Generation of a stepwise prostate carcinogenesis model

A study of epithelial to mesenchymal transition and malignant transformation of prostate primary epithelial cells

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I. Ke XS*, **Qu Y***, Goldfinger N, Rostad K, Hovland R, Akslen LA, Rotter V, Øyan AM, Kalland KH. Epithelial to mesenchymal transition of a primary prostate cell line with switches of cell adhesion modules but without malignant transformation. **PLoS ONE**, 2008;3(10):e3368. PMID:18852876. * equally contributed to this work

II. Ke XS, Li WC, Hovland R, **Qu Y**, Liu RH, McCormack E, Thorsen F, Olsen JR, Molven A, Kogan-Sakin I, Rotter V, Akslen LA, Oyan AM, Kalland KH. Reprogramming of cell junction modules during stepwise epithelial to mesenchymal transition and accumulation of malignant features *in vitro* in a prostate cell model. **Exp Cell Res**. 2011;317 (2):234-47. PMID:20969863.

III. Qu Y, Li WC, Hellem MR, Rostad K, Popa M, McCormack E, Oyan AM, Kalland KH, Ke XS. MiR-182 and miR-203 induce mesenchymal to epithelial transition and self-sufficiency of growth signals via repressing SNAI2 in prostate cells. **Int J Cancer**. 2013 doi: 10.1002/ijc.28056. PMID:23354685.

IV. **Qu Y,** Oyan AM, Liu RH, Hua YP, Jigang Zhang, Hovlan R, Popa M, , Brokstad KA, Molven A, Liu XJ, Simon R, Lin B Zhang WD, McCormack E, Kalland KH, Ke XS. Generation of prostate tumor-initiating cells is associated with elevation of reactive oxygen species and IL6/STAT3 signaling. **Manuscript**.

ABBREVIATIONS

EMT	epithelial to mesenchymal transition
miRNAs	microRNA
MET	mesenchymal to epithelial transition
ROS	reactive oxygen species
IL6	interleukin 6
STAT3	Signal transducer and activator of transcription 3
PCa	prostate cancer
PSA	prostate specific antigen
CRPC	castrate resistant prostate cancer
SCs	stem cells
TACs	transit amplifying cells
AR	androgen receptor
PIA	prostatic inflammatory hyperplasia
PIN	prostatic intraepithelial neoplasia
CSC	cancer stem cell
TICs	tumor initiating cells
GF	growth factor
EGF	epidermal growth factor
IGF	insulin-like growth factor
TGF	transforming growth factor
VEGF	vascular endothelial growth factor
TNF	tumor necrosis factor
ECM	extracellular matrix
hTERT	human telomerase transcriptase
BPH	benign prostatic hyperplasia
FCS	fetal calf serum
GFP	green fluorescent protein
CIC	cancer initiating cell

ABSTRACT

Despite progress in recent years, prostate cancer continues to be one of the most prevalent causes of cancer-related mortality and morbidity in men in Western countries. A major clinical challenge of prostate cancer therapy is the current inability to readily distinguish between tumors with low and high aggressivity concerning ability to further invasion and metastasis. Understanding the mechanism of tumor initiation and progression is critical in diagnosis and treatment of prostate cancer patients. However, optimal prostate cancer initiation and progression models have been lacking. Previous malignantly transformed prostate cells were mainly established by exposure of benign prostate cells to strong external carcinogens or introduction of oncogenes or viral elements. These kinds of prostate cancer models are not optimal for representative studies on prostate carcinogenesis in vivo. Therefore, it is extremely valuable to establish a human prostate carcinogenesis model based on more physiological conditions.

The present translational study mainly focuses on the establishment of a stepwise prostate carcinogenesis model based upon human primary basal epithelial EP156T cells that were derived from prostate benign tissues in a patient following radical prostatectomy for prostate cancer. **Paper I** established an epithelial to mesenchymal transition (EMT) model. EP156T cells were adapted in long-term confluent culture, and cells with reduced contact inhibition appeared and had undergone EMT without malignant features, the new cells were named EPT1 cells. In **Paper II** EPT2-D5 cells were generated based on focus formation in confluent EPT1 cell monolayers and cloning in soft agar. EPT2-D5 cells had acquired *in vitro* malignant features such as focus formation, colony formation in soft agar, higher resistance to apoptosis and independence of exogenous growth factors. However, EPT2-D5 cells failed to form xenograft tumors in mice. **Paper III** explored the roles of microRNAs (miRNAs) in EMT using the EMT model established in Paper I. Two miRNAs (miR-203 and miR-182) were found to induce both mesenchymal to epithelial transition (MET) and self-sufficiency of growth signals via repressing SNAI2 in prostate cells. **Paper IV** reported the complete malignant transformation of prostate benign epithelial cells.

EPT2-D5 cells were adapted in protein free medium and generated prostate spheres that efficiently initiated subcutaneous tumors and subsequent large primary prostate tumors and metastasis in nude mice. Cells isolated from subcutaneous tumors, primary tumors and metastases were named EPT3, EPT3-PT1 and EPT3-M1 cells, respectively. Furthermore, a ROS/IL6/STAT3 cascade was found critical in the EPT3 tumor initiation and progression.

The present prostate stepwise carcinogenesis model is unique because all the cells were obtained under physiological conditions and cells at different stages harbor distinct phenotypes that are commonly utilized as markers for tumor initiation and metastasis *in vitro* and *in vivo*. An exact passage record has been secured, and the EP156T, EPT1, EPT2 and different EPT3 cells of the model can be propagated indefinitely as seemingly rather stable cell types in subconfluent cultures. The identification of the ROS/IL6/STAT3 cascade in EPT3 tumor initiation and progression provides a good opportunity for therapeutic development of anti-cancer drug targeting of the ROS/ IL6/STAT3 pathway.

INTRODUCTION

Prostate and prostate cancer

Epidemiology

Prostate cancer (PCa) is the most frequently diagnosed malignancy among adult males in Western countries. The lifetime risk of developing prostate cancer is 17% (Prostate cancer-UK incidence statistics). Most cases of PCa will go undetected until the person dies from unrelated causes at old age (1). In Norway, it is the second leading cause of cancer related death (Cancer in Norway, 2010).

There are at least three major factors contributing to the occurrence of PCa: age, heredity and environment. The incidence of PCa increases with age, and it is estimated that 80% of men by age 80 have cancer cells in their prostate (Prostate cancer - UK incidence statistics). Regarding heredity, it has been shown that different populations may carry prostate cancer-susceptibility alleles at different frequencies. The environment factors include air, water, food and lifestyle (2). The incidence varies dramatically in different countries and more than 2/3 of cases are diagnosed in developed countries (Prostate cancer - UK incidence statistics). However, the higher incidence found in developed countries is not only caused by the true incidence, it is largely based on the wider use of prostate specific antigen (PSA) screening tests and biopsy follow-up that began from the 1980s in those regions (Cancer in Norway, 2010). A recent examination of age-standardized incidence rates (ASIR) of PCa in Asian countries found a clear trend of increasing PCa ASIRs in the four countries examined (China, Japan, Korea and Singapore) (3), indicating the increasing world health burden posed by this disease.

Current management of patients diagnosed with PCa is effective. However, cancer recurrence with castrate resistant prostate cancer (CRPC) and subsequent metastasis lead to poor survival outcome (1). Current therapy consists of removal of the entire prostate gland (radical prostatectomy) or radiation toward the gland, but is associated with side-effects such as incontinence and impotence that may adversely affect the quality of life (4). How to screen for and treat prostate cancer therefore pose difficult

dilemmas, suggesting that there is a dire need for novel mechanistic understanding of cancer progression and novel prognostic and predictive markers to guide treatment or watchful waiting.

Histology

The prostate is a male gland and the size increases with age. In young men it is the size of a walnut. The prostate is located in front of the rectum and underneath the urinary bladder (5). The development of the prostate in man is according to a ductal-acinar formation program without discernible lobular structures. To simplify it, the classic work of McNeal defined human prostate as having four anatomically and clinically distinct zones, corresponding to central, periurethral, transition and peripheral zones, together with an anterior fibromuscular stroma (5,6). Up to 70% of prostate adenocarcinomas arise in the peripheral zone (PZ), about 20% arise from the transitional zone (TZ) and only 1-5% occur in the central zone (CZ) (5,6).

According to the morphological characteristics, functional significance and relevance to carcinogenesis , there are at least three distinct cell types at the histological level within the human prostate pseudostratified epithelium: basal, luminal and neuroendocrine cells (7-9) (Figure 1). Some researchers also include two other kinds of inter-related cell types: stem cells (SCs) and transit amplifying cells (TACs) (9,10).

The predominant secretary luminal cells form a continuous layer of polarized columnar epithelium. They are a kind of differentiated androgen-dependent cells that produce prostatic secretary proteins, such as prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP). At the molecular level, luminal cells are characterized by their expression of androgen receptor (AR), cytokeratin 8 (KRT8) and 18 (KRT18) and CD57 (11-13). The second major epithelial cell type is the basal cell that is located between luminal cells and the underlying basement membrane. Basal cells do not produce PSA and PAP, they express KRT5, KRT14 and CD44, and occasionally a low level of AR (11-13). Neuroendocrine cells only make up a small percentage of the normal prostatic epithelium. Cytokines secreted by neuroendocrine cells play a role in the regulation of epithelial cell proliferation and differentiation (9, 14).

Stem cells (SCs) have tissue-regenerative capacity to replenish prostate cells that are continuously shed into the lumen of the gland and are therefore critical for prostate homeostasis. SCs isolated from the basal compartment are able to differentiate into luminal and neuroendocrine cells, and form whole epithelial structures (15,16). Other researchers demonstrated that a population of castration-resistant luminal cells expressing the homeobox transcription factor Nkx3.1 (termed CARNs) can generate prostatic tissue with basal, luminal and neuroendocrine cells in the castrated mouse prostate (16). Cells co-expressing both basal and luminal cell markers, sometimes even neuroendocrine cell markers are classified as transit amplifying cells (TACs) or intermediate cells (17). TACs probably represent progenitor cells that are in the process of differentiation, but not yet completely finished (Figure 1) (9). The features of TACs and the luminal compartment are among several remaining unclear issues regarding prostate stem cells and their differentiation and maturation.

