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STUDIES ON THE MECHANISMS OF CARCINOGENESIS AND CANCER CELL DEATH

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II. Zeng J*, Wang L*, **Li Q**, Li W, Björkholm M, Jia J, Xu D. FoxM1 is up-regulated in gastric cancer and its inhibition leads to cellular senescence, partially dependent on p27 kip1. J Pathol. 2009 2009;218:419-27

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*Equal Contribution

LIST OF ABBREVIATIONS

Abl	Abelson murine leukemia viral oncogene homolog 1
AMPK	5' AMP-activated protein kinase
AMP	Adenosine monophosphate
AIF	Apoptosis Inducing Factor
Apaf-1	Apoptotic protease activating factor 1
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
AREs	Androgen Response Elements
Atf4	Activating transcription factor 4
ATG	Autophagy-related gene
ATP	Adenosine triphosphate
Bad	Bcl-2-associated death promoter
Beclin1	Coiled-coil myosin-like BCL2-interacting protein
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BMDC	Bone Marrow Derived Cell
CAF	Cancer Associated Fibroblast
Csk1	Cyclin-dependent kinase activating kinase1
DISC	Death-inducing signaling complex
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCL14	Chemokine (C-X-C motif) ligand 14
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial-Mesenchymal Transition
ERG	ETS-Related Gene
ERK	Extracellular signal-Regulated Kinases
ETV1	ETS translocation variant 1
ETV4	ETS translocation variant 4
FIP200	Focal adhesion kinase family interacting protein of 200kD
FoxM1	Forkhead box protein M1
FOXO	Forkhead box-O
GLUT4	Glucose Transporter 4
GRB2	Growth factor receptor-bound protein 2
GSK3	Glycogen Synthase Kinase 3
H3K9me	Histone H3 lysine 9 methylation
HATs	Histone Acetyltransferases
HCC	Hepatocellular Carcinoma
HDACs	Histone Deacetylases
HER2	Human Epidermal Growth Factor Receptor 2
HGF	Hepatocyte Growth Factor
Hint1	Histidine triad protein
H.pylori	*Helicobacter pylori*
hTER	Human Telomerase RNA
hTERT	Human Telomerase Reverse Transcriptase
IGF-1	Insulin-like Growth Factor 1
IGFR	Insulin-like Growth Factor 1 Receptor

IL-6	Interleukin 6
JaK	Janus Kinase
KAI1	Kangai 1
KIS	Kinase Interacting Stathmin
LAMP-2	Lysosome-associated membrane protein 2
LC3	Microtubule-associated protein 1A/1B-light chain 3
LC3-II	LC3-phosphatidyl ethanolamine conjugate
Lck	Lymphocyte-specific protein tyrosine kinase
LHRH	Luteinizing hormone-releasing hormone
LKB1	Liver kinase B1
LOX	Lysyl Oxidase
LOXL2	Lysyl Oxidase Like 2
3-MA	3-methyladenine
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen Activated Protein Kinase
Mcl-1	Myeloid cell keukemia-1
MDM2	Mouse double minute 2 homolog
MEK	Mitogen-activated protein kinase/ERK kinase
MMP	Matrix Metalloproteinase
mTOR	Mammalian Target of RapaMycin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NRTK	Non-Receptor Tyrosine Kinases
PAS	Phagophore Assembly Site
PCR	Polymerase Chain Reaction
PDGFR	Platelet-Derived Growth Factor Receptor
PE	Phosphatidyl Ethanolamine
Ph+	Philadelphia chromosome-positive
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase
PSA	Prostate-specific antigen
PTEN	Phosphatase and Tensin homolog
Rb	Retinoblastoma protein
RBP2	Retinoblastoma binding protein 2
RheB	Ras homolog enriched in brain
ROS	Reactive oxygen species
RT	Reverse transcriptase
RTK	Receptor Tyrosine Kinase
RUNX3	Runt-related transcription factor 3
SA-β-gal	Senescence-associated beta-galactosidase
SAHF	Senescence-associated heterochromatic foci
SDF-1	Stromal cell-derived factor-1
SH	Spectrin Homology
Shh	Sonic hedgehog homolog
Skp2	S-phase kinase-associated protein 2
SnoRNAs	Small nucleolar RNAs
SOS	Son of Sevenless
Sp1	Specificity Protein 1

SQSTM1	Sequestosome 1
STAT	Signal transducer and activator of transcription
SV40	Simian Virus 40
TGF-β	Transforming Growth Factor β
TKI	Tyrosine Kinase Inhibitor
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TRF	Telomere Repeat binding Factor
TRF1	Telomere Repeat binding Factor 1
TRF2	Telomere Repeat binding Factor 2
TSC2	Tuberous Sclerosis protein 2
Tyk2	Tyrosine kinase 2
VEGF	Vascular Endothelial Growth Factor
VEGFR	Vascular Endothelial Growth Factor Receptor
VHL	Von Hippel-Lindau
ULK1	Unc-51-like kinase 1

ABSTRACT

Gastric cancer is the second most common cause of cancer-related mortality in the world. The mechanisms of gastric carcinogenesis are not fully elucidated. Telomerase and its catalytic subunit telomerase reverse transcriptase (hTERT) are aberrantly activated in gastric cancer cells and their activation disrupts a major malignant transformation barrier, namely cellular senescence and contributes to immortalization of gastric cells. In order to get further insights into the mechanisms of gastric carcinogenesis, we examined the role of two novel factors. In **study I**, the expression and function of the ATPase Reptin in gastric cancer were assessed. Reptin was up regulated in gastric cancer samples and required for the transcription of the *hTERT* gene by cooperating with c-Myc. Depletion of Reptin impaired the clonogenic potential of gastric cancer cells. In **study II,** the role of the transcription factor FoxM1 in gastric cancer was evaluated. Forkhead box protein M1 (FoxM1) was overexpressed in gastric cancer samples. Its inhibition led to cellular senescence and loss of clonogenic potential of gastric cancer cells. The induction of senescence was mediated by the $p27^{kip}$ signaling pathway. *hTERT* transcription and telomerase activity were also inhibited by FoxM1 depletion. In summary, these studies show the aberrant expression and function of Reptin and FoxM1 in gastric cancer and their potential as targets for gastric cancer therapy.

Prostate cancer is one of the most frequent malignancies and the second leading cause of cancer-related deaths in men in western countries. Its development is associated with the over-activation of androgen receptor- and tyrosine kinase-dependent signaling cascades in prostate cells. One of the biggest obstacles in the clinical management of prostate cancer is the development of resistance to hormone deprivation therapy. Several tyrosine kinase inhibitors (TKIs), including the multi-tyrosine kinase inhibitor sorafenib, are now in clinical trials as novel therapeutics for prostate cancer. In **study III**, we examined the mechanisms of cell death induced by sorafenib in prostate cancer cell lines. It was found that sorafenib can induce caspase-dependent cell death in the prostate cancer cell lines, 22RV1 and PC3. Importantly, we found that different signaling cascades were targeted by sorafenib in 22RV1 and PC3 cells that may determine the cytotoxic efficacy of the drug. Furthermore, the maximal cytotoxic efficacy of this TKI was attenuated by the induction of cytoprotective autophagy in these cell lines. Combination of sorafenib with the Bcl-2 antagonist ABT737 enhanced the cytotoxic efficacy of sorafenib. Interestingly, this combination can reverse the protection mediated by primary cancer associated fibroblasts against sorafenib-induced cell death in prostate cancer cells. In **study IV,** ATG5-independent autophagy in cancer cells was described. Treatment with sorafenib can induce mitochondria depolarization and damage in these cells. The ensuing induction of autophagy restored partially the mitochondria potential but did not rescue the cells from death. In fact, we found that the induced autophagy was cytotoxic due to lack of expression of the autophagy key regulator ATG5. Interestingly, loss of expression of ATG5 was also observed in prostate cancer tissue samples. In summary, these studies provide further insights on the mechanisms of cell death induced by the TKI sorafenib in the prostate cancer setting.

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1. Introduction

1.1 Hallmarks of cancer

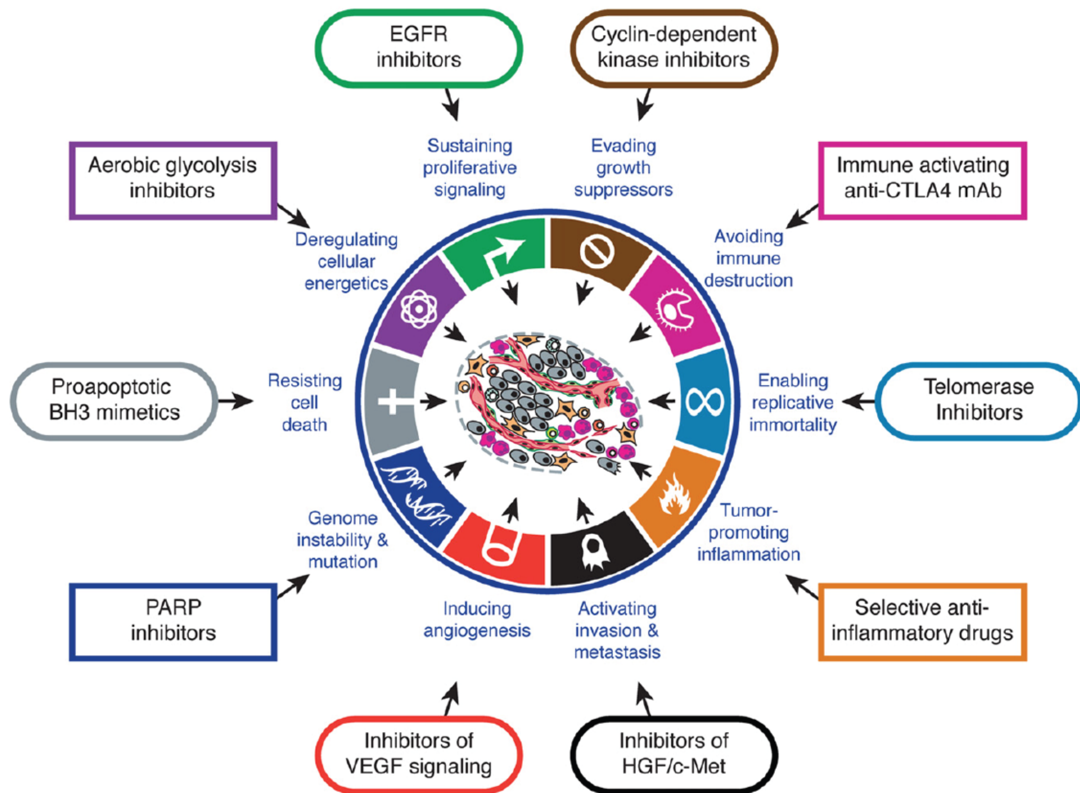

Figure 1. Hallmarks of cancer. Reprinted from Hanahan and Weinberg, 2011, with permission from Elsevier.

The four studies in this thesis have focused on certain hallmarks of gastric and prostate cancer. In 2011, Hanahan and Weinberg revised their original set of hallmarks of cancer and introduced to the six original hallmarks (i.e. sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis) two emerging hallmarks (i.e. deregulated cellular energetics and avoiding immune destruction) and two enabling characteristics (i.e. genomic instability and tumor promoting inflammation).[1]

Solid tumors are neoplasms composed of proliferating tumor cells. **Sustained proliferation** is the most prominent characteristic of cancer cells. Sustained growth of tumor cells can be due to several reasons: deregulated autocrine and paracrine proliferation signals, overexpression of growth factor receptors (e.g. overexpression hepatocyte growth factor receptor c-Met in gastric cancer) or activating mutations of downstream factors (e.g. phosphatidylinositol 3-kinase (PI3K) mutation in prostate cancer). Furthermore, loss of negative-feedback loops further promotes the constitutive proliferation of cancer cells (e.g. loss of phosphatase and tensin homolog (PTEN) in prostate cancer).[1]

Evading growth suppressors is another strategy utilized by cancer cells to sustain constitutive proliferation. Retinoblastoma gene Rb and p53 are two important growth suppressors which can be inactivated in tumor cells by different mechanisms. Loss of expression of proteins regulating cell cycle check points promotes tumor development. In prostate cancer, for example, loss of $p27^{kip}$ can lead to inhibition of senescence and progression from prostatic neoplasia to prostate cancer.[2]

Resistance to cell death induced either by intracellular or extracellular cytotoxic stimuli is a major hallmark of carcinogenesis and tumor progression. There are different modes of cell death: apoptosis (type I programmed cell death), autophagy (type II programmed cell death), necrosis (type III programmed cell death) and mitotic catastrophe. Tumor cells can avoid apoptosis by up-regulating oncogenic pathways such as PI3K-AKT pathway that inactivate downstream cell death effectors [e.g. Bcl-2-associated death promoter (Bad), caspase-9] or by overexpressing anti-apoptotic Bcl-2 members such as B-cell lymphoma 2 (Bcl-2), myeloid cell leukemia-1 (Mcl-1) and B-cell lymphoma-extra-large (Bcl-xL). Importantly, induction of cell death is an important strategy to eliminate tumor cells by anti-cancer treatments. Thus, one mechanism of acquisition of resistance to cancer treatment is the inactivation of the cell death pathways. This topic will be further discussed in the chapter 1.9.

Enabling replicative immortality allows cancer cells to achieve infinite growth. The role of telomerase in the acquisition of unlimited proliferative potential has been well established. This topic will be further discussed in the chapter 1.3.

