From DEPARTMENT OF ONCOLOGY-PATHOLOGY
Karolinska Institutet, Stockholm, Sweden

STUDIES ON THE MECHANISMS OF SORAFENIB-INDUCED CELL DEATH

Pedram Kharaziha

Karolinska Institutet

Stockholm 2014

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Printed by Universitetsservice US-AB

ISBN 978-91-7549-626-9

“There is only one thing that makes a dream impossible to achieve: the fear of failure…

… But this fear evaporates when we understand that our life stories and the history of the world were written by the same hand.”

The Alchemist by *Paulo Coelho*

To my wife ***Ghazaleh,***

my son ***Parham,***

and my parents ***Mohammad Hossein*** *and* ***Sedigheh***

LIST OF SCIENTIFIC PAPERS

I. **Kharaziha P*,** De Raeve H*, Fristedt C, Li Q, Gruber A, Johnsson P, Kokaraki G, Panzar M, Laane E, Osterborg A, Zhivotovsky B, Jernberg-Wiklund H, Grandér D, Celsing F, Björkholm M, Vanderkerken K, Panaretakis T. Sorafenib has potent antitumor activity against multiple myeloma in vitro, ex vivo, and in vivo in the 5T33MM mouse model. Cancer Res. 2012 Oct 15;72(20):5348-62

II. Anders Ullén, Marianne Farnebo, Lena Thyrell, Salah Mahmoudi, **Pedram Kharaziha**, Lena Lennartsson, Dan Grandér, Theoharis Panaretakis, Sten Nilsson. Sorafenib induces apoptosis and autophagy in prostate cancer cells in vitro, International Journal of Oncology. 2010 Jul;37(1):15-20.

III. **Kharaziha P***, Rodriguez P*, Li Q, Rundqvist H, Björklund AC, Augsten M, Ullén A, Egevad L, Wiklund P, Nilsson S, Kroemer G, Grander D, Panaretakis T, Targeting of distinct signaling cascades and cancer-associated fibroblasts define the efficacy of sorafenib against prostate cancer cells, Cell Death and Disease (2012) 3, e262

IV. **Pedram Kharaziha**, Sophia Ceder, Patricia Rodriguez, Vladimir Gogvadze, Lena Lennartsson, Ann-Charlotte Björklund, Boris Zhivotovsky, Dan Grandér, Lars Egevad, Sten Nilsson, Theocharis Panaretakis. Atg5-independent autophagy promotes sorafenib-induced necroptosis. Manuscript in preparation.

*Equal Contribution

Related publications not included in thesis:

I. Helene Rundqvist, Martin Augsten, Anna Strömberg, Eric Rullman, Sara Mijwel, **Pedram Kharaziha**, Theocharis Panaretakis, Thomas Gustafsson, Arne Östman Effect of Acute Exercise on Prostate Cancer Cell Growth , PLoS ONE ,2013, 8(7): e67579.

II. **Pedram Kharaziha**, Sophia Ceder, Claire Sanchez and Theocharis Panaretakis Multitargeted therapies for multiple myeloma. Autophagy ,Volume 9, Issue 2 February 2013.

III. **Kharaziha P***, Ceder S*, Li Q, Panaretakis T. Tumor cell-derived exosomes: A message in a bottle. Biochim Biophys Acta. 2012 Aug;1826(1):103-11.

IV. Panaretakis T., **Kharaziha P.**, Laane E., Pokrovskaja K. and Grandér D. Autophagy as the main means of cytotoxicity by glucocorticoids in hematological malignancies. Autophagy. 2009 Nov;5(8):1198-200.

V. Laane E., Tamm K.P., Buentke E., Ito K., **Kharaziha P.**, Oscarsson J., Corcoran M., Björklund A.C., Hultenby K., Lundin J., Heyman M., Söderhäll S., Mazur J., Porwit A., Pandolfi P.P., Zhivotovsky B., Panaretakis T., Grandér D. Cell death induced by dexamethasone in lymphoid leukemia is mediated through initiation of autophagy. Cell Death Differ. 2009 Jul;16(7):1018-29 (Erratum in: Cell Death Differ. 2009 Jul;16(7):1071).

*Equal Contribution

LIST OF ABBREVIATION

3-MA	3-Methyladenine
4E-BP1	4E-binding protein 1
Abl	Abelson murine leukemia viral oncogene homolog
ADT	androgen-deprivation therapy
AID	activation-induced deaminase
ALL	acute lymphoblastic leukemia
AMACR	α -Methylacyl-CoA racemase
AMBRA1	autophagy/Beclin-1 regulator1
AML	acute myeloid leukemia
APRIL	a proliferation-inducing ligand
ATG	autophagy-related gene
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
BAFF	B-cell activating factor
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X
BCL-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma-extra large
BCMA	B-cell maturation antigen
BH3	Bcl-2 homology domain 3
Blk	B-cell lymphocyte kinase
BMSC	bone marrow stromal cell
BPH	benign prostate hypertrophy
BRCA1/2	breast cancer 1/2
CAF	Cancer associated fibroblasts
CHEK2	checkpoint kinase 2
CDK2	Cyclin-dependent kinase 2
CIP4	Cdc42-interacting protein 4
COX2	cyclooxygenase 2
CpG	cytosine guanine
CRPC	castration-resistant prostate cancer

CSF-1	colony-stimulating factor- 1
c-Kit	stem cell factor receptor
CSK	C-terminal Src kinase
CYLD	Lys63-deubiquitylating enzyme cylindromatosis
CYP3A4	cytochrome P450 3A4
DEPTOR	DEP domain-containing mTOR-interacting protein
DFCP1	double FYVE-domain-containing protein 1
DFSP	dermatofibrosarcoma protuberance
DISC	death-inducing signaling complex
DNA	deoxyribonucleic acid
DR5	death receptor 5
DRE	digital rectal examination
EBRT	external-beam radiotherapy
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF3	eukaryotic initiation factor 3
ELK1	ETS domain-containing protein
ERK	extracellular signal-regulated kinase
Ets/Bmx	endothelial/epithelial tyrosine kinase
ETS	*E*-twenty-six specific
ETV6	ets variant 6
FADD	FAS-associated death domain protein
FAK	focal adhesion kinase
FAP	fibroblast-activated protein
FDA	food and drug administration
FER	Feline sarcoma-related
FES	feline sarcoma oncogene/fujinami avian sarcoma viral oncogene homolog
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FIP200	FAK family kinase-interacting protein of 200 kDa
Flk-1	fetal Liver Kinase 1

flt	fms-related tyrosine kinase
FSP1/S100A4	fibroblast-specific protein-1
GIST	gastrointestinal stromal tumors
Glut 1	glucose transporter 1
GPCR	G-protein-coupled receptors
GRB2	growth-factor-receptor-bound protein 2
GSTP1	glutathione S-transferase π
Hck	hemopoietic cell kinase
HER2	human epidermal growth factor receptor 2
HIF-1α	hypoxia-inducible factors1 alpha
HMGB1	high mobility group box 1
HPC1	hereditary Prostate Cancer 1
IAP	inhibitor of apoptosis family of proteins
ICAD	inhibitor of caspase activated DNase
IGF1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor-1 receptor
IGFBP1	insulin-like growth factor binding protein
IKK	activate IκB kinase
IL-6	interleukin 6
InsRR	insulin receptor-related protein
IR	insulin receptor
IRS	insulin receptor substrate
JAK	Janus kinase
JNK	c-jun NH kinase
KDR	kinase insert domain-containing receptor
KLF6	Kruppel-like factor 6
KRAS	Kirsten rat sarcoma viral oncogene homolog
LC3	microtubule-associated protein 1A/1B-light chain 3
LIR	LC3-interacting region
MAPK	mitogen-activated protein kinase
Mcl-1	myeloid leukemia cell 1
MDM2	mouse double minute 2 homolog
MEK	mitogen-activated protein kinase kinase

MGUS	monoclonal gammopathy of undetermined significance
MIP-1α	macrophage inflammatory protein-1α
mLST8	mammalian lethal with SEC13 protein 8
MM	multiple myeloma
mSIN1	mammalian stress-activated protein kinase -interacting protein
MST-1	mammalian STE20-like kinase 1
mTOR	mammalian target of rapamycin
mTORC	mTOR complex
MVD	microvessel density
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NBS1	Nijmegen breakage syndrome1
NFκB	nuclear factor kappa B
NG2	neuron-glial antigen-2
NGF	nerve growth factor
NRTK	non-receptor tyrosine kinase
NSCLC	non-small cell lung cancer
OPG	Osteoprotegerin
p87PIKAP	PI3Kγ adaptor protein of 87 kDa
PABPC	polyA binding protein
PAK	p21-activated kinase
PB1	Phox and Bem1 domain
PC	plasma cells
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PDGFR	platelet-derived growth factor receptor
PDK1	phosphoinositide-dependent protein kinase-1
PH	pleckstrin homology
PI3K	phosphatidylinositol 4,5-bisphosphate 3-kinase
PIA	prostatic inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PIP	phosphatidylinositol-4-phosphate
PIP2	phosphatidylinositol-4, 5-bisphosphate

PIP3	PtdIns (3,4,5)P3
PITX2	paired-like homeodomain transcription factor 2
PKC-α	protein kinase C alpha
PPAR γ	peroxisome-proliferator-activated receptor-gamma
PRAS40	proline-rich AKT1 substrate 1 40
PROTOR-1	Protein Observed With Rictor-1
PSA	prostate-specific antigen
PSCA	prostate stem cell antigen
PtdIns	phosphatidylinositol
PUMA	p53 upregulated modulator of apoptosis
Pyk2	proline-rich tyrosine kinase 2
RANK	receptor activator of NFκB
RANKL	receptor activator of NFκB ligand
Raptor	regulatory-associated protein of mTOR
RB	Retinoblastoma
RET	REarranged during Transfection
Rictor	rapamycin insensitive companion of mTOR
RIP	receptor-interacting serine/threonine-protein kinase
Rock1	Rho-associated kinase 1
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RTKi	RTK inhibitor
SCF	stem cell factor
SCID	severe combined immunodeficiency
SCLC	small-cell lung cancer
SDF1α	stromal cell-derived factor α
SGK	serum- and glucocorticoid-regulated kinase
SH2	Src homology 2
SH3	Src homology 3
Shc	Src homology 2 domain containing transforming protein1
SMA	smooth-muscle actin
SMM	smoldering multiple myeloma

SNARE	soluble N-ethylmaleimide-sensitive fusion attachment protein receptor
sor	Sorafenib
STAT	signal transducer and activator of transcription
SYK	spleen tyrosine kinase
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
Tec	Tec protein kinase
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TnK	tyrosine kinase nonreceptor
TP53	tumor protein53
TRADD	TNFR1-associated death domain protein
TSC1	tuberous sclerosis 1
TSG	tumor suppressor genes
Tyk 2	tyrosine kinase 2
UBA	ubiquitin-associated
UGT1A9	UDP glucuronosyltransferase
ULK	Unc-51 like autophagy activating kinase
UVRAG	UV radiation resistance associated gene
VAMP7	vacuolar morphology protein7
VAMP8	vacuolar morphology protein8
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VMP1	vacuole membrane protein 1
VTI1B	vesicles transport through interaction with t-SNARE homolog 1B
WIPI	WD-40 repeat-containing protein that interacts with PtdIns
XIAP	X-linked inhibitor of apoptosis protein

Abstract

In 2008, 26% of all deaths were cancer-related, making this group of diseases the second leading cause of death in the EU countries. Derailment of tyrosine kinase signaling is one important pre-requisite towards tumorigenesis. Small molecular inhibitors of receptor tyrosine kinases (RTKs) are a new type of targeted therapy and they are increasingly used as a core component of personalized cancer therapy. The main aim of this thesis is to investigate the anti-cancer effects of the multi tyrosine kinase inhibitor (TKI) sorafenib in hematological and solid tumors.

In the first study, we found that sorafenib is particularly effective in inducing cell death in a panel of human myeloma cell lines. We investigated the mode of cell death induced by sorafenib and found that this TKI induces both caspase dependent and caspase independent cell death. Furthermore, sorafenib induces autophagy in some human myeloma cell lines, myeloma patient samples and mouse myeloma cells and co-treatment of myeloma cells with sorafenib and autophagy inhibitors potentiates the cytotoxic efficacy of sorafenib. Importantly, sorafenib induced cell death in freshly isolated $CD138^{+}$ multiple myeloma cells from newly diagnosed patients chemotherapy naïve as well as bortezomib resistant patient samples. We investigated the efficacy of sorafenib in the 5T33MM mouse myeloma model and found that this TKI lead to significantly increased survival, reduced tumor growth and decreased serum M component.

In the pertaining studies we investigated the efficacy of sorafenib against prostate cancer cell lines. In the second study we demonstrated that sorafenib caused a dose-dependent decrease in cell viability in two hormone refractory and one hormone responsive prostate cancer cell lines.

In the third study we further investigated the signaling cascades inhibited by sorafenib leading to cell death in prostate cancer cell lines (22Rv1 and PC3). Activation of caspases and downregulation of Mcl-1 are seen in both cell lines. However we found that distinct upstream signaling cascades are activated in these two prostate cancer cell lines which are differentially affected upon treatment with sorafenib. In 22Rv1, ERK1/2 is constitutively phosphorylated and active whereas in PC3 cells it is not active. In contrast, Src and AKT were constitutively active in PC3 cells but not in 22Rv1 and treatment with sorafenib could inhibit these kinases in PC3 cells. In both cell lines, sorafenib induces autophagy and inhibition of autophagy potentiates the cytotoxic efficacy of sorafenib. PC3 and 22Rv1 cells could further be rescued from sorafenib-induced cell death when co-cultured with cancer associated fibroblasts. This protection could be overcome by co-treatment with ABT737 (a Bcl-2/Bcl-xL inhibitor), suggesting that these anti-apoptotic proteins are, at least in part, responsible for the rescuing phenotype observed upon co-culture with cancer associated fibroblasts.

In a fourth study we found that even though DU145 cells do not express *ATG5* they undergo autophagy upon treatment with sorafenib or bafilomycin A1. Interestingly, we showed that sorafenib-induced autophagy in DU145 cells is cytotoxic and the cell death observed could be inhibited by the exogenous re-constitution of Atg5 expression. We found that treatment with molecular or chemical inhibitors of RIPK1 suppressed the observed cell death. Collectively our data suggest that in Atg5-deficient cells autophagy is cytotoxic and the ensuing cell death is executed by the necroptotic program.

In summary, these data identify some molecular mechanisms and requirements for the successful usage of sorafenib as a putative anti-cancer treatment against multiple myeloma and prostate cancer.

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1. Introduction

Cancer reminds us of chaos and anarchy, however I deeply believe that malignant cells are not simply impaired cells with aberrations in biological process within the body. They acquire the power to survive, proliferate and resist cell death intelligently. They exploit other cells to support them by and secreting growth factors. They can also evade the immune system effectively. In this chapter, I describe more about cancer, tyrosine kinase signaling in cancer and their inhibitors as cancer treatment.

1.1 Cancer

Cancer, with 26% of all deaths in 2008, is the second leading cause of death in the EU countries (1). Scientists estimate that our body consists of 10^{13} cells and any of them can potentially initiate a tumor. The total number of cells during a human lifetime is estimated to be about 10^{16} that indicates a turnover of 10 million cells per second (2). A neoplasm can appear if any of these cells escape from the strict mechanisms of cell proliferation, survival or death.

In a follow up from their landmark paper from 2000, Hanahan and Weinberg have complemented the six main hallmarks of cancer plus two emerging hallmarks and two enabling characteristics (3).

1. Sustaining proliferative signaling

One of the most important characteristics of a cancer cell is to maintain chronic proliferation without dependency on external growth factors (3). Normal tissues have tightly regulated growth that control cell growth and entry to division cycles. The majority of these growth factors bind to cell-surface receptors which typically contain intracellular tyrosine kinase domains. These domains transduce signals via signaling pathways that control cell cycle, growth, survival and energy metabolism. Cancer cells deregulate these signaling cascades by different strategies, for example prostate cancer cells can acquire an autocrine loop of vascular endothelial growth factor (VEGF)-VEGF receptor 2 (VEGFR2) to escape growth control (4). Similar mechanisms are also shown in multiple myeloma (MM) (5, 6). Another mechanism is activating mutations of growth factor receptors that result in continuous transduction of mitogenic signals to the cells. Cai *et al.* showed four new mutations in the epidermal growth factor receptor (EGFR) gene that increases cell growth and invasion of prostate cancer cell lines via constitutive and hyperactive tyrosine phosphorylation and cause activation of the mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 3 (STAT3) and AKT pathways (7).

2. Evading growth suppressors

In addition to the ability of self-sufficiency in growth signals, cancer cells must overcome the signals that negatively regulate cell proliferation (3). Usually products of tumor suppressor genes (TSGs) are responsible for these negative feedbacks. Two of the most known examples of TSGs are the tumor protein 53 (TP53) and the retinoblastoma-associated (RB) proteins. RB

integrates extracellular and intracellular signals and determines whether or not a cell continues through the cell proliferation cycle. Impairment of the RB pathway, by mutations or deletion, leads to the loss of this critical gatekeeper of cell cycle progression, thus allowing cancer cells to divide continuously. The TP53 protein senses intracellular stress signals which come from for example excessive damage to the genome or abnormal concentrations of nucleotides, growth promoting signals, low glucose or oxygen. The TP53 protein temporarily pauses progression of the cell cycle until these stress conditions have been normalized, and if this cannot be achieved it activates apoptotic cell death (3).

3. Resisting cell death

A cancer cell does not only has to modify normal cellular growth pathways to grow and proliferate widely, but also to evade cellular death pathways (3). This acquired resistance to apoptosis has been shown in many types of cancers (8-11). This phenomenon is also reported to be involved in resistance to anti-cancer therapy. There are known mechanisms for evasion of cell death including: 1) disruption of the balance of pro-apoptotic and anti-apoptotic proteins 2) reduced caspase function and 3) impaired death receptor signaling (12).

4. Enabling replicative immortality

In 1961 Leonard Hayflick showed that most normal human cells can divide between 60 to 70 times (13, 14). This is because of shortening of telomeres at the ends of chromosomes. This promotes cellular senescence, a non-proliferative but viable state, or mitotic catastrophe, which leads to apoptosis after a certain number of cell divisions. Telomerase adds repeated segments of telomere to the ends of chromosomes. It is almost absent in non-immortalized cells but expressed in 90% of immortalized cells (2, 3).

5. Development of sustained angiogenesis

Tumors, like normal tissues, require sustained delivery of nutrients and oxygen, as well as removal of CO_2 and metabolic byproducts. The maximal distance that oxygen, nutrients and waste can diffuse from or to a blood vessel is 1 to 2 millimeters and tumors cannot grow larger unless they induce new blood vessels, a process known as angiogenesis. Angiogenesis is induced at an early stage in tumor development (15). The “angiogenic switch” is controlled by inducers, like VEGF and hypoxia-inducible factors1 alpha (HIF-1α), and inhibitors like thrombospondin-1. Both hypoxia and oncogenic signaling can activate the pro-angiogenic switch through increased expression of VEGF or decreased degradation of HIF-1α (16). Malignant tumor cells also need the angiogenesis as a route for distant metastasis (13).