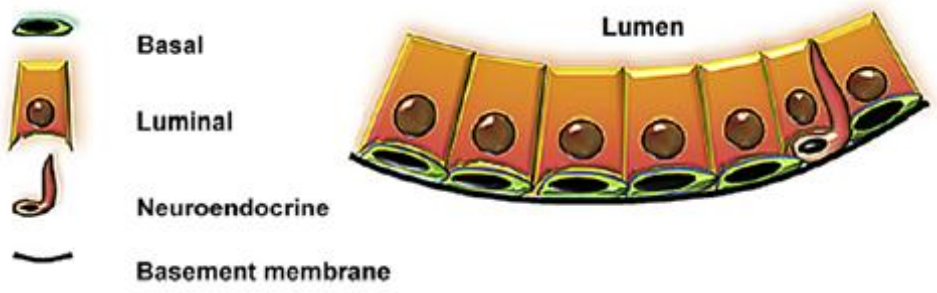

Figure 1. Three cell types in adult prostate epithelium. Shown are luminal cells (orange), basal cells (green) and basement membrane (black) from fluid-filled lumen to outside. Neuroendocrine cells (red) are typically found in the basal layer with neurite-like extensions that can approach the luminal layer. The figure is adapted from (16).

Most PCa are adenocarcinomas sharing numerous common features with other epithelial cancers, *e.g.* breast cancers. The pathologists diagnose PCa based on neoplasia of luminal cells and the absence of basal cell markers, combined with impaired underlying basement membranes. Luminal cells have traditionally been considered as the main origin of PCa since more than 95% of prostate cancer cells

predominantly exhibit luminal cell markers, However, recent experiments show evidence that basal cells may be the cell of origin of PCa (18-20).

Progression of prostate cancer

It always takes several years to gradually develop prostatic malignant tissues from normal tissues. Between the two extreme situations, completely normal and highly malignant, there are a broad spectrum of intermediate tissues with different morphologies and properties regarding cell markers, metabolism and aggressive abilities. Some anthropogenic distinct stopping points along this path have been used to depict this long story: normal epithelium, neoplastic, adenocarcinoma and metastatic (Figure 2).

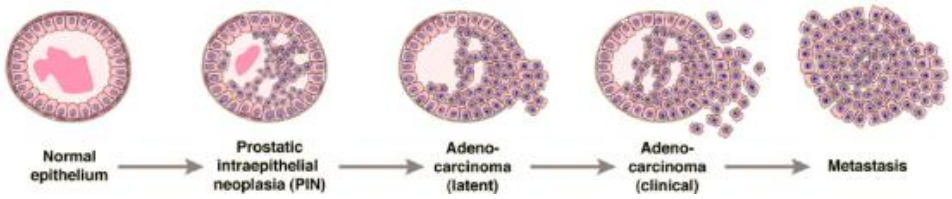

Figure 2. Model of human prostate cancer initiation and progression. Stages of progression are shown. The figure is adapted from (1).

In prostate cancer, the early pre-malignancy stage appears to be associated with the dysplasia state which starts with proliferative inflammatory atrophy (PIA). Some of the cases may stop here while others may progress to prostatic intraepithelial neoplasia (PIN). These early lesions may be initiated by exposure to various inflammatory and carcinogenic agents, oxidative stress and DNA damage (1,21,22). When cancer suppressor proteins become lost at later stages, prostate primary cancer appears. The worst clinical situation typically is bone metastasis (23).

The treatment of PCa can be prostatectomy or irradiation. Most PCa are androgen-dependent and respond to androgen-ablation therapy. However, some cancers finally become “castration resistant” and progress despite anti-androgen therapy. PCa develop into this stage via multiple mechanisms: most involving the AR and others bypassing it. Therapy for disseminated anti-androgen-resistant PCa is palliative and does not

increase survival. Currently the clinical treatment options are limited to chemotherapy, steroids, radiation and the new radixin therapy against bone metastasis (24).

Prostate tumor initiating cells and cancer stem cells

It has been known for long time that cancer cells in a tumor are not equally contributing to the tumor formation and have different potential to reconstitute the tumor upon transplantation (25-28), which gave rise to the concept of cancer stem cells (CSCs), that a tumor is hierarchically organized with a distinct fraction of tumorigenic CSCs generating the bulk of the non-tumorigenic cells (29). The concept of CSCs is often used interchangeably with tumor initiating cells (TICs) in the literature, but distinctions have been defined: CSCs are able to reconstitute a tumor that is identical to the parental tumor from which they were isolated and can be serially xenotransplanted indefinitely, while the most important feature of TICs is ability to regrow the tumor from which they were isolated, it is not necessary for TICs to generate a hierarchically organized tumor (15). Yet another concept is the cell of origin of cancer that represents the normal cell type from which malignant cells were derived.

In prostate, luminal cells were traditionally considered as the origin of PCa. Korsten *et al.* reported this for a subset of luminal cells expressing the progenitor markers Trop2 and Sca-1 (30). Murine castration-resistant Nkx3.1-expressing cells efficiently initiated prostate carcinoma following androgen-mediated regeneration upon deletion of Pten gene (20). On the other hand, independent evidence supports that PCa arise from normal basal AR^- stem cells. For example, putative basal CSCs with a $CD44^+\alpha2\beta1integrin^{high}CD133^+$ phenotype have been isolated from human PCa biopsies (31) . Goldstein *et al.* showed that basal cells with $CD49f^{hi}Trop2^{hi}$ isolated from human primary prostate tissue can recapitulate the histological and molecular features of human PCa by cooperation with AKT/ERG/AR signaling (32). Liao *et al.* reported that basal $Lin^-Sca\text{-}1^{high}CD49f^{high}$ cells have the capacity to form tumor spheres *in vitro* and xenograft tumors *in vivo* (33). Additionally, a small population of TRA-1-60^+ $CD151^+$ $CD166^+$ TICs expressed basal cell markers and did not express the luminal marker AR (34).

There is therefore not yet consensus regarding the origin of human prostate cancer and CSCs. Actually, it is not necessary to have only one cell-of-origin of prostate cancer and CSCs since different genetic alterations may simultaneously transform different target cells and different clinical sub-types of cancer may arise from different cell types (35) .

Prostate cancer models

One big obstacle in prostate cancer research is the lack of relevant preclinical models to understand the mechanism of prostate carcinogenesis and to develop effective preventive and therapeutic interventions. Currently, the prostate cancer models mainly include human cancer cell lines, human prostate malignant transformation models and transgenic mouse models.

Human prostate cancer cell lines

To represent *in vitro* models of prostate cancer at different stages, numerous attempts have been done to establish cell lines from human prostate carcinomas. Although cells from prostate carcinomas have proven to be one of the most difficult cell types to establish as stable cell lines, there are approximately 30 putative human prostate cell lines that have been isolated from different stages of PCa. An overview of site of origin and molecular patterns of the 15 most used prostate cancer cell lines and references is summarized in Table 1. Among these cell lines, 22Rv1, CWR-R1 and PC-346C were derived from a primary tumor (36,37). LNCaP and LAPC-4 were established from lymph node metastases (38,39), while MDA PCa 2a, MDA PCa 2b, PC-3 and VCaP were isolated from bone metastases (40-42) and DuCaP and DU145 from brain metastases (43, 44).

Whilst these prostate cancer cell lines have greatly promoted the field of prostate cancer research, it is difficult to examine the multistep development process of cancer in xenografts since most of these cell lines were isolated from metastatic lesions and represent the histology and metastatic patterns of human cancers at an advanced stage. Most importantly, the lack of benign counterparts of these cancer cells limits the use in studying the mechanism of prostate cancer initiation.

TABLE 1 *Site of origin and molecular characterization of human prostate cancer cell lines. The table is adapted from (45) .*

Cell line	Site of origin	Ref	K5	K14	K8	K18	p63	CGA	NSE	VIM	AR
DU 145	Brain metastasis	(43)	+	-	+	+	-	-	-	+	-
1013L	Primary, transitional cell carcinoma	(46)	+	-	+	+	-	-	-	-	-
PC3	Bone metastasis	(40)	+	-	+	+	-	-	+	+	-
LNCaP	Lymph node metastasis	(38)	-	-	+	+	-	-	-	-	+
UM-SCP-1	Primary squamous cell carcinoma	(47)	+	+	+	+	+	-	-	-	-
NCI-H660	Lymph node metastasis	(48)	-	-	+	+	+	+	+	-	-
PC-346C	Primary, xenograft		-	-	+	+	-	-	-	-	+
LAPC-4	Lymph node metastasis, xenograft	(39)	+	-	+	+	-	-	-	-	+
MDA PCa 2a	Bone metastasis	(41)	+	-	+	+	-	-	-	-	+
MDA PCa 2b	Bone metastasis	(41)	+	-	+	+	-	-	-	-	+
22Rv1	Primary, xenograft CWR22R-2152	(36)	-	-	+	+	-	-	-	-	+
PSK-1	Primary, SCC	(49)	-	-	+	+	-	-	+	+	-
VCaP	Spinal cord metastasis, xenograft	(42)	-	-	+	+	-	-	-	-	+
DuCaP	Dura metastasis, xenograft	(44)	-	-	+	+	-	-	-	-	+
CWR-R1	Primary, xenograft CWR22R	(37)	-	-	+	+	+	-	-	-	+

K5: cytokeratin 5, **K14**: cytokeratin 14, **K8**: cytokeratin 8, **K18**: cytokeratin 18, **p63**: tumor protein p63, **CGA**: chromogranin A, **NSE**: neuron specific enolase, **VIM**: vimentin, **AR**: androgen receptor.

Human prostate malignant transformation models

Considering the disadvantages of human cancer cell lines described above, several malignant transformations of human prostate primary epithelial cells were established by radiation (50) or chemical treatment, such as cadmium (51,52), N-nitroso-N-methylurea (51,52), or introduction of virus elements, such as the SV40 early region (53), HPV-18 and v-Ki-ras (54,55). However, most incidences of prostate cancer patients are not likely due to exposure to such strong external carcinogens considering it is mainly a disease of aging.

