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# Tumor Microenvironment and Cellular Stress

## Signaling, Metabolism, Imaging, and Therapeutic Targets

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# Preface

The tumor microenvironment has long been recognized for the critical roles it plays in both promoting the malignant progression of solid tumors and modifying the response of solid tumor cells to cytotoxic or targeted therapy. This book comprises 12 chapters that provide critical insights into how changes in the tumor microenvironment affect tumor metabolism, cell stemness, cell viability, genomic instability, immune modulation, and metastasis. In addition, there is also a chapter devoted to magnetic resonance imaging techniques used to visualize changes in tumor invasion, angiogenesis, and inflammation. The work described in these chapters was presented at the first Aegean meeting on the Tumor Microenvironment and Cellular Stress held in Crete, Greece, in October 2012.

Most solid tumors have a microenvironment that differs from their normal tissue counterpart because of malformed vasculature that is insufficient to be able to adequately perfuse tumor tissue. The inadequacy of the vasculature supply to tumors leads to areas that are hypoxic, or low in oxygen. One of the major changes that a tumor cell must surmount is the metabolic changes imposed by a low oxygen environment. Chapters 1, 5, and 10 describe how hypoxic tumor cells adapt and respond to decreased oxygen levels. Hypoxic tumor cells rely heavily on glycolysis, increasing their uptake of glucose by select glucose transporters, increasing the expression of glycolytic genes, inhibiting mitochondrial respiration, and increasing their levels of lactate. The roles of the hypoxia-inducible transcription factors HIF-1 and HIF-2 in regulating these metabolic changes are nicely described in Chapt. 1. The metabolism of glutamine is also discussed, especially as it relates to lipid synthesis. Perhaps the most interesting aspect of this chapter is the proposed therapeutic approaches that may be used to exploit the metabolic changes induced by the hypoxic tumor microenvironment. Chapter 5 describes research on the mitochondria voltage-dependent anion channel (VDAC1). This channel is located in the outer mitochondrial membrane and is responsible for the transfer of a large number of charged and uncharged molecules through changes in membrane voltage. It is interesting to note that hypoxia induces the expression of a C-terminal truncated form of VDAC1

(VDAC1-ΔC). This truncated form is associated with a high output of adenosine triphosphate and resistance to chemotherapy, suggesting that targeting this truncated form of VDAC1 may increase the chemosensitivity of tumor cells. Chapter 10 presents a in-depth summary of the hypoxia-inducible microRNA miR-210, which, among other functions, serves to regulate mitochondrial metabolism and oxidative stress. miR-210 is a robustly induced microRNA under hypoxic conditions, and its role in regulating metabolism may in fact be one of its most critical functions under low oxygen conditions. Taken together, these chapters present a comprehensive picture of metabolic changes induced by hypoxia and the genes that control them.

The importance of niches as regions of tumors that can modulate the growth and aggressive nature of tumor cells is a new concept in the study of the tumor microenvironment. Hypoxia and cancer stem cells are the subjects addressed in Chap. 2. In particular, the concept that hypoxia can modify the "stemness" of a tumor cell is a consequence of hypoxia inducing a niche where tumor stem cells can be arrested in an undifferentiated state through interactions with undifferentiated stromal cells. Furthermore, this chapter proposes that cancer stem cells located in the hypoxic niche may exist in a different state than other cancer stem cells based on analysis of stem cell markers. Hypoxia can affect the generation of cancer cell stemness through the HIF transcription factor as well as chromatin-modifying genes. This is an intriguing hypothesis that is supported by a growing amount of literature and has important therapeutic implications for tumor progression and responses to therapy. Chapter 3 discusses the role of hypoxia in promoting tumor cell metastasis through the increased expression of genes regulating the invasion of tumor cells trough the basement membrane, intravasation of tumor cells into the circulation, survival of tumor cells in circulation, extravasation of tumor cells out of circulation and into tissue, and colonization of a new tissue. The authors also include a circumspect and relevant analysis of hypoxia and the formation of the premetastatic niche, which is formed by tumor cells secreting factors that increase the ability of tumor cells to grow in a site distant from the primary tumor. The role of hypoxia and the genes and proteins it regulates in the formation of the premetastatic niche represents new targets for therapeutic intervention. Thus, these two chapters present new functions for hypoxia in regulating tumor stemness and the premetastatic niche.

Hypoxia has long been recognized as an impediment for cytotoxic therapies such as ionizing radiation and chemotherapy. This information is reviewed well and expanded on in Chap. 7. The role of the microvasculature, tumor stroma, the extracellular matrix, and resident and infiltrating immune cells in influencing the responsiveness of tumors to radiotherapy are described in a logical and clinically relevant manner. This chapter also makes the point that to date there has yet to be a successful targeted therapy against hypoxic tumor cells. The challenge of developing a targeted therapy against hypoxic tumor cells represents the focus of Chap. 6. The chapter is a must-read because it relates the history of developing agents to tackle the hypoxic problem, starting with hypoxic sensitizers, moving on to hypoxic cytotoxins, and ending with targeting the hypoxia response pathway. A different approach to selectively targeting hypoxic tumor cells is brought forth in Chap. 8 through the concept of "synthetic lethality." This concept of cell killing is based on

work with lower eukaryotes, which showed that a mutation in one of two different genes had no effect on cell survival, but if both genes were mutated at the same time, lethality would result. This chapter focuses on the process of autophagy and the genes and pathways that regulate this process. There is ample discussion of autophagy in tumor progression and resistance to therapy. The intriguing concept of activating autophagy to induce cell death and under what circumstances that would be effective is presented in a concise manner. The poster child for targeted therapy has been anti-angiogenic therapy. First described by Folkman and his colleagues many decades ago, anti-angiogenic therapy received approval from the US Food and Drug Administration for treating solid tumors both as a monotherapy and in combination with other agents. Chapter 4 presents the events that led to the development of anti-angiogenic therapy, its current success, and, most important, the reasons underlying the development of resistance to anti-angiogenic therapy. The presentation of a number of possible approaches to overcome the resistance to anti-angiogenic therapy, such as targeting pro-angiogenic myeloid cells, is the most exciting aspect of this chapter. Without question, understanding the mechanism of resistance to anti-angiogenic therapy will drive the next generation of therapeutics that should exhibit more durable benefits. These chapters present an up-to-date picture of the importance of hypoxia in cytotoxic therapy and targeted therapy.

While the conventional thinking is that hypoxic tumor cells are resistant to cytotoxic radiotherapy and chemotherapy based on a reduction of free radical formation and cessation of cell cycling, a different point of view is presented in Chap. 9. This chapter relates the concept that hypoxic cells are deficient in homologous recombination and prone to exhibit gene amplification and chromosome instability. These findings suggest that hypoxia inhibits genome maintenance and integrity and can promote tumor aggressiveness by increasing genomic instability. By understanding the mechanisms underlying the repair deficiency induced by exposure to hypoxia, the potential to selectively target hypoxic tumor cells using synthetic lethality could be a new avenue for fruitful investigation. This chapter provides several examples of how this form of synthetic lethality could be developed for therapy.