6. Activating invasion and metastasis

Metastasis is a complex process driven by the “invasion-metastasis cascade” and includes invasion through basement membranes, intravasation into blood and lymph nodes, extravasation and colonization of micrometastasis (17).

7. Reprogramming of energy metabolisms

This emerging hallmark was first mentioned by the Nobel Prize winner Otto Warburg in 1931. He postulated that even in the presence of sufficient oxygen, most cancer cells predominantly produce energy by glycolysis rather than oxidation of pyruvate in the mitochondria (18).

8. Evasion of immune system

The second emerging hallmark is evasion from immune destruction. Tumor cells manage to avoid detection and destruction by immune system (3).

9. Genomic instability

Individuals who have inherited disorders in deoxyribonucleic acid (DNA) repair system that leads to accumulation of genetic alterations and consequently genomic instability, are more prone to develop cancer compared to healthy ones. Despite the diversity in genome alternation in different tumor type, a wide range of defects in proteins which are responsible for DNA maintenance and repair are known. Then genomic instability is an enabling characteristic of cancer cells (3).

10. Tumor promoting inflammation

For a long time it has been known that white blood cells infiltrate into tumors and it is the first clue of linkage between inflammation and cancer. It is known that the risk of developing cancer also increases in some chronic inflammatory diseases like Barrett esophagus, hepatitis B, chronic pancreatitis and ulcerative colitis. Inflammation plays a critical role in cancer development (13).

1.2 Tyrosine kinase signaling in cancer

Protein kinases catalyze the transfer of a terminal phosphate group from a nucleoside triphosphate (generally adenosine triphosphate (ATP)) to the hydroxyl group on a serine, threonine or tyrosine of a protein (19). Because of the release of a large amount of energy, this reaction is unidirectional. Eukaryotic kinases are named based on the amino acid modification, i.e. serine/threonine kinases or tyrosine kinases. This post-translational modification is reversed by another class of enzymes called protein phosphatases. About 2% of the human genome encodes for 500 protein kinases and it signifies the importance of protein kinases in human biology (20). Protein kinase can modify up to 30% of all human proteins and they are involved in the regulation of cellular pathways, especially those involved in signal transduction and regulating cellular activity (21). Tyrosine kinases are classified into two subgroup: Non-receptor tyrosine kinase (NRTK) and receptor tyrosine kinase (RTK).

1.2.1 Non-receptor tyrosine kinase

So far around 32 non-receptor tyrosine kinases have been identified in human cells (22). Non-receptor tyrosine kinases are involved in a variety of signaling processes, including T- and B-cell activation, mitogenesis, cytoskeleton restructuring, differentiation, adhesion, migration and cell death (23, 24).

The majority of the NRTKs are cytoplasmic enzymes but some of them are found attached to the cell membrane by amino acid modifications like myristoylation or palmitoylation and some of them, like Abelson murine leukemia viral oncogene homolog (ABL) and Feline sarcoma-related (FER), are found in the nucleus (25, 26). They have a modular structure which comprises of several domains which bind to each other. Apart from the tyrosine kinase domain they contain protein-protein, protein-lipid, and protein-DNA interaction domains. Src homology 2 (SH2) and Src homology 3 (SH3) domains are the most common protein-protein interaction domains in this family which enable them to interact with both upstream and downstream signaling molecules (27). A hundred amino acid constitute the SH2 domain that binds to phosphotyrosine and the SH3 domain is composed by 60 amino acid and makes a polyproline type II helix. Pleckstrin homology (PH) domain binds to phosphatidylinositol (PtdIns) lipids, which are found in the plasma membrane and can lead to the activation of the Phosphatidylinositol 4,5-bisphosphate 3-kinase (PI3K) pathway. Janus kinase (JAK) homology domains target JAK family protein to the cytoplasmic part of cytokine receptors. Integrin-binding- and a focal adhesion-binding domains are two other examples of protein-protein interaction domains. Other examples are the DNA-binding domain and F actin–binding domain, two domains which are found in for instance in ABL (23).

It is possible to classify NRTKs based on structural similarities into several families: ABL, C-terminal Src kinase (CSK), focal adhesion kinase (FAK), feline sarcoma oncogene/fujinami avian sarcoma viral oncogene homolog (FES), Janus kinase (JAK), Src, spleen tyrosine kinase (SYK), Tec protein kinase (Tec), tyrosine kinase nonreceptor (TnK) (28).

- **Src Family:** Src is the prototype of the Src protein tyrosine kinase family members, and it contains several proteins like B-cell lymphocyte kinase (Blk), hemopoietic cell kinase (Hck) and Src (28). Src has dual functions, as a scaffold molecule to assemble signaling complexes and as a tyrosine kinase. Src family members anchor to the cell membrane through the N-terminal region whereas the SH3, SH2 and kinase regions in C-terminal which contains two critical tyrosine residues (Tyr-416 and Tyr-527) that regulate the Src activity (29). The phosphorylated Tyr-527 at the C-terminal interacts with the SH2 domain which folds Src to a closed bundle. Tyrosine phosphatases dephosphorylate Tyr-527 and opens up inactive Src. Various tyrosine kinases phosphorylate Src on the Tyr-416 which is located within the kinase domain, resulting in an increase in enzyme activity. Some proteins like PDFG and FAK can bind to the SH2 domain and activate Src (30). This can be achieved by either direct contact between Src and its potential substrate or relocalization of Src inside the cell to have close proximity to potential substrate (31). Active Src has been shown to promote survival, proliferation, angiogenesis and invasion pathways (32). Src can also activate downstream tyrosine kinases such as ABL (33) or promote formation of the osteoclast podosome with proline-rich tyrosine kinase 2 (Pyk2) (34).

- **CSK:** CSK can phosphorylate Tyr-527 in the Src family and inactivate them (35, 36).
- **ABL:** ABL is an oncogene which is required for the development of leukemia's and its expression is induced by chromosome translocations or retroviruses (37). ABL1 and ABL2 are two members of ABL family that transduces extracellular signals which control proliferation, survival, migration, cellular polarity and invasion. ABL kinases are activated in breast-, lung-, colorectal-, gastric-, prostate cancer and melanoma cells.
- **FAK:** FAK was identified as a substrate for viral Src in 1992 and it controls cell motility (38). FAK does not contain SH2 or SH3 protein interaction domains. FAK is activated by growth factors and integrins during migration and recruits other focal contact proteins or their regulators to facilitate cellular movement. FAK transduces signal from growth factor- and integrin receptors to extracellular the signal-regulated kinase 2 (ERK2)/MAPK cascade and Rho family GTPases (38, 39).
- **FES:** The structure of FES consists of an N-terminal FCH (FES/FER/ Cdc42-interacting protein 4 (CIP4) Homology) domain, three coiled-coils regions, an SH2 domain and a C-terminal kinase domain. FES play a role in cell-cell and cell-matrix interactions (40).
- **JAK:** JAKs are involved in the regulation of several cellular functions including cell proliferation, differentiation, cell migration and apoptosis (41). In mammals, the JAK family comprises four members: JAK1, JAK2, JAK 3 and Tyrosine kinase 2 (Tyk2). Erythropoietin, growth hormone, interferons and interleukins bind to their receptors, where erythropoietin and growth hormone receptos homodimerize and interferon and interleukin receptos heterdimerize and as the result two JAKs which are bound to the cytoplasmic domain of these receptors phosphorylate each other and subsequently phosphorylate the receptors. These phosphorylated parts act as docking sites for the SH2 domains for STATs. Then STATs are phosphorylated by the JAKs and make homo- or heterodimers and translocate to the nucleus. The STAT dimers bind to the STAT responsive element in the promoter through their DNA-binding domains and repress or activate or transcription of target genes. The JAK/STAT signaling cascade thus facilitates the transduction of an extracellular signal to a transcriptional response (41-43).
- **SYK:** SYK plays a role in adaptive immune receptor signaling, cellular adhesion, innate immune recognition, osteoclast maturation, platelet activation and vascular development (44).
- **Tec:** Members of the Tec kinase family are the second largest class of NRTK. They participate in lymphocyte development and activation(45).
- **Tnk:** Tnk are involved in cell survival, tumor development and migration (46).

1.2.2 Receptor tyrosine kinase

In 1952 Rita Levi-Montalcini discovered the nerve growth factor (NGF) (47). She showed that NGF extracted from mouse tumors, can promote neurite outgrowth in chicken embryos. Stanley Cohen isolated and characterized epidermal growth factor (EGF). They won the Nobel Prize in Physiology or Medicine in 1986 for their work. Cohen and his colleagues also discovered the EGFR in 1978 (47). Now 58 RTKs are known in human which are categorized into 20 families (**Figure 1**) (48, 49). All RTKs have a similar molecular structure. An extracellular region which binds ligand, a single transmembrane helix and an intracellular

region which contains juxtamembrane regulatory region and tyrosine kinase domain (49). When growth factors bind to the RTKs, usually two RTK monomers dimerize. Some RTKs like Insulin-like growth factor 1 (IGF1) exist as oligomers in absence of its ligand. Binding of insulin induces conformational changes in the structure of the receptor dimer, which activates its kinase activity by autophosphorylation (50). After dimerization, the activated receptor phosphorylates tyrosines in adjacent RTK and the phosphorylated receptor activates or assembles intracellular signaling proteins (49). From here on, I will focus on the RTKs that are known to play a role in multiple myeloma or prostate cancer development and can be targeted by sorafenib.

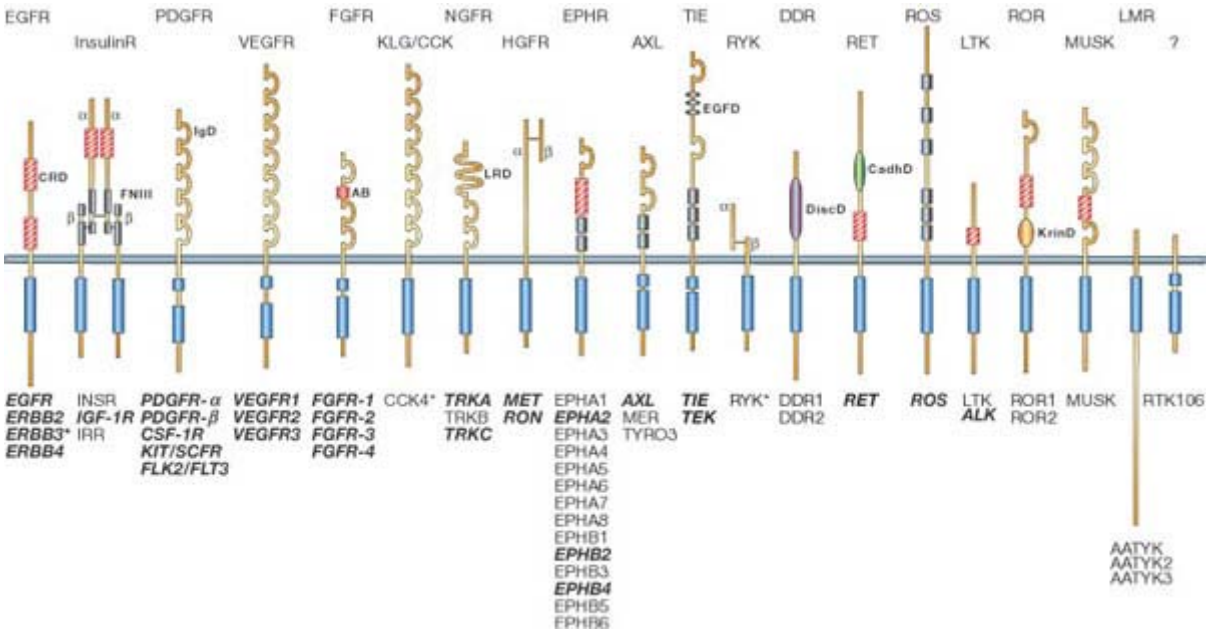

Figure 1. Human receptor protein-tyrosine kinases. The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. Reproduced with permission from Oncogenic kinase signaling, Peter Blume-Jensen and Tony Hunter, Nature 411, 355-365.

- **VEGFR:** VEGFR comprises three receptors, VEGFR-1 (fms-related tyrosine kinase 1(Flt-1)), VEGFR-2 (kinase insert domain-containing receptor (KDR) / Fetal Liver Kinase 1 (Flk-1)) and VEGFR-3 (Flt-4) (51). VEGF is the ligand for VEGFR. The VEGF-A gene is located on the short arm of chromosome 6 and consists of eight exons and seven introns and alternative exon splicing generates four different VEGF isoforms (52). VEGF belongs to the platelet-derived growth factor (PDGF) superfamily and it is a heparin-binding homodimeric glycoprotein (53). VEGF can promote endothelial cell survival and vascular permeability through activation of PI3K/AKT, endothelial cell migration through activation of p38 MAPK and endothelial cell proliferation through activation of the Ras/Raf/MEK/ERK signaling pathway (51).
- **FGFR:** This family of tyrosine kinase includes the 4 receptor fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3, and FGFR4) with 18 known ligands that can bind to them and transfer extracellular signaling into the cytoplasm through tyrosine kinase signaling (54). Several examples are known relating to cancer development due to dysregulation of FGFR signaling such as activating mutations found in 50% of bladder

cancer cases (55), prostate cancer and multiple myeloma (54), FGFR gene amplifications, a rare event associated with gastric-, lung- and breast cancer (56), chromosomal translocation, is frequently found in multiple myeloma (57) and increased autocrine and paracrine signaling, observed in melanoma and prostate cancer (54, 58). Deregulated FGF signaling can increase the activity of downstream signaling cascades which lead to tumorigenesis. For example in T cell lymphoma, the ets variant 6 (ETV6)-FGFR3 fusion protein activates PI3K signaling (59). Fibroblast growth factor (FGF) can also promote resistance to apoptosis by inducing the expression of anti-apoptotic proteins through PI3K/AKT and STAT signaling. It has been shown that FGF can induce the expression of B-cell lymphoma 2 (BCL-2), B-cell lymphoma extra-large (Bcl-xL), X-linked inhibitor of apoptosis protein (XIAP) and inhibitor of apoptosis family of proteins 1 (IAP1). FGF can induce proliferation and decrease apoptosis by MAPK signaling (54). In bladder cancer and multiple myeloma, the FGFR has been shown to activate the nuclear factor kappa B (NFκB) signaling pathway responsible for proliferation, survival and anti-apoptotic effects (60). It is also shown that FGFR activation can lead to increased invasion and angiogenesis (61).

- **PDFGR:** This family consists of PDGF receptor α and β, stem cell factor receptor (c-Kit), colony-stimulating factor- 1 (CSF-1) receptor, and Flt-3 (62). They are composed by five Ig-like domains in their extracellular part, a transmembrane domain, an intracellular juxtamembrane domain, a tyrosine kinase domain and a C-terminal tail. Their ligands are dimeric and induce receptor dimerization upon binding. The PDGFR family has two members, PDGFRα and PDGFRβ. PDGF is the ligand for PDGFR and it exists in both disulphide-bonded homodimer forms (PDGF-AA, -BB, -CC, -DD) and heterodimer form PDGF-AB. The Monomeric form of PDGF is inactive. Upon binding of the PDGF dimer to PDGFR, two PDGFRs dimerize and make several possible combinations; PDGFR-αα, -ββ and –αβ. All these receptors can be activated by PDGF-BB (63-65). Oncogenic activation of PDGFR includes: 1.chromosomal rearrangement: Increased expression of PDGFR after fusion with the gene encoding collagen 1A1 in dermatofibrosarcoma protuberance (DFSP) and constitutive activation of PDGFRα after rearrangement with FIP1L1 in chronic myelomonocytic leukemia, haves been reported leukemia (63, 66); 2. Point mutation: It has been demonstrated that point mutations in exon 9 or 11 of PDGFRα gene cause constitutive kinase activation of the PDGFRα by disrupting regulatory portions of PDGFRα which are fundamental in gastrointestinal stromal tumors (GIST) development (67). The PDGFRs can transduce the extracellular signal through downstream signaling like PI3K, STAT and Ras/Raf/MEK/ERK signaling pathways (64).
- **c-Kit receptor:** Following dimerization of the c-Kit receptor (CD117) by binding of dimeric ligands, PI3K/AKT, Src kinases, Ras/Raf/ERK pathway and phospholipase C and D can be activated and regulate cellular proliferation, differentiation and migration, tumor development and recurrence (62). Alterations in the c-Kit receptor is involved in pathogenesis or progression of acute myeloid leukemia, GIST, mastocytosis melanoma and small-cell lung cancer (SCLC) (62, 68).
- **Flt3:** Flt3 is expressed only in cells with high level of the CD34 antigen like early progenitors of hematopoiesis, B-lymphoid progenitor cells and monocytes (69). A mutation of the Flt3 gene which leads to the expression of a constitutively active

receptor was found in acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). PI3K/AKT and Ras/Raf/ERK signaling pathways are activated by Flt3 and this promotes cell mobilization, proliferation and survival (69, 70).

- **IGFR:** The IGF system consist of two tyrosine kinase receptors, the insulin receptor (IR) and the IGF-1 receptor (IGF-1R), and insulin receptor-related protein (InsRR) which are activated upon binding of four different ligands (insulin, proinsulin, IGF-I and IGF-II) and six regulatory binding protein (IGFBP1 - IGF-BP6) (71). The IGFBPs are transport proteins for IGF-I and IGF-II in the circulation and also regulate their access to the IGF-1R. Free IGF has a very short half-life while the bound fraction has a longer half life (72). There are many common effects of insulin and IGF-1 like stimulation of cell proliferation, glucose uptake and protein synthesis, decreased fat breakdown, induction of DNA and RNA synthesis and inhibition of apoptosis (73). The two main signaling pathways downstream of the IGFRs are the PI3K/AKT and Ras/Raf/MEK/ERK pathways which are coupled to IGFR through the insulin receptor substrate (IRS) and Src homology 2 domain containing transforming protein 1 (Shc) protein, respectively (71, 74). It has been shown that the IGF system is involved in the pathogenesis, progression and metastasis of breast-, prostate- and colon cancer (75).
- **Ret:** The REarranged during Transfection (RET) proto-oncogene encodes for the RET protein which is associated with papillary thyroid carcinoma (76). The RET receptor has four extracellular cadherin-like domains, the cysteine-rich region and tyrosine kinase domains. It has isoforms, RET9 and RET51 which is an anchoring site for docking proteins. Consequently these proteins can activate multiple signaling pathways, including the PI3K/AKT, Ras/Raf/ERK and Rac/c-jun NH kinase (JNK). Through these signaling pathways, the RET receptor plays a role in cellular motility, proliferation, differentiation and survival (77).