Transgenic mouse models

Except for the human prostate cancer cell lines described above, laboratory mice afford one of the best models for studying human cancer due to many advantages. Firstly, mice are as susceptible to cancer as humans (56). Secondly, most of the cancer-related genes with essential functions in carcinogenesis are structurally homologous in mouse and humans and are easy to manipulate (57). Finally, the relatively short gestation period and lifespan of mice favors establishment and passaging of new models.

Regarding prostate cancer, a number of transgenic mice models have been developed by genetic engineering, such as targeted gene deletions, mutations or insertions, which have provided a unique opportunity to study the function of manipulated genes in prostate carcinogenesis. Generally, gain of function models need to insert oncogenes to get overexpression, and loss of function models need complete or conditional genetic knockout of tumor suppressors. The manipulated genes in these models can be single or multiple, and the protein products can be hormone receptors, growth factors and receptors or key components involved in cell cycle, signaling pathways or genomic instability. The prostate phenotypes can be hyperplasia, prostate intraepithelial neoplasia (PIN), high-grade prostate intraepithelial neoplasia (HGPIN), locally invasive adenocarcinoma or metastatic carcinoma. A summary of the transgenic mouse models is shown in Table 2.

Table 2 *Transgenic models of prostate cancer. The table is adapted from (58) .*

Phenotype	Models	Genetic disruptions	Ref
Hyperplasia	PB-FGF7 (PKS)	FGF7	(59)
	C3(1)-bcl-2	Bcl-2	(60)
	PSA-CRExNkx3.1$^{f/f}$	Nkx3.1	(61)
	PB-FGFRiiib	FGFRiiib	(59)
	C3(1)-Polyoma virus Middle T	Polyomavirus middle T gene	(62)
	MMTV-wap	Whey acidic protein gene	(63)
	PB-Cre^{+}/Rb$^{loxp/loxP}$	Retinoblastoma	(64)
PIN	ARR$_2$PB-FGFR1	FGF receptor 1	(65)
	BK5-IGF1	IGF-1	(66)
	ARR$_2$PB-myc-PAI	Myc	(67)
	MPAKT model	Akt1	(68)
	PSA-Cre^{+}x Nkx3.1$^{+/flox}$	Nkx3.1	(61)
	PB-RAS	H-Ras	(69)
	PB-Cre4x Pten$^{loxp/loxp}$	PTEN	(70)
	PB-EcoRI	ECO:RI	(71)
	LPB-Tag/PB-Hepsin	Hepsin, p53, Rb	(72)
	TRAMP	p53, Rb	(73)
	LADY	p53,Rb	(74)
HGPIN	PB-mAR	Androgen receptor	(75)
	ARR$_2$PB-FGF8b	FGF8b	(76)
	PB-Cre4x Pten$^{loxp/loxp}$	Pten	(70)
	MMTV-Crex PTEN$^{loxp/loxp}$	Pten	(77)
Locally invasive adenocarcinoma	C3(1)-SV40 T/t	p53,Rb	(78)
	PB-Cre^{+}x APC$^{flox/flox}$	APC	(79)
	PB/Neu	HER-2/Neu	(80)
Metastatic carcinoma	PSP-KIMAP	p53,Rb	(81)
	Cryptidin-2/SV40 T	p53,Rb	(82)
	Fetal Gγ-globin	p53,Rb	(83)
	TRAMP	p53,Rb	(73)
	PTEN$^{+/-}$/TRAMP	Pten, p53, RB	(84)
	P53$^{-/-}$/Rb$^{-/-}$	p53, Rb	(85)
	Nkx3.1$^{-/-}$/Pten$^{+/-}$	Nkx3.1, Pten	(86)
	Pten$^{+/-}$/FGF8b	Pten, FGF8b	(87)

Despite the advantages mentioned above, limitations of mouse models in human cancer research should be noted. There are many differences between mouse and human, such as anatomy and structure of the prostate and other organs, immortalization kinetics and patterns of carcinogenesis, additionally, the origin of cancer is mainly mesenchymal cells in mice, but mostly epithelial cells in human

cancers (88). Therefore, mouse models should be used to complement rather than replace human studies, and a careful interpretation of findings obtained in mouse models is needed to better understand cancer pathogenesis in human (58) .

Hallmarks of cancer

To rationalize this complex process of cancer, Hanahan D and Weinberg RA tried to define the essential functional capabilities that normal cells must acquire to evolve progressively into malignant cells as the hallmarks of cancer (Figure 3) (89). Only if cancer cells acquire these hallmarks, they can maintain their deregulated survival, proliferation and dissemination, in another word, to become carcinogenic and ultimately invasive and metastatic (89).

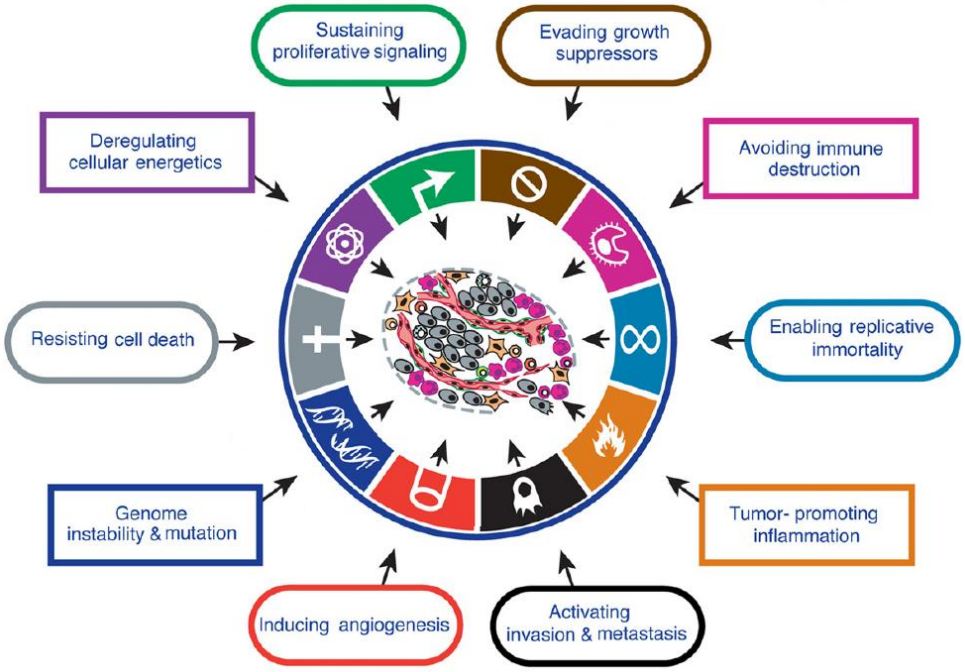

Figure 3. Schematic diagram of the hallmarks of cancer as proposed by Hanahan and Weinberg. The figure is adapted from (89).

Sustaining proliferative signaling

Cancer initiation as well as subsequent steps of cancer progression usually correspond to unlimited cell proliferation which depends on sustained proliferative signaling

(typically including EGF, IGF, TGF and VEGF) (90-92). Cancer cells can obtain this hallmark via different mechanisms: some cancer cells can produce growth factors (GFs) by themselves and display autocrine proliferative stimulation (93); some cancer cells possess elevated amounts of or structurally altered receptor proteins. Both can boost signal capacity or facilitate GF ligand independent activation. As a result, cancer cells become masters of their own fate, proliferate infinitely and finally become malignant (89).

Evading growth suppressors and resisting cell death

To maintain tissue homeostasis, organisms must keep the balance between survival and death. Deregulated proliferation alone is not sufficient for malignancy, while too little death may lead to cancer formation (94). Apoptosis is programmed cell death and can exclude the damaged cells and serves as a main barrier against cancer (95-97). TP53 works as the master guardian of apoptosis. When cells encounter a variety of physiologic stress such as anoxia and damage to the genome, functional TP53 can sensor the DNA damage and promote cell cycle arrest, apoptosis or senescence (98).

Enabling replicative immortality

Cells in most normal cell lineages can only pass through a pre-determined and limited number of division cycles, and then irreversibly enter into replicate senescence. Such kind of "cell generational clock" is an anti-cancer mechanism and determined by telomerase activity (1). Telomeres are located at the end of chromosomes and prevent end-to-end fusion of chromosomes. In non-immortalized cells, telomeres will be progressively shorter and eventually tend to malfunction after repeated DNA replication (99,100). Telomerase, a specialized DNA polymerase adding telomere repeat segments to the ends of telomeric DNA, is almost absent in normal differentiated cells but at functionally significant levels in many cancer cells. Cancer cells with functional telomerase manage to maintain telomeric DNA at lengths which are sufficient to escape senescence or apoptosis (89, 101,102).

Inducing angiogenesis

Cancer propagation and progression require high density of vessels to supply nutrients and oxygen and to evacuate waste products (103). The cancer-associated *de novo* vasculature formation termed angiogenesis satisfies these needs. Cancer angiogenesis differs from the physiologic angiogenesis by the aberrant vascular structure, altered endothelial and pericyte interaction, abnormal blood flow, increased permeability and delayed maturation (2,103,104,105). The onset of angiogenesis is under the control of the so-called "angiogenic-switch", which depends on the balance of the pro-angiogenic and anti-angiogenic factors (106,107). Previous work has identified sets of pro-angiogenic (VEGF, VEGF receptors PAI-1, PDGF-B, TIE-2 receptor and MMPs) and anti-angiogenic molecules (endostatin, TNFα, TGFβ and TP53) (103,106-110).

Activating invasion and metastasis

Invasion and metastasis are responsible for more than 90% of solid cancer associated mortality. At the time of death the majority of cancer cells are found in metastases rather than in the primary tumor (2). The invasion-metastasis cascade involves series of linked sequential steps: "seeds" in primary location weaken cellular adhesion and acquire increased motility, local invasion, intravasation (entry into and survival in the circulation), transport, extravasation (exit from the circulation), micrometastasis formation, colonization and new cancer initiation in distant organs (111,112). The primary tumor releases millions of cells but only a tiny minority of these cells have the capacity to initiate metastatic cancer. It only happens when certain cells of the primary cancer ("the seed") adapt to the environment in certain secondary organs (the "soil"). This non-random pattern of metastasis depends on cross-talk between selected cancer cells and specific microenvironments (111, 113-115).

