtome.

Molecular Biology Skills

Western Blot; Immuno-precipitation; qRT-PCR; Molecular Cloning; ELISA.

Animal Skills

Rodent Model of Prostate Inflammation; Drug Administration (I.P. / S.C); Animal Handling and Welfare; Tissue Dissection.

Computer Skills

Statistical Software (SPSS/Graphpad Prism); Flowjo; Microsoft Office; Adobe Photoshop; Derwent Patent Engine.

Honors and Awards

2015 Paradise Travel Award

2015 Presentation Award for John P. Donohue Urological Research Symposium

2014 Travel Award for Society for Basic Urologic Research

2004 Excellent Students Representative Award, China Agriculture University

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Teaching Experience

2008 Teaching Assistant in biochemistry experiments.

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