1.2.3 Downstream signaling cascades

1.2.3.1 The PI3K/AKT signaling pathway:

The class I phosphatidylinositol 3-kinases (PI3Ks) is the most extensively investigated family of the three different classes of PI3Ks and it can be divided into Class IA PI3Ks which are activated by receptor tyrosine kinases and class IB PI3Ks which are activated by G-protein-coupled receptors (GPCRs) (78, 79). Class IA PI3Ks are composed of three isoforms of the p110 catalytic subunit (p110α, β and δ) and three isoforms of the p85 (p85 α, β and δ) regulatory subunit (**Figure 2**) (78). Phosphatidylinositol, phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4, 5-bisphosphate (PIP2) are substrates of Class IA PI3Ks. Class IB PI3Ks consist of the p110γ catalytic subunit and a p101 regulatory subunit or its homologues p84 or PI3Kγ adaptor protein of 87 kDa (p87PIKAP) (80).

Binding of ligand to RTKs leads to the phosphorylation of the C-terminal tail of RTK allowing for the recruitment of the inactive p85-p110 complex to the receptor. The catalytic subunit of p110 is thus brought in close proximity to its lipid substrates in the plasma membrane. The inhibition of p85 on p110 kinase can be relieved by the RTK-p85 interaction which induces conformational changes in p85-p110 complex. Ras is another important signaling molecule, activated downstream of RTKs and which promotes the activation of the p110 subunit.

Phosphatidylinositol 3-kinase (PI3K) phosphorylates PtdIns (4,5)P2 (PIP2) to form the second messenger PtdIns (3,4,5)P3 (PIP3). PIP3 can be dephosphorylated by PTEN. Generation of PIP3s in the plasma membrane leads to the recruitment of AKT and its upstream activator 3-phosphoinositide-dependent protein kinase-1 (PDK1) which phosphorylates AKT on Thr308 and activates it. PDK2 can phosphorylate AKT at Ser473 (78). In general, AKT activation has three main downstream effects:

- **Cell survival:** Activated AKT inhibits apoptosis by several mechanisms (78, 81); 1) first AKT phosphorylates Bcl-2-associated death promoter (BAD) protein, a pro-apoptotic member of BCL2 family. BAD can make a heterodimer with the anti-apoptotic survival factor Bcl-xL but phosphorylated BAD cannot interact with Bcl-xL. 2) AKT also phosphorylates and inactivates caspase-9; 3) AKT can phosphorylate and activate IκB kinase (IKK) followed by degradation of this NFκB inhibitor, which leads to the activation of the NFκB survival pathway; 4) AKT can phosphorylate mouse double minute 2 homolog (MDM2) which leads to enhanced degradation of the pro-apoptotic tumor suppressor 53.
- **Cell proliferation:** AKT promotes cell proliferation by affecting cell-cycle machinery (82). p21 is an protein which can make complex with proliferating cell nuclear antigen (PCNA) and inhibit DNA replication. p21 is also binds to Cdk2 and inhibit cell cycle progression. It mainly inhibits the activity of cdk2 complexes and negatively modulates cell cycle progression. AKT phosphorylates the p21 at Thr145 and it has two effects, first it prevents the complex formation of p21 with PCNA and second decrease of binding of p21 to Cdk2. These effects promote cell cycle progression (83).
- **Cell growth:** The mammalian target of rapamycin (mTOR), a serine/threonine kinase, is a central regulator of cell growth and its activity regulated by AKT (78). mTOR is part of two complexes, mTOR complex 1(mTORC1) and mTORC2 which can be stimulated by interleukin 6 (IL-6), insulin and other growth factors. mTORC1 consist of mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with SEC13 protein 8 (mLST8), proline-rich AKT1 substrate 1 40 (PRAS40) and DEP domain-containing mTOR-interacting protein (DEPTOR). Tuberous sclerosis 1 (TSC1) and TSC2 are negative regulators of the mTOR activity. AKT can phosphorylate TSC2 followed by dissociation of the TSC1/2 complex from mTORC1, leading to its activation. AKT can also phosphorylate and inactivate PRAS40 which is a negative regulator of mTORC1. In addition, activated AKT increases the expression of amino acid and nutrient transporters like Glucose transporter 1 (Glut1) in the cell membrane leading to the activation of mTORC1. On the other hand mTOR can also activate AKT via mTORC2. The mTORC2 components include mTOR, rapamycin insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase -interacting protein (mSIN1), PROTOR-1, DEPTOR and mLST8 which regulate Protein kinase C alpha (PKC-α), Serum- and glucocorticoid-regulated kinase (SGK) and AKT. mTOR has been involved in sensing ATP level (78, 84-87).

  Under basal physiological conditions, inactive p70 S6 kinase makes a complex with eukaryotic initiation factor 3 (eIF3) (88). After growth factor stimulation, mTORC1 binds to the eIF3-S6K complex and detaches S6K from the mTORC1-eIF3 complex. mTORC1-eIF3 complex associates with the 5'mRNA cap structure. Interaction between the scaffold protein eIF-4G with eIF-4F, eIF-4A and polyA binding protein (PABPC) makes up the cap-structure on mRNAs and initiates protein synthesis. The hypophosphorylated form of

4E-BP1 is attached to eIF-4E and inhibits the interactions of eIF-4G with eIF-4E. The phosphorylation of 4E-BP1 occurs sequentially at the Thr37 and Thr47, Ser65 and Thr70 leading to gradually enhanced activation of translation.

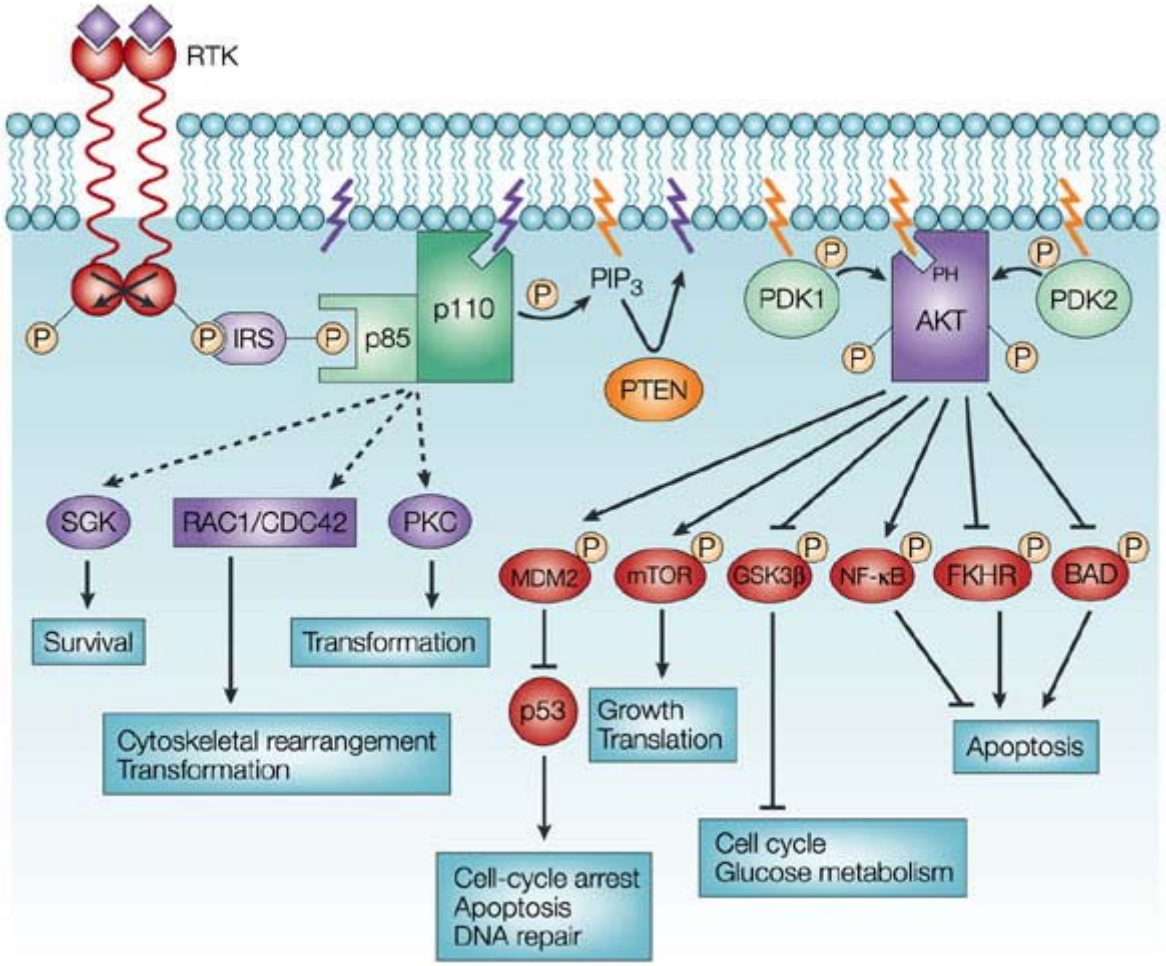

Figure 2. PI3K/AKT signaling pathway. Reproduced with permission from The phosphatidylinositol 3-Kinase–AKT pathway in human cancer, Igor Vivanco & Charles L. Sawyers, Nature Reviews Cancer 2, 489-501

1.2.3.2 The MAPK signaling cascade:

This signaling pathway consist of four family members p38, c-jun-N-terminal kinase (JNK), Extracellular signal-regulated kinase 1/2 (ERK 1/2) and ERK5 (89).

Ras/Raf/MEK/ERK:

Activation of some RTKs like PDGFR, VEGFR and FGFR causes binding of growth-factor-receptor-bound protein 2 (GRB2) to the cytoplasmic tails of the receptor (**Figure 3**) (89). Then the guanine-nucleotide exchange factor SOS docks to GRB2 and this complex replaces GDP with GTP- (in Ras) and activates Ras. Ras induces the kinase activity of Raf which phosphorylates and activates the mitogen-activated protein kinase kinase (MEK) by phosphorylation on two serine residues, Ser217 and Ser221. MEK has two isoform MEK1 and

MEK2. MAPK/extracellular signal-regulated kinase 1 (ERK1) is phosphorylated on Thr202 and Tyr204 and ERK2 is phosphorylated on Thr185 and Tyr187 by active MEK1/2. Active ERK1/2 has several cellular downstream effects like activation of several downstream kinases and activation of transcription factors including the peroxisome-proliferator-activated receptor-gamma (PPAR γ), ETS domain-containing protein (ELK1), ETS, signal transducer and activator of transcription 1 (STAT1) and STAT3. The overall effect of Ras/Raf/MEK/ERK activation is cell cycle progression and increase in cell motility.

AKT and SGK are furthermore negative- and PKC, SRC, p21-activated kinase (PAK), 14-3-3, adhesion of integrins to extracellular-matrix molecules positive inducers of this signaling pathway (89-91).

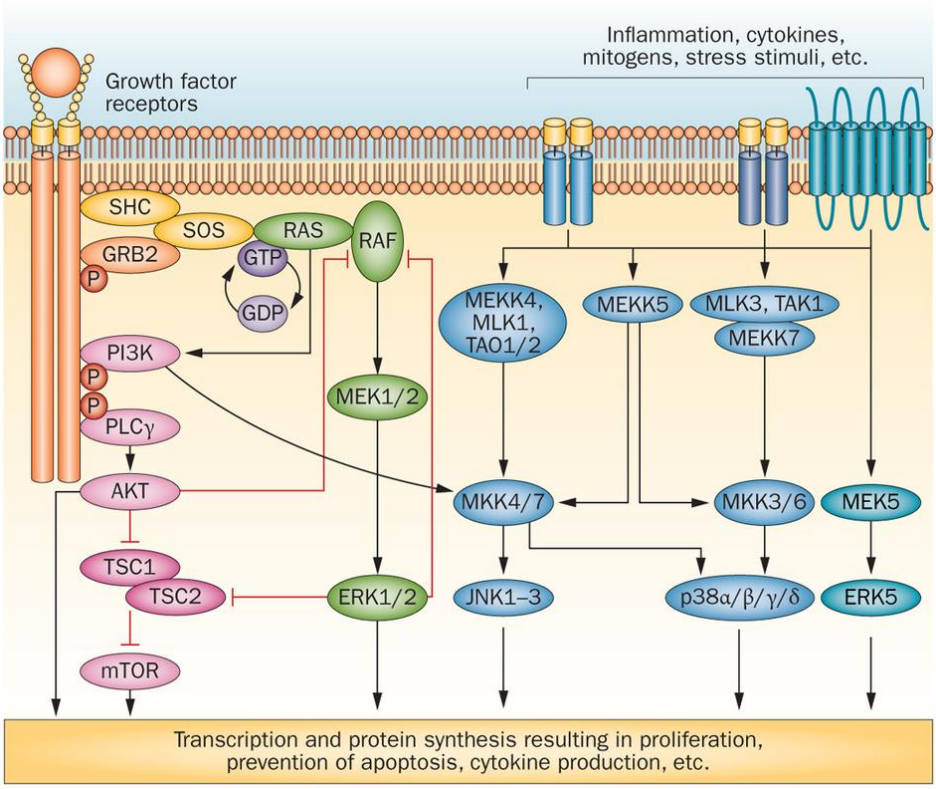

Figure 3. Ras/Raf/MEK/ERK pathway. Reproduced with permission from The clinical development of MEK inhibitors, Yujie Zhao& Alex A. Adjei, Nature Reviews Clinical Oncology, 11, 385–400 (2014).

1.3 Tyrosine kinase inhibitors in cancer treatment

1.3.1 Overview

Tyrosine kinase activity deregulation is widely observed in various cancer types thus making these kinases a suitable target for anticancer therapy.

There are three approaches to target tyrosine kinases:

1. Prevent the interaction between ligand and its receptor(s) by targeting the growth factors before binding to their receptor(s). One example is bevacizumab, a Food and Drug

Administration (FDA) approved humanized neutralizing immunoglobulin G1 against VEGF that inhibits the binding of VEGF to VEGFRs (92).

2. Prevent the interaction between ligand and its receptor by targeting the extracellular domain of RTKs. Trastuzumab is an example of a monoclonal antibody which targets the extracellular domain of the human EGFR2 (human epidermal growth factor receptor 2 (HER2 or ErbB-2)) protein (93).

3. Target the intracellular domain of RTKs. The majority RTK inhibitors (RTKis) belong to this approach and they are classified into four categories: Type I inhibitors, recognize the active conformation of the kinase and bind in and around the region occupied by the adenine ring of ATP (94, 95); Type II inhibitors occupy a hydrophobic site that is directly adjacent to the ATP binding pocket leading to an alteration in the conformation of the activation loop known as DFG-out. The type II RTKi recognize the inactive conformation of the kinases (94, 95); Type III inhibitors are capable of inhibiting RTKs in an allosteric manner, binding at an allosteric site which is located outside the ATP-binding site. This group has the highest degree of kinase selectivity. CI-1040, the inhibitor of MEK1 and MEK2, belongs to this group (95); Type IV inhibitors are covalent inhibitors. Usually they make an irreversible covalent bond with a cysteine residue in the kinase active site. HKI-272, an EGFR inhibitor, is one example of such a covalent inhibitor (96).

1.3.2 Sorafenib

Bayer and Onyx initiated a collaboration to discover new agents to target the Ras/Raf/MEK/ERK pathway. High-throughput screening for Raf1 kinase inhibitory activity led to identification of sorafenib (BAY 43-9006, Nexavar®) (**Figure 4**) (97). Sorafenib is available as tablets made by Bayer under trade name of Nexavar®. Each tablet contains sorafenib tosylate (274 mg) equivalent to 200 mg of sorafenib (98).

The recommended dose of sorafenib is 400 mg orally twice daily. Treatment should continue until there are no clinical benefits or toxicity occurs. In the case of adverse reactions, the dosage can be reduced to 400 mg per day or 400 mg per every other day (99).

The empirical formula of sorafenib tosylate is C21H16ClF3N4O3 x C7H8O3S.

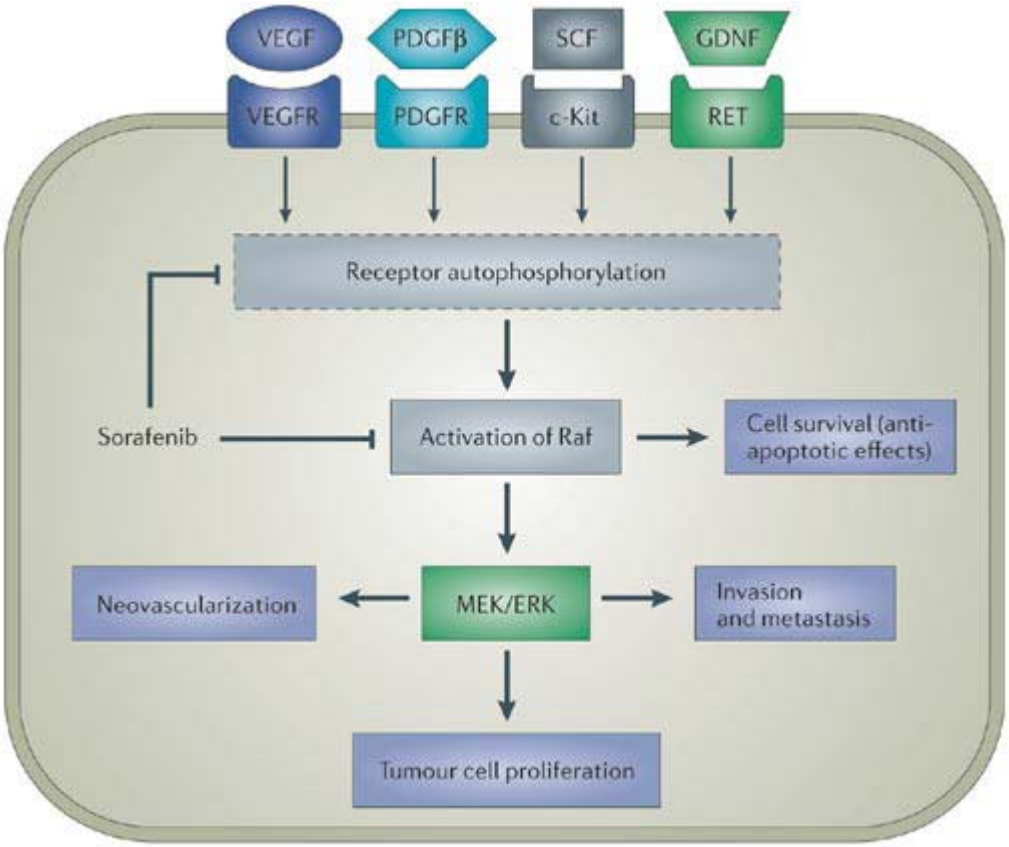

Figure 4. Cellular targets of sorafenib. Reproduced with permission from Discovery and development of sorafenib: a multikinase inhibitor for treating cancer. Scott Wilhelm, Christopher Carter, Mark Lynch, Timothy Lowinger, Jacques Dumas, Roger A. Smith, Brian Schwartz, Ronit Simantov & Susan Kelley, Nature Reviews Drug Discovery 5, 835-844 (October 2006).