Genome instability and mutation

Genome instability and mutations are considered to be both the reasons and results of cancer. Mutations and damages within the genome happen spontaneously during the life span. Normal cells have an extraordinary DNA-maintenance machinery to detect and resolve defects so the accumulated mutations are usually very low during each generation. Cells with unrepaired mutations may enter into a state of quiescence or

apoptosis. Cancer cells with unstable genomes generate random mutations at much higher frequency, including chromosomal rearrangements and deletions. Such genetic changes can affect the DNA-maintenance machinery (*e.g.*TP53 and RB) or activate telomerase and imprinted genes. It may precipitate the rest of hallmark capabilities (116).

Reprogramming energy metabolism

Normal cells process glucose mainly via glycolysis under anaerobic conditions but not under aerobic conditions. Different from this, cancer cells utilize glycolysis for their glucose metabolism in both conditions and produce much more energy all the time (117,118). Such reprogramming of energy metabolism in cancer cells fuels the enormous augmentation of energy requirement needed for accelerated cell growth and division and makes cancer progression possible (89).

Cancer-promoting inflammation and evading immune destruction

Cancer has been considered as "wounds that do not heal" (119). Epidemiology data showed that up to 20% of cancers are linked to chronic inflammation (120). In healthy organisms the immune system works as a significant barrier to cancer formation and progression by destructing and eliminating transformed cells. Cancer cells can evade such immune destruction by disabling key components which are dispatched by the immune system (121,122). An inflammatory microenvironment can elevate both the genomic mutation rates and the proliferation rates of mutated cells, through affecting the production of cytokines, chemokines, GFs, prostaglandins, reactive oxygen and nitrogen species. Some target genes of these products are well-known key mediators of cancer progression, such as Bcl2, TP53, cyclin D1 and D2, as well as c-Myc (123). During the late metastasis stage, inflammation can promote the intravasation by increasing the vascular permeability, and lots of inflammatory factors participate in the pre-establishment of localized and colonized microenvironment (123).

Epithelial to mesenchymal transition

The majority of human tumors arise from epithelial tissues. Epithelial cells are tightly connected laterally by specialized membrane junction structures such as tight junctions,

desmosomes, gap junctions and adherens junctions and form an aligned apical–basolateral polarized layer through their association with basement membranes. Epithelial cells can only move away from their nearest neighbors along the basal surface by assistance of the locally distributed adhesion molecules including certain cadherins and integrins, but they do not detach and move away from the epithelial layer and do not invade into the extracellular matrix (ECM) under normal conditions (124,125). In cell culture epithelial cells grow as clusters in a cobblestone-like monolayer that maintain tight cell–cell adhesions with their neighbors.

Different from epithelial cells that typically exert tissue specific functions, mesenchymal cells play a supporting role in epithelial tissue (126). They do not express the same kinds of strong cell surface adhesion molecules as epithelial cells, so they usually exhibit a front-back end polarity. Mesenchymal cells rarely contact the neighboring cells directly and have higher motility. If contacts exist between neighboring mesenchymal cells, they are focal and transient, and are not typically associated with a basal lamina. In cell culture, mesenchymal cells have spindle-shaped, fibroblast-like morphology and tend to be highly mobile and migrate individually or together (127,128).

Epithelial to mesenchymal transition (EMT) refers to a cellular program by which cells shed their epithelial features and acquire mesenchymal features (Figure 4) (129). Epithelial cells may be plastic and thus able to move back and forth between epithelial and mesenchymal states via EMT and the reverse process MET (130-134). EMT was first described in three-dimensional culture of corneal epithelial cells in the laboratory of Elizabeth Hay in 1982 and has since been implicated in numerous embryonic states and pathologies including fibrotic disease and carcinogenesis (135). In mammals, experimental work on epithelial cell plasticity mainly follows the trail of two broad interests, metaplasia and EMT (136).

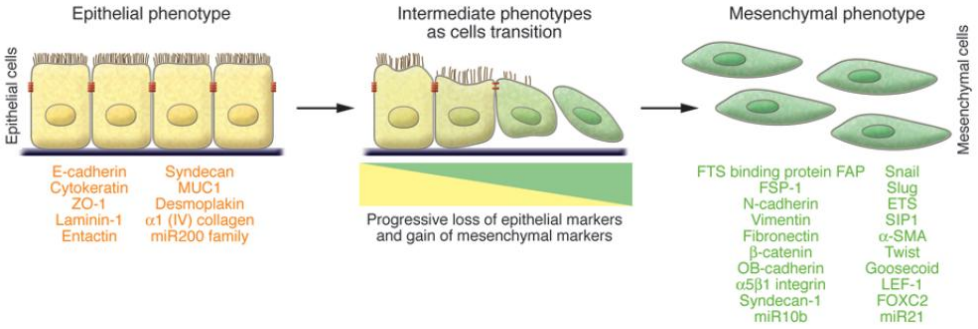

Figure 4. A model of functional transition of polarized epithelial cells into mobile mesenchymal cells. Several widely accepted epithelial and mesenchymal cell markers are shown. Cells that have passed only partly through an EMT and harbor both distinct markers are defined as an intermediate phenotype of EMT. The figure is adapted from (126).

Characterizations of EMT

The morphological changes during EMT include abolishment of epithelial cell polarity and acquirement of fibroblast-like shape and motility. In cell cultures we can find the switch from tightly connected cobblestone-like monolayer of epithelial cells to spindle-shapes of mesenchymal cells with migratory protrusions (126, 134, 136).

At the molecular markers level, functional loss of the adherens junction protein (E-cadherin) is considered to be the hallmark of EMT. There exists a direct correlation between loss of E-cadherin expression and loss of epithelial phenotype, but E-cadherin loss alone is not sufficient for EMT (137-139). Loss of cytokeratin (intermediate filament) expression, while acquiring weakly mesenchymal intercellular adherens (N-cadherin) and gain of the mesenchymal marker vimentin (intermediate filament), PDGF receptor and integrin $\alpha_V\beta_6$, as well as the secretion of proteases (MMP2 and MMP9) (126, 136,140-142) are additional but variable requirements. A number of transcription factors, including SNAILs, TWISTs, ZEBs, FOXC2 and Goosecoid are identified as EMT markers via regulating E-cadherin, N-cadherin and integrins directly or indirectly (141,143,146,147). Interestingly, these transcription factors all induce the mesenchymal phenotype, while the investigation of epithelial inducing transcription factors has lagged behind, although some, such as GRHL2, ELF3, ELF5

and p63, have been discovered (148). The functional changes of EMT associate with transition from the stationary epithelial cells to the motile mesenchymal cells. The reorganization of cytoskeletal proteins and production of ECM-degrading enzymes together enable mesenchymal cells to migrate individually and invade underlying ECM. Some of them acquire a heightened resistance to apoptosis partially based on the mesenchymal stem cell features (141).

EMT and cancer invasion and metastasis

The relevance of EMT to human physiological processes *in vivo* was long debated (125,149-152). However, human cancer pathological specimens have provided accumulating evidence for its relevance to carcinogenesis (153,154). The last step of cancer progression is invasion and metastasis. During the invasion and metastasis cascade, some epithelial cancer cells from the primary location must modify their phenotypic features to overcome all the barriers and survive, and finally initiate secondary cancer formation. Some researchers consider EMT as the first step of metastasis (155). When transformed epithelial cancer cells activate embryonic programs of epithelial plasticity and acquire access to EMT, they generate various mesenchymal derivatives that have enhanced invasive and migratory abilities through three main intrinsic modifications: weakened cell to cell cohesion, enhanced ability to degrade ECM and facilitated cell motility due to modified cytoskeleton (Figure 5) (155). Relying on these acquired abilities mesenchymal cells leave primary cancer nodules and invade into surrounding stroma, some of them intravasate to become circulating tumor cells (CTCs) (131, 137,155) .

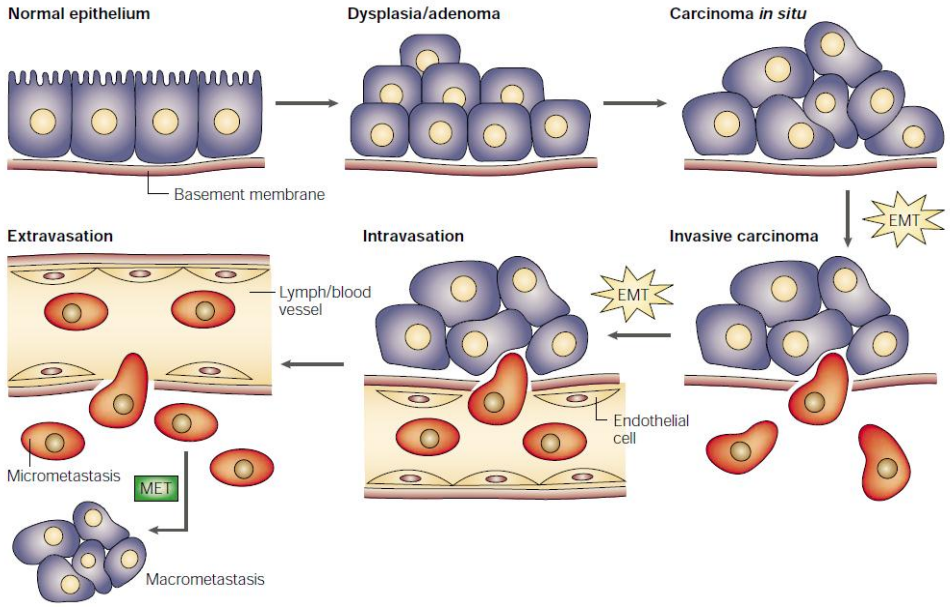

Figure 5. EMT and cancer progression. Normal epithelia can give rise to a local adenoma. Further transformation leads to carcinoma in situ, and further local dissemination of carcinoma cells can be induced by EMT. The EMT transformed cells can move into the circulation and be transported to distant organs. At secondary sites, a new carcinoma can be formed through MET. The figure is adapted from (137).