Infinite growth and sustained proliferation of cancer cells lead to the expansion of the tumor mass. This also increases the demand of tumor cells for oxygen and nutrient supply. Tumor cells can only grow to 1-2 mm^3 without angiogenesis. During carcinogenesis, **angiogenesis** is induced in order to sustain nutrient and oxygen supply to the neoplastic mass. Vascular endothelial growth factor (VEGF) is one of the key regulators of angiogenesis.[3] It is involved in the carcinogenesis of different types of cancer and is a target for anti-cancer treatment.

Activating invasion and metastasis is involved in the later steps of tumor progression. Several mechanisms such as epithelial–mesenchymal transition (EMT) can contribute to the acquisition of mobility of tumor cells. Tumor cells break the barrier of basal membrane, degrade extracellular matrix, migrate and establish colonies at distant metastatic sites.

Genomic instability is characteristic of most human cancers.[4, 5] Genomic instability can be caused, for example, by base pair mutations and chromosomal deletions or translocations. Base pair mutation or depletion silences tumor suppressor gene, and DNA damage repair machinery. Chromosome translocation can lead to aberrant oncogene expression. TMPRSS2-ERG created by gene fusion is a key factor in prostate cancer development.

The paradigm that **tumor promoting inflammation** can increase the predisposition of certain malignancies is nowadays widely accepted. Pro-carcinogenic inflammation can be caused by infections. For example, *H.pylori* infection is associated with the development of gastric cancer.[6] The progression from inflammation to carcinogenesis can be due to different reasons, for example DNA damage caused by reactive oxygen specifies (ROS) generated by *H.pylori*, infiltrating inflammatory cells and growth factor-driven cell proliferation.

Reprogramming of cancer cell metabolism is an emerging hallmark of cancer.[7] In cancer cells, metabolism is switched from oxidative phosphorylation to glycolysis which is 18 times less efficient in terms of ATP production. The reprogramming of cancer cells to obtain their energy from alternative energy sources such as aerobic glycolysis is known as the Warburg effect.[8] One of the main signaling pathways involved in this reprogramming is the PI3K-AKT/mammalian target of RapaMycin (mTOR) which is activated in a variety of human malignancies including gastric and prostate cancer. In cancer cells, several key metabolic enzymes are involved in tumor development. For example, hexokinase II which is required for the phosphorylation of glucose to glucose-6-phosphate can promote tumor cell survival by suppressing cell death.[9]

Immune surveillance is a crucial mechanism to eradicate malignant cells. Cancer cells have devised several ways to **evade immune destruction**. For example, by impairing antigen presentation e.g. mutations of the antigen; secreting immunosuppressive factors like transforming growth factor β (TGF-β)[10] and interleukin 10[11].

In the first two studies on gastric cancer, two novel factors Reptin and FoxM1 are examined, which provide insights into the mechanism of gastric cancer development. In the two studies on prostate cancer we delineated the cell death mechanisms induced by the multi-tyrosine kinase inhibitor sorafenib.

1.2 Gastric cancer

1.2.1 Introduction

Gastric cancer is a neoplasm derived from the stomach. It is the fourth most common cancer type and the second highest cause of cancer-related mortality in the world.[12] Japan, China, Latin America, parts of Eastern Europe have more gastric cancer-related deaths compared to other areas in the world. The geographic pattern of gastric cancer incidence is related with socio-economic state, and infection of *H.pylori*.[13] Predisposing risk factors of gastric cancer include *H.pylori* infection, smoking, obesity and diet. For example salty diet and low fruit/vegetables intake can increase the risk of gastric cancer. Furthermore, males have higher frequency of developing gastric cancer than females.[14] Genetic polymorphisms are also associated with gastric cancer.[15]

Most of gastric cancers are adenocarcinomas. Gastric adenocarcinomas can be divided into two major histological types- diffuse type and intestinal type.[16] The intestinal type of

gastric cancer develops from chronic gastritis to intestinal metaplasia to dysplasia and finally invasive carcinoma.

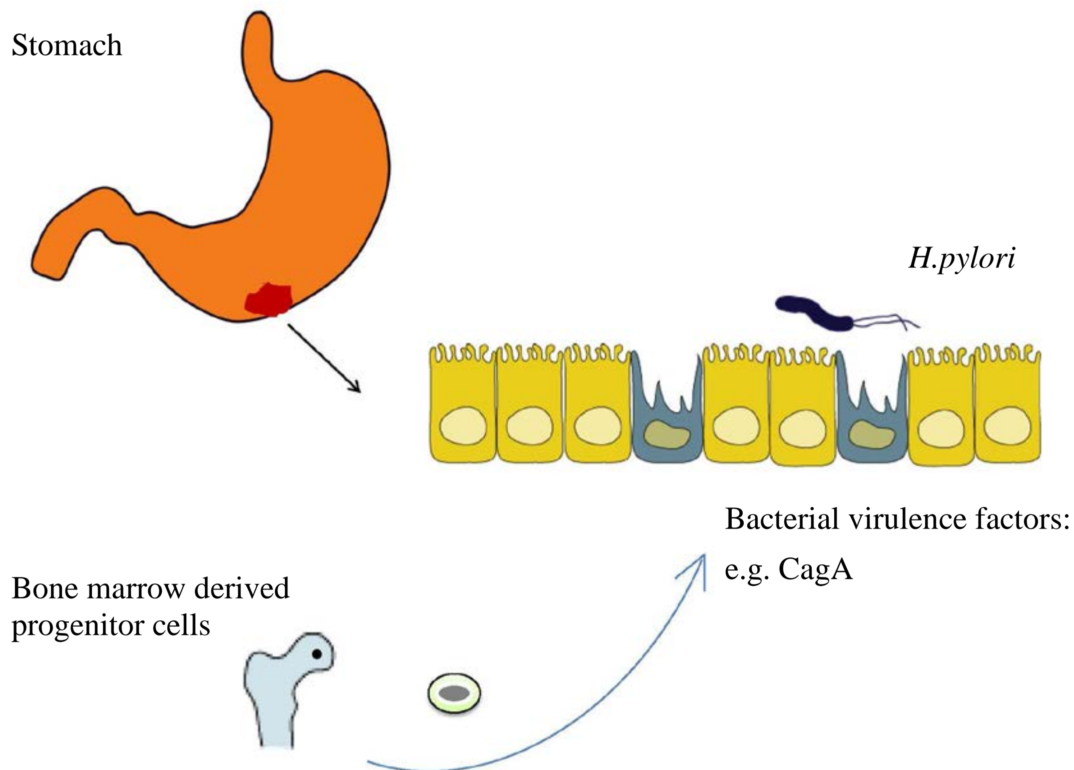

Figure 2. Role of *H.pylori* in gastric cancer. *H.pylori* can induce gastric cancer by secreting bacterial virulence factors and recruiting bone marrow-derived progenitor cells.

Gastroscopy examination is a useful tool in the early detection of gastric dysplasia and gastric adenocarcinoma. The prognosis of early gastric cancer is relatively good with 70-95% five-year survival rate. Clinical stage IV gastric cancer with metastases is associated with poor prognosis. Surgical resection is the primary treatment modality for gastric cancer. Alternative or additional treatments include adjuvant chemotherapy and radiotherapy.

1.2.2 Molecular mechanisms of gastric cancer

The mechanisms of gastric cancer development are not fully elucidated yet. Inflammation, genetic and epigenetic regulations of oncogenes and tumor suppressor genes are thought to play roles in the pathogenesis of gastric cancer.[17]

Development of gastric cancer is closely related to the chronic inflammation caused by *H.pylori* infection.[6] *H.pylori* is a pathogen that colonizes the stomach of more than half of the human population. Persistent infection with *H.pylori* can induce gastritis and is associated with the development of peptic ulcer, gastric mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma.[6] Mechanisms of *H.pylori* induced gastric cancer include bacterial virulence factors, chronic inflammation, and recruitment of bone marrow progenitor cells. *H.pylori* secretes several bacterial virulence factors

including CagA which can activate the nuclear factor kappa B (NF-κB) signaling cascade. Bone marrow-derived progenitor cells (BMDC) were proposed to be the origin of gastric cancer cells in gastric adenocarcinoma induced by *H.pylori* infection.[18] These undifferentiated precursor cells were recruited to repair the lesion caused by *H.pylori* infection and further contributed to the development of dysplasia and intraepithelial neoplasia lesions. [18]

Deregulation of oncogenes and tumor suppressor genes is also implicated in the development of gastric cancer. Loss of adenomatous polyposis coli (APC) tumor suppressor gene is one of the early events during gastric carcinogenesis and found in 20% of differentiated gastric carcinomas.[19] Runt-related transcription factor 3 (RUNX3) is a tumor suppressor which is frequently down-regulated in gastric cancer.[20] In 45% to 60% of human gastric cancer, RUNX3 is silenced due to gene deletion or hyper-methylation of its promoter region.[20] There are multiple mechanisms by which the loss of RUNX3 expression promotes gastric cancer. RUNX3 has been shown to induce apoptosis of gastric cancer cells by activating Bim under cooperation with the PI3K-AKT signaling pathway component FoxO3a/FKHRL1.[21, 22] RUNX3 also suppresses cell proliferation by activating $p21^{cip1}$ signaling.[23]

Oncogene activation plays an essential role in the pathogenesis of gastric cancer. The overexpression of c-Met, the receptor of hepatocyte growth factor (HGF), correlates with advanced stages of gastric cancer and worse prognosis.[24] Compared to tumors without liver metastases, c-Met was expressed at higher level in stage IV gastric cancer with liver metastasis.[25] The human telomerase enzyme catalytic subunit hTERT is also up-regulated during the development of gastric cancer.[26] In more than 90% of gastric cancer tissue telomerase activity was detected. [26]

1.3 Telomerase

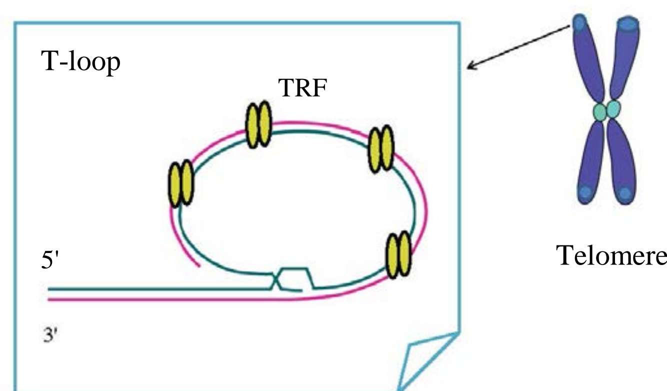

Figure 3. Structure of the telomere. Telomere is a structure in the end of eukaryotic chromosome. Telomere struture is charisterised by a T-loop. It is formed by the DNA 3´-end overhang intruding into telomere duplex DNA. TRF (Telomere repeat binding factor) as telomere binding protein binds with telomere duplex DNA and regulates telomere length.

1.3.1 Introduction of telomere and telomerase

The telomere is a complex located at the end of eukaryotic chromosomes. It is comprised of conserved TTAGGG tandem repeats and telomere binding proteins. The physiological function of telomere is to sustain chromosome stability. Because of the end replication problem of conventional DNA polymerase, the lagging strand of DNA gets shorter in the 5´ end during each round of cell division. When the erosion of telomeres reaches the critical point, cells go to a permanent quiescence state or senescence. Senescent cells cannot re-enter the cell cycle, do not proliferate but are metabolically active. Loss of the telomere can cause chromosome end-to-end fusion which is recognized as a DNA damage signal. So the telomeres can serve as a biological clock and their length reflects the replicative potential of the cells. Telomere DNA is bound by single and double strand telomere binding proteins. Telomere repeat binding factor 1 (TRF1) and telomere repeat binding factor 2 (TRF2) proteins bind with double strand telomere DNA and are negative regulators of the length of the telomere.[27] Telomere structure is characterized by a D-loop and T-loop which are formed by single strand 3´-end overhang intruding into telomere duplex DNA.[28] This cap structure can stabilize and protect chromosome ends.[28] The structure of telomere is demonstrated in **Figure 3.**

Telomerase is an RNA-dependent DNA polymerase, composed of two essential subunits: the RNA template (hTER) and the catalytic subunit hTERT. Telomerase activity is silent in most human somatic cells because of the tight repression of hTERT expression. In up to 90% of human malignancies, telomerase is re-activated. Telomere length can be maintained by telomerase dependent and independent (alternative telomere-lengthening) mechanisms.[29]

Telomerase RNA component

Telomerase RNA template is essential for the enzyme activity of telomerase. Reconstituting telomerase RNA component hTER with hTERT can restore the telomerase activity in vitro.[30] Unlike hTERT, hTER is expressed in most cell types including normal cells. hTERT mRNA expression level is often related to telomerase activity, for example in the case of gastric cancer.[26] But hTER levels do not necessarily correlate with telomerase activity.