1.3.2.1 Pharmacological actions:

Sorafenib is a type II inhibitor of Raf-1 (C-Raf), B-Raf, V600E B-Raf, VEGFRs, PDGFRβ, FGFR1, c-Kit, Flt-3 and RET (97). Sorafenib cannot inhibit MEK1, ERK1, protein kinase B, protein kinase A, protein kinase Cα, protein kinase-Cγ, EGFR, HER2/neu and IGFR1 (97, 100, 101).

Apart from targeting tyrosine and serine/threonine kinases, sorafenib has also been shown to modulate the protein stability of complex I components of the mitochondrial electron transport chain leading to loss of mitochondrial membrane potential and caspase- independent cell death (102).

Sorafenib can also induce apoptotic cell death via a mitochondria-dependent oxidative stress mechanism. Sorafenib induces rapid production of reactive oxygen species (ROS) which leads to the depletion of intracellular glutathione. This effect is independent of the kinase inhibition capacity of sorafenib (103).

1.2.3.2 Indications

Currently sorafenib is FDA approved for three indications (104). On December 20, 2005, the FDA approved sorafenib tosylate for the treatment of patients with advanced renal cell carcinoma (RCC) and on November 16, 2007 for the treatment of patients with unresectable hepatocellular carcinoma (HCC) and finally for the treatment of locally recurrent or metastatic, progressive differentiated thyroid carcinoma (DTC) refractory to radioactive iodine treatment on November 22, 2013.

1.2.3.3 Pharmacokinetics

Sorafenib reaches the peak of plasma levels in three hours following oral administration with a mean elimination half-life of approximately 25-48 hours (98). The mean bioavailability of oral sorafenib is 38-49%. Bioavailability of sorafenib is reduced by 29% when consumed with a high-fat meal. Steady state for plasma concentrations of sorafenib are reached within 7 days. Almost 99.5% of sorafenib binds to human plasma protein (98, 105). Sorafenib is metabolized by two metabolic pathways in the liver and kidney. Cytochrome P450 3A4 (CYP3A4) (M2) oxidizes the pyridine N-oxide ring of sorafenib and UDP glucuronosyltransferase 1(UGT1A9) (M7) conjugates sorafenib with glucuronic acid. Seventy seven percent of the administrated sorafenib dose is excreted in feces (50% as unchanged drug) and 19% in urine as glucuronide conjugates (106).

1.2.3.4 Toxicity

The most notable acute adverse events related to sorafenib include asthenia (weakness), rashes and hand-foot skin reaction (HFSR), diarrhea and arterial hypertension (107). The other frequent adverse reactions to sorafenib are infection, lymphopenia, anorexia, hypophosphatemia, hemorrhage, nausea, vomiting, constipation, dry skin, alopecia, arthralgia, pain, fever, weight loss, increased amylase and increased lipase (107).

The mechanisms underlying the adverse effects of sorafenib are not clear but it has been postulated that VEGFR inhibition is responsible for hypertension and hypothyroidism, and skin toxicity is caused because of secretion of sorafenib by the eccrine glands of the skin (96).

1.2.3.5 Sorafenib in cancer treatment

Based on registered data (https://clinicaltrials.gov) on 2014-08-04, there are 638 clinical trials with sorafenib, out of which 10 trials are in phase IV, 68 in phase III, 358 in phase II, 199 in phase I, 1 in phase 0 and 2 of them not mentioned. All phase IV clinical trials are in patients with hepatocellular- or renal cell carcinoma. Sorafenib as an anticancer treatment is in phase 3 trials in cancers like hepatocellular carcinoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), acute myeloid leukemia, desmoid tumors or aggressive fibromatosis, melanoma, thyroid, breast and pancreatic cancer.

1.2.3.5.1 Sorafenib in multiple myeloma treatment

In a panel of human myeloma cell lines we have found responsiveness to sorafenib which we further showed similar efficiency in an in vivo mouse model as well as freshly isolated $CD138^+$ multiple myeloma cells from newly diagnosed patients (108). Ramakrishnan and colleagues have also investigated the effect of sorafenib in multiple myeloma and they reported that sorafenib is toxic for both cell lines and patient samples (109). Udi and colleagues have also reported that sorafenib induces apoptosis in myeloma cell lines (110). It reduces CD138-expression and induces actin depolymerization in these cell lines, which can disrupt the microenvironment support of tumor cells. Another recent study depicts induction of caspase-dependent apoptosis in myeloma cell lines and patient samples treated with sorafenib. They have also shown in the MM.1S cell line, that co-treatment of sorafenib with the pan caspase inhibitor Z-VAD-fmk induces necroptosis (111).

Seven clinical trials using sorafenib in myeloma are ongoing as follows:

1. "A phase I/II study of the Raf kinase/VEGFR inhibitor sorafenib in combination with the mTOR inhibitor RAD001 (Everolimus) in patients with relapsed non-Hodgkin lymphoma, Hodgkin lymphoma, or multiple myeloma", ClinicalTrials.gov identifier: NCT00474929. This study is ongoing, but not recruiting participants.

2. "Phase I/II trial of sorafenib and weekly Bortezomib in the treatment of patients with relapsed or refractory multiple myeloma", ClinicalTrials.gov identifier: NCT00536575. This study is ongoing, but not recruiting participants.

3. "Phase I/II study of sorafenib, Lenalidomide, and Dexamethasone in relapsed/refractory multiple myeloma", ClinicalTrials.gov identifier: NCT00687674. Due to study design and toxicity, this study has been terminated.

4. "A Phase II trial of BAY 43-9006 (sorafenib) (NSC-724772) in patients with relapsing or resistant multiple myeloma", ClinicalTrials.gov identifier: NCT00253578. This study has been completed.

   In this latter investigation, the researchers looked for overall response rate in patients with a confirmed diagnosis of refractory or relapsed (RR) multiple myeloma (MM) with measurable monoclonal protein. Patients were treated with 400 mg oral sorafenib twice a day for 28-day treatment cycles. Three of eighteen eligible patients experienced grade 4 toxicities: one with thrombocytopenia, one with anemia, and one with renal failure. Four of the eighteen eligible patients were removed from study because of toxicity in three patients and one for personal reason. No partial or complete responses were observed in this study and authors suggested combination therapy of sorafenib with standard medications (112).

5. “Pharmacokinetic and phase I study of sorafenib (BAY 43-9006, NSC 724772, IND 69896) for solid tumors and hematologic malignancies in patients with hepatic or renal dysfunction”, ClinicalTrials.gov identifier: NCT00118170. This study has been completed.

6. “A phase I study of the Raf Kinase/VEGFR inhibitor BAY 43-9006 in combination with the proteasome inhibitor PS-341 in patients with advanced malignancies”, ClinicalTrials.gov identifier: NCT00303797. This study has been completed.

   Three renal, three lung, two pancreatic cancer and one patient from each of the following cancer type, breast, adrenal gland, melanoma, spindle cell tumor, chronic lymphocytic leukemia and multiple myeloma were enrolled to this study to define the toxicity and the maximum tolerated doses of the combination of sorafenib and bortezomib. The author concluded that the combination of sorafenib and bortezomib was well tolerated (113).

7. “A phase I study of Lenalidomide in combination with Bevacizumab, sorafenib, Temsirolimus, or 5-fluorouracil, Leucovorin, Oxaliplatin (FOLFOX) in patients with advanced cancers”, ClinicalTrials.gov identifier: NCT01183663. This study is ongoing, but not recruiting participants.

1.2.3.5.2 Sorafenib in prostate cancer treatment:

We demonstrated that sorafenib induces cell death in both hormone refractory and hormone responsive prostate cancer cell lines. Sorafenib also decreases ERK phosphorylation and induces autophagy in these cells (114). We have also analyzed sorafenib induced cell death in greater detail in some prostate cancer cell lines (115). Huang and colleagues have reported that sorafenib decreases ERK phosphorylation and mitochondrial depolarization in prostate cancer cell lines (116). They also showed sorafenib induces cytochrome *c* release from mitochondria and caspase 3 activation. In another study the effect of sorafenib on rats that were inoculated with the prostate carcinoma cell line MLLB-2 was investigated. The authors showed that treatment with 10 mg per kg of sorafenib via gastric gavage reduces number of endothelial and proliferating cells and induced the number of apoptotic cells (117-119). Su and colleagues showed that sorafenib induces apoptosis in both androgen receptor positive and negative prostate cancer cells. It causes downregulation of the anti-apoptotic protein myeloid leukemia cell 1 (Mcl-1) and AKT phosphorylation. Sorafenib also decrease expression of androgen receptor and PSA levels in androgen-sensitive cell lines (120).

Current clinical trials regarding of administrating of sorafenib in prostate cancer are:

1. “A phase II study of sorafenib (Nexavar®) prior to radical prostatectomy in patients with high-risk localized prostate cancer”, ClinicalTrials.gov Identifier: NCT00466752.This study has been completed.

2. “A phase II study of BAY 43-9006 (sorafenib) in metastatic, androgen-independent prostate cancer”, ClinicalTrials.gov Identifier: NCT00090545. This study has been completed and were published. The authors reported that sorafenib is relatively well tolerated in androgen independent prostate cancer patients (121) and shows moderate activity in treatment for metastatic castration-resistant prostate cancer (122).

3. “Phase I/II study of sorafenib concurrent with androgen deprivation and radiotherapy in the treatment of intermediate- and high-risk localized prostate cancer”, ClinicalTrials.gov Identifier: NCT00924807, This study has been terminated by sponsor.

4. “Phase II study of sorafenib (Bay 43-9006) and Docetaxel in metastatic prostate cancer”, ClinicalTrials.gov Identifier: NCT00619996, This study has been completed.

5. “Open-label, multicenter, phase I trial in order to determine the safety and pharmacokinetics of BAY43-9006 in combination with Docetaxel as first-line treatment in metastatic hormone refractory prostate cancer patients”, ClinicalTrials.gov Identifier: NCT00405210, This study has been completed. In this study, the researchers increased the dose of sorafenib and combined it with Docetaxel and prednisone. They concluded that 400 mg sorafenib twice per day can be combined with Docetaxel and prednisone (123).

6. “Mitoxantrone, Prednisone plus sorafenib in Taxane-refractory metastatic hormone refractory prostate cancer (HRPC)”, ClinicalTrials.gov Identifier: NCT00452387, This study has been terminated due to early stopping rule.

7. “A phase II study of BAY 43-9006 (NSC 724772; CTEP IND# 69,896) in patients with hormone refractory prostate cancer”, ClinicalTrials.gov Identifier:NCT00093457,This study has been completed. In this study patients with a pathologic diagnosis of adenocarcinoma of prostate and PSA equal or higher than 10 μg/l have been received 400 mg sorafenib twice daily continuously for 4 weeks. Twenty eight patients were enrolled and PSA was decreased in one patients more than 50% and in five patients less than 50 percent. The authors concluded that sorafenib has limited activity regarding PSA as an indicator of response to treatment (124).

8. “Phase I study investigating the safety and feasibility of combining Imatinib Mesylate (Gleevec) with sorafenib in patients with androgen-independent chemotherapy-failure prostate cancer”, ClinicalTrials.gov Identifier: NCT00424385, This study has been completed.

9. “Phase I/II study to evaluate the ability of sorafenib in overcoming resistance to systemic chemotherapy in androgen-independent prostate cancer (AIPC)”, ClinicalTrials.gov Identifier: NCT00414388, This study has been completed.

10. “A Phase II study of BAY 43-9006 in combination with Bicalutamide in patients with chemo-naïve hormone refractory prostate cancer”, ClinicalTrials.gov Identifier: NCT00430235.This study has been completed.

11. “Phase I study of sorafenib, Pemetrexed, and Cisplatin for the treatment of advanced solid tumors”, ClinicalTrials.gov Identifier: NCT00703638.This study has been completed.

12. “A Phase I study of Lenalidomide in combination With Bevacizumab, sorafenib, Temsirolimus, or 5-fluorouracil, Leucovorin, Oxaliplatin (FOLFOX) in patients with advanced cancers” , ClinicalTrials.gov Identifier: NCT01183663, This study is ongoing, but not recruiting participants.

1.4 Cell death and autophagy

1.4.1 Apoptosis:

Apoptotic cell death can be started in three ways: via the intrinsic (mitochondrial), the extrinsic (death receptor-mediated) and granzyme B pathways (**Figure 5**). In all of them activation of a protease is the first execution step (125).

Intrinsic pathway:

Sustained cellular stresses such as DNA damage, viral infection, transcriptional/translational inhibition, protein misfolding and growth-factor deprivation can initiate the intrinsic pathway (125, 126). Induction of Bcl-2 homology domain 3 (BH3)-only proteins such as BIM and p53 upregulated modulator of apoptosis (PUMA) or inactivation of some BCL-2 family members like Bcl-2, Bcl-xL and Mcl-1, leads to oligomerization of Bcl-2-associated X (Bax) and Bcl-2 homologous antagonist killer (Bak) on the mitochondrial membrane which in turn promotes cytochrome *c* release and mitochondrial fission (126, 127). Bax is mainly present as an inactive soluble monomeric protein in the cytosol of healthy cells while Bak is constitutively integrated in the mitochondrial outer membrane (126). These events are followed by formation of apoptosomes and activation of caspase-9 and then caspase-3 and caspase-7 (127). Activation of caspase-3 and caspase-7 mediates proteolysis of caspase substrates like catenin, Rho-associated kinase 1 (Rock1), lamins, mammalian STE20-like kinase 1(MST-1) (128), and further inhibitor of caspase activated DNase (ICAD) and p75 which leads to cell detachment, nuclear fragmentation, membrane blebbing, chromatin condensation, DNA degradation, accumulation of ROS and deactivation of high mobility group box 1 (HMGB1) (125).

The extrinsic pathway

Binding of extrinsic death ligands of the tumor necrosis factor (TNF) family to their receptors, such as CD95, death receptor 5 (DR5) or TNF receptor 1 (TNFR1) recruits the proteins of receptor-associated death-inducing signaling complex (DISC) (125). DISC comprises of an adapter like FAS-associated death domain protein (FADD) or TNFR1-associated death domain protein (TRADD), and zymogen caspase-8. Subsequent to caspase-8 activation, via a proximity induced model, caspase-3 and caspase-7 are activated downstream, which in turn execute apoptosis in targeted cell. BH3 interacting-domain death agonist (BID), a pro-apoptotic protein, may also be cleaved and activated by active caspase-8 (129, 130).

The granzyme B pathway

Cytotoxic T lymphocytes release lytic granules that contain granzyme B and perforin. Granzyme B is internalized by the mannose 6-phosphate/insulin-like growth factor II receptor (131). Granzyme B internalization is also facilitated by perforin and initiates apoptosis by activation of caspase-8 and caspase-3 or cleavage of BID that triggers mitochondrial cytochrome *c* release and apoptosome formation (132).

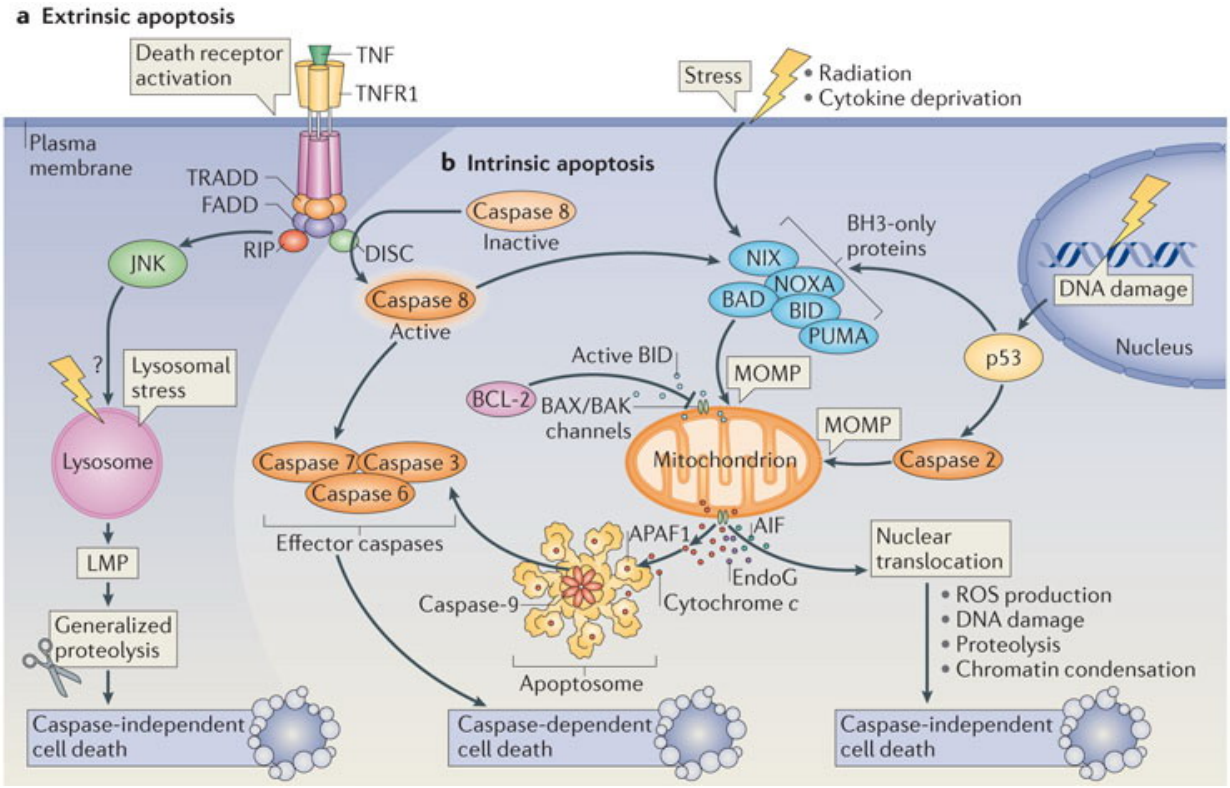

Figure 5. Apoptotic cell death. Reproduced with permission from Self-consumption: the interplay of autophagy and apoptosis. Guillermo Mariño, Mireia Niso-Santano, Eric H. Baehrecke & Guido Kroemer, Nature Reviews Molecular Cell Biology 15, 81–94 (2014).