EMT and resistance to cell death and senescence

When cancer cells enter into the vascular bed and tend to be CTCs, they will meet a lot of strong apoptotic inducing signals such as deficiency of cell adhesion, physical stress and immune cell interaction (155). EMT provides cancer cells with the ability to conquer these barriers and evade cell death and senescence. Constitutive expression of EMT inducers can maintain the mesenchymal and invasive phenotype while ensuring the survival of micrometastatic cells by suppressing two safeguard mechanisms against cancer: premature senescence and apoptosis. For example, Snail can down-regulate caspases directly and antagonize TP53 (156); Twist can antagonize Myc, abrogate TP53 and RB or inhibit p16/ink4a and p21/cip (157, 158), Zeb1 also protects mouse embryonic fibroblasts from senescence (159). This suggests that abrogation of apoptosis and senescence may be general EMT associated mechanisms.

EMT and resistance to chemotherapy and immunotherapy

Cancers undergoing EMT may resist conventional chemotherapy. For example, colon and ovarian carcinoma cell lines that underwent EMT are resistant to oxaliplatin and paclitaxel (154,160,161). Over-expressed Twist in human cancer cell lines confers resistance to paclitaxel, taxol and vincristine (162,163). Moreover, Twist depletion partially reversed multi-drug resistance in breast cancer cells and cisplatin resistance in lung cancer (164,165). Similarly, Snail1 and Snail2 provide mammary tumors and melanoma with resistance to paclitaxel, adriamycin and radiation (156,166).

EMT and prostate cancer

Metastases have lethal consequences in PCa and are associated with EMT. The Gleason grading system is used to evaluate the malignant degree and is the basis for clinical treatment. Increasing Gleason score is associated with EMT related morphology and biomarker changes, including progressive loss of epithelial cells and gain of mesenchymal cells (139). There is evidence that transformed epithelial and mesenchymal cells co-exist in prostate phyllodes tumor and carcinosarcomas (167, 168). However, many aspects of EMT in human prostate cancer remain unsettled on the basis of pathologic examination, due to the lack of unambiguous markers, possible stromal staining leading to misinterpretation and the lack of longitudinal evaluation (139) .

On the other hand, a number of studies have reported EMT in prostate cancer cell lines. For example, overexpression of CAV1 and ID1 or MMP14 induced EMT in LNCaP cells (169,170). EGF treatment induced EMT in DU145 cells (171). Depletion of PDEF (Prostate-derived ETS factor) or treatment by BMP7 induced EMT in PC3 cells (172). However, there are some contradictory observations among these studies, such as both EMT and MET induction were reported in PC3 cells (172-174).

MicroRNAs

MicroRNAs (miRNAs) are evolutionary conserved tiny non-coding RNAs that target mRNAs (175). They are 18–25 nucleotides in length and regulate gene expression using a sequence-specific fashion defined by Watson–Crick complementarities. The

targeted mRNA sequences will be degraded when miRNA–mRNA complementarity is perfect, and with imperfect complementarity translation of target mRNAs will be blocked. Regardless of which event occurs, the result is a decrease in proteins made from the targeted mRNA (176).

MiRNAs and cancer progression

MiRNAs work as cancer oncogenes or suppressors and mutations of these miRNAs are also associated with carcinogenesis (177-179). During cancer initiation, anomalous miRNAs are involved in excessive proliferation and apoptosis prevention. For example, let-7 negatively regulates RAS and MYC and acts as a cancer suppressor (180-184). Regarding angiogenesis, mir-125 and mir-17/20/106 regulate VEGF-A and VEGFR1/HIF-1A, respectively (185,186). MiRNAs also act as master regulators during invasion and metastasis (187). The deregulated miRNAs in metastatic cancers are referred to as metastamir (187,188).

MiRNAs and EMT

MiRNAs contribute to cancer also through regulating EMT and MET programs by direct or indirect effects on EMT related factors (187). MiR-9 may initiate EMT in breast cancer by targeting E-cadherin (189). MiR-200 family members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 regulate EMT by directly targeting the mRNA of ZEB1/ZEB2 or TGF-β (190-192). Aberrant miR-103/107 is associated with metastasis and poor outcomes in breast cancer patients (193). Additionally, EMT related miRNAs are verified to contribute to therapy resistance: reintroduction of miR-200c, a negative regulator of EMT, restores chemotherapeutic sensitivity in ovarian cells (155,194).

Reactive oxygen species and oxidative stress

Reactive oxygen species (ROS) are chemically reactive molecules naturally produced during cellular metabolism of oxygen (195). Increased ROS has traditionally been associated with pathological conditions involved in infection or aging due to tissue injury or DNA damage. Thus, excessive production of ROS or decreased ability to detoxify the reactive intermediates can cause the cell to experience oxidative stress and

the elevated ROS may play a role in many diseases, including cancer (196). Recent studies reveal an essential role of ROS in processes associated with proliferation, apoptosis and senescence resulting from the activation of signaling pathways (197).

ROS include a wide range of molecules. Superoxide, hydrogen peroxide and hydroxyl radicals are the most well studied ROS in cancer. Increasing evidence has shown that ROS participate in multiple steps of cancer progression: (1) promote cellular transformation by generating gene mutations and structure alterations in initiated cell populations; (2) increase cell proliferation and/or decrease cell apoptosis and introduce further DNA mutations (196,197); (3) contribute to uncontrolled cancer cell survival and proliferation by influencing lots of key signaling cascades involved in cell survival, proliferation, apoptosis and cycle progression (197-199); (4) strengthen cancer metastasis by increasing the migration and invasion of cancer cells, as well as angiogenesis (195).

ROS and prostate cancer are tightly associated because prostate cancer is mainly an age-related malignancy and multiple epidemiological and clinical studies indicate oxidative stress as one of the major aging-associated influences on prostate carcinogenesis (196,200). In addition, high ROS status is found critical for the malignant phenotype of PC3 and DU145 prostate cancer cell lines (201). A number of molecular links have been found among oxidative stress, aging and inflammation (202). However, direct evidence and the mechanism of ROS-mediated oxidative stress involved in prostate initiation and progression remain to be elucidated (196).

IL6/STAT3 signaling

Inflammation is a widely accepted important factor in cancer (89). Among the many putative inflammation-related factors, pro-inflammatory cytokines, especially interleukin-6 (IL6), have many physiologic roles and have been implicated in a number of types of cancer (203).

IL6 is elevated in the sera of patients with metastatic prostatic carcinoma and it is a widely recognized marker of prostate cancer (203-205). Serum levels of IL6 were significantly elevated in patients with clinically evident hormone refractory disease

comparing the IL6 levels to those in normal controls, prostatitis, benign prostatic hyperplasia (BPH) and localized and recurrent disease, suggesting that IL6 may be a surrogate marker of the androgen independent phenotype (204,205). In a study of incidence of abnormal circulating levels of IL6 in patients with well-characterized, advanced, hormone refractory prostate cancer prior to suramin therapy, a direct comparison of the high and low serum IL6 groups show that elevated IL6 levels are strongly correlated with objective measures of morbidity including decreased hematocrit, hemoglobin, and serum cholesterol, and increased white blood cell count and serum lactate dehydrogenase levels, all without clinical infection (204,205).

IL6 belongs to the ‘‘IL6 type cytokine’’ family including leukemia inhibitory factor (LIF), IL11, ciliary neurotrophic factor (CNTF), cardiotrophin-1(CTF1) and oncostatin M (OSM) (203). They exert multifunctional actions via the signal transducer gp130, LIF receptor and OSM receptor. Signal transducer and activator of transcription 3 (STAT3) is the central mediator of IL6 type cytokine signaling pathway and executes most of the proliferative and survival effects (206). They mediate tumorigenesis by protecting cells from apoptosis and promoting cell cycle progression (G1 and G2/M) (207). They also regulate angiogenesis and cancer induced immunosuppressive functions (208). Extensive studies have demonstrated the central role of STAT3 in IL6-type cytokine signaling in prostate cancer (209-211), suggesting a potential role of the IL6/STAT3 cascade in prostate cancer progression.

BACKGROUND AND AIMS

Background

Understanding the mechanisms of tumor initiation and progression is critical in diagnosis and treatment of prostate cancer patients with metastases. Though multiple factors and processes have been implicated in prostate cancer progression (1), the trigger for initiation of prostate malignancy is still a topic of debate, which is partially due to the situation that good cancer initiation and progression models of this cancer type have been lacking. Previous human prostate carcinogenesis models were mainly established by exposure of benign prostate cells to strong external carcinogens or introduction of oncogenes or viral elements. However, most of the clinical prostate cancers have developed without exposure to such kinds of strong external carcinogens, and these kinds of prostate cancer models are therefore likely to lack important aspects of prostate carcinogenesis *in vivo*. Therefore, it is extremely valuable to establish a prostate carcinogenesis model based on more physiological conditions.

The EP156T cell line is a successfully immortalized prostate epithelial cell line achieved by transduction of human telomerase reverse transcriptase (hTERT) without over-expression of viral oncogenes or other exogenous oncogenes (212,213). The EP156T cell line exhibits a significant pattern of authentic prostate epithelial cell specific features. In Matrigel 3-dimensional cultures it is able to differentiate into glandular buds that closely resemble the structures formed by primary prostate epithelial cells in vivo (212,213). The EP156T cell line may serve as a unique experimental platform to perform several studies, including cell-cell interactions in an authentic prostate microenvironment, prostate epithelial cell differentiation, and most importantly, the complex multistep process leading to prostate cell transformation towards cancer (212, 213).

General aims

In this study, the main aim was to develop a prostate cancer initiation and progression model based on physiological adaptation and selection without exposure to external carcinogens or introduction of oncogenes or virus elements. The second aim was the

systematic characterization of the model including functional abilities, gene expression, microRNA expression and genetic profiling. The final and most important aim was to identify key regulators and factors in prostate cancer initiation and progression, thus providing targets for therapeutic development of anti-cancer drugs.