1.3.2 Regulation of telomerase

The expression and enzyme activity of telomerase is tightly regulated in normal somatic cells. Telomerase activity is controlled at different levels.[29, 31, 32]

1.3.2.1 Regulation by transcription factors

hTERT is, primarily, regulated at the transcriptional level. The transcription factor binding sites in the hTERT core promoter region include E-Box (5-CACGTG-3), GC-Box (GGGCGG) and others.[33, 34] C-Myc is one of the key regulators for *hTERT* transcription.[33, 34] C-Myc forms heterodimers with Max and bind to the E-box activating downstream target genes such as *hTERT*. When Max forms heterodimer with Mad, it

antagonizes the role of c-Myc/Max complex and inhibits *hTERT* expression.[35] Transcription factor Sp1 is another positive regulator of telomerase; it can bind to the GC-boxes on the *hTERT* promoter and regulate *hTERT* transcription.[36] The core promoter of *hTERT* harbors at least five GC-boxes. Mutations in all five GC-boxes completely inhibited the *hTERT* promoter activity.[37] p53 is an important suppressor of *hTERT* transcription.[38] It was shown that the inhibition of telomerase activity by p53 requires the cooperation of transcription factor Specificity Protein 1 (Sp1).[39, 40] Steroid hormones can regulate *hTERT* expression. Estrogen is the most well studied steroid hormone in the regulation of *hTERT*. Estrogen can directly trans-activate the *hTERT* gene, [41] or act through the AKT signaling cascade. [42] Estrogen deficiency can lead to inhibition of telomerase activity and shorter telomere lengths.[43]

1.3.2.2 Regulation by epigenetic modifications

In order to organize and package DNA in the nucleus, the DNA strand is wound around histones. There are five families of histone proteins, including H1/H5, H2A, H2B, H3 and H4. Modification of the histone tails can change chromatin conformation and regulate gene transcription. hTERT expression can be modified by different epigenetic mechanisms including acetylation, methylation, phosphorylation and ubiquitination.[44] Different enzymes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs) are implicated in the regulation of *hTERT* transcription.[45] Histone acetylation and deacetylation change histone charges and accessibility of DNA with transcription factors. Histone deacetylation can repress *hTERT* transcription in normal human cells.[46] HDAC inhibitor trichostatin A can upregulate hTERT expression.[47] Histone demethylase Retinoblastoma binding protein 2 (RBP2), by interacting with Mad1, is recruited to the *hTERT* promoter where it induces histone H3-K4 demethylation and thereby inhibits the transcription of *hTERT*.[48] Ge *et al.* found that Mitogen activated protein kinase (MAPK)-dependent phosphorylation of histone H3 is important in the induction of *hTERT* transcription and activation of telomerase.[49]

1.4 ATPase Reptin

Reptin is an AAA+ ATPase that belongs to the ATPase family associated with several cellular processes such as DNA replication and gene expression regulation.[50] It is the mammalian homolog of DNA helicase RuvB which functions in the Holliday junction migration.[51] Reptin is involved in the assembly and stability of telomere complex mediated by its ATPase activity.[52] Using a TAP tag approach and mass spectrometric analysis, Reptin and Pontin were identified in the complex of telomerase. They were associated with telomerase in a cell cycle-dependent manner. Knocking down Reptin decreased telomerase activity.[52] The physical association of Reptin with telomerase was also reported in budding yeast.[53] When telomerase is not recruited to the telomere ends, Reptin still exists together with the telomere chromatin.[53]

Reptin often forms hexamers and dodecamers with Pontin, another member of AAA+ family of ATPases.[54] There are controversial reports regarding the assembly of Reptin and Pontin. One study demonstrated that they were organized as dodecamers made from homo-hexamers of Reptin and Pontin.[55] There was also a report showing that they exist as mixed hexamers.[56]

Reptin and Pontin are overexpressed in some types of cancer.[57, 58] For example, by comparing human hepatocellular carcinoma (HCC) tumor tissues with corresponding normal tissues, it was found that Reptin together with Pontin were overexpressed.[58, 59] Also Pontin was reported to be overexpressed in colon cancer.[57]

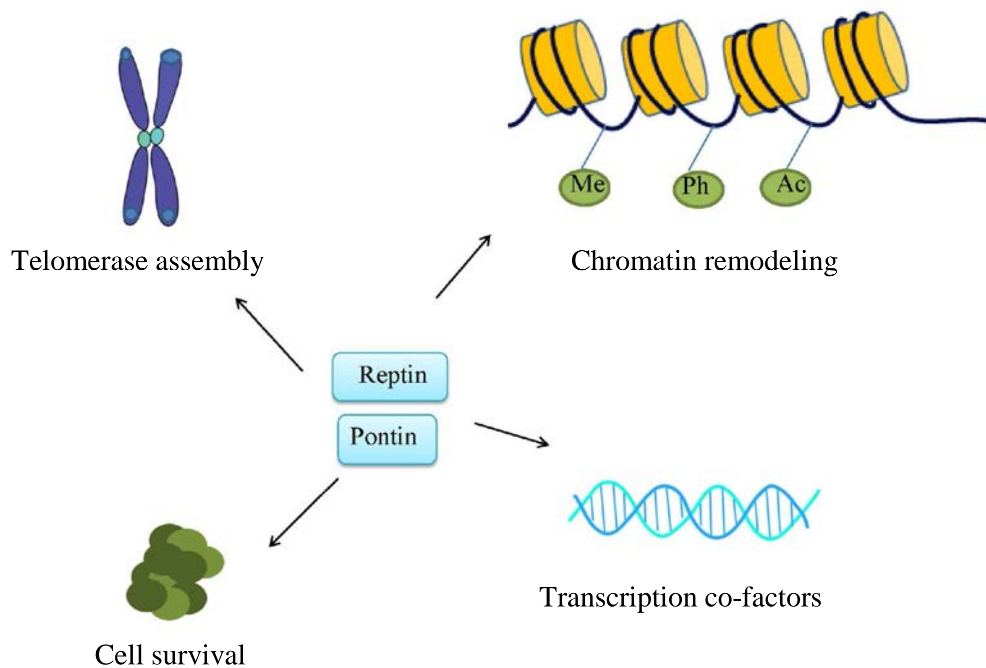

Figure 4. Functions of Reptin. Reptin forms complex with Pontin and functions in telomerase assembly, chromatin remodeling, and cell survival and transcription regulation.

The function of Reptin and Pontin can also be independent of their ATPase activity. They can act as transcription co-factors by interacting with transcription factors. The regulation of target genes by Reptin and Pontin can occur through two different mechanisms, transcriptional regulation and chromatin remodeling (**Figure 4**).

1.4.1 Reptin/Pontin interact with different transcription factors

Reptin and Pontin as the co-factors of c-Myc were shown in different models.[60, 61] For example, Reptin and Pontin interact with Myc during the wing development of Drosophila. [60] Bellosta *et al*. reported that in drosophila d-Myc interacts with Reptin and Pontin in vitro. Reptin functions antagonistically with Pontin regarding the interaction with d-Myc. Also by interacting with Myc, Reptin regulates cell proliferation in the early development of Xenopus.[61] Reptin and Pontin are required for the oncogenic transformation activity of c-Myc.[62] Mutation of Pontin ATPase motif can inhibit the

transformation ability of c-Myc. Reptin and Pontin can interact with transcription factors beta-catenin and tumor suppressor histidine triad protein (Hint1). [63, 64] Rottbauer *et al.* showed that Reptin and Pontin can regulate heart growth in zebrafish via the beta-catenin pathway.[63]

1.4.2 Pontin/Reptin and epigenetic regulation

Reptin and Pontin are subunits of different chromatin remodeling complexes, for example in yeast the INO80 remodeling complex[65] and the TIP60 histone acetylase complex[66]. As chaperones they participate in the assembly of INO80 complex. [67] In the TIP60 complex, Reptin and Pontin are essential for the ATPase activity of the complex.[66]

By recruiting different chromatin remodeling complexes, Reptin and Pontin can antagonistically regulate the expression of the tumor metastasis suppressor Kangai 1 (KAI1). [68] In metastatic prostate cancer cells, Reptin and histone deacetylase HDAC were recruited together with beta-catenin to inhibit KAI1. In non-metastatic tumor cells, HAT Tip60 and Pontin chromatin remodeling complex were recruited to the KAI1 promoter.[68] Pontin can regulate the oncogenic properties of beta-catenin by chromatin remodeling.[69] Depletion of Pontin inhibited beta-catenin target gene expression. [69] Reptin and Pontin can regulate the process of nonsense-mediated mRNA decay which is involved in cancer development. [61, 70, 71] Reptin and Pontin are also implicated in the synthesis of small nucleolar RNAs (snoRNAs).[72]

1.5 Senescence

1.5.1 Introduction

Cellular senescence is an irreversible cell growth arrest. Senescent cells remain viable and metabolically active, which distinguish them from dead cells. Senescence can be triggered by different stimuli such as DNA damage, telomere attrition, and oncogene activation. Senescence can be divided into three categories: premature senescence, replicative senescence and oncogene-induced senescence. Premature senescence is induced by stress signals such as DNA damage. Replicative senescence is triggered by the successively shortened telomeres. Oncogene-induced senescence is caused by aberrant activation of oncogenes, for example, overexpression of Ras can trigger senescence and block excessive proliferation. Because senescent cells cannot re-enter the cell cycle, it acts as a barrier for the immortalization and transformation of cells.

Senescent cells can be identified by characteristic cell morphology with enlarged cytoplasm and can be positively stained for beta-galactosidase at pH6. Several cell cycle check point proteins are involved in and up-regulated during senescence, such as p53, $p21^{cip1}$ and $p16^{INK4a}$. Senescence is also associated with formation of senescence-associated heterochromatic foci (SAHF).[73]

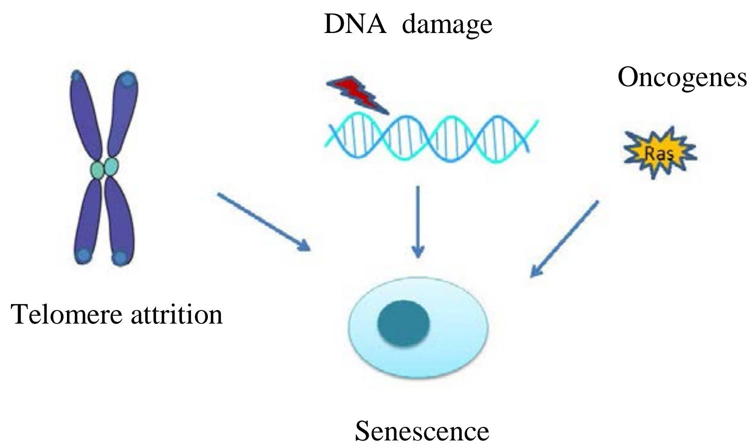

Figure 5. Types of senescence. Senescence can be induced by telomere attrition, DNA damage, and oncogene activation.

1.5.2 Regulation of senescence

Overexpression of oncogenes, for example Ras, can induce senescence to counteract the excessive pro-proliferation signals. INK family proteins $p19^{ARF}$ and $p16^{Ink4a}$ are implicated in mediating oncogene-induced senescence. Oncogene activation can activate $p19^{ARF}$; whereas $p19^{ARF}$ inhibits mouse double minute 2 homolog (MDM2) which can inhibit p53 accumulation through proteasomal degradation. Oncogene activation can also activate $p16^{Ink4a}$. $p16^{Ink4a}$ can inhibit cyclin-dependent kinase CDK4/6 and induce cell cycle arrest by regulating the tumor suppressor protein Rb. Hypophosphorylated Rb is at its active state, E2F target genes are silenced, and cells go to senescence.[74] Rb can also mediate the formation of heterochromatin by methylation of histone H3 lysine 9 (H3K9me) and induce senescence.[74]

DNA damage-induced by chemotherapeutic drugs (e.g. etoposide) can cause DNA double strand break. p53 is activated and it in turn activates downstream $p21^{cip1}$. DNA damage-induced senescence can also be mediated through $p16^{Ink4a}$.[75]

Replicative senescence is induced by progressive telomere shortening. Telomere attrition can cause chromosomal end to end fusions. These will be recognized as double-strand breaks and trigger the DNA damage repair machinery. P53-p21 and $p16^{INK4a}$-Rb signaling are essential regulators in mediating replicative senescence. In many human tumors, cell cycle checkpoint proteins for example p53 and INK4 families are silenced by mutations or promoter methylation which compromise the senescence barrier.

1.5.3 Role of senescence in cancer

Senescence is an important suppression mechanism in the premalignant lesion step. In prostate cancer, cellular senescence induced by cell cycle check point protein $p27^{kip1}$ up-regulation was shown to be a barrier during the development from prostatic hyperplasia and prostatic intraepithelial neoplasia to invasive tumor.[2] Senescence is an initial barrier during lymphoma development.[76] Histone methyltransferase Suv39h1 is required for Rb mediated methylation of histone H3 lysine 9 and senescence associated heterochromatin

formation.[77] Braig *et al.* found that Eμ-N-Ras transgenic mice with Suv39h1 heterozygous lesions developed lymphoma at a significantly earlier phase than N-Ras-transgenic wild-type mice. It was demonstrated that the senescence gatekeeper can delay the onset of oncogenic Ras-induced malignancy. [76]

However, senescence can also be an important cancer promoting mechanism. In response to anti-cancer drugs, senescence can lead to therapeutic resistance. Senescent cells resting in G1 cell cycle will be resistant to anti-cancer drugs targeting proliferating cells. Furthermore, senescent cells may form a favorable microenvironment for tumor progression.[78] It was shown in the co-culture setting that senescent fibroblasts can promote the proliferation of prostate epithelial cells.[78] Conditioned medium from the senescent fibroblasts also promotes the growth of prostate epithelial cells. The tumorigenic effect of senescent fibroblasts was postulated to be mediated by paracrine factors such as growth factors or inflammatory factors secreted by these growth-arrested fibroblasts.[78]

1.6 Transcription factor FoxM1

FoxM1 is member of the Forkhead transcription factor family. FoxM1 has three spliced isoforms: FoxM1a, FoxM1b, and FoxM1c. FoxM1b and FoxM1c are actively involved in regulating cellular proliferation. FoxM1 is expressed in embryonic tissues and proliferating cells but silent in terminally differentiated cells. In malignant cells, FoxM1 expression is up-regulated. FoxM1 expression can be up-regulated by the oncogenes c-Myc and Ras;[79] whereas inhibited by tumor suppressors such as p53,[80] and p19[81]. FoxM1-induced anchorage-independent cell growth on soft agar can be inhibited by p19.[81] FoxM1 activates transcription factors c-Myc and c-Fos that promote cell proliferation. The target genes of FoxM1 also include cell cycle checkpoint proteins during G1-S transition,[82] S phase progression,[83] G2-M transition[83] and M phase progression.[84]

1.6.1 Role of FoxM1 in human cancer

FoxM1 is deregulated in a variety of human tumors, for example lung cancer,[85] prostate cancer,[86] glioblastoma,[87] basal cell carcinomas.[88] Elevated expression of FoxM1 is correlated with the degree of malignancy. Thus, in prostate adenocarcinomas, [86] cervical cancer, [89] glioblastoma, [87] and non-small cell lung cancers, [85] FoxM1 is expressed at higher levels at more advanced tumor stages.