1.4.2 Necroptosis

Another regulated cell death which results in cellular leakage is necroptosis (133, 134). A DISC is formed following the binding of ligand to TNFR1. Lys63-deubiquitylating enzyme cylindromatosis (CYLD) removes ubiquitin from Receptor-interacting serine/threonine-protein kinase 1 (RIP1) which leads to recruitment of RIP1, RIP3, FADD, TRADD and caspase-8. In this complex, caspase-8 inactivates RIP1 and RIP3 by proteolytic cleavage and promotes apoptosis. If caspase-8 is deleted, depleted or inhibited, this complex cannot proceed to apoptosis and instead, causes programmed necrosis or necroptosis. The role of FADD and TRADD is not clear in necroptosis. Necrostatin 1 and necrostatin 3 are small molecules that inhibit the kinase activity of RIP1, thereby inhibiting necroptosis (133, 134).

1.4.3 Autophagy

Autophagy is a highly conserved process from yeast to mammals in which portions of cytosol and aberrant organelles are sequestered by a double-membrane vesicles, termed the phagophore. The phagophore is elongated and closed, which is called autophagosome (F**igure 6**) (135, 136). Autophagosomes are fused with lysosomes to breakdown the macromolecules and recycle them.

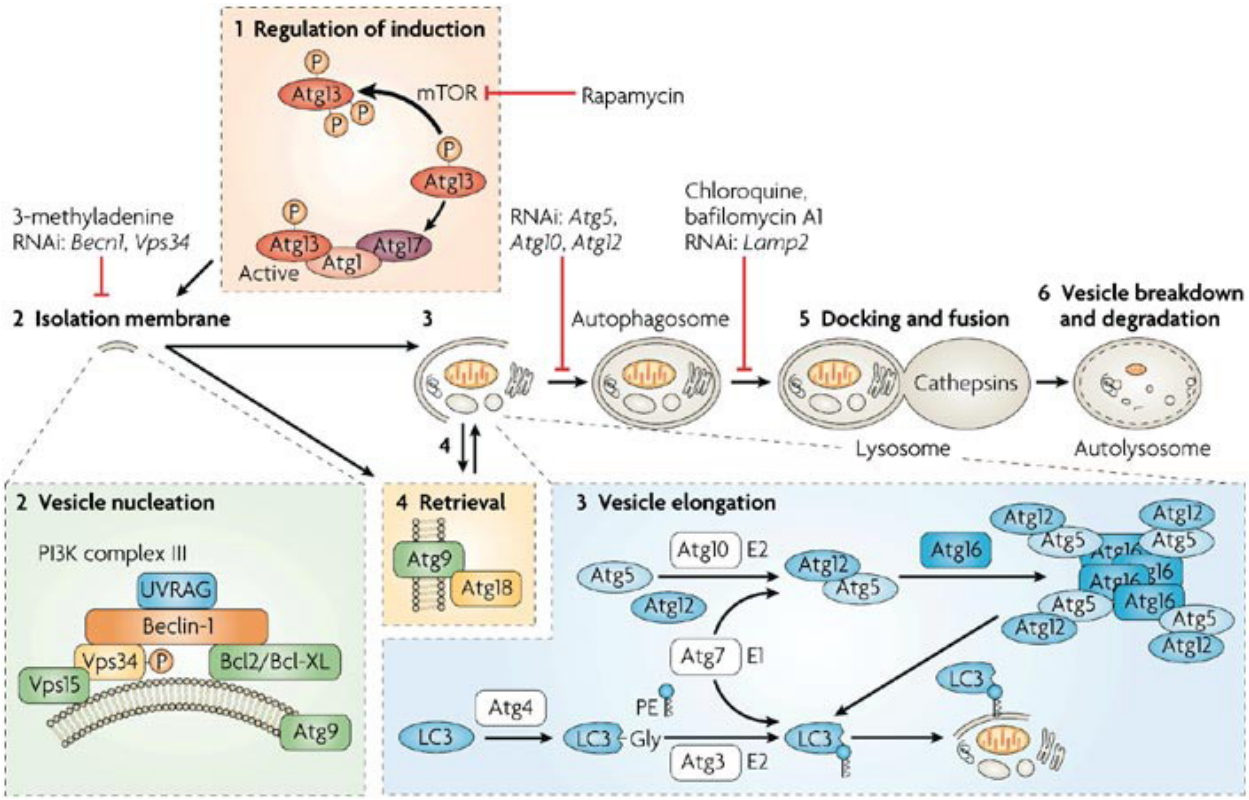

Figure 6. Process of autophagy. Reproduced with permission from Self-eating and self-killing: crosstalk between autophagy and apoptosis, M. Chiara Maiuri, Einat Zalckvar, Adi Kimchi & Guido Kroemer.Nature Reviews Molecular Cell Biology 8, 741-752.

The core machinery process of autophagy in mammalian cells can be divided into five steps (137):

1. **Initiation of autophagy:**

Unc-51 like autophagy activating kinase 1/2 (ULK1/2), FAK family kinase-interacting protein of 200 kDa (FIP200), autophagy-related gene13 (ATG13) and ATG101 form a stable complex in both nutrition-rich and starvation condition (138). In nutrition-rich conditions mTORC1 binds to the complex through direct interaction between raptor and ULK1/2 and phosphorylates both ULK1/2 and ATG13, thereby inhibiting ULK1/2 kinase activity. Upon starvation, mTORC1 is released from the complex leading to that the mTORC1-dependent phosphorylation sites (such as S638 and S758) in ULK1/2 rapidly become dephosphorylated by unknown phosphatases. Activation of ULK1/2 leads to its autophosphorylation and further phosphorylation of ATG13 and FIP200 that translocate the whole complex to the pre-autophagosomal membrane (139).

2. **Vesicle nucleation:**

The Beclin 1–Vps34 complex includes several protein such as Beclin 1, Vps34, Vps15, Autophagy/Beclin 1 Regulator1 (AMBRA1), UV radiation resistance associated gene (UVRAG), Rubicon and ATG14L. The Beclin 1–Vps34 complex is recruited to the autophagosome formation site by the activated ULK1 complex via phosphorylation of AMBRA1 (140). The activated ULK1 complex, also phosphorylates Ser14 on Beclin 1 enhancing the activity of the ATG14L containing Vps34 complex (141). Vps34 complex kinase activity produces PtdIns(3)P which binds to effector proteins like double FYVE-domain-containing protein 1 (DFCP1) and WD-40 repeat-containing protein that interacts with PtdIns (WIPI) and recruits them to promote autophagosome formation (142). AKT inhibits autophagy by direct phosphorylation of Ser234 and Ser295 of Beclin 1(143). EGFR inhibits autophagy via phosphorylation of Tyr229, Tyr233, and possibly Tyr352 on Beclin 1 leading to decrease Beclin 1-associated VPS34 kinase activity (144). BCL2 or Bcl-xL can bind to the BH3 domain of Beclin 1 and inhibits autophagy (137).

3. **Retrieval**

ATG9 and vacuole membrane protein 1 (VMP1) are two transmembrane proteins which undergo recycling between the Golgi, endosomes and autophagosomes (137). Atg9 transfers membrane components which required for phagophore expansion. VMP1 might function as a transmembrane protein to recruit Beclin 1 (145, 146).

4. **Vesicle elongation**

The vesicle elongation process compromises of two ubiquitin-like conjugation systems. ATG7, an E1−like enzyme, is present in both pathways. ATG3 and ATG10 are E2−like enzymes (147). The first pathway includes ATG7, ATG10, ATG16 and ATG12 that is

covalently bound to ATG5 (147, 148). ATG16 is required for the translocation of this complex to isolation membranes (149). The second pathway includes ATG3 and ATG7 and the conversion of microtubule-associated protein 1A/1B-light chain 3-I (LC3-I) (the soluble form) to LC3-II (autophagic vesicle-associated form) by conjugation of phosphatidylethanolamine (PE) to LC3-I (150). Both the inner and outer membrane of the autophagosomes has LC3-II (151). LC3-II is used as a marker of autophagy. ATG4 is a protease that cleaves LC3 to form LC3-I (152). Ubiquitin-binding proteins are involved in the recognition of autophagy targets such as p62 which contains a carboxy-terminal ubiquitin-associated (UBA) domain, Phox and Bem1 domain (PB1) and an LC3-interacting region (LIR). p62 acts as an adapter between ubiquitinylated autophagy substrates (e.g. protein aggregates, organelles) and the autophagic machinery (153). The E3 ligase parkin and p62 have been implicated in autophagy of non-functional mitochondria (154). p62 also plays an important role in autophagic degradation of soluble proteins (155).

5. Fusion with lysosomes and degradation

Soluble N-ethylmaleimide-sensitive fusion attachment protein receptors (SNAREs), including vacuolar morphology protein 7 (VAMP7), vacuolar morphology protein 8 (VAMP8), and vesicles transport through interaction with t-SNARE homolog 1B (VTI1B), are involved in docking and fusion of the autophagosomes to lysosomes (156).

1.5 Multiple Myeloma

Multiple myeloma (MM) accounts to 1% of all cancers and is the second most common hematological malignancy (157). Multiple myeloma is characterized by accumulation of monoclonal, terminally differentiated B cells (plasma cells), in the bone marrow and the production of either complete immunoglobulins (most frequently IgG or IgA) or only immunoglobulin light chains (either kappa or lambda but not both of them) (158). Multiple myeloma is diagnosed based on the presence of such clonal plasma cells in the bone marrow, the monoclonal immunoglobulin chains in serum (M protein), and clinical indications of end organ damage such as hypercalcemia, renal failure, anemia and lytic bone lesions (CRAB) (159).

1.5.1 Risk factors for multiple myeloma

The most common risk factors associated with multiple myeloma are (160):

1. ***Age*****:** incidence rate increase after the age of 40 and most diagnosed patients are more than 65 years old. Only 2% of patients are diagnosed when they are younger than 40 years old.
2. ***Ethnicity*****:** myeloma has a higher incidence in African American.
3. ***Gender*****:** Myeloma risk is elevated in men.
4. ***Obesity*****:** Obese persons have a higher risk to develop myeloma.

5. ***Family history***: People with a positive family history of lymphatohematopoietic cancers have a higher risk of myeloma.
6. ***Radiation***: Exposure to radiation increases the risk of myeloma.

1.5.2 Genetic Abnormalities

Specific genetic changes of multiple myeloma do not play a role in diagnosis but they can be used to assign patients to different risk groups which determine choice of treatment and prognosis (161). Multiple myeloma is classified into two groups: hyperdiploid and non-hyperdiploid, which have almost the same number of patients. Hyperdiploid patients usually have better survival compared to non-hyperdiploid patients. Hyperdiploid multiple myeloma has extra copies of chromosomes while non-hyperdiploid is characterized by translocations of the *IgH* locus (14q32) with other chromosomal parts like 11q13, 4p16, 16q23. The t(11;14)(q13;q32) translocation, fusing *IgH* with *CCND1* gene is the most frequent translocation which is associated with low levels of serum monoclonal proteins, low plasma cell proliferation and good prognosis. The t(4;14)(q16;q32) and the t(14;16)(q32;q23), fusing *IgH* with *CCND1* gene , translocations are associated with poor prognosis with the former having aggressive clinical features. Other abnormalities like deletion of 17p, detected in 10% of newly diagnosed cases, and chromosomes 13 deletion, found in 50% of cases at the time of diagnosis, are also common in multiple myeloma.

1.5.3 Pathogenesis

Myeloma rise out of a specific population of plasma cells (PCs) in the bone marrow which is termed myeloma cancer stem cells (162). The myeloma cancer stem cells are not well defined but there is a report showing clonogenic myeloma cells expressing the B cell surface antigens (CD19, CD20, CD22 and CD45) but not CD138. These cells have capacity to later become mature CD138+ plasma cells which produce circulating M protein and myeloma colonies (162).

Genes for variable regions of the heavy and light chains of antibodies in B-cell progenitors rearrange to start the development of B cells. This process called V(D)J recombination (163). PCs proliferate and differentiate at the pre-germinal-center after primary exposure of mature B cells to antigen. These PCs are short lived and usually secrete IgM. Somatic hypermutation of IgH and IgL V(D)J sequences occurs in antigen-activated lymphoblasts that enter a germinal center. Some cells, which express high levels of antigen receptor, are selected and generate memory B cells or post-germinal-center plasma cells. Finally IgH switch recombination occurs in more differentiated cells, and these non-proliferating cells stay in the bone marrow. Terminally differentiated normal plasma cells highly express the CD138 antigen (164-168).

Multiple myeloma progression start from germinal center B cells and is developed further to monoclonal gammopathy of undetermined significance (MGUS), smoldering multiple myeloma (SMM), intramedullary myeloma, extramedullary myeloma and finally independent myeloma cell line (**Figure 7**). Almost all cases of multiple myeloma proceed from the premalignant state MGUS (169). It is estimated that the prevalence of MGUS is 3.2% in people

older than 50 years and the prevalence is affected by age and sex. The risk of progression of MGUS to multiple myeloma is approximately 1% per year (170). MGUS is characterized by the clonal expansion of plasma cells in the bone marrow, less than 10%, and monoclonal protein in serum (serum M protein), less than 3 g/dL, and without any clinical manifestations or other laboratory abnormalities related to monoclonal gammopathy (171). Hyperdiploid and non-hyperdiploid abnormalities which are seen in myeloma are also detected in MGUS. Patients with MGUS must be examined for detection of early signs of disease progression. Smoldering myeloma is characterized by the presence of plasma cells in the bone marrow equal or more than 10%, monoclonal protein in serum (serum M protein) equal or more than 3 g/dL and without any clinical manifestations or other laboratory abnormalities related to monoclonal gammopathy (171). The risk of progression of SMM to MM is 10% per year for the first 5 years, then decreases to 3% per year for the next 5 years (172).

Kuehl and Bergsagel divide pathogenesis of MGUS and myeloma into three phases: in the early phase primary *IgH* translocation, hyperdiploidy, and chromosomes 13 deletion take place which dysregulate the cyclin D1 gene (173, 174). The second phase is associated with v-myc avian myelocytomatosis viral oncogene homolog (MYC) overexpression, sometimes Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation and chromosomes 13 deletion. The third phase is associated with increased proliferation and genomic instability and the cells are less dependent on the bone marrow microenvironment. Activation of the NFκB pathway and inactivation of TP53, P18 and RB1 have also been reported (173, 174).

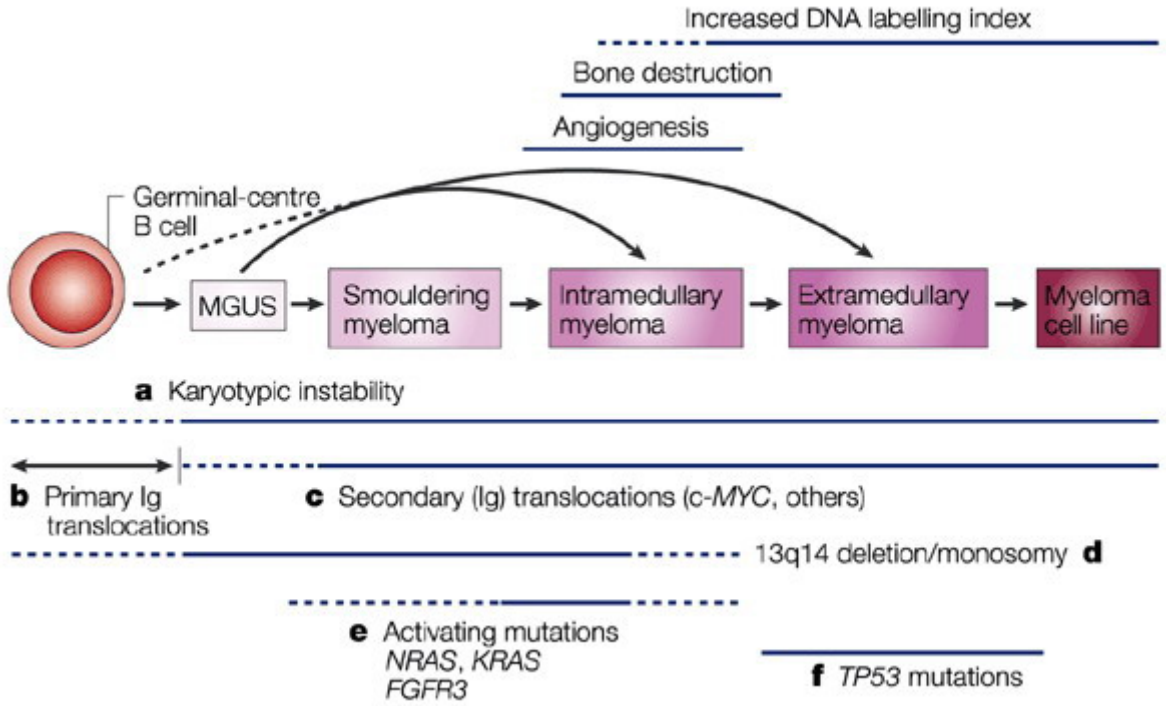

Figure 7. Multiple myeloma development. Reproduced with permission from Multiple myeloma: evolving genetic events and host interactions, W. Michael Kuehl & P. Leif Bergsagel. Nature Reviews Cancer 2, 175-187 (March 2002).

1.5.4 Signaling cascades involved in multiple myeloma pathogenesis

The four main signaling cascades that have been shown to be activated in multiple myeloma are PI3K/AKT/mTOR, JAK/STAT3, Ras/Raf/MEK/ERK and NFκB (175).

These signaling pathways were generally described before in the thesis and here I will describe their specific special role in myeloma pathogenesis.

- **The PI3K/AKT/mTOR signaling pathway**

PI3/AKT/mTOR activation leads not only to increased survival, proliferation and migration but also to the overexpression of anti-apoptotic proteins in myeloma cells (175). Several growth factors activate this signaling pathway. IGF-1 and insulin are survival and proliferation inducers in most primary myeloma cells as well as cell lines. The IGF-1R is expressed aberrantly by myeloma cells but not in normal plasma cells (176, 177). IGF-1 is secreted by octeoclasts in the bone marrow and its level is high in bone marrow of multiple myeloma patients (176, 178). CD138 is a hallmark of myeloma cells CD138 mediates adhesion of myeloma cells to collagen and myeloma cell-cell adhesion which is very important in myeloma growth and development. Many growth factors such as HGF , VEGF and FGF2 interact with heparan sulfate of CD138 (179).

- **The JAK/STAT3 signaling pathway**

One of the main growth factors for myeloma is IL-6 (180) which can directly activate both the JAK/STAT3 and Ras/Raf/MEK/ERK signaling pathways by binding to its receptor, gp130 (181). The receptor gp130 phosphorylates the tyrosine (Tyr99) on JAK which leads to the recruitment and phosphorylation of mainly Tyr705 in STAT3 and Tyr701 in STAT1. Subsequently active STAT dimers are formed and translocated to the nucleus initiating the transcription of genes that are promote expression of anti-apoptotic protein like Mcl-1 and Bcl-xL in the myeloma cells (181-184). Phosphorylated JAK also phosphorylates Shc which leads to the activation of the Ras/Raf/MEK/ERK pathway which induces proliferation of myeloma cells (183).