Tumor hypoxia has often tried to escape immune surveillance. An important pathway in immune surveillance against cancer has been the complement system. Cancer cells have, unfortunately, developed inhibitory mechanisms against complement activation that allow them to escape immune attack from the body and impede the activity of monoclonal antibody-directed therapy. This Chapter 11 describes our most up-to-date understanding about the activation and function of the complement system in human tumors and the paradoxical role of complement in promoting tumor growth in the face of an inflammatory signal. The impact of inflammation and hypoxia due to poor perfusion and increased interstitial fluid pressure in tumors can be visualized using magnetic resonance imaging. Chapter 12 describes the elegant use of tracking dyes or contrast agents to follow the fate of cells in tumors and their interactions with other components of the tumor microenvironment such as stroma, blood vessels, and immune cells. Imaging of the tumor microenvironment is useful for tumor staging as well as monitoring the efficacy of therapy directed at a specific tumor compartment.

We are grateful to all the authors who contributed these outstanding chapters to this book. We are also grateful to all the staff at Springer, especially Portia Wong, Development Editor, as well as Fiona Sarne and Gregory Baer, who have worked dilligently to get this book to publication. Their assistance has been greatly appreciated.

We hope that this book entices all those who do not study the tumor microenvironment to consider working in this field.

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# Contents

# Chapter 1
# Hypoxia and Metabolism in Cancer

**Karim Bensaad and Adrian L. Harris**

**Abstract** Interest in targeting metabolism has been renewed in recent years as research increases understanding of the altered metabolic profile of tumor cells compared with that of normal cells. Metabolic reprogramming allows cancer cells to survive and proliferate in the hostile tumor microenvironment. These metabolic changes support energy generation, anabolic processes, and the maintenance of redox potential, mechanisms that are all essential for the proliferation and survival of tumor cells. The metabolic switch in a number of key metabolic pathways is mainly regulated by genetic events, rendering cancer cells addicted to certain nutrients, such as glutamine. In addition, hypoxia is induced when highly proliferative tumor cells distance themselves from an oxygen supply. Hypoxia-inducible factor 1α is largely responsible for alterations in metabolism that support the survival of hypoxic tumor cells. Metabolic alterations and dependencies of cancer cells may be exploited to improve anticancer therapy. This chapter reviews the main aspects of altered metabolism in cancer cells, emphasizing recent advances in glucose, glutamine, and lipid metabolism.

**Keywords** Cancer • Hypoxia • Metabolism • Glycolysis • Glutaminolysis • Mitochondrial respiration • Lipids • Therapy • Synthetic lethality

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## 1.1 Introduction

### *1.1.1 Metabolism in Normal and Cancer Cells*

Cancer is a genetic disease involving numerous pathways that are mainly induced by gain of function mutations that activate oncogenes or loss of function mutations, which inhibit tumor suppressor genes. These changes primarily lead to the dysregulation of cell proliferation. More than a decade ago, in an influential article titled the "Hallmark of cancer," Hanahan and Weinberg (2000) organized these pathways into six major biological capabilities acquired during the multiple steps of cancer development. These researchers recently published an up-to-date version of their initial article, describing reprogramming of energy metabolism as one of two new emerging hallmarks of cancer (Hanahan and Weinberg 2011). Cancer cells gain an undeniable survival advantage by reprogramming metabolism to respond to environmental stress, a process known as metabolic transformation. Metabolism can be described as the pathways required for the maintenance of life within a living cell. In metabolism, some substrates are broken down to yield energy while other substances necessary for cell survival are synthesized. The amount of adenosine triphosphate (ATP) required for cell proliferation is, surprisingly, not radically different from that required for resting cells (Kilburn et al. 1969). Cancer cells undergo metabolic reprogramming as their energy requirement increases to fuel increased growth and proliferation. It is important to note that tumor cells also have high need of carbon building blocks, which are provided by glucose, glutamine, and fatty acids, and cofactors (nicotinamide adenine dinucleotide phosphate [NADPH] and nicotinamide adenine dinucleotide [NADH]) for growth and proliferation in the changing tumor microenvironment.

Otto Warburg, a German biochemist and Nobel laureate, first observed increased glycolytic flux and lactate production in tumor ascites (Warburg 1956). Under aerobic conditions, normal cells mainly produce energy through mitochondrial oxidative phosphorylation using pyruvate derived from glucose through the glycolytic pathway. Under anaerobic conditions, through a process called the Pasteur effect, energy is essentially provided by glycolysis, and pyruvate is mostly converted to lactate that is preferentially exported from the cells. Most cancer cells undergo a metabolic shift toward glycolysis to produce energy and toward anabolic pathways using metabolic intermediates from the glycolytic pathway to synthesize proteins and lipids independently of oxygen availability. These different processes favorably promote rapid growth and proliferation of tumor cells (Cairns et al. 2011). In cancer cells, the upregulation of glycolytic flux, with lactate production from pyruvate, even in the presence of abundant oxygen, is now known as the Warburg effect (Koppenol et al. 2011; Vander Heiden et al. 2009). The Warburg effect is the molecular basis of the diagnostic tumor imaging technique called fluorodeoxyglucose positron emission tomography ($^{18}$F-FDG-PET), which allows fluorodeoxyglucose metabolism in tissues with high metabolic activity, such as most types of tumors, to be assessed.

Substrates other than glucose can be used in the mitochondria for energy production, including glutamine as well as long-chain fatty acids (LCFAs) (Dang 2012; Locasale and Cantley 2011). In this chapter, we describe these various metabolic pathways, their regulation under oxygen deprivation, and their importance in the development of cancer.

### *1.1.2 Hypoxia and Cancer*

Oxygen is an essential molecule for cell survival because it is used as the final acceptor in mitochondrial respiration for energy production. Hypoxia refers to lower-than-normal oxygen conditions, with oxygen ($O_2$) concentrations around 21 % (150 mmHg) in ambient air and 2–9 % (around 40 mmHg) in most healthy mammalian tissues. Hypoxia is defined as less than 2 % $O_2$, whereas anoxia (or severe hypoxia) is defined as less than 0.02 % $O_2$ (Bertout et al. 2008). Low oxygen availability is associated with inflammation (Murdoch et al. 2005), necrosis, and/or abnormal neovascularization. In addition, highly proliferative cancer cells can outgrow their blood supply and trigger hypoxia. In the latter situation, hypoxia has a major role in metabolic reprogramming of tumor cells and is also considered to be a hallmark of cancer (Hanahan and Weinberg 2011). Hypoxia is thought to promote invasiveness and metastasis (Harris 2002). The hypoxic environment of tumors leads to the stabilization of hypoxia-inducible factors (HIFs).