FoxM1 plays critical roles in the regulation of proliferation, tumorigenesis, angiogenesis, and invasion of tumor cells.[83] The roles of FoxM1 in human cancers are illustrated in **Figure 6**. The growth promoting function of FoxM1 in cancer cells is described in different types of cancers, for example in both in vitro and in vivo models of prostate cancer[86], and lung cancer.[85] Depletion of FoxM1 in tumor cells, for example in prostate cancer cells,[86] lung cancer cells[85] and glioblastoma cells[87] inhibits the anchorage-independent growth of tumor cells on soft agar and impairs the tumor formation ability of cancer cells in nude mice.[87]

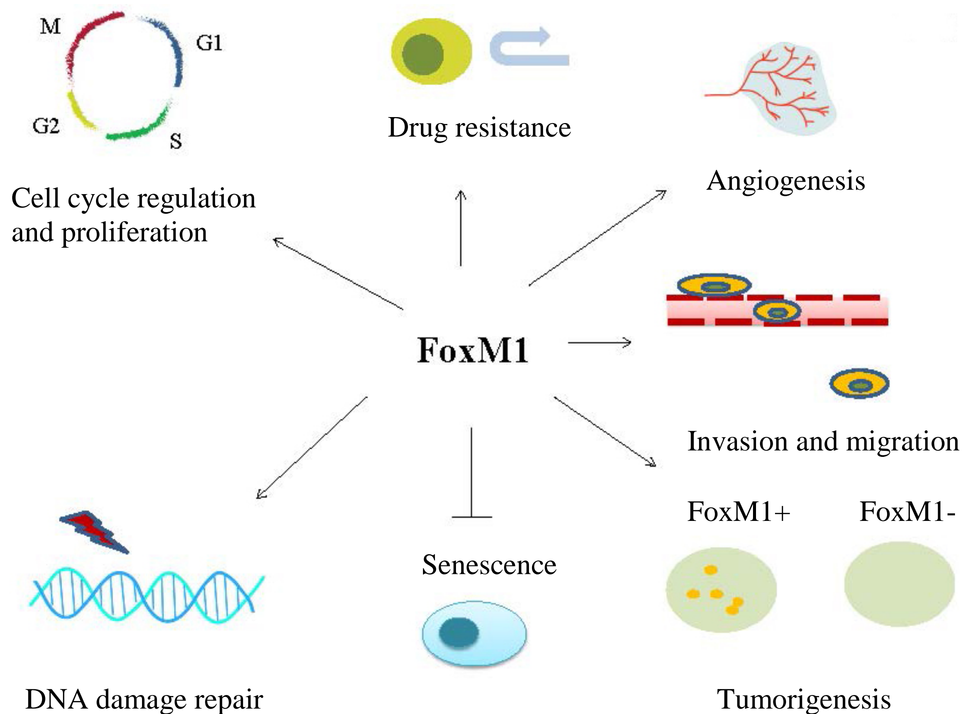

Figure 6. Roles of FoxM1 in human cancer. FoxM1 exerts its functions in cell cycle regulation, DNA damage repair, angiogenesis, invasion and migration, tumorigenesis, drug resistance and inhibition of senescence.

In certain cancers, for example prostate cancer[86] and lung adenocarcinomas[90], FoxM1 promotes tumor progression. FoxM1 overexpression promotes the metastasizing capacity of prostate cancer cells.[91] In transgenic adenocarcinoma of the mouse prostate (TRAMP) and Lady transgenic mouse models of prostate cancer, overexpression of FoxM1 can accelerate the tumor development.[86] In hepatocellular cancer cells, FoxM1 stimulates the expression of lysyl oxidase (LOX) and lysyl oxidase-like 2 (LOXL2) to prepare a pre-metastatic niche for HCC cells.[92] In glioma cells, FoxM1 promotes angiogenesis of tumor cells by up-regulating VEGF expression.[93] Matrix metalloproteinase (MMP) can degrade basement membrane and extracellular matrix to facilitate the metastasis of tumor cells. In pancreatic cancer cells, FoxM1 enhances the invasion and angiogenesis of cancer cells by inducing the expression of matrix metalloproteinase 2 and 9 (MMP2 and MMP9).[94] Similar effects of FoxM1 on MMP2 were reported in glioma cells.[95]

FoxM1 is also implicated in the resistance of tumor cells to chemotherapy. For example, FoxM1 overexpressing breast cancer cells are more resistant to cisplatin, [96] and trastuzumab treatment[97]. FoxM1 induced trastuzumab resistance in breast cancer cells is associated with the trastuzumab induced up-regulation of $p27^{kip}$. [97] It was also found that in gastric cancer cells, overexpression of FoxM1 can induce chemotherapeutic resistance of gastric cancer cells to docetaxel.[98]

1.6.2 Role of FoxM1 in cellular senescence

FoxM1 is also implicated in regulating cellular senescence. FoxM1-/- mouse embryonic fibroblast cells undergo premature senescence during their early passages.[82] This is mediated by the up-regulation of $p27^{Kip1}$. FoxM1 regulates $p27^{Kip1}$ expression through multiple mechanisms. FoxM1 stimulates the expression of SCF ubiquitin ligase complex subunit S-phase kinase-associated protein 2(Skp2) that mediates the ubiquitin proteasome degradation of $p27^{Kip1}$.[82, 99] FoxM1 also increases the nuclear export of $p27^{Kip1}$ by regulating growth factor-induced expression of kinase-interacting stathmin (KIS).[100] Depletion of FoxM1 can induce the nucleus accumulation of $p27^{Kip1}$. Consistently during pancreas development, depletion of FoxM1 increased the nuclear amount of $p27^{Kip1}$ and induced senescence in pancreatic beta cells. Oncogenic Ras can induce senescence by up-regulating ROS.[101] The study by Park *et al* described that ROS enrichment induced by Ras increased the expression of FoxM1. [79] Depletion of FoxM1 leads to oxidative stress induced senescence. [79] It suggested that up-regulation of FoxM1 can be an adaptive machinery of cancer cells. Also FoxM1 was found to be a substrate for cyclin dependent kinases CDK4 and CDK6. Its phosphorylation by CDK4 and CDK6 prevents the onset of senescence.[102]

FoxM1 is proposed as a potential target of cancer therapy. [103] Targeting FoxM1 can be restricted to cancer cells because FoxM1 is overexpressed in proliferating cells. There are studies reporting on potential inhibitors of FoxM1. Radhakrishnan *et al*. identified that antibiotic Siomycin A was a specific inhibitor of FoxM1.[104] This thiazole antibiotic targets FoxM1 and thereby induces apoptosis in human cancer cells.[105] The small molecule thiostrepton can inhibit the binding of FoxM1 to the promoter of its target genes.[106] PPARγ agonists such as thiazolidinediones and proteasome inhibitors like bortezomib also inhibit the expression of FoxM1.[107]

1.7 Prostate cancer

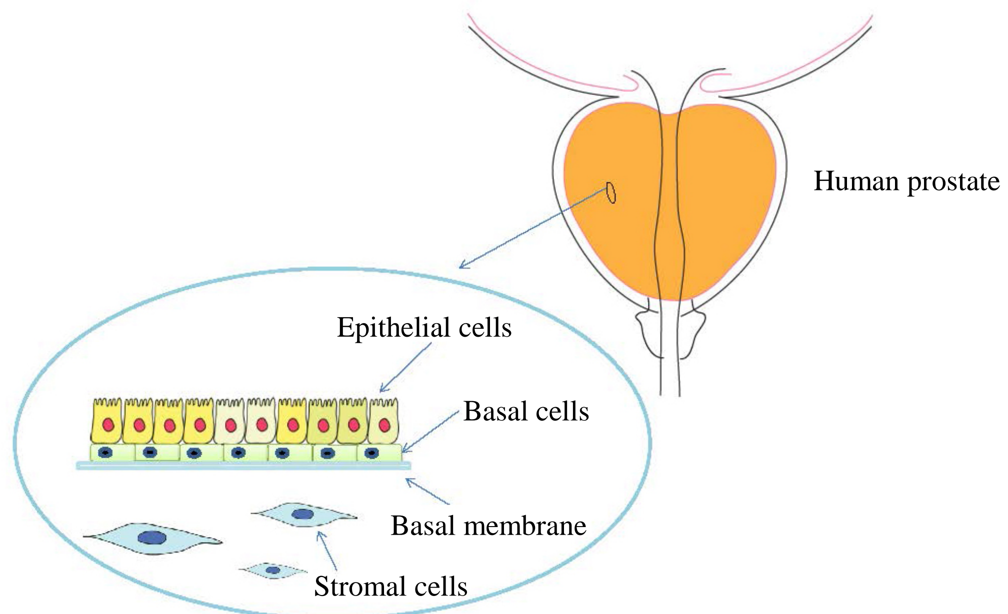

Figure 7. The Human prostate gland. It is mainly composed of epithelial cells, basal cells and stromal cells.

1.7.1 Introduction

The prostate gland's physiological function is to produce secretory proteins e.g. prostate-specific antigen (PSA) into the seminal fluid and facilitate the fertility of sperms. The prostate gland is divided into the peripheral zone, central zone and transition zone. The majority of prostate cancer derives from the prostate peripheral zone. Tumors arising from the central zone are relatively rare. Growth of prostate cells in the transition zone can cause benign prostatic enlargement and can also cause prostate cancer.

Prostate cancer is the most frequent malignancy and second leading cause of cancer related deaths in men in industrialized countries. [12] Several factors are associated with an increased risk to develop prostate cancer. This type of cancer usually afflicts elderly men. Ethnicity is another risk factor.[108] Thus, prostate cancer has a higher incidence in African-American men compared to Asian men. Diet, smoking, obesity are also correlated with the occurrence of prostate cancer.[108] Benign prostate hyperplasia is commonly observed in elderly men which can increase the probability of developing prostate cancer.[109]

1.7.2 Molecular mechanisms of prostate cancer

Androgens play an essential role in the development of normal prostate and in the development of prostate cancer. Androgen regulated signaling can be initiated by the binding of androgen ligand with androgen receptor (AR), a member of the steroid hormone receptor family. After binding with the ligand, ARs dissociate from heat shock proteins, translocate to the nucleus and bind with androgen response elements (AREs) in the promoter regions and regulate the expression of target genes.[110]

Prostate cancer can be divided into two types: castration sensitive prostate cancer and castration refractory prostate cancer. Castration refractory is common in the late stage of prostate cancer. Androgen withdrawal can cause apoptosis of hormone sensitive prostate normal cells and tumor cells at the early stage of prostate cancer.

AR is an important promoter of prostate cancer development. Several mechanisms can induce over-activation of AR signaling: overexpression of the receptor due to gene amplification, ligand independent activation of AR, mutation of the receptor which can decrease the specificity of the ligand binding and AR activation by tyrosine kinases. [111]

Loss of expression of the tumor suppressor gene PTEN is found in 20-30% of metastatic prostate cancer.[112] Loss of PTEN expression or activity in prostate cancer can occur by homozygous deletions, loss of heterozygosity and inactivating mutations. PTEN is an important negative regulator of PI3K-AKT signaling pathway by dephosphorylating phosphatidylinositol (3-5)-triphosphate (PIP3) to phosphatidylinositol 4, 5-bisphosphate (PIP2). Loss of PTEN leads to over-activation of PI3K-AKT signaling pathway. Prostate cancer cells with PTEN double allele mutation are resistant to hormone deprivation treatment.[113]

TMPRSS2-ERG is a key factor in prostate cancer development. The genomic alteration of TMPRSS2-ERG is created by fusion of androgen-regulated gene TMPRSS2 with ETS transcription factors ETS-Related Gene (ERG), ETS translocation variant 1 (ETV1), or ETS translocation variant 4 (ETV4). It exists in 40 to 80% of prostate cancers and its expression level increases with higher prostate cancer stages.[114]

1.7.3 Cancer associated fibroblasts in prostate cancer

Prostate stroma cells are implicated in the normal development and differentiation of prostate cells. Prostate cancer stromal compartment is composed of different components including CAFs, immune cells, macrophages, pericytes, endothelial cells and extracellular matrix. CAFs are the most prominent component of tumor stroma.[115]

CAFs are one of the major constituents of the tumor stroma.[116, 117] The role of CAFs in prostate cancer development has been demonstrated and recapitulated by various co-culture and recombination experiments.[117] The reciprocal interaction between cancer associated fibroblasts and tumor epithelial cells can be mediated by paracrine factors growth factors, chemokine, cytokines and other mediators.