Specific aims

Paper I

The aim was to establish a prostate malignant transformation model based on EP156T cells. EP156T cells are human prostate primary epithelial cells with basal cell features, and they were isolated from benign tissue of a patient and immortalized by overexpression of hTERT in collaborator Professor Varda Rotter's group. The strategy was to adapt EP156T cells in long-term confluent culture and select cells with loss of contact inhibition.

Paper II

We wanted to promote malignant features of the EPT1 cells established in Paper I. EPT1 cells were derived from EP156T following epithelial to mesenchymal transition (EMT) but without additional malignant features. The strategy was to adapt EPT1 cells in long-term confluent culture and to select cells with the ability to override quiescence and form focus in monolayer culture.

Paper III

The aim was to understand the roles of miRNAs in EMT and its reversal (MET). The strategy was to profile the differentially expressed miRNAs in epithelial EP156T cells and mesenchymal EPT1 cells by miRNA microarrays and examine the roles of the most significantly changed microRNAs by loss of function and gain of function in EP156T cells and EPT1 cells.

Paper IV

We wanted to achieve full malignant transformation of the EPT2 cells established in paper II. EPT2 cells had obtained the abilities to form foci in confluent monolayer culture, to form robust colonies in soft agar, to be more resistant to apoptosis and to be

independent of exogenous growth signals. However, EPT2 cells failed to form tumors in immunodeficient mice, suggesting that full transformation had not been achieved. The strategy was to adapt EPT2 cells in protein free medium and test the tumorigenicity of cells that are growing independently of exogenous growth factors. Furthermore, it was an aim to start to define the critical molecular mechanisms associated with tumorigenicity and metastasis.

METHODOLOGICAL CONSIDERATIONS

Cell culture, cell selection and adaption

EP156T, EPT1 and EPT2 cells were cultured in MCDB153 medium supplemented with MEM non-essential amino acids solution, hydrocortisone, triiodothyronine, insulin, transferrin, sodium selenite, testosterone, EGF, bovine pituitary extract and fetal calf serum (FCS). EPT3, EPT3-PT1 and EPT3-M1 cells were grown in Hams F12 medium containing 5% FCS. Prostate cancer PC3 and DU145 cell lines were grown in Hams F12 medium and DMEM medium containing 10% FCS, respectively. All cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C.

To select cells with loss of contact inhibition, EP156T cells were allowed to grow to full confluence at passage 43 (p43) in 96-well plates. Cells were maintained in the same plates and the medium was changed every 3 days until distinct morphological changes appeared at around twelve weeks later. We chose loss of contact inhibition as the phenotype in selection of malignant cells because it is a widely accepted property to distinguish cancerous cells from normal cells. Contact inhibition is the natural process of ceasing growth once normal epithelial cells abut on each other or reach confluence in culture. Immortalized cell lines are still subject to contact inhibition even when they are able to proliferate indefinitely. This process is tightly regulated by cell communication and it is a mechanism to prevent uncontrolled growth and maintain tissue homeostasis (214), while cancer cells typically demonstrate loss of contact inhibition and continue to grow even when they reach confluence (215). To select cells with the ability to form focus in monolayer culture, EPT1 were kept growing at full confluence in standard culture medium. The medium was changed every third day without trypsinization until foci formed 8 weeks later. After reaching confluence, EPT1 cells continue dividing for 2 weeks and enter a resting state referred to as quiescence. Overriding quiescence indicates the ability of cells to undergo uncontrolled proliferation and give rise to tumors (216). To adapt EPT2-D5 cells in protein free medium, the complete medium was replaced with basic Hams F12 medium without serum (protein free medium). The protein free medium was changed

every 3 days until spheres were generated. The ability to grow in protein free medium indicates self-sufficiency of growth signals, which is the first of the ten hallmarks of cancer (89). Collectively, we chose these simple but useful physiological selection and adaption strategies in this study.

Functional assays for malignant features *in vitro*

In this study, assays for *in vitro* malignant features included cell proliferation, cell migration, cell invasion, foci formation, anchorage independent growth and self-sufficiency of growth signals. Cell proliferation assay is a colorimetric method for determining the number of viable cells in proliferation using a tetrazolium compound Assay (MTS). For confluent culture, the viable cells were determined by cell counting since MTS assay is dependent on cellular metabolic activity and not suitable for quiescent cells in confluent culture. Cell migration and invasion were determined in chamber assays utilizing basement membrane-coated inserts to assay the migrative or invasive properties of examined cells. Wound healing assay was also used to determine cell migration. Foci formation was examined in confluent monolayer culture. Anchorage independent growth is a classic malignant feature *in vitro* and it was evaluated by culturing cells in soft agar. Self-sufficiency of growth signals was measured by adapting cells in protein free medium, the dependence of cells on extra growth factors was represented as the relative proliferation of the test group (in protein free medium) compared with the control group (in complete medium).

Functional assays for malignant features *in vivo*

Generation of tumor and metastasis in mice model is the gold standard for fully transformed malignant cells. The malignant capacity *in vivo* was evaluated by xenograft tumor formation in mice. The cells were tested in subcutaneous tumor growth and subsequently in orthotopic xenograft tumor formation. The metastasis was tracked by bioluminescence images employing Optix® MX2 Time-Domain Molecular Imager or In Vivo MS FX PRO. The development and progression of tumors were monitored by bioluminescent imaging. All experiments were approved by the

Norwegian Animal Research Authority and conducted according to The European Convention for the Protection of Vertebrates Used for Scientific Purposes.

Gene expressing analysis

Gene expression was analyzed at the mRNA and protein levels. At the mRNA level, the methods included genome wide profiling using the Agilent DNA microarray platform and real-time quantitative PCR using TaqMan assays. DNA microarray is a powerful technique that provides an overview of transcriptional patterns of all the genes in the cells. It is extremely advantageous in looking for critical regulators in certain processes. At the protein level, methods included immunoblotting (Western blot) to quantify the expression level, immunofluorescence and immunohistochemistry to visualize the expression and location of proteins in cells and tissue sections. For secreted proteins, we used proteomic analysis and enzyme-linked immunosorbent assay (ELISA) techniques to detect or measure the proteins in cell culture supernatants.

For miRNAs expression, the methods were genome-wide miRNA microarray techniques combined with real-time qPCR using using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). MiRNA microarrays detected the expression of all the registered miRNAs and provided a list of the significantly changed miRNAs in certain experiments. The TaqMan MicroRNA Assays used miRNA-specific looped RT primer and miRNA-specific forward PCR primer to accurately detect mature miRNAs.

Genetic profiling

A serious problem in cell culture experiments is cross-contamination and cell line misidentification (217) . We verified the genetic identity of the cell lines in this study by various genetic characterizations, such as karyotyping, DNA microsatellite and DNA copy number analysis. Chromosome karyotyping was performed by the standard Giemsa staining procedure and metaphase spreads were analyzed. DNA fingerprinting was performed using the AmpFlSTR Profiler Plus PCR Amplification Kit (Applied Biosystems). Samples were run and allele sizes were interpreted on an ABI 3100 Genetic Analyzer with Gene Mapper v3.7 software. DNA copy number analysis was

performed using Affymetrix Genome-Wide human SNP arrays to examine acquired genomic copy number changes and loss of heterozygosity of all the cell lines.

Loss of function and gain of function analysis

We characterized the roles of regulators or signaling pathways by loss of function and gain of function assays. For loss of function analysis, we used RNA interference technique to knock down the mRNA expression (**Paper III**). By RNAi technique, the mRNAs of target gene are destroyed by the designed small RNAs that are perfectly complementary to the target mRNAs (218). We introduced the small RNAs to cells by transduction of lentiviral vectors carrying the small RNA coding sequences. Three small RNA expression clones were used for each target mRNAs. RNAi is a simple and widely used knock-down technique, the limitation is that less significant knock-down phenotypes may occur due to remaining undestroyed mRNAs. To evaluate the function of IL6, a neutralizing antibody was used to block the interaction between IL6 and IL6 receptor (**Paper IV**). To block STAT3 signaling pathway, several chemical inhibitors that have been demonstrated in the literatures were used for loss of function analysis (**Paper IV**).

For gain of function analysis, we overexpressed the interesting genes using third generation lentiviral systems. The advantages of lentiviral vectors include high transduction rate and efficient integration into the host genome. The positive transduced cells were purified by the expression of fluorescent protein encoded by the vectors. To examine gain of function of IL6, we used conditioned medium of D5HS cells with high secretion of IL6. A limitation of this conditioned medium is the short time that it can be used (usually 1 day) because it has been used for 3 days already in D5HS cells to accumulate the high IL6 level.

Characterization of EMT

We characterized EMT features in multiple aspects: 1) Morphological observation. In monolayer culture, epithelial cells have a very clear and round boundary with individual cells abutting on each other in a uniform array, while mesenchymal cells have a much longer and irregularly scattered cell shape and varied in composition and

density. In three dimensional cultures epithelial cells form spheroids with lumen-like structures, mesenchymal cells grow as loose cell clusters and migrate as individual cells within the gel. 2) Functional assays, such as trans-well migration and invasion assay, wounding healing assay. Compared to epithelial cells, mesenchymal cells have higher abilities of migration and invasion. 3) Examination of molecular markers of EMT by DNA microarray, real-time qPCR and immunoblotting. The EMT markers include epithelial cell markers (e.g. E-cadherin and P-cadherin), mensenchymal cell markers (e.g. FN1 and FBN1), as well as EMT regulators such as SNAI11/2, ZEB1/2 and TWIST1/2. EMT can be considered when all the morphological changes, functional changes and molecular markers are evidenced.