- **The Ras/Raf/MEK/ERK signaling pathway**

This pathway is induced by the activation of IGF-1R and IL-6R as described above. Activation of this signaling pathway leads to increased proliferation of myeloma cells (175).

- **The *NFκB* signaling pathway**

Tumor necrosis factor alpha (TNF-α) family members like BAFF and APRIL are very important in multiple myeloma pathogenesis (185). These molecules bind to the TNF family receptors including B-cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and activate the NFκB, PI3K/AKT and Ras/Raf/MEK/ERK signaling pathway which is important in survival and proliferation of

normal and malignant B cells. Activation of these signaling cascades also up-regulate the Mcl-1 and Bcl-2 anti-apoptotic protein levels in myeloma cells (185-187).

1.5.5 Role of the microenvironment in myeloma

Apart from the end stage of multiple myeloma, myeloma cells cannot grow outside of the bone marrow, indicating the importance of the bone marrow microenvironment for the myeloma cell survival and proliferation **(Figure 8**) (188). The direct interaction between bone marrow cells and multiple myeloma cells leads to growth, survival, migration of myeloma cells and osteolysis (188-190) and neovascularization by secreting growth factors, cytokines, and extracellular vesicles (191, 192). The major players in myeloma pathogenesis in the bone marrow are bone marrow stromal cells (BMSCs), osteoblast, osteoclasts and extracellular matrix (ECM).

Attachment of multiple myeloma cells to extracellular matrix (ECM) and BMSC mediates the homing of myeloma cells in bone marrow. VLA-4 on myeloma cells binds to the vascular cell adhesion molecule 1(VCAM-1) on BMSCs which activates the NFκB pathway and increases IL-6 secretion from BMSC (193). Subsequently, IL-6 increases the secretion of VEGF and TNFα from myeloma cells which in turn upregulate IL-6 secretion from BMSC via a paracrine loop (194, 195). Recently Wang and colleagues showed that BMSC-derived exosomes increase the multiple myeloma cell growth and induce drug resistance to bortezomib by influencing the activation of several pathways such as JNK, p38, p53, and AKT (191). Other soluble factors like VEGF, IGF1, stromal cell-derived factor α (SDF1α), B-cell activating factor (BAFF), a proliferation-inducing ligand (APRIL) and HGF are also secreted by BMSC and play an important role in myeloma pathogenesis (175). Bone marrow microvessel density (MVD) is consistently increased in active myeloma. This is primarily due to the secretion of angiogenesis promoting factors like VEGF and metalloproteinases from myeloma cells (175, 196). Adhesion of myeloma cells to ECM through binding of β1-integrin to fibronectin has an important role in the multi-drug resistance phenotype. It can be mediated via the nuclear accumulation of Spy1 and $p27^{Kip1}$ (197, 198).

Lytic bone lesion and bone resorption which is seen in myeloma is caused by two mechanisms: first the binding of the myeloma cells to BMSCs causing the increased production of receptor activator of NFκB ligand (RANKL). RANKL binds to the receptor activator of NFκB (RANK) on the osteoclast precursors and induces their differentiation to osteoclasts. On one hand RANK signals via NFκB and JNKs pathways increase osteoclastic bone resorption and osteoclast survival. On the other hand, it decreases the secretion of osteoprotegerin (OPG) from BMSCs. OPG is a soluble decoy receptor for RANKL (199). The second mechanism for bone resorption is mediated by the secretion of macrophage inflammatory protein-1α (MIP-1α) from myeloma cells. MIP1α induces osteoclast formation (200). Decreased activity of osteoblasts has also been reported in multiple myeloma (201).

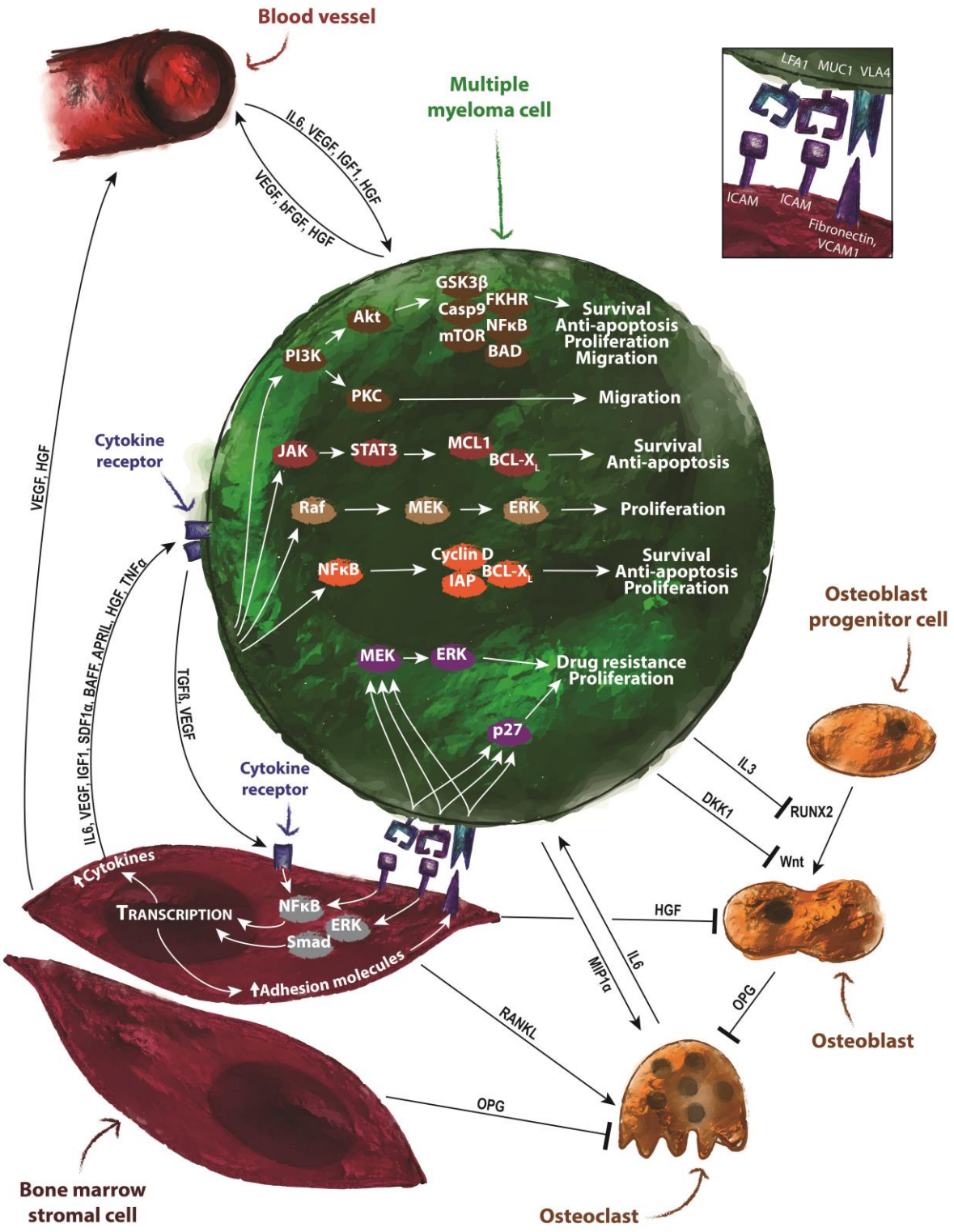

Figure 8. Interaction of multiple myeloma cells in their bone marrow microenvironment. Modified with permission from Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. Teru Hideshima, Constantine Mitsiades, Giovanni Tonon, Paul G. Richardson & Kenneth C. Anderson, Nature Reviews Cancer 7, 585-598 (August 2007)

1.5.6 Clinical and laboratory finding:

The clinical manifestations of myeloma are hypercalcemia (≥11 mg/dL), renal failure due to nephropathy or hypercalcemia and elevated serum creatinine (in 50% of patients at the time of diagnosis), pallor, weakness and fatigue because of anemia (in 70% of patients at the time of diagnosis), bone pain (in 60% of patients at the time of diagnosis) usually in the back and the ribs (202). These four manifestations (CRAB) are major difference between MGUS and multiple myeloma. Other symptoms and signs are radiculopathy, infection and organ infiltration. Laboratory findings of multiple myeloma are normocytic normochromic anemia, rouleaux formation in peripheral blood smear, single narrow peak in the serum protein electrophoresis, monoclonal protein in the serum while two-thirds of cases are kappa light chain and one-thirds are lambda. Usually more than 10% of the bone marrow cells are monoclonal plasma cells and the cytoplasm of these cells contains only kappa or lambda chain. Myeloma cells are positive for CD38 and CD138 and two-thirds of these express CD56. Lytic lesions, osteoporosis and pathologic fracture are seen by radiography. The most common bone involvements are vertebra, skull and thoracic cage.

1.5.7 Treatment

Multiple myeloma is considered as an incurable but manageable disease with a median survival of 3-4 years (203). The treatment of multiple myeloma involves seven steps:

1. Diagnosis and determination of need for therapy

   It is necessary to show that a monoclonal plasma cell process is ongoing and that it is in an active phase which needs treatment.

2. Risk stratification

   Three risk factors must be considered in the treatment of myeloma: age, renal function and type of genetic abnormalities.

3. Induction therapy

   Based on the eligibility of patients for autologous stem cell transplantation, initial therapy includes lenalidomide and dexamethasone (RD), bortezomib and dexamethasone (VD), cyclophosphamide, bortezomib and dexamethasone (CyBroD or VCD), bortezomib, lenalidomide and dexamethasone (VRD), melphalan, prednisolone and thalidomide (MPT), melphalan, prednisolone and lenalidomide (MPR) and finally melphalan, prednisolone and bortezomib (VMP).
4. Consolidation therapy
5. Maintenance therapy
6. Monitoring for relapse
7. Supportive care, including biphosphonates used for reducing the bone problem (202).

1.5.8 Mouse models of multiple myeloma

The role of animal models has a significant impact on our understanding of mechanisms of diseases and the discovery of new treatments. There are several different mouse models of

multiple myeloma like LAG λ-1 (204) model or MOPC315.BM (205). Here I describe some common models.

- **The 5T series**

Jiri Radl observed in the 1970s that C57BL/KaLwRij mice sometimes developed myeloma when they grow old. He isolated and injected these myeloma cells into young mice and found that threy infiltrate bone marrow and injected mice developed multiple myeloma (206). He repeated this process several times and established a new mouse models of myeloma which is known as the 5TMM syngeneic mouse model. Localization of myeloma cells and increased angiogenesis in bone marrow, positive correlation between serum paraprotein and myeloma stage and osteolytic bone lesions are some of the main characteristics of this models and resemble closely the human disease. The 5T2MM and 5T33MM are two well characterized models with the latter being more aggressive. Signs of myeloma are seen after 12 weeks post-injection and are found in the in the bone marrow and spleen demonstrating that these myeloma cells are dependent on the bone marrow stromal cells for growth and survival (207). This model has been used to study myeloma cell homing and efficiency of new treatments (208).

- **SCID models**

There are several experimental systems of immortalized or primary human cells injected subcutaneously into severe combined immunodeficiency (SCID) mice (209-212). Urashima and colleagues implanted human fetal bone grafts in SCID mice and then injected human myeloma cells directly into the graft. In this model, human bone grafts can provide an appropriate niche for human myeloma cells which need interaction with the human bone microenvironment (213). Primary human myeloma cells are solely dependent on the engrafted human bone and there are no myeloma cells found in any of the mouse organs. The SCID-hu model is suitable to investigate the natural history of multiple myeloma regarding bone resorption or angiogenesis and efficacy of new treatment (214).

- **Transgenic mouse models**

It was observed that mice with several genetic abnormalities can develop a myeloma-like disease. Chesi and colleagues showed that misdirecting the activity of activation-induced deaminase (AID) to a conditional MYC transgene can cause multiple myeloma Vk*MYC mice (215). Carrasco *et* al. also showed that transgenic mice with Eμ-directed expression of the XBP-1 spliced isoform (XBP-1s) displays an MGUS and myeloma phenotype (216). The Jackson Laboratory also created several transgenic mice such as B6.129S1-Irf4^{tm1Rdf}/J, B6; 129S6-Lig4^{tm1Fwa}/Kvm, C3.B6-Tg(Fabp1-Ccnd1)4Rdb/J, C57BL/6-Tg(Fabp1-Ccnd1)4Rdb/J and FVB.129S2(B6)-Ccnd1^{tm1Wbg}/J which develop multiple myeloma (217).

1.6 Prostate cancer

According of Globocan report, prostate cancer comprises 15% of all cancers diagnosed in men worldwide (218). It is the second most common cancer and the fifth leading cause of cancer-related deaths in men. In 2012 around 1.1 million men (2 men every minute) were diagnosed with prostate cancer worldwide. Ratio of highest to lowest incidence in the world is more than 25-fold. The highest rates (approximately 70% of cases) occur in Australia, New Zealand, Northern America, and in Western and Northern Europe and the lowest rate are found in Eastern- and South-Central Asia.

The most common form of prostate cancer is moderately differentiated adenocarcinoma which in 70 to 80% of cases and arises in the peripheral zone of prostate gland (13).

1.6.1 Risk factors for prostate cancer

1. ***Age***: Based on Globocan report, prostate cancer is never seen in men younger than 15 years old which shows the critical role of androgen hormone in prostate cancer development (218). The incidence of prostate cancer increases dramatically from 60 years of age with the highest rate in men over 75 years of age.

2. ***Family history*****:** It was reported that men who have a first-degree male relative (i.e. father, brother, son) with a history of prostate cancer have a 2- to 3-fold increased risk of prostate cancer (219). There are some reports that suggest associations between genetic variants in genes such as Hereditary *Prostate Cancer 1 (HPC-1), breast cancer 1/2 (BRCA1/2), homeobox B13 (HOXB13)* (which are more common in Scandinavian men), *Nijmegen breakage syndrome1 (NBS1), checkpoint kinase 2 (CHEK2)* and *PALB2* and prostate cancer but it needs further investigations (220).

3. ***Physical activity*****:** Physical activity may decrease the risk of prostate cancer by decreasing levels of total and free testosterone, reducing obesity, and enhancing immune function (219).

4. ***Diet*****:** There are some evidences that diet and lifestyle plays a role in prostate cancer pathogenesis (221). Red meat, well cooked and processed meat increase the risk of developing prostate cancer by producing heterocyclic amines and heme compounds. Excessive consumption of milk and dairy products is also consider as risk factor of prostate cancer due to increase fat intake and blood levels of IGF-1 and a decrease in circulating 1,25 dihydroxyvitamin D3.

5. ***Smoking*****:** Because smoking is a known source of carcinogens, it is suggested that smoking is a risk factor for prostate cancer (222). A meta-analysis report enrolling 21,579 prostate cancer cases showed that current smokers had higher risk of fatal prostate cancer and heavy smokers had around 30% greater risk of death compared to nonsmokers.

6. ***Alcohol*:** Most studies cannot show any association between alcohol consumption and prostate cancer, but increased alcohol intake is associated with the risk of high-grade prostate cancer in patients which use dutasteride, a 5α-reductase inhibitor (223).

7. ***Sexual activity*:** Because prostate cancer tumorigenesis is associated with infection and inflammation in the prostate gland, it is possible that sexual activity increases the risk of prostate cancer (224). There are some reports indicating that starting sexual intercourse at an early age and higher number of sexual partners increases the risk of prostate cancer. It is also shown that higher frequency of ejaculation decreases the risk of prostate cancer but association of sexual activity and prostate cancer needs further investigation.

1.6.2 Pathogenesis of prostate cancer

A multi-step progression pathway was suggested for prostate cancer development (**Figure 9**) (225). It is initiated from normal prostate tissue damage by different mutations like *hereditary prostate cancer 1* (*HPC1*) gene mutation, oxidative damage, dietary and environmental factors, infection and inflammation in prostate tissue which leads to prostatic inflammatory atrophy (PIA). Prostatic intraepithelial neoplasia (PIN) is characterized by loss of heterozygosity (LOH) involving chromosome 8p21 and the transcription factor *NKX3.1* as a putative tumor suppressor gene in prostate cancer, overexpression of MYC, up regulation of glutathione S-transferase π (GSTP1) in response to increased oxidative stress, increase expression of α - Methylacyl-CoA racemase (AMACR) and decrease in telomere length. Progression of PIN towards prostate cancer is associated with loss of 10q (phosphatase and tensin homolog (PTEN)) and 13q (RB), abnormal methylation of the Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A), and a reduced level of p27 that may be secondary to PTEN loss, increased activity of telomerase and prostate stem cell antigen (PSCA). Loss of 17p (p53) and decreased activity of E-cadherin is further associated with metastasis in prostate cancer (225-227).

1.6.3 Role of NRTK in prostate cancer:

- **Src family:**The Src family consists of nine members which play an important role in pathogenesis and metastasis of prostate cancer (228). Src makes a complex with Fak and Etk which interact and enhance the effect of each other. These complexes are involved in the activation of the androgen receptor (AR), EGFR, IGFR and VEGF mediated signaling in prostate cancer. Src activity is enhanced in hormone refractory prostate cancer and in a closed loop process, Src can activate AR as well as being a downstream target of the AR. Src is involved in growth, migration and metastasis of prostate cancer cells (229). FYN can regulate cyclooxygenase 2 (COX2) by direct phosphorylation. COX2 in turn is involved in the initiation and progression of prostate cancer (230).
- **TEC family:**This family comprises of four members. Etk/Bmx expression is increased in prostate cancer and its overexpression leads to PIN in mouse model (231, 232). The levels of Etk/Bmx are also increased in castration-resistant prostate cancer (CRPC) (231, 232). Etk is further involved in IL6-dependent induction of neuroendocrine differentiation in prostate cancer cell lines (233).