Growth factors are the most well-known regulators of the interaction between tumor cells and tumor stroma. For example, HGF from fibroblast cells can bind with c-Met on tumor cells and promote metastasis of tumor cells.[118] Chemokines secreted by the tumor stroma also play a role in cancer progression. Stromal cell-derived factor 1 (SDF-1) secreted by CAFs participates in recruiting endothelial progenitor cells to promote angiogenesis of breast cancer.[119] SDFs secreted by CAFs bind with Chemokine (C-X-C motif) ligand 12 (CXCL12) receptor on breast cancer cells and promote the growth of breast cancer cells by paracrine signals.[119] The role of cytokines in tumor microenvironment is well

established. For example, Interleukin 6 (IL-6) derived from bone marrow stromal fibroblasts has been shown to promote the growth of prostate cancer cells.[120]

The potential roles of CAFs in prostate cancer are illustrated in **Figure 8**; i) CAFs can promote the progression of prostate cancer cells from immortal phenotype to tumorigenic phenotype. In a co-culture study, Olumi *et al.* described that normal prostatic fibroblasts and CAFs exhibited different effect on initiated human prostatic epithelial cells which were transformed by simian virus 40 (SV40).[121] Co-culturing of prostate epithelial cells with prostate CAFs led to the onset of prostatic intraepithelial neoplasia in the prostate epithelial cells. In contrast, the phenomenon was not observed when prostate cancer cells were co-cultured with normal prostate fibroblasts; ii) CAFs can promote the proliferation of prostate cancer cells. It was found that insulin-like growth factor-1(IGF-1) from prostate stroma can promote the growth of prostate cancer cells.[122] Insulin-like growth factor-1 receptor (IGF-IR) abrogation on prostate epithelial cells stimulates Extracellular signal-regulated kinases (ERK) tyrosine kinase signaling and increase proliferation of prostate cancer cells;[122] iii) CAFs can promote the occurrence of EMT and acquisition of stem cell-like characteristics in prostate cancer cells.[123] In a study by Giannoni *et al*, it was found that the bi-directional interaction between prostate cancer cells and CAFs can stimulate EMT of tumor cells.[123] Fibroblast cells secrete MMP which can facilitate EMT of cancer cells and contributes to the acquisition of stem cell-like characteristics in prostate cancer cells;[123] iv) cancer cells need to acquire mobility to migrate to surrounding tissues. CAFs can promote this ability in prostate cancer cells. Augsten *et al.* demonstrated by co-culture experiments and an animal model that Chemokine (C-X-C motif) ligand 14 (CXCL14) secreted by prostate cancer associated fibroblast can promote the growth of prostate cancer xenografts, increased tumor angiogenesis and macrophage infiltration.[124]

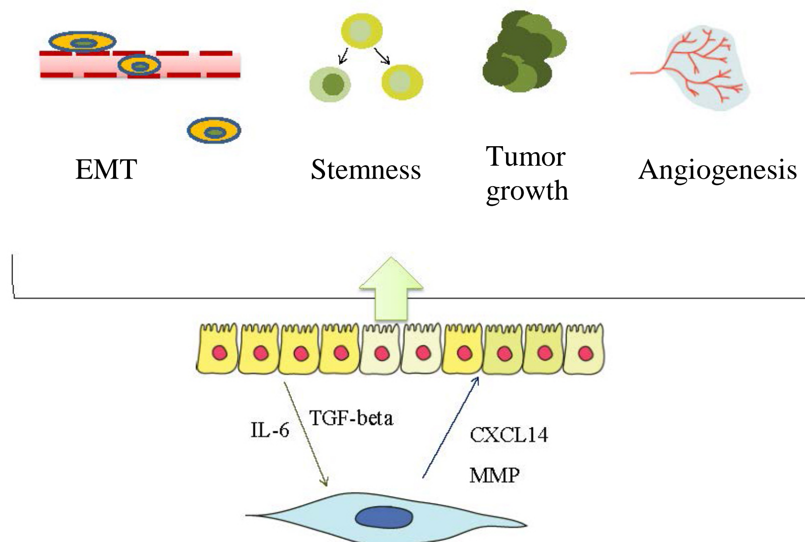

Figure 8. Roles of cancer associated fibroblast in prostate cancer. Through reciprocal crosstalk between prostate cancer cells and prostate tumor stroma, CAFs can regulate several hallmarks of prostate cancer including tumor proliferation, angiogenesis, EMT and acquisition of

stem cell traits. TGF-beta: transforming growth factor beta IL-6: Interleukin 6 CXCL14: Chemokine (C-X-C motif) ligand 14 MMP: matrix metalloproteinase.

1.7.4 Stages and treatments of prostate cancer

Prior to the initiation of prostate cancer treatment several factors have to be considered: disease´s stage, type of prostate cancer, patients´ overall health condition and life expectancy. The stage of prostate cancer is an important predictor of the patients´ response to therapy. The most commonly used method is Gleason score grading system.[108] It is valued according to the prostate tissue morphology under the microscope. It comes from the sum of two scores. The first grade score is assigned to the most common tumor pattern under the microscope. The second grade score is assigned to the next most common tumor pattern. Gleason score is the sum of the two grades and it ranges from 2 to 10. Higher Gleason scores predict a worse prognosis.

Treatment modalities of prostate cancer include “watchful” waiting, surgery, androgen deprivation therapy, radiotherapy, chemotherapy, cryo-therapy and palliative therapy. If the expected life span of the patients is less than 10 years, patients usually undergo watchful waiting. Surgical management including radical prostatectomy is performed in patients who are in good health condition. Prostate and nearby lymph nodes are removed during surgery. Androgen deprivation therapy can be conducted at the early stage and late stage of prostate cancer. Androgen deprivation can be achieved by chemical castration or surgical castration. Different factors in the androgen signaling axis can be targeted by hormone deprivation therapy. Luteinizing hormone-releasing hormone (LHRH) agonists can inhibit the production of testosterone by inhibiting the release of luteinizing hormone.[125] Inhibitors of the enzyme 5-reductase can inhibit the testosterone conversion step. Androgen receptor can also be a direct therapeutic target. For example anti-androgen MDV310 is an AR antagonist.[126] It can inhibit receptor ligand interaction by targeting receptor´ ligand binding domain. Deprivation of hormone induces apoptosis of prostate cancer cells. But most of patients will relapse after hormone deprivation therapy. Currently treatment for castration resistant prostate cancer patients is still a big challenge in the clinic. Mitoxantrone plus a glucocorticoid provide palliative benefit to the patients but does not improve the survival of the patients.[127] Docetaxel which is the first chemotherapy drug that was approved for this indication can improve the median survival of hormone refractory metastatic prostate cancer patients.[128] The improvement, however, is only 2.4 months with this regimen.[128] Development of suitable therapeutic methods for castration-resistant prostate cancer are warranted.

1.8 Tyrosine kinase signaling

1.8.1 Tyrosine kinases

Tyrosine kinases are a family of kinases that can be categorized to receptor tyrosine kinases (RTK) and non-receptor tyrosine kinases (NRTKs). Vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), endothelial growth factor receptor (EGFR) are some of the RTKs that promote tumor growth and metastasis. NRTKs also play a major role in tumor development and they include among others the Abl family (e.g. Abelson murine leukemia viral oncogene homolog 1(Abl)), the SRC family kinases (e.g. SRC, lymphocyte-specific protein tyrosine kinase (Lck), tyrosine-protein kinase Lyn (Lyn), tyrosine-protein kinase Fyn (Fyn)) and the JaK family (tyrosine kinase 2 (Tyk2), JaK1-3). Binding of ligands to the receptors on cellular membrane promotes receptor dimerization and auto-phosphorylation of the receptor intracellular spectrin homology (SH) domains. These domains act as docking sites for SH domain-containing proteins and recruit adaptor protein (e.g. growth factor receptor-bound protein 2 (GRB2), Son of Sevenless (SOS)) initiating a downstream signal transduction cascade that leads to the activation of several processes such as gene expression, proliferation and motility.

1.8.2 Tyrosine kinase signaling in prostate cancer

It was found that in both mouse prostate cancer models and clinical castration resistant prostate cancer samples tyrosine kinases are overexpressed or activated.[129, 130] The main tyrosine kinase signaling cascades activated in prostate cancer are the Raf-Mitogen-activated protein kinase/ERK kinase (MEK)-ERK, the PI3K-AKT and Janus kinase (JaK)-signal transducer and activator of transcription (STAT) signaling cascades **(Figure 9)**.[129, 131] The Ras-Raf-MEK-ERK pathway provides mitogenic signaling to the cells and can regulate diverse biologic functions in the cells including apoptosis and cell cycle progression.[132] The activation of the Raf-MEK-ERK pathway is important in the development of prostate cancer and correlates with higher Gleason score and poor prognosis of prostate cancer patients.[133, 134] [134] Signal transduction mutations in the Raf-MEK-ERK pathway is not common in prostate cancer. It is postulated that the activation of this mitogenic signaling in prostate cancer can be due to the aberrant activation of upstream factors such as the deregulated RTK and NRTK.[132, 135]

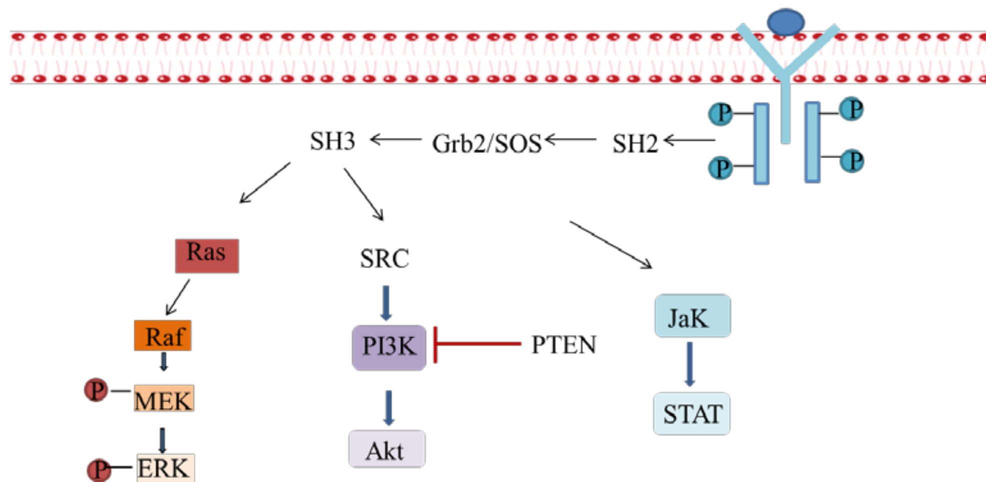

Figure 9. Tyrosine kinase signaling cascades. Ras-Raf-MEK-ERK, the PI3K-AKT and JAK-STAT signaling cascades are the main downstream signaling cascades activated by tyrosine kinases in prostate cancer.

In prostate cancer, the PI3K-AKT pathway promotes the growth,[136] invasion[137] and angiogenesis[138] of prostate cancer cells. In 30-50% of prostate cancers the PI3K-AKT pathway is activated primarily due to PTEN silencing or aberrant activation of upstream RTKs.[139, 140] It can confer cell death resistance to cancer cells by inhibiting the apoptotic activity of caspase 9 and Bad. mTOR is a downstream target of the PI3K-AKT pathway. It can induce phosphorylation of ribosomal p70S6K and eukaryotic initiation factor-4E binding protein (4E-BP) to initiate protein synthesis. Activation of PI3K signaling can also inhibit glycogen synthase kinase 3(GSK3) which can mediate stabilization and nuclear translocation of beta-catenin and induction of downstream target genes such as cyclin D and c-Myc.

There is also an extensive cross-talk between the Raf-MEK-ERK pathway and PI3K-AKT. PI3K kinase can be directly activated by Ras.[141] Conversely, over-activation of AKT signaling can lead to phosphorylation and inhibition of Raf.[142]

Aberrant activation of tyrosine kinases can confer androgen-independent growth signals to prostate cancer cells. Tyrosine kinase phosphorylation can enhance transcription and nuclear translocation of the AR.[143] The RTK insulin growth factor receptor (IGFR) and the NRTK SRC phosphorylate the AR, induce its nuclear translocation and thereby promote androgen-independent growth of prostate cancer cells.[144] Androgen receptor signaling is also regulated by the MAPK signaling pathway and usage of MEK inhibitors impairs androgen receptor signaling.[145] The PI3K-AKT pathway is involved in the progression from hormone sensitive to hormone refractory prostate cancer supported by evidence from both clinical and cell line data.[146, 147]

1.8.3 Tyrosine kinase inhibitor sorafenib

Sorafenib (BAY43-9006, Nexavar), is a multi-tyrosine kinase inhibitor that can target the RTKs VEGFR, PDGFR-β and EGFR as well as NRTK such as Src.[148] Sorafenib is a type II tyrosine kinase inhibitors, i.e. its inhibitory activity does not rely on the active conformation of the receptors.