Identification of target genes of miRNAs

For the most highly repressed targets of miRNAs, mRNA destabilization usually comprises the major component of repression (219). The strategy for identification of miRNA targets included bioinformatics prediction, reporter assay and loss of function assay. 1) The candidate targets of miRNAs were firstly identified by computational miRNA target prediction combined with the repressed genes by overexpression of miRNAs. 2) Direct interaction of miRNAs and target mRNAs was evaluated using luciferase reporter assays. The predicted target sequences were linked to the firefly luciferase gene in a reporter vector. Following co-transfection of miRNA expression vectors and reporter vectors into HEK293T cells, the direct interaction of miRNA and target sequences can be measured based on the activity of firefly luciferase in cells. To further demonstrate the targeting, point mutations were made in the predicted sequence in the reporter vector, a rescue of the firefly luciferase activity will verify the sequence-mediated miRNA targeting. 3) Functional targeting of miRNAs was verified by comparison of the phenotypes and affected genes in cells following knock-down of the target mRNAs and in cells overexpressing miRNAs. The phenotypes induced by overexpression of miRNAs should be phenocopied by knock-down of the target mRNA using RNAi technique. If the target mRNAs encode transcription factors, the changed gene expression patterns by over-expression of miRNAs should have significant overlap with the changed genes by knock-down of target mRNAs.

Measurement of ROS/IL6/STAT3 levels

Production of cellular ROS was measured using membrane permeable fluorescent dye 2',7',-dichlorofluorescin diacetate (DCFDA) followed by fluorescent microscopy or flow cytometry. It is important to have the same incubation time and interval time before examination of ROS in different experiments because the DCFDA intensities varied with different incubation time and decreased in time course after the dye was removed. ROS encompass a wide range of molecules including hydroxyl radicals, peroxides and superoxides. DCFDA is mainly sensitive to hydrogen peroxide (220), future work will examine the role of other reactive oxygen species.

The level of autocrine IL6 was detected by enzyme-linked immunosorbent assay (ELISA) and proteomic analysis of the secreted proteins in the culture supernatant. For ELISA, it is important to have cells in similar density and culture time to have comparable data among different experiments because the secreted IL6 levels depend on the cell number and the culture time. The STAT3 signaling activity was tracked by a green fluorescent protein (GFP) based pathway reporter. A tandem STAT3 targeting DNA motif was linked to the mini-promoter of GFP. When the reporter was transduced in cells, the activity of STAT3 signaling in the cells was reflected by the GFP expression that can be measured by fluorescent microscopy and flow cytometry. The STAT3 signaling activity was also determined by immunoblotting technique using an antibody against the STAT3 protein phosphorylated on Y705 (pSTAT3). STAT3 is activated by phosphorylation at tyrosine 705 (Y705) in response to IL6, which induces dimerization, nuclear translocation and DNA binding of STAT3 proteins (221,222). An antibody against total STAT3 was used as control to show that the detected STAT3 by pSTAT3 antibody is due to tyrosine phosphorylation.

Evaluation of tumor initiating cells

Tumor-initiating cell (TIC) is assumed to be a small fraction of the cancer cell population driving cancer initiation, maintenance and metastasis formation. We defined TICs in this study by xenograft tumor implantation. Firstly, D5HS spheres, EPT2-D5 cells in complete medium and EPT2-D5 cells in protein free medium for three days were tested in parallel. Only mice injected with D5HS spheres formed

tumors, indicating the enrichment of TICs in D5HS spheres. Secondly, small populations of EPT3-M1 cells with high and low STAT3 activities were tested for tumorigenicity in serially diluted cell numbers, much fewer cells were needed for $STAT3^{high}$ cells than $STAT3^{low}$ cells to generate tumor and metastasis, demonstrating higher enrichment of TICs in EPT3-M1 cells with high STAT3 activity.

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RESULTS

In **Paper I** we have established an EMT model in prostate cells. In selecting for cells with loss of contact inhibition, the prostate primary epithelial EP156T cells were observed to undergo EMT accompanied by loss of contact inhibition after about 12 weeks in confluent culture. The changed new cells were named EPT1. EMT of EPT1 was characterized by striking morphological changes and increased invasion and migration compared with the original EP156T cells. Gene expression profiling showed extensively decreased epithelial markers and increased mesenchymal markers in EPT1 cells, as well as pronounced gene expression switches of modules involved in cell adhesion and attachment. However, EPT1 cells were sensitive to serum or growth factor withdrawal and not able to grow in an anchorage-independent way in soft agar, suggesting lack of several malignant features *in vitro*.

In **Paper II** we maintained cells in saturation density cultures to select for cells overriding quiescence. Foci formed repeatedly following around 3 - 8 weeks in confluent EPT1 monolayers. Cells picked from the foci were named EPT2 and were cloned in soft agar. Cells derived from a clone with name EPT2-D5 was used as representative in further studies. EPT2-D5 cells formed robust colonies in soft agar, a malignant feature present neither in EP156T nor in EPT1 cells. EPT2-D5 cells showed additional malignant traits *in vitro*, including higher ability to proliferate following confluence, higher resistance to apoptosis and lower dependence on exogenous growth factors than EP156T and EPT1 cells. Microarray profiling revealed a set of cell junction modules that changed stepwise from EP156T to EPT1 and to EPT2-D5 cells. These findings provide a novel cell model in which EMT emerges independently of transformation and is associated with subsequent accumulation of malignant features in prostate cells.

In **Paper III** we profiled miRNA expression and found that miR-203 and miR-182 were highly expressed in EP156T cells and became completely repressed in the progeny EPT1 cells following EMT without malignant transformation. Forced re-expression of miR-203 or miR-182 in EPT1 cells induced MET features including increased epithelial traits in both two and three dimensional cultures and decreased

ability of migration and invasion. Simultaneously, miR-203 and miR-182 made EPT1 cells resistant to apoptosis induced by growth factor withdrawal and provided cells with the ability of self-sufficiency of growth signals, a well-recognized oncogenic feature. Gene expression profiling showed high overlap of the affected genes by miR-203 and miR-182. Many up-regulated epithelial genes and down-regulated mesenchymal genes were found in both EPT1-203 and EPT1-182 cells. SNAI2 was identified as the common target of miR-203 and miR-182. Knock-down of SNAI2 in EPT1 cells mimicked the phenotypes of re-expression of miR-203 or miR-182 regarding both MET and self-sufficiency of growth signals. Furthermore, considerable overlaps were found between the changed genes following miR-203 or miR-182 expression and those affected by knock-down of SNAI2. P-cadherin was identified as the downstream target of SNAI2. We conclude that miR-203 and miR-182 induce both MET and growth factor independent growth via repressing SNAI2 in prostate cells.

In **Paper IV** we achieved fully malignant transformed prostate cells and identified a ROS/IL6/STAT3 cascade critical to prostate cancer initiation and progression. When premalignant EPT2-D5 cells were adapted in protein-free medium, numerous tight spheres were generated in monolayer culture. In contrast to EPT2-D5 cells, the prostate spheres (D5HS) efficiently formed large subcutaneous tumors and subsequent metastasis *in vivo*, thus verifying the ability of tumor initiation of D5HS. Gene expression profiling revealed that a number of growth factors and cytokines, especially the pro-inflammatory cytokine IL6, were significantly activated in D5HS. The essential roles of IL6 and the downstream STAT3 signaling were confirmed by neutralizing antibody, chemical inhibitors and pathway reporter. Additionally, elevated ROS, so called oxidative stress, were produced upon protein depletion and were required for the activation of IL6/STAT3 in D5HS. Importantly, a positive feedback loop was found between ROS and IL6. The association of ROS/IL6/STAT3 in tumor initiation was further demonstrated by examination of xenograft tumors and tumor derived cells and diluted cell implantation. Consistently, inflammation signatures were found in tumor spheres and metastasis cells, indicating a causal role of inflammation in prostate progression.

In this study, for the first time, we demonstrated the intrinsic association of ROS and IL6/STAT3 in prostate carcinogenesis. The high levels of ROS/IL6/STAT3 cascade in this carcinogenesis model will benefit understanding of the mechanism of prostate cancer initiation and progression as well as therapeutic development of anti-cancer drug targeting of the IL6/STAT3 pathway.

DISCUSSION

Prostate carcinogenesis model

A barrier in prostate cancer research is the lack of a good carcinogenesis model of this cancer type. Previously, malignant transformation of human prostate cells were established by radiation (50) or chemical treatment (51,52) or introduction of virus elements (54,55). However, most incidences of prostate cancer patients are not likely due to the exposure to such strong external carcinogens considering it is mainly a disease of aging, and these kinds of prostate cancer models may not be representative of prostate cancer *in vivo*.

In contrast to above models based on such strong external carcinogens, this study presents an *in vitro* carcinogenesis model based on biological adaption and selection in long-term saturation density culture and protein free medium culture without addition of any external inducers. EPT1 cells were generated by selection of cells with loss of contact inhibition (**Paper I**). Contact inhibition is a natural process of ceasing growth when cells abut on each other or reach confluence in culture (215). Loss of contact inhibition is associated with decrease of cell to cell junctions and is required for increased invasion and migration of metastatic cells (223). EPT2-D5 cells were generated by selection of cells with increased ability to override quiescence in confluent culture (**Paper II**). Overriding quiescence is considered as an important mechanism of cancer initiation *in vivo* (216), which represents a proliferative advantage and favors selection of tumor cells (216). Finally, EPT3 tumors were initiated from tumor spheres that were produced by adapting EPT2-D5 cells in protein free medium (**Paper IV**). Growth in protein free medium, also called self-sufficiency of growth signals, is the first of six capacities of cancer cells (89). Most importantly, the increased ROS in protein free culture has a well-recognized major aging-associated influence on prostate carcinogenesis (200). Collectively, the stepwise establishment of this prostate carcinogenesis model based on such kinds of physiological adaption has generated a unique and attractive model for prostate cancer research.