HIFs are dimeric protein complexes that consist of an α-subunit (HIF-1α or HIF-2α) and a β-subunit (HIF-1β). HIF-1α is expressed ubiquitously, whereas HIF-2α, also known as endothelial PAS domain protein 1 (EPAS1), was initially detected in endothelial cells but is also selectively highly expressed in a smaller number of tissues (Patel and Simon 2008). HIF-1α and HIF-2α activities are regulated by levels of oxygen. Under normoxic conditions, both of these proteins are degraded by the proteasome machinery. HIFs are targeted for ubiquitination by oxygen-sensitive prolyl-hydroxylases (PHDs) and the von Hippel–Lindau (VHL) tumor suppressor protein. In normoxia, Factor Inhibiting HIF-1 (FIH) also leads to inactivation by hydroxylation of HIFs. In hypoxic conditions, there is stabilization of HIF-1α and HIF-2α because hydroxylases, the VHL tumor suppressor protein, and factor-inhibiting HIF-1 are all inhibited by low oxygen availability. When stabilized, HIFs can bind to specific regulatory elements in the promoter of their target genes and induce their expression (Semenza 2012).

HIF-1α and HIF-2α are differentially regulated by the $NAD^+$-dependent deacetylase sirtuin (SIRT) 1, a known stress-activated factor. SIRT1 is activated by elevation of the $NAD^+$-to-$NADP^+$ ratio and directly couples $NAD^+$ hydrolysis to the deacetylation of numerous transcription factors and cofactors, including HIFs. As a consequence, SIRT1 directly links metabolic status to gene expression by acting as a redox sensor, and it plays an important role in various pro-survival and metabolic activities (Haigis and Yankner 2010; Schug and Li 2011). SIRT1 deacetylates

specific lysine residues in HIF-1α and HIF-2α proteins, resulting in opposite downstream outcomes (Dioum et al. 2009; Laemmle et al. 2012; Lim et al. 2010). During normoxia, SIRT1 binds to HIF-1α and deacetylates it at Lys674. This deacetylation inactivates HIF-1α transactivation function by blocking p300 recruitment. During hypoxia, SIRT1 activity is reduced because of decreased $NAD^+$ levels associated with increased glycolytic flux. This results in HIF-1α retaining its acetylation status and remaining activated (Lim et al. 2010). Therefore, if glycolysis is inhibited and, as a consequence, $NAD^+$ levels are increased, even under hypoxia, SIRT1 is activated and results in HIF-1α inhibition (Lim et al. 2010). SIRT1 has a surprising opposite effect on HIF-2α: deacetylation stimulates activity of HIF-2α during hypoxia (Dioum et al. 2009).

Tumor hypoxia is mainly caused by defective vasculature in fast-growing solid tumor tissues, leading to diminished supply of oxygen and nutrients. This local lack of nutrients and oxygen triggers the formation of new blood vessels in the growing tumor. HIF-1α initiates angiogenesis by inducing Vascular Endothelial Growth Factor (VEGF, also know as VEGF-A) and many other angiogenic factors such as stromal-derived factor 1 (SDF1), placental growth factor (PGF), platelet-derived growth factor B (PDGFB), and angiopoietin 1 and 2 (ANGPT 1 and 2) (Chen et al. 2009; Hickey and Simon 2006; Rey and Semenza 2010). Tumor neovasculature is poorly developed and effective and thus leads to nutrients shortage and hypoxic stress. Adaptation to these conditions of intermittent hypoxia is essential for the survival and progression of cancer.

## 1.2 Glucose Metabolism

### *1.2.1 Glycolysis in Normal and Cancer Cells*

Glucose is transported from the circulation into cells via glucose transporters; it then is phosphorylated to form glucose-6-phosphate (G6P). G6P is then further phosphorylated and, after a series of reactions, is broken up into dihydroacetone-phosphate (DHAP) and glyceraldehyde-3-phosphate (G3P), which is converted to glycerol-3-phosphate for lipid synthesis or sequentially transformed to produce pyruvate. Pyruvate can be converted to acetyl-coenzyme A (CoA) in the tricarboxylic acid (TCA) cycle in the mitochondria or converted to lactate in the cytosol. G6P can take an alternative metabolic pathway, the pentose phosphate pathway (PPP), which generates ribose-5-phosphate for nucleotide synthesis and the byproduct NADPH for reductive biosynthesis. Finally, G6P can also be converted to glycogen for storage (Fig. 1.1).

**Fig. 1.1** (continued) glycogen phosphorylase liver form; *ROS* reactive oxygen species; *R5P* ribose-5-phosphate; *SFA* saturated fatty acid; *SCD1* stearoyl-CoA desaturase 1; *SDH* succinate dehydrogenase; *SCO2* synthesis of cytochrome C oxidase 2; *TIGAR* TP53-inducible glycolytic and apoptotic regulator; *TCA* tricarboxylic acid; *TPI* triose-phosphate isomerase; *TG* triglyceride; *UDP-GlcNAc* UDP-N-acetylglucosamine