Sorafenib can exert its function by several mechanisms. Firstly, it can induce apoptosis in tumor cells. The apoptosis induction by sorafenib can be primarily mediated by down-regulation of anti-apoptotic member of the Bcl-2 family Mcl-1 through translational inhibition.[149-151] It can also be mediated by inhibition of the mitogenic signaling through targeting Raf.[149] The Raf-MEK-ERK signaling pathway is the main target of sorafenib.[148] The inhibitions of MAP kinases by sorafenib and apoptosis induction were shown in different cancer cells including prostate cancer,[152] hepatocellular carcinoma,[153] breast cancer,[148]colon cancer [148] and multiple myeloma[150]. The induction of tumor cell apoptosis in different tumor models by sorafenib was established, including in PLC/PRF/5 HCC xenografts model[153], MDA-MB-231 breast cancer model[148] and multiple myeloma[150]. Secondly, sorafenib can inhibit tumor angiogenesis by targeting VEGFR, PDGFR-β tyrosine kinase signaling in renal cell carcinoma,[154] melanoma xenograft.[155] Sorafenib is approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of renal cell carcinoma and hepatocellular carcinoma. With regard to prostate cancer, there are several clinical trials using sorafenib in the treatment of castration resistant prostate cancer.[156-158]

The molecular mechanisms of sorafenib-induced cell death in prostate cancer are not clearly elucidated in the prostate cancer setting. We have previously shown that sorafenib can induce apoptosis and autophagy in prostate cancer cell lines,[152] but the molecular targets of sorafenib in prostate cancer are not known.

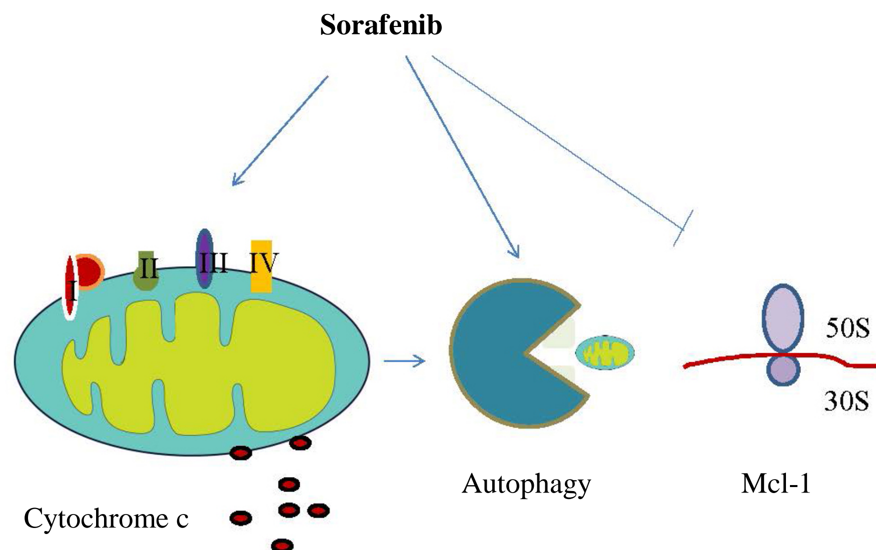

Figure 10. Mechanisms of sorafenib. Sorafenib can exert its function by inducing apoptosis through Mcl-1 inhibition and induction of autophagy.

1.8.4 Resistance to tyrosine kinase inhibitor

Resistance to TKIs is a big challenge that needs to be overcome in tumor therapy.[159-161] Several mechanisms can lead to the acquisition of resistance to tyrosine kinase inhibitors. Mutations on the TKI binding site in tyrosine kinase are the most frequent cause of resistance to TKIs. One amino acid residue mutation in ATP binding sites of tyrosine kinase receptor can cause the loss of binding of the TKI to the receptor. For example, a T674I mutation was reported to cause imatinib resistance.[162] Protective signals from the microenvironment or overexpression of anti-apoptotic members can also cause resistance to TKIs. Combination of TKIs with chemotherapy might overcome the drug resistance to a certain extent and increase treatment efficiency.

1.9 Cell death

1.9.1 Caspase-dependent cell death

Caspase-dependent cell death mainly refers to the type I programmed cell death, apoptosis. Apoptosis plays important roles during embryonic development and is involved in the balance regulation between cell death and cell proliferation. Caspase-dependent cell death can be divided into two categories, the extrinsic and intrinsic cell death pathway (**Figure 11**). The extrinsic pathway is mediated by the binding of tumor necrosis factor (TNF) or Fas ligand to their death receptors tumor necrosis factor receptor (TNFR) and Fas receptor. After binding with the ligands, intracellular death domain of the receptor recruits TNF receptor-associated death domain and Fas-associated death domain to form death-inducing signaling complex (DISC) which will activate the apical pro-caspase 8. Caspase 8 activation leads to the activation of the downstream effector caspases -3, -6 and -7.

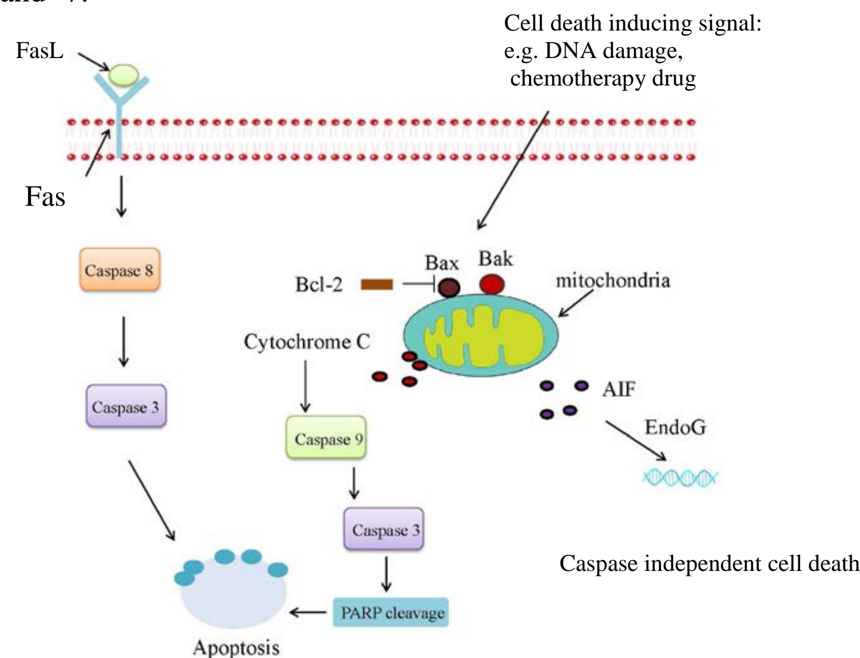

Figure 11. The apoptotic signaling cascades. Apoptosis can be executed by the extrinsic pathway and the intrinsic pathway.

The intrinsic cell death pathway is mediated by the mitochondria. It can be triggered by cell death stimuli such as DNA damage, chemotherapeutic drug treatment, ionizing radiation, Adenosine triphosphate (ATP) depletion and growth factor withdrawal. Upon activation, the pro-apoptotic Bcl-2 family members Bax and Bak translocate from the cytoplasm to the mitochondria to form pores on the outer mitochondrial membrane and cause mitochondrial membrane permeabilization.[163] Cytochrome c is released from the inner mitochondrial membrane into the cytoplasm which is an important hallmark of apoptosis. Cytochrome c together with apoptotic protease activating factor 1 (Apaf-1) and pro-caspase 9 forms the "apoptosome", which main function is the activation of caspase-9. Capase-9 in turn activates the downstream effector caspase-3, -6 and -7.

The apoptotic machinery is regulated by the Bcl-2 family proteins. There are three groups of Bcl-2 family members: the pro-apoptotic members, the anti-apoptotic members and the BH3-only proteins. Bcl-2, Mcl-1, Bcl-xL are the main members of anti-apoptotic Bcl-2 family. Anti-apoptotic Bcl-2 members form complexes with the pro-apoptotic Bcl-2 members such as Bak and Bax to inhibit their activation. The BH3-only proteins (Bid, Bim, Bad, Noxa, and Puma) can competitively bind with anti-apoptotic Bcl-2 members through their BH3 domain and release the pro-apoptotic Bcl-2 members to induce apoptosis. BH3 only protein Bim have three splice variants: Bim S, Bim L, and Bim EL. [164] Bim S is the most potent one in inducing cell death.[164] Bim can be regulated by Forkhead box O (FOXO) transcription factor which is downstream substrate of PI3K-AKT signaling pathway. Bad can be phosphorylated at serine residues 112 and 136.[165] Phosphorylated Bad is sequestered by the signal transducer protein 14-3-3 and cannot activate apoptosis. The PI3K-AKT and the Raf-MEK-ERK pathways have been shown to induce the phosphorylation of Bad.[165]

1.9.2 Caspase-independent cell death

There are different modes of caspase-independent cell death. [166] They include the apoptosis inducing factor (AIF) mediated caspase-independent cell death, autophagy and mitotic catastrophe. Caspase-independent cell death is characterized by cell demise in the absence of caspase activation. AIF is a key factor in caspase-independent cell death.[167] AIF is in the inner mitochondrial membrane space under normal conditions. In the presence of cell death signals, AIF undergoes proteolytic cleavage and translocates from mitochondria to the nucleus where it causes large scale DNA fragmentation through endonuclease G. [167] AIF mediated caspase-independent cell death cannot be inhibited by the pan-caspase inhibitor Z-VAD.

1.9.2.1 Autophagy

There are three types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. "Macroautophagy" will be referred to as "autophagy" in this thesis. Autophagy is an evolutionarily conserved mechanism for cells to maintain homeostasis. Autophagy can be triggered by different stimuli: nutrient starvation, cytotoxic drug and metabolic stress. Autophagy has two important functions. One is the degradation of

protein aggregates and organelles. Another function of autophagy is the recycling of nutrients. When cells are confronted with metabolic stress, cytoplasmic components such as ribosomes and mitochondria can be degraded to amino acids and fatty acids. These metabolites can be re-used in the synthesis of proteins and lipids.

The process of autophagy occurs in four phases: nucleation, elongation, fusion and degradation. After initiation of autophagy, proteins and organelles are sequestered by the phagophore at the phagophore assembly site (PAS). Elongation and closure of phagophore leads to the formation of the autophagosome. Fusion of the autophagosomal outer membranes with lysosomes gives rise to the autophagolysosomes. Engulfed proteins and cellular organelles are degraded by proteases in lysosomes. The process of autophagy is demonstrated in **Figure 12B**. The process of autophagy is executed by several evolutionarily conserved proteins complexes. Autophagy initiation is regulated by two protein complexes: mammalian homolog of yeast Atg1 (ULK1) protein complex and Class III PI3K protein complex (**Figure 12A**). Members of the ULK1 protein complex include ULK1, ATG13, and focal adhesion kinase family interacting protein of 200 kD (FIP200). The ULK1 kinase complex plays an essential role in autophagy initiation. Members of class III PI3K protein complex include Coiled-coil myosin-like BCL2-interacting protein (Beclin1) and Vps34. Beclin1 is a key regulator of autophagy. [168] For example, AKT can inhibit the initiation of autophagy by phosphorylation of Beclin1.[168] Lipidation of microtubule-associated protein 1A/1B-light chain 3 (LC3) is an important step in the elongation phase of autophagy (**Figure12C**). Two ubiquitin like protein conjugation systems ATG5-ATG7-ATG12 and LC3 are involved in the autophagosome formation phase. LC3 is firstly cleaved on c-terminal by ATG4 protease to form the cytoplasmic soluble LC3-I. LC3-I is conjugated with phosphatidyl ethanolamine (PE) and form LC3-phosphatidyl ethanolamine conjugate (LC3-II), a reaction that is catalyzed by the E1 ubiquitin enzyme like protein ATG7. ATG5 is a key regulator of LC3 lipidation. ATG12 is conjugated to ATG5 by E2 ubiquitin like protein Atg10. ATG5-ATG12 further forms a high molecular weight complex with ATG16. ATG5-ATG12-ATG16 facilitates the binding of LC3-PE to the autophagosome membrane.[169] P62 (sequestosome 1 (SQSTM1)) is an adaptor protein in the autophagy process. It has both ubiquitin binding and LC3 binding domains. By binding to both ubiquitinated proteins and LC3, it can transport the cargo into autophagosomes for degradation. If autophagy is impaired, p62 cannot be degraded and accumulated in the cytoplasm. This will lead to DNA damage by induced oxidative stress which can promote tumorigenesis.[170]

Finally in the late stage of autophagy, autophagosome fuses with lysosomes**.** The contents of the autophagosome are degraded by acidic hydrolases in lysosome. Lysosome-associated membrane protein 2 (LAMP-2), an integral lysosome membrane protein, is essential for the autophagolysosome formation.[171] In LAMP-2 knockout mice, autophagolysosome formation is impaired. [171] Defect of the late stage autophagy leads to the accumulation of autophagosomes.