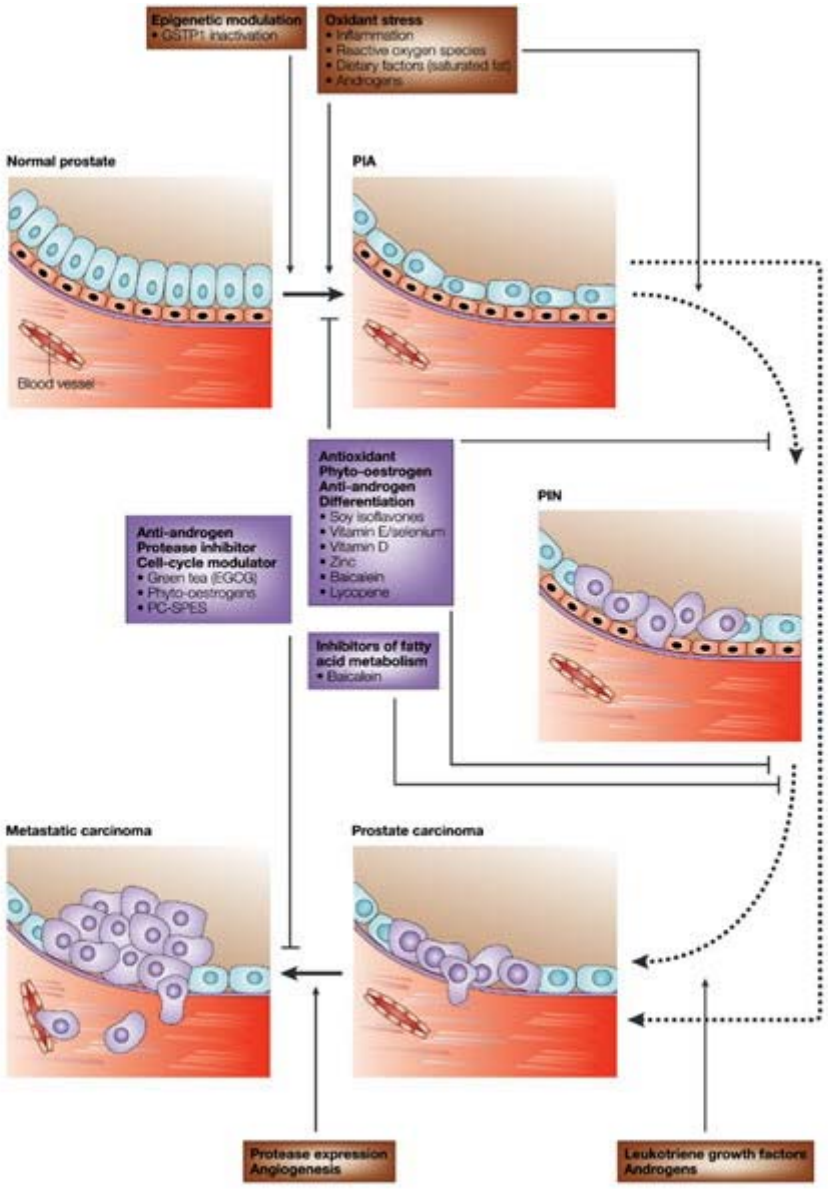

Figure 9: Prostate cancer development. Reproduced with permission from Unconventional therapy for prostate cancer: good, bad or questionable? Peter S. Nelson & Bruce Montgomery, Nature Reviews Cancer 3, 845-858 (November 2003).

- **FAK family:**FAK expression is elevated during prostate cancer pathogenesis (234). Integrins, neuropeptides, chemokines and growth factors stimulate FAK which leads to migration, growth, cell polarity, adhesion and metastasis through Src, PI3K, RhoGTPase, and p130Cas pathways. FAK can increase VEGF transcription through ERK1/2 and affects angiogenesis and apoptosis (28).
- **JAK family:** Binding of IL-6 to its receptor can activate JAK1 and STAT3 in prostate cancer cells, which is known to be important in progression of hormone sensitive to the hormone refractory situation (235). The concentrations of IL-6 in the serum of patients with hormone sensitive prostate cancer is less than in patients with hormone refractory prostate cancer. The IL-6 receptor/JAK/STAT3 cascade also increase the proliferation rate of

prostate cancer cell lines and AR-negative cells such as DU145 and PC3 having higher level of activated STAT3 than AR-positive cells like LNCaP.

1.6.4 Role of RTK in prostate cancer

- **EGFR**:The human epidermal growth factor receptor (HER) family comprises of four type 1 transmembrane receptors: EGFR, HER2 (ErbB2), HER3 (ErbB3) and HER4 (ErbB4) (236). They undergo homodimerization or heterodimerization with another family member upon binding of the ligand and activate downstream signaling pathways like the ERK1/2 and the phosphatidylinositol 3-kinase (PI3K)/AKT pathways. Overexpression of EGFR, HER2 and HER3 are associated with progression from localized to metastatic prostate cancer and to the androgen-independent state (236-238).
- **FGFR:**The FGFR family consists of four receptors. They can activate the Raf/Ras/ERK, PI3K/AKT, PLCγ and STAT pathways. Normal epithelial cells of prostate express multiple FGF receptors. While there is enough evidence to support the role of FGFR-1, FGFR-3 and FGFR-4 in prostate cancer initiation and progression, the role of FGFR-2 is not clear. Twenty two FGFs were identified so far in the human proteome, some of which have an autocrine and/or a paracrine role in prostate cancer (239, 240).
- **HGFR:**Stromal cells produce HGF in normal tissues and HGF/c-Met signaling usually occurs through paracrine mechanisms (241). c-Met can activate downstream signaling molecules like the Src kinase, PI3K/AKT/mTOR and Ras/Raf/MEK/ERK which promote cellular proliferation, survival and motility, resistance to apoptosis and metastasis. A c-Met/HGF paracrine loop is responsible for increased c-Met signaling in prostate cancer. Half of primary prostate tumors and almost all bone metastases have high expression of c-Met.
- **IGF-1R:**Different mechanisms are involved in the regulation of IGF-1R transcripts in prostate cancer cells such as defective BRCA1 and Kruppel-like factor 6 (KLF6), epigenetic changes of paired-like homeodomain transcription factor 2 (*PITX2*) gene which is an upstream regulator of *IGF-1R* gene expression and IGF-1R translocating to the nucleus (242, 243). BRCA1 regulates the IGF-IR expression in an AR-dependent manner in prostate cancer. The IFG-1R activates PI3K/AKT and Raf/Ras/MEK/ERK. IFG-1R can also facilitate the metastatic potential of prostate cancer cells by transactivation of IGF-IR with IL-6 and induction of epithelial to mesenchymal transition (244). Increased level of IGF-1R, IGH-1 and IGF-2 has been reported in advanced prostate cancer (242, 243).
- **PDGFR:**The PDGFR is detected in 88% of prostate cancer primary tumors and 80% of the metastases. It has been reported that PDGF-BB can upregulate Mcl-1 through activation of β-catenin and hypoxia-inducible factor (HIF)-1α in metastatic prostate cancer (245, 246).

1.6.5 Signaling cascades activated by RTKs and NRTKs in prostate cancer

PI3K/AKT/mTOR is one of the major survival pathway which is activated in prostate cancer. Loss-of-function mutation or deletion of PTEN leads to an increase in AKT phosphorylation levels in prostate cancer (247). This downstream pathway is activated both with NTRK and RTK. The other important signaling pathway is Ras/Raf/ERK which causes cell growth, malignant transformation and drug resistance (248).

1.6.6 The role of the microenvironment in prostate cancer

The tumor microenvironment consist of both a cellular component including malignant cells, fibroblasts, inflammatory cells, adipocyte, vessel-related cells, mesenchymal stem cells and a non-cellular component including factors such as collagen, elastin and glycoproteins forming the extracellular matrix (ECM) (**Figure 10**). Stromal support is necessary for prostate tumor formation. Tumor stroma becomes reactive during progression of prostate cancer from PIN to metastatic disease and it is also important in development of androgen resistance (249).

- **Involvement of the extracellular matrix (ECM)**

ECM comprises of collagen, elastin, fibronectin, laminin, and polysaccharide. ECM proteins interact with integrins which regulate the attachment of epithelial cells to the basement membrane. Integrins which are expressed on malignant cells and activated endothelial cells interact with ECM and recruit FAK, activate Src and PI3K and finally activate the MAPK signaling pathway (249).

- **Cellular components**

Inflammation is known one of the hallmark of cancer and the role of inflammation and inflammatory cells in prostate cancer is well-described (250). The tumor vasculature differs from normal vessel in many aspects like aberrant vascular structure, different endothelial-cell–pericyte interactions, increased permeability and delayed maturation (251). It has been recently shown that bone marrow mesenchymal stem cells which infiltrate prostate tissue cause an increase in the number of prostate cancer stem cells and promotes the metastatic ability (252). Cancer associated fibroblasts (CAFs) characterized by expression of smooth-muscle actin (SMA), fibroblast-activated protein (FAP), fibroblast-specific protein-1 (FSP1/S100A4), neuron-glial antigen-2 (NG2) and the PDGF b receptor. Inactive resident fibroblast or fibroblast precursors are thought to be the cells of origin of CAF (253).

- **Molecular pathways in stromal-epithelial crosstalk**

Androgen signaling: Stromal cells can convert testosterone to the more potent androgen DHT or they can produce it locally from cholesterol or by conversion of adrenal androgens, by CYP17 which leads to the activation of the MAPK signaling pathway (249). AR signaling is also involved in the osteoblastic metastatic lesions. Osteoblasts, osteoclasts and bone marrow stromal cells express AR and they can respond to androgens and increase the growth of prostate cancer cells via increased local production of androgens and androgen‐independent crosstalk of AR with growth factor pathways (249, 254, 255).

FGF signaling: FGFs and their receptors control the development of the prostate gland during embryogenesis and maintain normal tissue homeostasis in the adulthood (239). An FGF signaling crosstalk between stromal and epithelial prostate tissue plays a role in increasing cellular proliferation, motility and invasiveness, resistance to apoptosis, treatment and enhancement of angiogenesis. Different FGFs are responsible for this crosstalk (256).

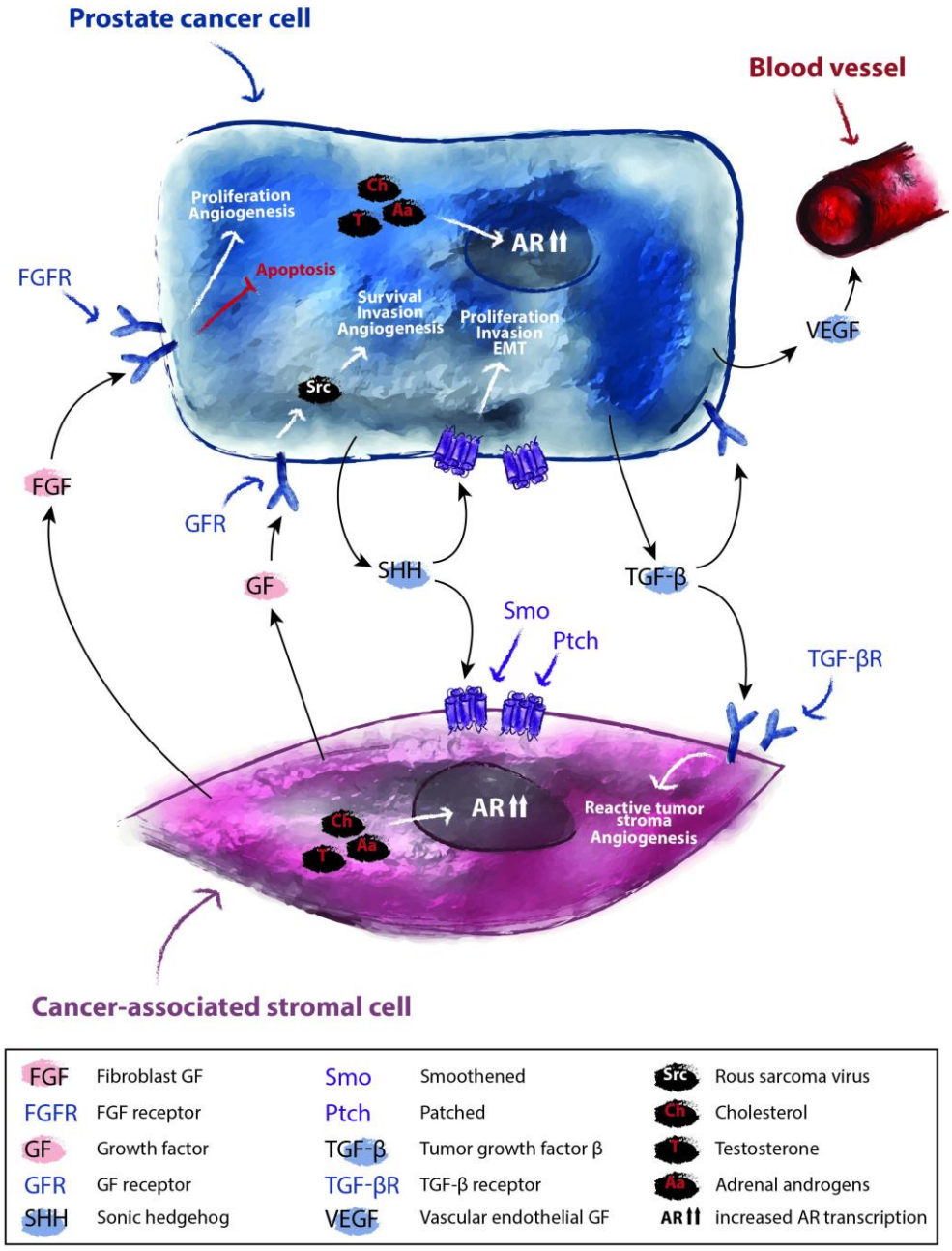

Figure 10. Interaction of prostate cancer cells in their bone marrow microenvironment. Modified with permission from Therapeutic targeting of the prostate cancer microenvironment Maria Karlou, Vassiliki Tzelepi & Eleni Efstathiou. Nature Reviews Urology 7, 494-509, September 2010)

Src signaling: Src family kinases (SFKs) are essential for the interaction between malignant cells and tumor-associated bone stromal cells. Varkaris *et al.* have shown that these kinases promote bone remodeling during metastasis (257). Activation of Src leads to an increase in the secretion of VEGF from tumor cells, which leads to not only increased proliferation in tumor associated endothelial cells in a paracrine manner but also promotes tumorigenesis in an autocrine manner.

1.6.7 Clinical and laboratory findings

Most of the patients are asymptomatic at the early-stage prostate cancer (258). Locally advanced or metastatic cases show symptoms like obstructive or irritative voiding and bone pain. In the presence of metastasis to the vertebral column, symptoms of back pain as well as of cord compression such as paresthesias and weakness of the lower extremities and urinary or fecal incontinence may be seen. It is possible to detect irregular hard nodules on digital rectal examination (DRE) if prostate cancer arise in the peripheral zone (258). PSA levels above 4.0 ng/mL (4 µg/L) is also associated with higher chance of prostate cancer. Prostate-specific antigen (PSA) can increase in benign prostate hypertrophy (BPH), urinary tract infections, and prostatitis. On the other hand, PSA level can be lower than 4.0 ng/mL (4 µg/L) in 15% of prostate cancer patients (259). Since PSA has a limited positive predictive value, finding new prognostic and diagnostic biomarkers in prostate cancer is necessary. Other emerging biomarkers are PSA velocity and isoforms, human kallikreins, hypermethylation of certain cytosine guanine (CpG) dinucleotide islands, presence of the product of the *TMPRSS2–ERG* fusion gene, RNA biomarkers like PCA3 and Alpha-methylacyl CoA racemase (AMACR) and prostate-specific membrane antigen (PSMA) (260).

1.6.8 Treatment

- **Low risk prostate cancer:**

By definition, low-risk prostate cancer is characterized by a Gleason Score of 6 or less and a PSA value less than 10 ng/ml (261, 262). About 50% of cases found by prostate cancer screening is diagnosed as low-risk. However, about one third of men diagnosed with low-risk disease have a high-grade cancer which could not be detected by the needle biopsy. A small proportion of low-grade cancers have molecular alterations which allow them to rapidly progress to aggressive disease. Low-risk prostate cancer should be managed by active surveillance with reservation of radical treatment for cases with higher-risk disease or possibly in younger patients. Active surveillance involves serial PSA assessments and biopsies from zones which are usually under-evaluated. If treatment is necessary, focal therapy or prostatectomy and radiotherapy are possible therapeutic options.

- **High risk prostate cancer:**

There are no common definitions for high-risk prostate cancer (263). American Urological Association defines it by a preoperative PSA greater than 20 ng/ml, and/or a preoperative Gleason score between 8 and10, and/or clinical stage ≥ T2c, while the European Association of Urology defines high-risk prostate cancer with the same condition but clinical stage ≥T3a. The purpose of treatment is a local control of cancer and the prevention of metastasis. It is well known that combination of androgen-deprivation therapy (ADT) and external-beam radiotherapy (EBRT) is better than either of these approaches alone. The treatment of clinically localized disease is initiated with neo-adjuvant therapy and then adjuvant therapy after surgery/radiotherapy. Another option for patients with high-risk prostate cancer is radical prostatectomy. Three to six months after prostatectomy, it is possible to start adjuvant radiotherapy in men with a high risk of local recurrence of cancer when the PSA value is

undetectable (<0.2 ng/ml) and based on pathological features from the prostatectomy sample. In patients with a rising or detectable PSA after radical prostatectomy, salvage EBRT may be administered. Pre-chemotherapy with Sipuleucel-T (the first therapeutic cellular immunotherapy), Abiraterone (a 17 α-hydroxylase/C17,20 lyase (CYP17A1) inhibitor) or Radium-223 is followed by Docetaxel (a microtubule inhibitor) as first line chemotherapy therapy and Abiraterone, Cabazitaxel (a microtubule inhibitor) or Radium-223 as post-chemotherapy treatment.

2. Aims of the studies

Derailment of tyrosine kinase signaling is known to be the driving force in different types of cancers. Small molecules are a new category of receptor tyrosine kinase inhibitors that are increasingly used in a personalized cancer therapy setting. The main aim of this thesis is to evaluate the anti-cancer effects of the multi TKI sorafenib. To achieve this purpose we worked on both a hematologic malignancy, multiple myeloma and a solid tumor, prostate cancer.

3. Results and discussion

Paper I: Sorafenib has potent antitumor activity against multiple myeloma in vitro, ex vivo, and in vivo in the 5T33MM mouse model

Multiple myeloma is a neoplastic B-cell disorder characterized by the activation of multiple of tyrosine kinase signaling cascades that induce 3 main downstream pathways, the Ras/Raf/MEK/ERK1/2, PI3K/AKT, and the JAK/STAT3. Targeting of multiple activated tyrosine kinase signaling cascades with chemical inhibitors maybe a promising therapeutic strategy against multiple myeloma.

In this study, we treated a panel of human myeloma cell lines comprising of U-266, LP1, OPM-2, NCI-H929, RPMI-8226, and Karpas 620 with different concentration of sorafenib. We found that apart from one cell line, U-266 which is relatively resistant to Sorafenib (50% cell death after 72 hours of treatment with 10 μM Sorafenib), the rest of the cell lines are sensitive within 24 hours of treatment with 10 μM sorafenib. This sorafenib concentration is a clinically relevant dose. We also showed that sorafenib induces apoptotic cell death characterized by mitochondrial depolarization, cytochrome c release, Bak and caspase-3 activation, and nuclear condensation/fragmentation. Our findings are in agreement with other sorafenib-myeloma studies (109-111).

We further investigated apoptotic cell death and found that caspase-3 and caspase-7 were activated even after 8 hours treatment. The pan-caspase inhibitor z-VAD-fmk could inhibit cell death in U-266 and RPMI-8226 but not in the rest of cell lines. That indicates that sorafenib can induce both caspase-dependent and caspase-independent cell death in myeloma cell lines. We also reported that the apoptosis-inducing factor (AIF) is released from mitochondria into the cytoplasm in cell lines which undergo caspase-independent cell death, possibly providing an explanation on the mechanism of cell death in these cell lines.