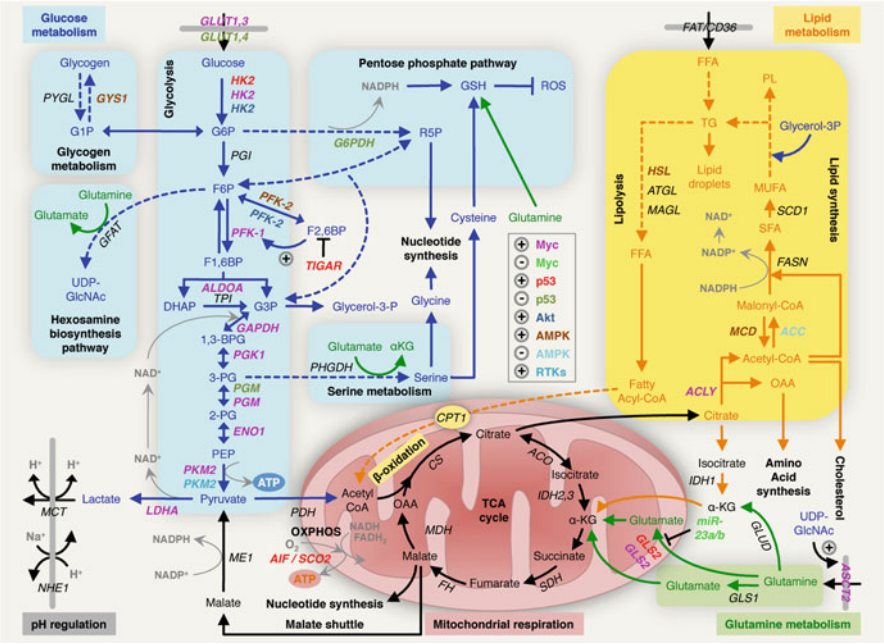


**Fig. 1.1** Regulation of cancer metabolism pathways by oncogenes and tumor suppressors. Metabolic enzymes are regulated by oncogenes – *Myc*, *Akt*, and receptor tyrosine kinases (*RTKs*) – and the tumor suppressors 5′ adenosine monophosphate-activated protein kinase (*AMPK*) and *p53*. Key metabolic pathways are represented within colored boxes: *blue* indicates pathways linked to glucose metabolism (glycolysis, pentose phosphate pathway, glycogen metabolism, hexosamine biosynthesis pathway, and serine metabolism); *pink* represents mitochondrial respiration, *green* represents glutamine metabolism, and *yellow* indicates lipid metabolism (lipid synthesis, lipolysis, and β-oxidation). pH regulation contributes to the control of intracellular acidity. The enzymes involved in metabolic pathways regulated by oncogenes or tumor suppressors are shown in *bold* and *colored* as indicated above. A *circled plus* or *minus* represents a positive or negative regulation by the indicated oncogenes or tumor suppressors. *Dashed arrows* represent multiple reaction pathways. *ACC* acetyl-CoA carboxylase: *α-KG* α-ketoglutarate; *ACLY* ATP citrate lyase; *ACO* aconitase; *ALDOA* aldolase A; *ATP* adenosine-5′-triphosphate; *ATGL* adipose triglycerides lipase; *AIF* apoptosis-inducing factor; *CoA* coenzyme A; *CS* citrate synthase; *DHAP* dihydroxyacetone phosphate; *ENO1* enolase 1; *FASN* fatty acid synthase; *FAT/CD36* fatty acid translocase; $FADH_2$ flavin adenine dinucleotide; *FFA* free fatty acid; *F1,6BP* fructose-1,6-bisphosphate; *F2,6BP* fructose-2,6-bisphosphate; *F6P* fructose-6-phosphate; *FH* fumarate hydratase; *GFAT* glucosamine fructose-6-phosphate amidotransferase; *GAPDH* glyceraldehyde 3-phosphate dehydrogenase; *GLS* glutaminase; *GLUD* glutamate dehydrogenase 1; *GSH* glutathione; *G1P* glucose-1-phosphate; *G3P* glyceraldehyde 3-phosphate; *G6P* glucose-6-phosphate; *G6PDH* G6P dehydrogenase; *GLUT* glucose transporter; *GYS1* glycogen synthase 1; *HK2* hexokinase 2; *HSL* hormone-sensitive lipase; *IDH* isocitrate dehydrogenase; *LDHA* lactate dehydrogenase A; *MCD* malonyl-CoA decarboxylase; *MCT* monocarboxylate transporters; *MDH* malate dehydrogenase; *ME1* malic enzyme 1; *miR* microRNA; *MAGL* monoacylglycerol lipase; *MUFA* monounsaturated fatty acid; *NHE1* $Na^+/H^+$ exchange protein 1; *NADH* nicotinamide adenine dinucleotide; *NADPH* nicotinamide adenine dinucleotide phosphate; *OAA* oxaloacetate; *OXPHOS* oxidative phosphorylation; *PDH* pyruvate dehydrogenase; *PEP* phosphoenolpyruvate; *PFK-1* phosphofructokinase 1; *PFK-2* 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; *PGI* phosphoglucose isomerase; *PGK1* phosphoglycerate kinase 1; *PGM* phosphoglycerate mutase; *PHGDH* phosphoglycerate dehydrogenase; *PKM2* pyruvate kinase M2; *PL* phospholipids; *PYGL*

The enzyme 6-phosphofructo-1-kinase (PFK-1) regulates a key step in glycolysis by controlling the conversion of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (F1,6BP). Four different genes (*pfkfb1–4*) encode another enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2), which has an essential role in regulating PFK-1 activity. PFK-2 is a bifunctional enzyme with both kinase and bisphosphatase activities that are catalyzed at different sites on each subunit of this protein. The kinase domain of this enzyme is localized within the $NH_2$-terminal part of the enzyme, and the bisphosphatase domain is in the COOH-terminal region. PFK-2/FBPase-2 regulates both the synthesis (through its kinase function) and the degradation (through its phosphatase function) of intracellular fructose-2,6-bisphosphate, the most potent positive allosteric activator of PFK-1. PFK-1 activity is increased in tumors and activated by oncogenes. Similarly, fructose-2,6-bisphosphate levels are also increased in tumors (Bartrons and Caro 2007; Yalcin et al. 2009).

Driver genetic mutations can directly regulate metabolic enzymes (Fig. 1.1). Oncogene status can drive some of these metabolic changes in tumor cells. Oncogenic Myc, oncogenic Ras, and Akt kinase (also known as protein kinase B (PKB)) promote an increase in glycolytic flux by upregulating the transcription of various metabolic genes (Levine and Puzio-Kuter 2010). *Myc* was the first oncogene to be linked to increased glycolysis in cancer cells, through the direct activation of almost all glycolytic enzymes, and lactate dehydrogenase A (LDHA), which converts pyruvate to lactate (Shim et al. 1997). Oncogenic Ras induces glycolysis by enhancing the stability of *Myc* (Sears et al. 1999). Akt kinase stimulates glycolytic flux through activation by mutated phosphoinositide 3-kinase (PI3K) (Elstrom et al. 2004). Mutations in tumor suppressor genes can also influence the glycolytic rate. More than 50 % of human tumors contain a mutation or deletion of the tumor suppressor gene *p53*. In addition to its role in cell cycle arrest and cell death, several recent studies have revealed a major role for *p53* in the regulation of metabolism (Maddocks and Vousden 2011; Vousden and Ryan 2009). *p53* can inhibit glycolysis by repressing the expression of the glucose transporters GLUT1 and GLUT4 and the glycolytic enzyme phosphoglycerate mutase (Kondoh et al. 2005; Schwartzenberg-Bar-Yoseph et al. 2004). p53 also induces the expression of the TP53-inducible glycolytic and apoptotic regulator (TIGAR). TIGAR is involved in the regulation of PFK-1 activity, the key enzyme in the glycolytic pathway (Bensaad et al. 2006, 2009). Therefore, TIGAR inhibits glycolysis and induces the PPP, leading to the removal of intracellular reactive oxygen species (ROS).