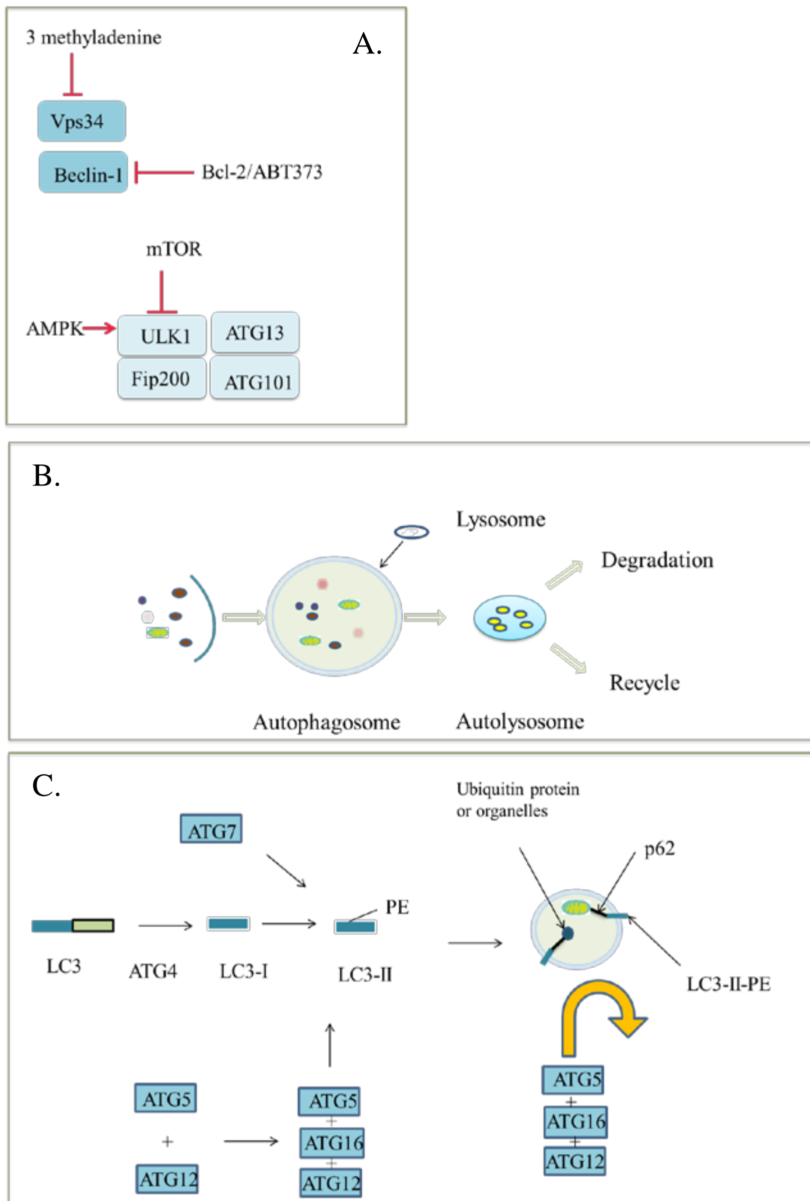

Figure 12. Process of autophagy. A. initiation phase of autophagy B. autophagy process C. LC3 lipidation process.

1.9.2.2 Regulation of Autophagy

1.9.2.2.1 Autophagy regulation by PI3K-AKT-mTOR signaling

PI3K-AKT-mTOR signaling is the major regulatory pathway of autophagy.[172] Growth factors and nutrient conditions regulate the initiation of autophagy via the PI3K-AKT-mTOR signaling pathway. Under nutrient rich condition, PI3K gets activated and phosphorylates AKT which in turn phosphorylates and inhibits tuberous sclerosis protein 2 (TSC2). In the absence of activated TSC2, Ras homolog enriched in brain (RheB) is phosphorylated and activated which in turn will activate mTOR. mTOR inhibits the initiation of autophagy by phosphorylating and repressing ULK1. Under harsh metabolic

conditions (e.g. starvation, hypoxia) PI3K-mTOR pathway is inhibited, allowing for the autophagic machinery to be activated. [173] It was recently found that AKT can directly phosphorylate Beclin1 independently of mTOR thereby inhibiting autophagy and promoting oncogenesis.[168]

1.9.2.2.2 Regulation of autophagy by metabolic stress

Autophagy can be triggered by metabolic stress which is frequently observed in cancer cells. The initiation of autophagy process is regulated by 5' AMP-activated protein kinase (AMPK), an energy sensor in the cells. AMPK is a serine tyrosine kinase which can sense the level of adenosine monophosphate (AMP) and ATP. Increased AMP: ATP ratio can trigger the activation of AMPK by the serine threonine kinase liver kinase B1 (LKB1). Activated AMPK phosphorylates and inhibits mTOR thus allowing for the initiation of autophagy.

1.9.2.2.3 Connection between apoptosis and autophagy

There is an extensive cross-talk between apoptosis and autophagy. One convergence point is Beclin1.[174] Beclin1 has a BH3-only protein domain which can bind with anti-apoptotic Bcl-2 family members.[175] Bcl-2 binds with Beclin-1 and inhibits autophagy. It was shown that inhibition of Bcl-2 expression by small interfering RNA in MCF-7 breast cancer cells lead to autophagic cell death.[176] ABT737, a BH3 mimetic, can induce autophagy because it can interrupt the complex between Bcl-2 and Beclin1.[177] Another convergence point is ATG5. [178] It is reported that ATG5 can be cleaved by calpains to a 24kD fragment which then acts as BH3-only protein interacting with Bcl-xL, allowing for the activation of Bax and the induction of apoptosis.[178]

1.9.3 Autophagy in cancer and chemotherapy

The role of autophagy in cancer is complex and context-dependent.[179] Autophagy has a tumor suppressive role in the early stages of tumor development. Beclin1 is a haploinsufficient tumor suppressor gene. In mice carrying Beclin1 monoallelic deletion, the potential of tumor development was increased.[175, 180] Furthermore, ~50% of human breast and prostate cancers patients have monoallelic deletion of Beclin1.[181, 182] One explanation for the tumor suppressive function of autophagy is that defective autophagy can lead to the accumulation of damaged organelles such as mitochondria, accumulation of ROS production and increased genomic instability.[183] The degradation of p62 by autophagy is another explanation for tumor suppressive role of autophagy.[170, 184] In autophagy defective tumors cells, p62 cannot be degraded and accumulates in the cells. Accumulation of p62 induces ROS which leads to DNA damage and tumorigenesis.

Autophagy also plays tumor promoting roles since it is a survival mechanism for cells. Autophagy can protect cancer cells from metabolic, oxidative stress or cellular damage induced by different stimuli such as hypoxia, starvation and anti-cancer drugs. Inhibition of autophagy can sensitize cancer cells to anti-cancer drug. LnCaP prostate cancer cells can utilize autophagy to survive from androgen deprivation treatment.[185] In breast cancer, inhibition of autophagy can reverse the resistance of tumor cells to tamoxifen treatment.

[186] Thus autophagy may also be a therapeutic target in the late stages of tumorigenesis.[187] The autophagy inhibitor chloroquine in combination with the TKI imatinib mesylate has shown better therapeutic efficiency in chronic myeloid leukemia both in vitro experiments and in clinic.[188] In prostate cancer, TKIs together with autophagy modulators emerge as a potential treatment strategy.[189]

2. Aims of the studies

The overall objective of the present study is to gain insights into the mechanisms of malignant transformation and mechanisms of drug-induced cell death, thereby contributing to a better understanding of oncogenic processes and the rational development of novel anti-cancer strategies. Specifically, the project is designed:

1. To define potential roles for transcription factor FoxM1 in regulating telomerase and senescence of gastric cancer cells.
2. To determine whether dysregulation of Reptin contributes to telomerase activation in these cancers.
3. To delineate the mechanisms of sorafenib-induced cell death in prostate cancer.
4. To examine the role of autophagy in prostate cancer response to therapy.

3. Results and discussion

3.1 Paper I

Reptin is overexpressed in gastric cancer and can regulate hTERT transcription

In order to assess the expression of Reptin in gastric cancer, Reptin expression was examined in clinical gastric cancer patient samples by immunohistochemistry and reverse transcriptase (RT)-polymerase chain reaction (PCR). Expression of Reptin in primary gastric cancer tissues was significantly higher compared to that in normal gastric tissues. The expression of Reptin and hTERT was in parallel with each other in clinical samples. The expression of Reptin was also detectable in a series of gastric cancer cell lines (AGS, BGC, HGC, and KATO-III).

In order to find out the role of Reptin in the pathogenesis of gastric cancer, Reptin was depleted by using a specific siRNA. Depletion of Reptin led to down-regulation of hTERT expression at the RNA level. Telomerase activity was also inhibited. Combined inhibition of Reptin and Pontin had synergistic inhibitory effect on hTERT mRNA level. However, Reptin and Pontin had an opposite effect on the hTERT promoter activity. Reptin can positively while Pontin negatively regulate the promoter activity. Studies have shown that Reptin and Pontin can have antagonistic regulation of target genes.[63, 190] It was also shown that overexpression of one of them can lead to down-regulation of the other one's protein level.[191] The antagonistic regulation by Reptin and Pontin could be a feedback loop that can regulate the target gene expression at a balanced level.

It was found that depletion of Reptin leads to the co-depletion of Pontin. This is in line with previous findings which indicated that the stability of Reptin and Pontin relies on each other.[52, 58] The co-depletion of Reptin and Pontin was also observed in human liver cancer cell line HepG2, breast cancer cells MCF7, prostatic cancer cells LnCaP and cervical cancer HeLa cells. [58] Silencing of Reptin by using siRNA did not change the Pontin mRNA level. [58]

In the current study, it was shown that inhibition of Reptin expression impaired the colony formation ability of gastric cancer cells. It was reported that silencing Reptin *in vivo* induced replicative senescence in human hepatocellular carcinoma in mice and inhibited the growth of explanted tumors. [192] Similarly, in renal cell carcinoma, depletion of Reptin can impair the clonogenic potential of renal carcinoma cells. Senescence was observed in the Reptin depleted renal cancer cells.[193] The growth inhibition role of Reptin could be partially explained by the down-regulation of telomerase, since telomerase is critical in sustaining proliferation. This was also consistent with the finding that Reptin is subunit of telomerase complex and required for its activity.[52] It was also reported that shRNA mediated depletion of p400 or knock down of p400 complex components Reptin and Pontin can induce senescence in primary human diploid fibroblasts as shown by

SAHF formation and senescent β-gal staining.[194] This report gives another explanation of the senescence induction by Reptin inhibition.

The hTERT promoter-luciferase reporter is a commonly used tool for study of telomerase transcription regulation. By this assay it was found that hTERT promoter activity was regulated by Reptin and the hTERT transcription needed the cooperation between c-Myc and Reptin. When the c-Myc binding cassette, E-box, was absent from the hTERT promoter region, regulation of hTERT by Reptin was attenuated. By performing immunoprecipitation experiments, we also confirmed the interaction of Reptin with c-Myc in gastric cancer cells. C-Myc is one of the key regulators of hTERT transcription. But E-box (5´-CACGTG) is not a specific binding site for c-Myc. It can also be bound by other transcription factors. It would be possible to confirm the specificity of c-Myc dependence in the regulation of hTERT by Reptin. There was a study showing that c-Myc N-terminus was responsible for the recruitment of Reptin and Pontin on their target gene promoters.[62] To approach this issue, c-Myc N-terminus can be genetically mutated and the effect examined.

Main findings in Study I:

1. Reptin is over-expressed in primary gastric cancer samples.

2. Reptin is required for the transcription of the *hTERT* gene.

3. hTERT transcription regulation by Reptin requires its cooperation with c-MYC.

3.2 Paper II

FoxM1 is overexpressed in gastric cancer and its inhibition leads to senescence

Extensive studies from both human tumor cell lines and patients´ samples establish that FoxM1 is aberrantly activated in most human malignancies. Our study provided evidence that FoxM1 is overexpressed in gastric cancer. FoxM1 was significantly overexpressed in gastric cancer patient’s samples examined by immunohistochemistry and RT-PCR and also in gastric cancer cell lines (AGS, BGC-823, HGC-27 and KATO-III). Similar results was repeated by another group showing that FoxM1b, one isoform of FoxM1, was expressed at significantly higher levels in the patients’ samples from gastric tumors and lymph node metastatic sites. It was also demonstrated that in the FoxM1 overexpressed animal model, FoxM1 can promote the growth and metastasis of gastric cancer cells.[195]

We found that siRNA mediated knockdown of FoxM1 induced senescence in gastric cancer cells and led to a diminished clonogenic potential. This is consistent with previous findings that knockdown of FoxM1 impaired the tumorigenesis of malignant cells.[85, 87]

P53-p21^{Cip1} and p16^{ink4a}-Rb signals regulating senescence were not affected by FoxM1 depletion. In all four tested cell lines, there were no differences in p21^{Cip1} expression level before and after knocking down of FoxM1. In contrast, p27^{kip} level was increased in all four cell lines. To verify the role of p27^{kip} in the senescence induction, p27^{kip} siRNA was

transfected into the gastric cancer cells. Deceased $p27^{kip}$ expression attenuated the effect of FoxM1 depletion. It further proves the regulatory axis that FoxM1 regulates Skp2 and Cyclin-dependent kinase activating kinase1 (Cks1) expression, [82] Skp2 as the upstream regulator of $p27^{kip}$ can mediate the ubiquitin proteasome degradation of $p27^{kip}$. FoxM1 depletion inhibits Skp2 and induces the accumulation of $p27^{kip}$. [82]

$P27^{kip}$ mediated senescence was reported in previous studies.[196] $p27^{Kip1}$ can be regulated by PTEN/PI3K-AKT signaling which is implicated in the carcinogenesis of many malignancies. Inhibition of PI3K-AKT signaling or overexpression of PTEN can increase the expression level of $p27^{Kip1}$.[197] It was shown that $p27^{Kip1}$ was a direct inducer of p53-independent senescence in mouse embryonic fibroblasts that did not express the suppressor VHL (von Hippel-Lindau).[198] The p53-independent $p27^{Kip1}$ mediated senescence regulation is also supported by another study showing that Skp2 inactivation induced senescence via Activating transcription factor 4(Atf4), $p27^{Kip1}$ and $p21^{Cip1}$ but independent of p53.[199]

hTERT is important for senescence prevention by maintaining telomere length in cancer cells. We examined the effect of FoxM1 depletion on expression of hTERT, and found in FoxM1-depleted gastric cancer cells that hTERT expression and telomerase activities were down regulated.

Main findings in Study II:

1. FoxM1 was up-regulated in gastric cancer cell lines and primary gastric cancer.

2. Depletion of FoxM1 induces senescence in gastric cancer cells.

3. Senescence triggered by FoxM1 inhibition is p53-and p16-independent and mediated by the enhanced expression of $p27^{kip1}$.