Since autophagy is a known cytoprotective mechanism we investigated whether multiple myeloma cells undergo autophagy in response to sorafenib. Sorafenib induced autophagy in some human myeloma cell lines, primary human myeloma cells and mouse myeloma cells. It is not clear whether sorafenib induced autophagy because of damaged mitochondria or due to the inhibition of tyrosine kinases. It is well established that damaged mitochondria are cleared by autophagy. Furthermore it is known that some tyrosine kinase inhibitors can induce autophagy primarily by shutting down the Src/PI3K/AKT/mTOR signaling cascade. Importantly, Co-treatment with sorafenib and 3-Methyladenine (3MA) which inhibits autophagy in early phase and chloroquine which inhibit autophagy in late stage, enhanced the cytotoxic efficacy of sorafenib.

To elucidate the importance of signaling pathways involved in sorafenib-induced cell death, we examined the PI3K/AKT and Raf/Ras/MEK/ERK pathways. In OPM-2 cells, which are PTEN-null, the constitutively active AKT is partially inhibited by sorafenib. In the rest of the cell lines phospho-AKT level were rather increased or did not change. Apart from the LP-1 cell line that does not express Bim, the Bim level were downregulated in response to sorafenib, indicating that Bim is not a mediator of sorafenib-induced cell death. Udi *et al.* and Ramakrishnan *et al.* have also showed decrease in ERK phosphorylation in other myeloma cell lines (109, 110).

Mcl-1 has been shown to be an important survival factor for multiple myeloma (264). The cytoprotective effect of the Ras/Raf/MEK/ERK signaling cascade is partly mediated by the inhibition of degradation and subsequent stabilization of Mcl-1 proteins levels. Mcl-1 protein level were downregulated in all myeloma cell lines and did not change by using a MEK inhibitor, U0126. This indicates that the observed Mcl-1 downregulation by sorafenib is independent of the Ras/Raf/MEK/ERK pathway.

Sorafenib induced cell death in freshly isolated $CD138^+$ multiple myeloma cells from newly diagnosed patients. To mimic the bone marrow microenvironment effect, we co-cultured myeloma cells with BMSCs. BMSC were not found to exert any effect on sorafenib-induced cell death in U-266 cells but were protective in RPMI-8226 and potentiating cell death in OPM-2 cells. Sorafenib was very potent in inducing cell death in all of the patient samples. While BMSCs cells protected the $CD138^+$ primary multiple myeloma cells from spontaneous cell death in the co-culture setting, primary cells, when cultured, were still sensitive to sorafenib treatment.

In line with the ability of sorafenib to potently downregulate Mcl-1 protein levels, we found that the combination of sorafenib with the Bcl-2 antagonist, ABT-737, potentiates sorafenib-induced cell death on some cell lines and even overcomes BMSC protection in RPMI-8226 cells. This combination is also very effective in patient samples. We also demonstrated that sorafenib is effective in both a bortezomib resistant cell line and primary samples.

We further investigated the effect of sorafenib in the 5T33MM myeloma mouse model in collaboration with Professor Karin Vanderkerken at Vrije Universiteit Brussel. Mice treated with either sorafenib (60 mg/kg oral gavage daily) or vehicle for three weeks. Mice treated with sorafenib showed significantly increased survival, reduced tumor growth and decreased serum M component. Treatment with sorafenib significantly decreased microvessel density by 60% in the bone marrow of treated 5T33MMvv mouse, compared with vehicle-treated mice. No adverse side effects or toxicity were observed as evaluated by behavior, body weight, histologic, and hematologic examinations. Sorafenib was also found to inhibit ERK1/2 phosphorylation, induced caspase activation and autophagy in multiple myeloma cells isolated from the bone marrow of the 5T33MM mice.

Paper II: Sorafenib induces apoptosis and autophagy in prostate cancer cells in vitro

The activation of both receptor tyrosine kinases (RTK) and that of nonreceptor tyrosine kinases (NRTK), such as Src and LCK, has been well described. Because of the critical role of RTK and NRTK in prostate cancer, they constitute prospective therapeutic targets. Sorafenib, a multi-tyrosine kinase inhibitor maybe a potentiate drug that can be used in prostate cancer treatment.

We demonstrated that incubation with sorafenib for 72 h caused a dose-dependent decrease in cell viability in two hormone refractory (PC3 and DU145) and one hormone responsive (22Rv1) prostate cancer cell lines. While 22Rv1 was the most sensitive cell line, DU145 cells showed an intermediate sensitivity and PC3 cells were the least sensitive. Sorafenib decreases mitochondrial membrane potential ($\Delta\Psi m$) in all cell lines and induces apoptosis.

Overexpressing the anti-apoptotic Bcl-2 protein in DU145 cells inhibits sorafenib-induced apoptosis. We also demonstrated that sorafenib induces autophagy and inhibits ERK phosphorylation in these prostate cancer cell lines.

Paper III: Targeting of distinct signaling cascades and cancer-associated fibroblasts define the efficacy of Sorafenib against prostate cancer cells

Following the demonstration that sorafenib is effective in prostate cancer, we investigate the mechanisms that sorafenib is effective against prostate cancer cell liens.

In this study we investigated the mechanisms of sorafenib induced cell death in non-metastatic (22Rv1) and metastatic (PC3) prostate cancer cell lines, showing that 22Rv1 cells are more sensitive than PC3 cells. Treatment with sorafenib induced a modest decrease in mitochondrial membrane potential ($\Delta\Psi m$) in 22Rv1 but almost complete decrease in PC3. Activation of caspases, cleavage of Bax and PARP and processing of AIF are seen in 22Rv1 after treatment with sorafenib but in PC3, there was only a small increase in active caspase-7 and PARP cleavage after treatment.

We determined whether and to which extent sorafenib might mediate its pro-apoptotic action by inhibiting the Ras/Raf/MEK/ERK pathway. In 22Rv1 ERK1/2 is constitutively phosphorylated but not in PC3 and consequently Sorafenib can only inhibit ERK activation in 22Rv1 cells. Chemically inhibition of MEK1 with U0126 by itself induced cell death in 22Rv1 cells. Constitutive activation of MEK1 by MEK1 overexpression with a MEK1-DD construct, attenuated the Sorafenib-induced inhibition of ERK1/2 phosphorylation and Bad dephosphorylation. However it did not alter Sorafenib-induced Mcl-1 downregulation and cleavage. MEK1-DD overexpression significantly inhibited Sorafenib-induced cell death in 22Rv1 cells. These data suggest that 22Rv1 cells require an active Ras/Raf/MEK/ERK pathway to survive and interrupting this pathway by sorafenib leads to the activation of Bad and consequent cell killing. Oh *et al.* has also shown decrease in ERK phosphorylation in 22Rv1 and LNCaP cell lines (120).

In PC3 cells, PTEN is not expressed leading to an uninhibited and constitutively active PI3K/AKT pathway. However, sorafenib inhibits phosphorylation of Src and AKT in PC3 cells. On the other hand overexpression of constitutively active AKT construct protects PC3 from sorafenib-induced cell death. Knockdown of Bim partially protected PC3 cells from sorafenib-induced killing, supporting an involvement of the PI3K/AKT/Bim axis in Sorafenib-mediated PC3 cell death. Oh et al has also reported the similar finding (120).

Treatment of 22Rv1 and PC3 with sorafenib induces LC3 lipidation, p62 degradation, and LC3-GFP^+ cytoplasmic foci formation, all of which are characteristics of autophagy. Transient knockdown of Atg5 in 22Rv1 and PC3 leads to potentiation of sorafenib-induced cell death that confirms the cytoprotective role of autophagy in this context.

Sorafenib downregulates Mcl-1 in a time and dose-dependent manner and co-treatment of with sorafenib and ABT737 significantly improved the efficacy of the therapy. On the other hand overexpression of Mcl-1 has a protective effect against Sorafenib. These data indicate that the

anti-apoptotic Bcl-2 family members Mcl-1, Bcl-2 and Bcl-xL protect prostate cancer cells from sorafenib-induced cell death.

Co-culture of PC3 and 22Rv1 with CAFs protect them against sorafenib induced cell death. However, treatment with sorafenib plus ABT737 re-established the sensitivity of 22Rv1 and PC3 cells to cell killing in spite of the presence of CAFs. As it has been mentioned before, 22Rv1 cells require an active Ras/Raf/MEK/ERK pathway to survive and that interrupting this pathway by sorafenib leads to the activation of Bad and consequent cell death. The presence of CAFs prevents the sorafenib-induced inhibition of ERK1/2 phosphorylation in 22Rv1 cells. In PC3 cells AKT phosphorylation is decreased after sorafenib treatment which leads to cell death but AKT phosphorylation and Bcl-xL protein levels were sustained in the presence of CAFs, thus providing survival signals for PC3 to resist sorafenib-induced cell death.

Paper IV: Atg5-independent autophagy promotes sorafenib-induced necroptosis

During studying the role of autophagy in sorafenib-induce cell death in prostate cancer cell lines, we found that autophagy is cytoprotective in PC3 and 22Rv1cell lines but it is cytotoxic in DU145 cell line.

In this study we found that the DU145 cell line does not express *ATG5,* which is necessary for LC3 lipidation. Despite the lack of Atg5 expression in DU145 cells, treatment with sorafenib induces autophagy and revealed intracellular structures characteristic of autophagosomes. Formation of LC3-GFP^{+} foci and RFP foci in stably transfected DU145 with LC3-GFP and LC3-GFP-RFP respectively, are evidences for induction of Atg5-independent autophagy. We showed the similar effect in MEF *Atg5-/-* cells treated with Sorafenib. We found that neither the pancaspase inhibitor zVAD.fmk nor the caspase-9 inhibitor LEHD.fmk could block sorafenib induced cell death. We could not detect caspase-3 activity or cleavage of the caspase substrate PARP in DU145 cells treated with sorafenib. Moreover, there were no changes observed in Bak, Bax, Bcl-xL and AIF protein levels, nor could any activation of Bak and Bax be detected.

Knockdown of ULK1 in DU145 cells leads to a potent decrease of sorafenib-induced cell death. Furthermore, stable transfection of DU145 cells with Beclin 1 shRNA constructs protected these cells from sorafenib-induced cell death. Importantly reconstitution of Atg5 expression in DU145 cells rescued them from sorafenib-induced cell death. Collectively these data demonstrate that sorafenib induced autophagy in DU145 cells is cytotoxic due to a lack of Atg5 expression and either inhibition of early autophagy or restoration of Atg5 expression protects the cells from sorafenib-induced cell death.

Treatment of DU145 cells with the RIPK1 inhibitor, necrostatin 1 (Nec1), or transient knockdown of RIPK1, inhibited sorafenib-induced cell death but had no effect in PC3 cells, which undergoes a caspase-dependent cell death. Importantly, ectopic expression of Atg5 in DU145 cells also protected from sorafenib-induced cell death and co-treatment with necrostatin 1 did not further inhibit cell death. Immunocytochemical staining and proximity ligation assay (PLA) of DU145 cells for p62 and RIPK1 revealed that upon treatment with sorafenib there is an increase in co-localization between these two proteins. Knocking down of p62 in DU145

cells decreased the basal levels of RIPK1 protein suggesting that p62 protects RIPK1 from degradation. These data indicate that the lack of Atg5 expression is important for the activation of necroptosis.

4. Conclusions and Future perspectives

Paper I:

Sorafenib alone or in combination with other chemicals like the Bcl-2/Bcl-xL antagonist (ABT737) or early or late stage autophagy inhibitors (3MA and chloroquine) is cytotoxic in multiple myeloma cell lines, primary human myeloma samples, mouse myeloma model and even both bortezomib resistant cell line and patient sample.

Paper II:

Sorafenib induces autophagy and apoptosis in prostate cancer cell lines.

Paper III:

Despite differential downstream signaling pathways activated in prostate cancer cell lines, sorafenib can induce apoptosis in both a hormone refractory (PC3) and a hormone responsive cell line (22Rv1). Cytoprotective autophagy was detected after sorafenib treatment of prostate cancer cell lines which shows important role of autophagy in the efficacy of anti-cancer treatment. Mcl-1 inactivation is further required for sorafenib-induced cell death. Co-treatment with ABT737 significantly increased sorafenib efficacy, and this combination can overcome the protective effect of the microenvironment in prostate cancer.

Paper IV:

Sorafenib can induce necroptosis in Atg5-deficient prostate cancer cell line. In this case autophagy is cytotoxic and co-treatment with an autophagy inducer may increases the efficacy of sorafenib.

In these translational studies, we found that sorafenib is effective in multiple myeloma in different modalities. Since sorafenib is used in clinic and we showed that it is effective not only in multiple myeloma cell lines and patient samples but also in myeloma mouse model, our finding provides a good rational to use sorafenib in clinical trial. Sorafenib can be used as a single agent or with combinational therapy based on individual characteristic of myeloma patient.

We need further investigation to determine the upstream targets of sorafenib in prostate cancer cell lines and testing the efficiency of sorafenib in prostate cancer mouse model.

5. Acknowledgments

This thesis is the result of around five years of reading, thinking, running different experiment, challenging, repeating, optimizing, interpreting the result, repeating again and again, success and mistakes, writing, disappointment, hope and the most important of all connecting with great people especially in department of oncology-pathology. I want to express my sincere gratitude to all the people that I have met or been working with during my PhD study at the department of oncology-pathology.

I would like to say my thanks to my main supervisor **Docent Theocharis Panaretakis** for the opportunity to do my PhD in his lab and helping me to prepare my thesis. My co-supervisor **Professor Dan Grandér**, which helped me generously to come to Sweden. I started my studies on cancer in his lab around 6 years ago and I have learned many things from him. As I heard from others and experienced myself many times, he is a real problem solver not only in science but also in personal problems. During these years every time I needed help, he is the first person that I trust and believe that he has the solution. His great personality and leadership make everything tolerable in the department. His teaching technique is unique. I hope I will stay for my postdoc in the department and learn more from him. My co-supervisor **Professor Sten Nilsson** which I learned a lot about prostate cancer from him and I hope learn more in the future. And my former co-supervisor **Professor Boris Zhivotovsky**, which I have never been participating in a course better than his course on apoptosis. He defines everything patiently and nobody becomes tired when listening to Boris even for hours. His course was the only course that I wished that never reaches the end. I learned a lot also in blue group meeting group on Tuesdays in IMM which Boris leads.

I have great opportunity to work in the same place that great scientists work and I learn a lot directly and indirectly. **Professor Arne Östman**, **Docent Bertrand Joseph** whose scientific achievement especially during recent years have been encouraging for junior scientists, **Professor Lars Holmgren** the former PhD student director, **Docent Andreas Lundqvist** the current PhD student director, **Docent Marianne Farnebo** who she is one of the most talented scientist I have ever met, **Docent Angelo De Milito**, which I hope to work with in future, **Docent Katja Pokrovskaja Tamm** who taught me many techniques when I came to the department, **Docent Maria Shoshan** and also **Docent Svetlana Bajalica Lagercrantz**, whose group I will hopefully join for my postdoc right after my dissertation.

Lotte who taught me many techniques when I came here and you always had time to help me. When I have problem in experiments, you are the first whom has the answer.

Lena who helped me a lot in prostate cancer experiments and for teaching me Swedish.

I want to say my special gratitude to three friends, **Ali** for the great time we have both at work and outside. You are a great man and because of your friendship I don't feel that I am far from home. Also for the long night that you stayed awake and went through my thesis and help me to edit and revise it. **Claire** who is working as postdoc in our group. For great time that we

have at work and lunch time. I will miss you at my dissertation, however we are in the communication era, maybe we can set up a webcam contact during the dissertation. And my dear colleague and Swedish teacher **Sophia**, whom we had a lot of adventures in tackling the problems. Even now that you are far from us, still you are helping me with drawing figures and designing the cover of my thesis. You did a great job and I wish all the best for you in your new group and life. I recommend everyone who needs scientific figures to talk with you first.

Thanks also to former member of the group **Patricia**, **Sebastian**, **Georgia**, **Maria** and of course **Qiao** that I never forget her. I wish you the best in your future life.

Neither would my study have been possible without co-authors of the publications. I want to say my thanks to all co-authors that I have not mentioned before for their great contribution in the publications.

Hendrik De Raeve, Charlotte Fristedt, Vladimir Gogvadze, Astrid Gruber, Edward Laane, Anders Österborg, Helena Jernberg-Wiklund, Fredrik Celsing, Karin Vanderkerken, Anders Ullén, Lena Thyrell, Lars Egevad, Peter Wiklund, Guido Kroemer.

I want to also say special thanks to **Anna-Maria**, the Queen, for lunch discussions and being a good friend. I hope you find a position and continue with your PhD.

I also would like to say some words with about other people in the department or other departments, being friend with you made my PhD easier than I expected. I hope I will stay at Karolinska Institutet for my postdoc and still enjoy communicating with you. Here I want to send my regards to **Mehdi**, **Sara**, **Kaveh**, **Amir**, **Omid**, **Mohammad**, **Hanif**, **Fariba**, **Hamid**, **Shahab**, **Per**, **Sofia** , **Martin**, **Johanna**, **Jens**, **My**, **Zheng**, **Linda**, **Mikael**, **Carolina**, **Ma**, **Lisa**, **Marianne**, **Rainer**, **Iulian**, **Iryna**, **Miguel**, **Edel**, **Linda**, **Erik**, **Sebastian**, **Lina**, **Naveen**, **Xianli**, **Satar**, **Hojir**, **Markus**, **Jeroen**, **Janna**, **Helene**, **Elin**, **Carina**, **Paola**, **Mao, Christos, Elizabeth, Matheus, Jason, Pinelopi, Ghazal, Shiva, Emma, Sanaz, Salah** and **Reza.**

I also want to express my appreciation to some people who are working behind the scene and without their help nothing goes forward. **Anne**, **Maria, Monica**, **Erika**, **Elle**, **Elisabeth**, **Sören**, **Eva-Lena**, **Ann-Gitt** and **Juan**.

I also want to thank my family and friends outside the lab. **Iman**, **Sima**, **Sam**, **Shirin**, **Afshin**, **Nasim**, **Hossein**, **Soudabeh, Maral**, **Azadeh** for their great help and warm-heart and kindness.

My parents Mohammad Hossein and Sedigheh and my brother, **Shahram** and my sister, **Mona** for your endless support during these years that I have been in Sweden.

My son, **Parham** that many times that you needed me, I was busy with my academic stuff and I miss your childhood time, even this summer with very nice weather I had to go to library and write my thesis instead of spending some time with you.

And finally my wife **Ghazaleh** that without your support I could never manage to come to Sweden and start my PhD. This is a long way and you took the difficult part to take care of family and I took the easiest part to study. I hope in future I have the opportunity to compensate your help.

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