The increased uptake of glucose and its conversion into lactate causes lactate accumulation and intracellular acidification. While acidification of the tumor microenvironment promotes tumor cell invasion and metastasis formation, intracellular pH must remain alkaline for cancer cells to survive (Chiche et al. 2010). Several mechanisms have been implicated in the pH regulation of cancer cells (Fig. 1.1). The levels of lactate in the cytosol are dependent on the regulation and expression of monocarboxylate transporters (MCTs) on the membrane of tumor cells. Lactate transport across the plasma membrane through MCTs is coupled to the symport of protons ($H^+$) (Halestrap and Wilson 2012). Expression of MCT1 and MCT4 has

been shown to be elevated in several types of tumors as compared to normal tissues, and it correlates with poor prognosis and disease progression (Chen et al. 2010; Pinheiro et al. 2009, 2010). Furthermore, MCTs have a role in cellular pyruvate uptake to fuel mitochondrial respiration and support proliferation of breast cancer cells (Diers et al. 2012). Another pH regulation mechanism involves the $Na^+/H^+$ exchanger protein called NHE1. NHE1 has recently been shown to be important for tumor growth, cell migration, and metastasis formation (Amith and Fliegel 2013; Loo et al. 2012).

As previously mentioned, glycolysis can rapidly produce energy, especially under low oxygen tension, but cells also require precursors for biosynthesis for growth and proliferation, and reducing equivalents for antioxidant mechanisms. During the final step of glycolysis, phosphoenolpyruvate (PEP) is converted to pyruvate, a reaction driven by the rate-limiting enzyme pyruvate kinase (Fig. 1.1). There are two isoforms of pyruvate kinase, pyruvate kinase isozyme type M1 (PKM1) and pyruvate kinase isozyme type M2 (PKM2), which are differentially expressed in normal and cancer cells. While PKM1 is mainly expressed in normal tissue, PKM2 is mainly expressed in cancerous tissue (Christofk et al. 2008a, b). However, a more recent study has shown that both PKM1 and PKM2 are expressed in normal and cancer tissues, but PKM2 is the prominent isoform in cancer cell lines (Bluemlein et al. 2011). PKM2 can be phosphorylated by oncogenic tyrosine kinases. This leads to a switch from its active tetrameric form to a much less active dimeric form and therefore contributes to anabolic metabolism in proliferating cancer cells (Christofk et al. 2008a, b; Vander Heiden et al. 2010). PKM2 can also be inactivated by ROS and contributes to oxidative stress. This regulation contributes to cellular antioxidant response by increasing the flux of phosphorylated glucose through the PPP to generate NADPH and remove intracellular ROS (Anastasiou et al. 2011).

### *1.2.2 The Pentose Phosphate Pathway*

Cell metabolism can lead to the continuous generation of ROS that are to a large extent produced by mitochondria. The cellular response to ROS is complex and cell- and context-dependent, but in general it ranges from the stimulation of proliferation and migration in response to low levels of ROS, through genotoxic damage at intermediate levels, to the induction of senescence or cell death as ROS levels rise. These widely diverse responses to ROS also are reflected in their role in tumorigenesis, with evidence that modulation of ROS levels can have both promoting and suppressing effects on cancer progression (Gupta et al. 2012). There is a requirement for NADPH produced by the PPP to generate reduced glutathione (GSH) and thereby decrease the level of ROS (Fig. 1.1). GSH, a tripeptide with a free sulfhydryl group, is required to combat oxidative stress and maintain a normal reduced state in the cell (Kletzien et al. 1994). Oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH, which is generated by G6P-dehydrogenase, the rate-limiting enzyme of the PPP, and 6-phosphogluconate dehydrogenase. Glutathione

peroxidase reduces hydrogen peroxide to water by oxidizing GSH. As a consequence, the PPP plays an essential role in the protection from oxidative stress–induced apoptosis (Fico et al. 2004; Tian et al. 1999). The tumor suppressor *p53* activates G6P-dehydrogenase; thus inactivation of *p53* in cancer cells enhances PPP and increases NADPH production and biosynthetic processes (Jiang et al. 2011).

### *1.2.3 Hypoxia and Regulation of Glycolysis*

To adapt to oxygen deprivation, hypoxia alters metabolic pathways in a number of ways. It is well established that HIF-1α induces two dramatic alterations of cellular metabolism: induction of glycolysis and inhibition of mitochondrial respiration. Aerobic glycolysis (two molecules of ATP) is an ineffective way of generating energy, with 18-fold less ATP synthesized per molecule of glucose than the quantity that can be generated through the complete oxidation of glucose to carbon dioxide ($CO_2$) in the mitochondria (36 molecules of ATP). Induction of glucose uptake and glycolysis by hypoxia can result in rapid energy production that compensates for its low efficiency.

Cell metabolism is shifted toward glycolysis and the generation of anaerobic energy by the HIF-1α-induced expression of numerous glycolytic enzymes (Fig. 1.2), such as glucose transporters (GLUT1 and GLUT3), hexokinases (HK1 and HK2), PFK-1, and phosphoglycerate kinase 1 (PGK1). In addition, hypoxia strongly induces the expression of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and -4 (*PFKFB3* and *PFKFB4*) genes in several cancer cell lines via an HIF-dependent mechanism (Bobarykina et al. 2006; Minchenko et al. 2005; Obach et al. 2004). HIF-1α also upregulates LDHA, which regenerates $NAD^+$ to provide a continuous supply for glycolysis (Semenza et al. 1994). In addition, it has been shown that glycogen metabolism is upregulated in response to hypoxia through the induction of glycogen synthase 1 (GYS1) and glycogen phosphorylase liver form (PYGL) and that metabolism of glucose via glycogen sustains the PPP, leading to removal of ROS and cell proliferation (Favaro et al. 2012). Recent data suggest that in some cell types, HIF-1α also regulates the expression of the transketolase (TKT) and transketolase-like 2 (TKTL2) enzymes of the nonoxidative branch of the PPP (Zhao et al. 2010) (Fig. 1.2).

A recent study demonstrated a functional interrelationship between HIF-1α and PKM2 (Fig. 1.2). It was established in the early 1990s that hypoxia and HIF-1α mediate the transcription of PKM2, but not PKM1 (Semenza et al. 1994). PKM2 can act as a coactivator of HIF-1α by physical interaction in the nucleus (Luo et al. 2011). In addition, the direct binding of PKM2 to HIF-1α is strengthened through hydroxylation of PKM2 by PHD3. The silencing of PHD3 inhibits PKM2 coactivator function, reduces glucose uptake and lactate production, and increases oxygen consumption in cancer cells. The reciprocal positive regulation of PKM2, PHD3, and HIF-1α in a positive feedback loop supports an important role for PKM2 in the reprogramming of cancer metabolism during hypoxia (Luo et al. 2011; Luo and Semenza 2011).