4. Depletion of FoxM1 inhibits the expression of c-Myc and hTERT.

3.3 Paper III

Sorafenib induced prostate cell death by different mechanisms

Tyrosine kinase inhibitors are attracting more and more attention as potential therapeutic strategies against prostate cancer. Sorafenib can target multiple tyrosine kinase signalling cascades and therefore could be a good candidate. We have previously shown that sorafenib can induce cell death in prostate cancer cell lines.[152] In this study, we delineated the mechanisms of sorafenib-induced cell death in two model prostate cancer cell lines. 22RV1 cells, a hormone sensitive, non-metastatic prostate cancer cell line, was more sensitive to sorafenib and displayed faster cell death kinetics comparing to PC3 cells, a hormone refractory, metastatic prostate cancer cell line. We found that the different cytotoxic efficacy of sorafenib is due to the different signalling cascades targeted by sorafenib. In 22RV1 cells, the constitutively active Raf-MEK-ERK pathway was inhibited by sorafenib whereas in PC3 cells this pathway was not activated. In PC3 cells, the PI3K-AKT pathway was constitutively active and the cytotoxic activity of sorafenib

was mediated by inhibiting this pathway. In a recently published study it was also shown that sorafenib can induce cell death in PC3 and 22RV1 cells.[200] Their experiments indicate that the differential cell killing effect of sorafenib might be due to the differential expression of AR which can be inhibited by sorafenib treatment. [200]

Mcl-1 down regulation is an important mechanism of sorafenib-induced apoptosis. We found in this study that overexpression of Mcl-1 in prostate cancer cells protected them from sorafenib-induced cell death. Mcl-1 down-regulation potentiated prostate cancer cells to sorafenib treatment which is independent of Raf-MEK-ERK and PI3K-AKT signaling pathways. The subsequent study from Oh *et al.* also showed that the down regulation of Mcl-1 and inhibition of AKT signaling in sorafenib-induced apoptosis in prostate cancer cells.[200]

In the current study, it was also found that protective autophagy was induced in these prostate cancer cells. This is in line with one reported role of autophagy in chemotherapy, as an adaptive mechanism to protect cancer cells from chemotherapy. CAFs play multifaceted roles in the prostate cancer pathogenesis.[121, 124] It was found in study III that CAFs can protect 22RV1 and PC3 prostate cancer cells from sorafenib-induced cell death. This protection can be reversed by the combination treatment of sorafenib with ABT373.

Main findings in Study III:

1. Sorafenib induces apoptosis in two prostate cancer cells, 22Rv1 and PC3, with 22RV1 being more sensitive of the two.

2. Targeting of distinct signaling cascades in 22Rv1 and PC3 defines the efficacy of sorafenib.

3. In both PC3 and 22Rv1 cells, Mcl-1 depletion is required for the induction of cell death by sorafenib.

4. Primary CAFs protect the cancer cells from sorafenib-induced cell death, and this protection could be largely overcome by co-administration of the Bcl-2 antagonist ABT737.

3.4 Paper IV

ATG5 independent cytotoxic autophagy was found in prostate cancer cell

In this study we found that treatment with sorafenib-induced mitochondria depolarization and caspase-independent cell death in DU145 cells. Treatment with and early inhibitor (3-methyladenine (3-MA)) and a late inhibitor of autophagy (chloroquine) increased the amount of cells with dissipated mitochondria. Inhibition of autophagy initiation by using shRNA against Beclin1 and the early stage autophagy inhibitor 3-MA protected DU145 cells from sorafenib-induced cell death. Surprisingly, LC3 lipidation was not evident in DU145 cells due to the lack of ATG5 expression. Restoration of ATG5 expression partially re-constituted LC3 lipidation and partially rescued DU145 cells from sorafenib-

induced cell death. ATG5 lack of expression was found in 18% of prostate cancer patient samples. We speculated that autophagy was initiated to remove the damaged mitochondria, sustain the ATP level and the metabolic homeostasis of the cells. Surprisingly, autophagosome formation occurred in an ATG5-independent manner in sorafenib treated DU145 cells. Similar results were reported in etoposide induced ATG5 independent autophagy in MEFs cells.[201] Non-canonical Beclin1-independent autophagy was also observed in resveratrol-treated human breast cancer cells.[202]It was recently shown that alternative splicing of ATG5 may be the reason for the loss of ATG5 expression in DU145 cells.[201]

We found that manipulation of autophagy key regulators such as Ulk1 and Beclin1 can attenuate cell death induced by sorafenib. Further investigations are needed to describe the regulation of the non-canonical autophagy process. It is unknown if prostate cancer cells acquire another mode of autophagy for example chaperone-mediated autophagy to regulate the protein turn over while macroautophagy is defective. In MEFs, mitochondria and proteins were degraded during the ATG5-independent autophagy.[201]

In the current study we found that overexpression of PI3K-AKT signaling pathways suppressed the cytotoxic autophagy and led to the resistance of sorafenib in DU145 cells. The resistance to sorafenib can be reversed by the PI3K-inhibitor LY294002. Comparing to Raf/MEK/ERK pathway, PI3K-AKT signaling is more likely implicated in drug resistance of prostate cancer cells and is more promising for the targeted therapy. In prostate cancer patients, loss expression of ATG5 also exists. The loss expression of ATG5 has not been reported in prostate cancer patients before. It is warranted to know the mechanism of ATG5 loss in the patient samples. It was shown that in mice with ATG5 or ATG7 depletion, mice developed tumors in the liver.[203] It is interesting to know whether ATG5 loss of expression is correlated with tumorigenesis in prostate cancer patients.

Main findings in Study IV:

1. DU145 cells undergo autophagic cell death in response to sorafenib.
2. Loss expression of ATG5 is responsible for the cytotoxic autophagy.
3. Development of resistance to sorafenib is associated with the activation of the constitutive of the PI3K-AKT pathway and subsequent inhibition of this cytotoxic autophagy
4. Tissue microarray analysis revealed that 18% of prostate cancer patients do not express ATG5.

4. Future perspectives

4.1 Exploring the regulators of Reptin and targeting Reptin as therapeutic strategy

In study I, we showed that Reptin was overexpressed in gastric cancer and it was required for hTERT transcription. Inhibition of Reptin impaired the clonogenic potential of gastric cancer cells. These findings provide a rationale for targeting Reptin to treat gastric cancer.

Understanding the regulation and function of Reptin will provide us further choices in gastric cancer treatment. The upstream regulators for Reptin are poorly characterized. The mechanism of the up-regulation of Reptin and Pontin in cancer is an unknown question to answer. Gene amplification can be a possible reason. Pontin maps to chromosome 3q21 which is a hot spot for amplification in non-small cell lung cancer.[204] In gastric cancer, high level of amplification at chromosome 3q21 was also reported.[205] Reptin can also be regulated by epigenetic machinery. For example, Reptin can be modified by sumoylation in metastatic prostate cancer cells. Sumoylation of Reptin had a negative effect on tumor suppressor KAI1 expression and further regulated the metastasis potential of prostate cancer cells.[206] Reptin can also be modified by methylation. Methylated Reptin can suppress the expression of hypoxia-dependent target genes which was mediated by the transcription factor HIF1α.[207] In gastric cancer, epigenetic regulation of Reptin expression may also exist.

Except for regulating hTERT transcription and clonogenic potential of gastric cancer cells, there are no reports about other roles of Reptin in gastric cancer, for example in invasion and metastasis; in malignant transformation, in metabolism: Reptin is involved in the regulation of glucose transporter 4 (GLUT4)[208] which is implicated in the cancer development;[209] DNA repair and transcription regulation of oncogenic transcription factors.

Targeting telomerase by inhibiting the regulators of hTERT is an also interesting avenue to explore. It is shown in human hepatocellular carcinoma that targeting Reptin inhibited the tumor growth in xenografts.[192] Identification of small molecule inhibitors for Pontin provides promising indication for the exploration of inhibitors for Reptin.[210] It can be achieved by antagonizing its ATPase activity or by inhibiting its interactions with other proteins.

4.2 Investigating roles of FoxM1 in cancer and targeting FoxM1 in cancer therapy

In study II we showed that targeting FoxM1 reduced telomerase activity, c-Myc expression and led to cellular senescence in gastric cancer cells. Extensive evidence shows that FoxM1 can regulate a multitude of essential oncogenic functions. FoxM1 is associated with a number of hallmarks of cancer such as: angiogenesis, proliferation, senescence regulation and metastasis, tumorigenesis. It is of great interest to explore other

roles of FoxM1 in tumor pathogenesis. One of them is the link between FoxM1 and regulation of metabolism. Sufficient metabolites are the prerequisite of cell proliferation. The link between metabolism and proliferation regulator FoxM1 may be involved in the coordination of signals during carcinogenesis. Preliminary evidence is as follows. Firstly, FoxM1 expression can be triggered by oxidative stress in the cells. Metabolism process is one major source of the reactive oxygen species produced inside the cells. FoxM1 might be involved in metabolism regulation. Secondly, link between oncogenic metabolic signaling and FoxM1 signaling is shown by experimental study. There is a study showing glycolysis enzyme phosphoglycerate dehydrogenase implicated in tumorigenesis[211] can promote the proliferation and invasion of glioma cells through stabilizing FoxM1.[212] Further investigation is necessary to decipher the interaction mechanism.
Third, it is interesting to know the mechanism of FoxM1 up regulation in gastric cancer. In human squamous cell carcinoma, up-regulation of FoxM1 is an early event during carcinogenesis.[213] And nicotine from tobacco consumption can promote the effect of FoxM1 in malignant transformation of human oral keratinocytes. [213] *H.pylori* infection is associated with the carcinogenesis of gastric cancer. Experimental investigation is needed to understand if *H.pylori* is implicated in the aberrant activation of FoxM1 during early stage of gastric cancer development. The hints are as follows. Firstly, oxidative stress caused by *H.pylori* infection plays key role in pathogenesis of gastric cancer.[214] Oxidative stress can induce the expression of FoxM1.[79] FoxM1 can promote proliferation of gastric cancer cells and regulate c-Myc and telomerase activity as shown in the current study. Secondly, FoxM1 can be regulated by Gli-transcription factors in sonic hedgehog (Shh) signaling.[88] *H.pylori* infection can cause the activation of Shh signaling by secreting virulence factor CagA.[215] FoxM1 is likely a downstream factor in the *H.pylori* infection process.

Meanwhile, it is warranted to develop therapeutic methods for targeting FoxM1. There are studies showing the potential of targeting FoxM1 in animal models. For example, FoxM1b deficient mice are resistant to developing hepatocellular carcinoma induced by carcinogen.[81] In human pancreatic liver and colon cancer cells, inhibition of FoxM1 can increase the sensitivity of tumor cells to DNA damage agents induced cell death. It is promising to develop treatment strategy by inhibiting FoxM1.[216] Combination of chemotherapeutic drug together with FoxM1 inhibitor can be a method. As shown in the current study, FoxM1 inhibition can cause permanent cell cycle arrest which is one of the major aims in tumor therapy.

4.3 Combination therapy based on sorafenib and targeting tumor microenvironment

Combination of tyrosine kinase inhibitor with other treatments such as chemotherapy can increase the treatment efficiency of tyrosine kinase inhibitors. In the current study, the co-administration of sorafenib with Bcl-2 antagonist ABT737 was found to be more efficient in killing both non-metastatic and metastatic prostate cancer cells than sorafenib alone. It was also shown that the treatment efficiency of sorafenib was dramatically enhanced

when combined with chemotherapy drug.[217] The synergistic effect of sorafenib with ABT737 was also reported in treatment of hepatocellular cancer cells, [218] chronic myelogenous leukemia and Philadelphia chromosome–positive (Ph+) acute lymphoblastic leukemia cells.[219] The current study provides an important piece of evidence for tyrosine kinase combination therapy.

Tumor microenvironment is participating in the regulation of most hallmarks of cancer. Components of the tumor microenvironment including activated fibroblasts, recruited macrophages and remolded extracellular matrix components actively contribute to the epithelial cell-stroma cell crosstalk. One important role of tumor stroma is to provide protection to tumor cells against chemotherapy. Targeting tumor microenvironment has become a novel therapeutic strategy. Understanding the contributors is the prerequisite of the specific treatment. Diverse growth factors, cytokines and chemokines can be involved in the protective effect transduced from cancer associated fibroblasts. Different strategies are proposed for targeting CAFs. Tyrosine kinase inhibitor is a promising candidate because the activation of fibroblasts in tumor stroma largely depends on the tyrosine kinas signaling pathways. The effect of sorafenib in reversing the tumor microenvironment protection was also shown in multiple myeloma.[150]

4.4 Understanding the regulation and role of non-canonical autophagy in cancer

The finding of ATG5-independent autophagy in prostate cancer cells may provoke the investigation of autophagy mechanism in other types of malignancies. DU145 cells are a suitable model for this investigation because they do not express ATG5. In ATG5-independent autophagy, several questions are left unanswered. For example, the membrane source of the ATG5-independent autophagy and how traffic of membrane structures is regulated. It has been found that in MEFs, the membrane source of ATG5-independent autophagosomes is the trans-Golgi network and late endosomes.[201] It is interesting to know the answer in tumor cells. It is reasonable to speculate that in prostate cancer cells *in vivo*, ATG5-dependent autophagy and ATG5-independent autophagy coexists. They might be induced by different stimuli under different contexts. They may also have different functions. Also different regulatory machinery can be involved in the regulations. To have a complete picture, further investigations are needed. Our study also indicates that defining the autophagy pattern in patients is important for the autophagy modulator therapy in clinic.

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