Hallmarks of the carcinogenesis model

There are 10 hallmarks of cancer according to Hanahan's and Weinberg's hypothesis: (**I**) sustaining proliferative signaling, (**II**) evading growth suppressors, (**III**) resisting cell death, (**IV**) enabling replicative immortality, (**V**) inducing angiogenesis, (**VI**) activating invasion and metastasis, (**VII**) genome instability and mutation, (**VIII**) reprogramming energy metabolism, (**IX**) cancer-promoting inflammation and (**X**) evading immune destruction (89). In this study, EPT3 tumors were generated by tumor spheres cultured in protein free medium. The ability to grow in protein free medium indicates self-sufficiency in growth signals (**hallmark I**), resistance to cell death induced by protein deprivation (**hallmark III**) and reprogrammed energy metabolism (**hallmark IX**); the serial passaging of the tumor spheres *in vitro* and xenograft tumor *in vivo* in long-term suggests the evasion of growth suppression (**hallmark II**) and replicative immortality (**hallmark V**); formation of large subcutaneous tumors demonstrates the ability to induce angiogenesis (**hallmark VI**); generation of extensive abdominal metastases following orthotopical injection of EPT3 cells suggests the activation of invasion and metastasis (**hallmark VII**); the increased genetic aberrations in EPT3-PT1 and EPT3-M1 cells suggest genomic instability and mutation (**hallmark VII**); the enrichment of inflammatory process in changed genes in tumor spheres and metastatic tumors indicates the cancer-promoting inflammation (**hallmark X**). The ability of the EPT3 tumor to evade immune destruction remains undetermined since the host mice are immunodeficient. Collectively, this prostate carcinogenesis model harbors 9 of the 10 hallmarks of cancer, thus it is a comprehensive cancer model and suitable for further prostate cancer research.

EMT in prostate cells

This work is the first report of an EMT model based on prostate primary epithelial cells. In prostate cancer cells, EMT has been described, including over-expression of CAV1 and ID1 or MMP14 in LNCaP cells (169,170), EGF treatment of DU145 cells (171), depletion of PDEF (173) or BMP7 treatment of PC3 cells (172). However, some of these studies cannot easily be reconciled, such as reports of both EMT and MET induction in PC3 cells (172-174). Long-term passages of these cell lines in different

laboratories may cause them to differ significantly from the original patient cells, and new models closer to prostate tissue are desirable.

EMT is not easily observed in histological examination of cancer tissue sections, even by experienced pathologists (224,225). An explanation may be that EMT may be transient during cancer progression (143) or occur only in a small subpopulation of the tumor cells, such as cells at the invasive front (150, 226-229) or cancer stem cells (152, 230,231). The successful establishment of an EMT model based on primary prostate cells with many traits of the prostate basal cell phenotype is important in light of a recent report on the significance of EMT in prostate cancer (232).

EMT and malignant transformation

EMT has been considered an event following malignant transformation to endow cancer cells with invasive and metastatic competence (125,233). EMT being the first step in the present prostate carcinogenesis model suggests that EMT can not only promote more aggressive cell biological traits in malignant cells, but also facilitate malignant features of benign prostate epithelial cells, which is supported by two observations that metastatic dissemination is a distinct early step in cancer progression, and the hypothesis was proposed that premalignant cells can enter the systemic circulation during these early stages and become sources of later metastatic tumors (143, 225).

The promotion of malignant features by EMT is also hinted to by studies of stem cells. Recent work demonstrated that EMT not only generates epithelial stem-like cells from normal epithelial cells, but also promotes the tumorigenicity of transformed cells in animals by inducing TICs (227,228), indicating crucial roles of EMT in the tumorigenic process. Our present study represents a distinct stepwise model in which cells first underwent EMT from untransformed cells and subsequently changed to cells with many malignant features and subsequently TICs, thus providing an alternative mechanism to explain carcinogenesis *in vivo*, since EMT is widely observed in body development and other physiological processes (234).

Dual roles of miRNAs in cancer-related processes

In **Paper III**, we found dual functions of miR-182 and miR-203 in EPT1 cells. MiR-182 and miR-203 were completely repressed during EMT from prostate epithelial EP156T cells to the progeny mesenchymal non-transformed EPT1 cells. Re-expression of miR-182 or miR-203 in EPT1 cells induced MET features and generated the ability to self-sufficiency of growth signals, a well-recognized oncogenic feature. Furthermore, SNAI2 was identified as a common target of miR-182 and miR-203. Knock-down of SNAI2 in EPT1 cells phenocopied EPT1-182 and EPT1-203 cells regarding both MET and self-sufficiency of growth signals.

It is interesting to find dual roles of miR-182 and miR-203 when they were re-expressed in EPT1 cells. Enforcing the epithelial phenotype and inhibition of migration and invasion suggest that both miRNAs are likely to suppress metastasis in tumor progression (151). Obtaining self-sufficiency of growth signals suggests oncogenic potential (89). Tumorigenesis is a multistep process, a certain factor can show different and even contradictory roles in different aspects of tumorigenesis, *e.g.* exemplified when miR-200s suppress early steps of metastasis by hindering tumor cell migration and invasion into the circulation from primary tumors, but enhance the late step of metastasis by promoting metastatic colonization at second sites (235). Both miR-203 and miR-200s were shown to be highly expressed in localized tumors and down-regulated in metastases, defining a dynamic two-stage model of miRNA expression (236,237). Additionally, knock-down of the key EMT regulators SNAI1, ZEB1 or TWIST2 in mesenchymal-like prostate cancer PC3 cells induced typical epithelial program and increased tumorigenicity of these cells (238), which fits with our findings that over-expression of miR-182/203 or knock-down of SNAI2 induced not only epithelial features but also an oncogenic property. The dual functions of these kinds of miRNAs and EMT regulators strongly indicate their complex mechanisms in tumor progression.

Adaption of cells in protein free medium

EPT3 tumor initiating cells were obtained by adapting pre-malignant EPT2 cells in protein free medium (**Paper IV**). Chemically defined serum-free medium is widely

used to culture tumor spheres or cancer initiating cells (239-241). However, these media are often supplemented with growth factors such as EGF/FGF and all cells are non-adherent in the cultures (239-241). In the current study, EPT2-D5 cells efficiently generated tumor spheres in medium completely free of serum and any other growth factor, suggesting an even higher independence of exogenous growth stimulation. Additionally, generation of D5HS spheres from single adherent cells provides a unique model to study the cell division and metabolism of tumor initiating cells.

Growth in protein free medium, so called self-sufficiency of growth signals, is the first of six capabilities required to convert a normal somatic cell into a cancer cell according to Hanahan's and Weinberg' outline (89). Although independence of exogenous growth factors is an inherent feature of EPT2 cells, comprehensive reprogramming was triggered when cells were adapted in protein free medium, as demonstrated by strikingly altered gene expression patterns, sphere formation, and most importantly, the ability to initiate tumorigenesis.

ROS/IL6/STAT3 and prostate cancer

Though multiple factors and processes have been implicated in prostate cancer progression (1), the trigger for initiation of malignancy is still a topic of debate. Prostate cancer is a mainly an age-related malignancy and multiple epidemiological and clinical studies indicated oxidative stress, caused by the imbalance of reactive oxygen species (ROS), as one of the major aging-associated influences on prostate carcinogenesis (196,200). In **paper IV**, the indispensable role of ROS in initiation of EPT3 tumor further supports the association of oxidative stress and prostate tumor progression.

IL6 is elevated in the sera of patients with metastatic prostatic carcinoma and it is a widely recognized marker of prostate cancer (203,204). Extensive studies have demonstrated the central role of STAT3 in IL6-type cytokine signaling in prostate cancer (209,210). In **Paper IV**, we confirmed the essential roles of IL6 and the downstream STAT3 signaling in tumor sphere formation and tumor initiation and progression.

As discussed above, there are abundant epidemiological and clinical studies indicating the correlation of prostate cancer to ROS and IL6/STAT3 signaling, respectively. In this study, for the first time, we demonstrated the intrinsic association of ROS and IL6/STAT3 in prostate carcinogenesis. Importantly, we observed a positive feedback loop between ROS and IL6 although the detailed mechanism is to be elucidated in future work.

CONCLUSIONS

In this study, we have established a complete and stepwise carcinogenesis model of prostate by physiological cell selection and adaption. The stepwise model consists of the prostate primary epithelial EP156T cells, mesenchymal non-transformed EPT1 cells, premalignant EPT2 cells, primary tumor derived EPT3-PT1 and metastasis derived EPT3-M1 cells. The common origin of these cell lines has been verified by DNA microsatellite and DNA copy number analysis. We have demonstrated the critical role of a ROS/IL6/STAT3 cascade in prostate cancer initiation and progression. Additionally, a positive feedback loop between ROS and IL6 has been found during prostate tumor sphere formation in protein free culture. Finally, miR-182 and miR-203 have been found to induce mesenchymal to epithelial transition features and self-sufficiency of growth signals via targeting the transcriptional suppressor SNAI2.

FUTURE PERSPECTIVES

In the present stepwise prostate carcinogenesis model, cells in each stage harbor distinct phenotypes that are commonly utilized as markers for cancer progression *in vitro*. EMT is a well-defined trait of cancer metastasis (125,137,150,224,234,242,243), while the ability to anchorage-independent growth has been connected with cancer cell aggressiveness and metastatic potential *in vivo* (244,245). Apoptosis is also important among multiple barriers to metastasis (246). Furthermore, growth autonomy and growth factor independent proliferation are additional characteristics of tumor metastasis *in vivo* (247). In this model, EPT1 cells have undergone complete EMT without the abilities to either anchorage-independent growth or growth factor independent proliferation. EPT2 cells have all of the above *in vitro* features, but still were unable to efficient carcinogenesis. EPT3-PT1 and EPT3-M1 cells are primary tumor and metastases derived cells and therefore represent the two important *in vivo* malignant features. Altogether, this study provides a unique model to study the mechanisms of these different properties of tumor metastasis *in vitro* and *in vivo*.

A ROS/IL6/STAT3 cascade has been found critical to cancer initiation and progression in this model. Both ROS and IL6 play central roles in inflammation networks (195). Indeed, inflammation signatures were found in both D5HS and metastatic EPT3-M1 cells in this carcinogenesis model, which provides a unique model for the study of the inflammation process and prostate carcinogenesis. Additionally, for the first time, we linked the intrinsic association of ROS and IL6/STAT3 in prostate carcinogenesis. Importantly, we observed a positive feedback loop between ROS and IL6. Future work will elucidate the detailed mechanism and the clinical implication of the ROS/IL6 loop. Finally, compared to prostate PC3 and DU145 cells, the higher levels of autocrine IL6 and phosphorylation of STAT3 in EPT3-M1 cells make this model attractive for therapeutic development of drug targeting the IL6/STAT3 pathway.

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