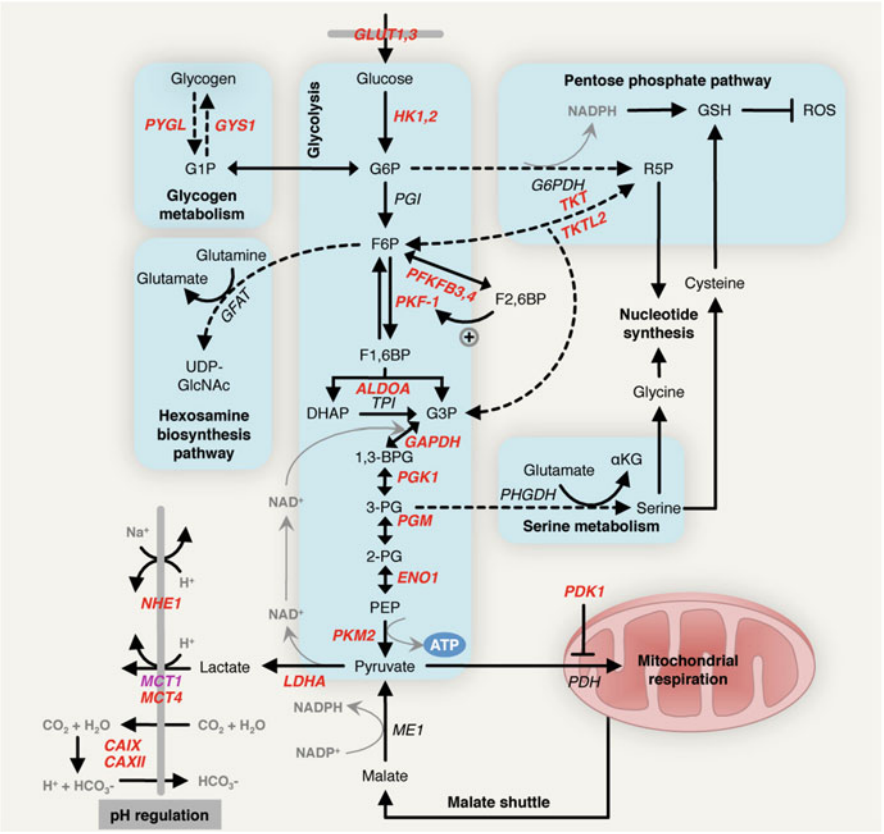


**Fig. 1.2** Control of glucose metabolism and pH regulation by hypoxia. Hypoxia-inducible factor (HIF)-1α regulates most of the enzymes involved in glycolysis and pH regulation. HIF-1α also inhibits mitochondrial respiration by inducing pyruvate dehydrogenase kinase 1 (*PDK1*), an inhibitor of pyruvate dehydrogenase (*PDH*). Metabolic enzymes regulated by HIF-1α are shown in *bold* and *red*. Metabolic enzymes regulated by hypoxia are shown in *bold* and *purple*. The pathways linked to glucose metabolism (glycolysis, pentose phosphate pathway, glycogen metabolism, hexosamine biosynthesis pathway, and serine metabolism) are represented within *blue boxes*. *Dashed arrows* represent multiple reaction pathways. *α-KG* α-ketoglutarate; *ALDOA* aldolase A; *ATP* adenosine-5′-triphosphate; *CAIX* carbonic anhydrase 9; *DHAP* dihydroxyacetone phosphate; *ENO1* enolase 1; *F1,6BP* fructose-1,6-bisphosphate; *F2,6BP* fructose-2,6-bisphosphate; *F6P* fructose-6-phosphate; *GFAT* glucosamine fructose-6-phosphate amidotransferase; *GAPDH* glyceraldehyde 3-phosphate dehydrogenase; *GSH* glutathione; *G1P* glucose-1-phosphate; *G3P* glyceraldehyde 3-phosphate; *G6P* glucose-6-phosphate; *G6PDH* G6P dehydrogenase; *GLUT* glucose transporter; *GYS1* glycogen synthase 1; *HK2* hexokinase 2; *LDHA* lactate dehydrogenase A; *MCT* monocarboxylate transporter; *ME1* malic enzyme 1; *NHE1* $Na^+/H^+$ exchange protein 1; *NADH* nicotinamide adenine dinucleotide; *NADPH* nicotinamide adenine dinucleotide phosphate; *PEP* phosphoenolpyruvate; *PFK-1* phosphofructokinase 1; *PFK-2* 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; *PGI* phosphoglucose isomerase; *PGK1* phosphoglycerate kinase 1; *PGM* phosphoglycerate mutase; *PHGDH* phosphoglycerate dehydrogenase; *PDH* pyruvate dehydrogenase; *PDK1* pyruvate dehydrogenase kinase; *PKM2* pyruvate kinase M2; *PYGL* glycogen phosphorylase liver form; *ROS* reactive oxygen species; *R5P* ribose-5-phosphate; *TKT* transketolase; *TKTL2* TKT-like 2; *TPI* triose-phosphate isomerase; *UDP-GlcNAc* UDP-N-acetylglucosamine

Enhanced uptake of glucose during hypoxia further increases the intracellular accumulation of lactate in cancer cells. In addition to pH regulation of cancer cells by MCTs and NHE1, which was described earlier, another mechanism involves the induction of the HIF-1α target gene carbonic anhydrase 9 (*CA9*) (Fig. 1.2). The protein CAIX prevents the acidification of hypoxic cells by catalyzing the reversible hydration of $CO_2$ into bicarbonate, thus removing $H^+$ from the cells and maintaining an alkaline intracellular pH (Tan et al. 2009). HIF-1α also regulates the expression of CAXII (Wykoff et al. 2000). It is notable that HIF-1α regulates the hypoxic induction of NHE1 (Rios et al. 2005; Shimoda et al. 2006) and MCT4 (Rademakers et al. 2011; Ullah et al. 2006) and that hypoxia induces the expression of MCT1 (Rademakers et al. 2011) (Fig. 1.2).

Hypoxia, in cooperation with oncogenes, can alter tumor metabolism to support cell proliferation and tumorigenesis. Oncogenic Ras induces glycolysis in part through the upregulation of HIF-1α (Pylayeva-Gupta et al. 2011). *Myc* increases HIF-1α expression, and both *Myc* and HIF-1α cooperate to induce metabolic changes (Dang et al. 2008; Qing et al. 2010). It is interesting that while HIF-1α inhibits *Myc* and arrests cell cycle progression, HIF-2α increases *Myc* activity and supports cell proliferation (Gordan et al. 2007, 2008). HIF-1α and HIF-2α have opposite functions on *Myc* activity by differential interactions with *Myc* cofactors (Gordan et al. 2008). The PI3K pathway through activation of Akt also has been implicated in the stabilization of HIF-1α (Elstrom et al. 2004; Robey and Hay 2009). Loss of tumor suppressors like *p53*, *PTEN*, or *VHL*, as well as the activation of oncogenes such as *Ras*, *SRC*, or *PI3K*, can induce HIF-1α independent of oxygen status (Bardos and Ashcroft 2004).

## 1.3 Mitochondrial Respiration

### *1.3.1 Mitochondrial Respiration in Normal and Cancer Cells*

Despite the well-known Warburg effect, it is clear that mitochondrial respiration persists in many cancers. Pyruvate produced by glycolysis can be converted to acetyl-CoA in the mitochondria and further converted into citrate during the TCA cycle; that citrate is sequentially converted to oxaloacetate to allow a new round of TCA cycling. This leads to the generation of high-energy electrons, $CO_2$, and carbon building blocks that can be used for anabolic processes.

A proposed hypothesis is that the increased glycolytic flux could be a consequence of the decreased mitochondrial function and that decreased oxidative phosphorylation might confer a selective advantage on tumor cells. Indeed, upregulation of glycolysis can lead to decreased ROS-induced damage through the reduction of mitochondrial oxidative phosphorylation (OXPHOS) and to increased anabolic metabolic pathways to support proliferation. These observations strongly suggest that decreased mitochondrial respiration may be advantageous for tumor growth.

The identification of mutations in genes encoding for mitochondrial enzymes in human cancers supports the idea that metabolic reprogramming can transform cells. Several mitochondrial enzymes that are components of the TCA cycle, such as succinate dehydrogenase (SDHB, SDHC, and SDHD) and fumarate hydrase (FH), have been shown to have tumor suppressor activities. Mutations in the corresponding genes are associated with familial predisposition to develop tumors (Gottlieb and Tomlinson 2005). Somatic mutations in isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) have been found in glioblastoma and acute myeloid leukemia (AML) (Kim and Liau 2012; Rakheja et al. 2012). Mutant IDH proteins acquire a neomorphic activity and catalyze the conversion of α-ketoglutarate to the oncometabolite 2-hydroxyglutarate (2HG) (Dang et al. 2009). Excessive accumulation of 2HG has been found in tumors carrying *IDH* mutations (Amary et al. 2011; Dang et al. 2009; Ward et al. 2010). 2HG inhibits DNA demethylases in AML and histone demethylases in glioma, thereby modulating gene expression in cancer cells by affecting epigenetic regulation to block differentiation and drive transformation (Figueroa et al. 2010; Lu et al. 2012; Sasaki et al. 2012; Xu et al. 2011) (Fig. 1.4). The role of oncometabolites on posttranslational modification of proteins could have an essential role in metabolic reprogramming in cancer cells and tumorigenesis.

In addition to its role in inducing glycolysis, *Myc* has been shown to induce genes involved in mitochondrial biogenesis (Li et al. 2005). In addition, the tumor suppressor p53 can regulate mitochondrial respiration as loss of *p53* in tumor cells prevents the expression of synthesis of cytochrome c oxidase 2 (SCO2), leading to the inhibition of mitochondrial respiration (Matoba et al. 2006) (Fig. 1.1). p53 also regulates the expression of the mitochondrial protein apoptosis-inducing factor (AIF) involved in oxidative respiration (Stambolsky et al. 2006).

### *1.3.2 Hypoxia and Mitochondrial Respiration*

The main role of metabolic adaptation under hypoxic conditions is to downregulate the amount of oxygen consumed during mitochondrial respiration, as most of oxygen consumption in normoxic cells is dedicated to OXPHOS (Papandreou et al. 2006). High glycolytic flux can also optimize the use of low oxygen concentrations in hypoxic cells for non-energy-generating reactions rather than mitochondrial respiration (Denko 2008). Reduction of oxygen consumption by mitochondria and the resulting decrease in the production of mitochondrial ROS have a protective role in cancer cell survival under hypoxia, even though ROS production by mitochondria is known to increase under hypoxia (Guzy et al. 2005; Guzy and Schumacker 2006). Through this mechanism, mitochondria can also regulate HIF-1α because ROS generated by mitochondrial complex III stabilizes HIF-1α by inhibiting PHD-mediated hydroxylation (Chandel et al. 2000; Guzy et al. 2007; Pan et al. 2007). Furthermore, it is well established that a sudden restoration of oxygen to hypoxic cells can cause substantial ROS accumulation and cell death (Prabhakar et al. 2010). The mitochondrial SIRT3 acts as a tumor suppressor because it suppresses HIF-1α and tumor growth by inhibiting the generation of mitochondrial ROS (Bell et al. 2011) (Fig. 1.3).

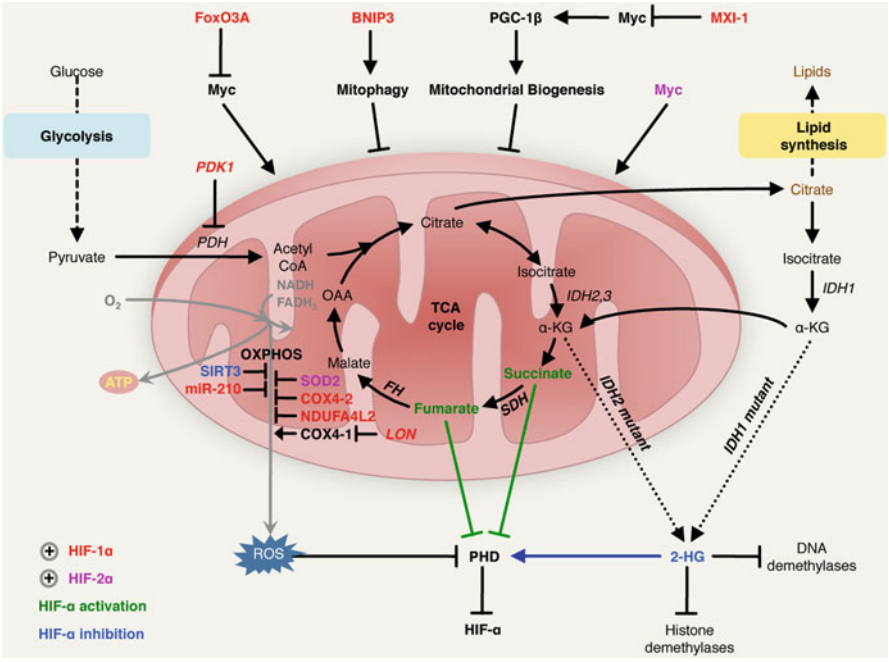


**Fig. 1.3** Interplay between hypoxia and mitochondria in cancer. Metabolic enzymes in the tricarboxylic acid (*TCA*) cycle, fumarate hydratase (*FH*) and succinate dehydrogenase (*SDH*), act as tumor suppressors because their mutations lead to the accumulation of fumarate and succinate (shown in *bold* and *green*). 2-Hydroxyglutarate (*2-HG*) is produced from α-ketoglutarate (*α-KG*) by the mutant forms of isocitrate dehydrogenase 1 and 2 (*IDH1 and IDH2*) enzymes that are found in cancer (*black dashed arrow*). The *circled plus* represents a positive regulation by hypoxia-inducible factor (HIF)-1α or HIF-2α. *ATP* adenosine-5′-triphosphate; *BNIP3* BCL2/adenovirus E1B 19 kDa protein-interacting protein 3; *CoA* coenzyme A; *COX* cytochrome c oxidase subunit; *FoxO3A* forkhead box 3A; *LON* LON, ATP-dependent protease La; *MXI-1* MAX-interacting protein 1; *miR* microRNA; *NDUFA4L2* NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2 (*NDUFA4L2*); *OAA* oxaloacetate; *OXPHOS* oxidative phosphorylation; *PGC-1β* peroxisome proliferator-activated receptor **γ** coactivator 1**β**; *PHD* prolyl hydroxylase; *PDH* pyruvate dehydrogenase; *PDK1* pyruvate dehydrogenase kinase; *ROS* reactive oxygen species; *SIRT3* sirtuin 3; *SOD2* superoxide dismutase 2

HIF-1α affects mitochondrial respiration by various mechanisms: first, by preventing the entry of acetyl-CoA into the TCA cycle, thus reducing NADH and $FADH_2$ production necessary for mitochondrial oxygen consumption. HIFs mediate the inhibition of mitochondrial respiration by activating pyruvate dehydrogenase kinase 1 (PDK1) (Kim et al. 2006a). PDK1 can phosphorylate serine residues in pyruvate dehydrogenase (PDH) and inactivate it (Fig. 1.3). Inactive PDH then is unable to drive the conversion of pyruvate to acetyl-CoA, therefore diverting pyruvate to lactate (Semenza 2010). Besides inducing PDK1, HIF-1α directly regulates the mitochondrial mass and the mitochondrial respiratory chain. HIF-1α blocks mitochondrial biogenesis by inducing MAX-interacting protein 1 (MXI-1), a repressor of *Myc*

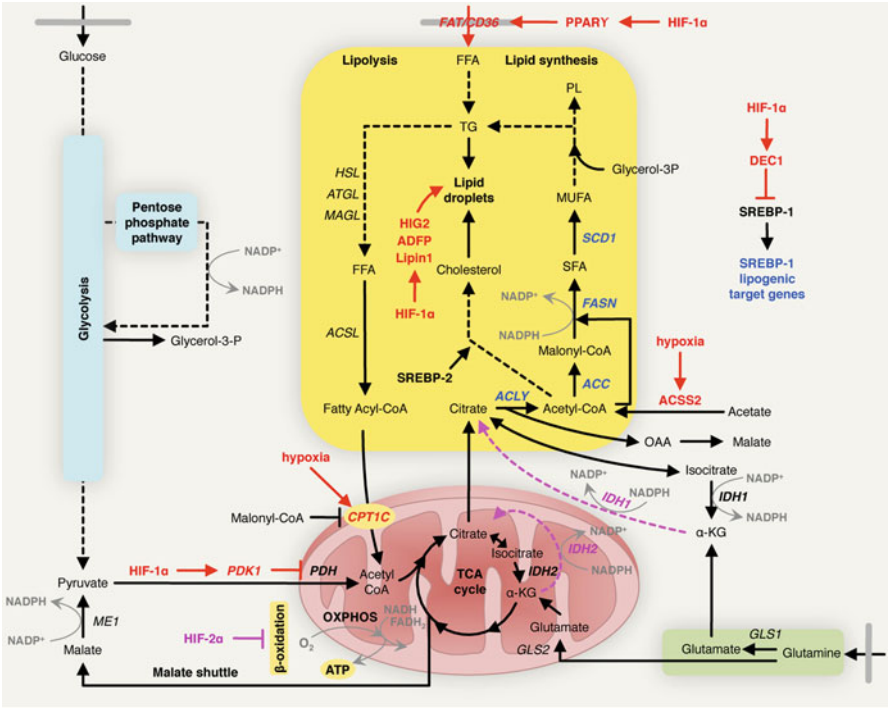


**Fig. 1.4** Control of lipid metabolism by hypoxia. Hypoxia-inducible factor (HIF)-1α inhibits lipid synthesis and induces fatty acid uptake and the accumulation of lipid droplets. HIF-2α inhibits β-oxidation. Reductive carboxylation of **α**-ketoglutarate (*α-KG*) by isocitrate dehydrogenase 1 and 2 (*IDH1* and *IDH2*) produces citrate for lipid synthesis in hypoxic cells (*pink dashed arrow*). Proteins regulated by hypoxia or HIF-1**α** are shown in *bold* and *red*. Proteins regulated by HIF-2α are shown in *bold* and *purple*. Proteins regulated by sterol response element-binding protein (*SREBP-1*) are shown in *bold* and *blue*. *Dashed arrows* represent multiple reaction pathways. *ACC* acetyl-CoA carboxylase; *ACSS2* acetyl-CoA synthetase 2; *ACSL* acyl-CoA synthetase; *ADFP* adipophilin; *ACLY* ATP citrate lyase; *ATP* adenosine-5′-triphosphate; *ATGL* adipose triglycerides lipase; *CoA* coenzyme A; *DEC1* differentiated embryo chondrocyte 1; *FASN* fatty acid synthase; *FAT/CD36* fatty acid translocase; $FADH_2$ flavin adenine dinucleotide; *FFA* free fatty acid; *GLS* glutaminase; *HSL* hormone-sensitive lipase; *HIG2* hypoxia-inducible protein-2; *ME1* malic enzyme 1; *MAGL* monoacylglycerol lipase; *MUFA* monounsaturated fatty acid; *NADH* nicotinamide adenine dinucleotide; *NADPH* nicotinamide adenine dinucleotide phosphate; *OXPHOS* oxidative phosphorylation; *PPARγ* proliferator-activated receptor γ; *PDH* pyruvate dehydrogenase; *PDK1* pyruvate dehydrogenase kinase; *PL* phospholipids; *SFA* saturated fatty acid; *SCD1*, stearoyl-CoA desaturase 1; *TCA* tricarboxylic acid; *TG* triglyceride

transcriptional activity by inhibiting Myc-MAX interaction (Fig. 1.3) and consequently repressing the transcription of peroxisome proliferator-activated receptor γ coactivator 1β (PGC-1β), which is involved in mitochondrial biogenesis (Zhang et al. 2007). A recent report suggests that HIF-1α directly induces forkhead box 3A (*FoxO3A*) and antagonizes *Myc* function, resulting in reduced mitochondrial respiration (Jensen et al. 2011). HIF-1α also activates mitochondrial autophagy (also called mitophagy)

to degrade mitochondria by inducing BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) (Zhang et al. 2008) (Fig. 1.3).

Hypoxia can downregulate OXPHOS through other mechanisms (Fig. 1.3). HIF-1α transcriptionally regulates the cytochrome c oxidase subunits COX4-1 and COX4-2. HIF-1α induces a switch from COX4-1 to COX4-2 by activating the transcription of the genes encoding for COX4-2 and LON, which induces the degradation of COX4-1 (Fukuda et al. 2007). This mechanism optimizes the efficiency of OXPHOS at low $O_2$ concentrations to ensure efficient ATP production with generation of fewer ROS in hypoxic cells. HIF-1α also upregulates the transcription of the microRNA miR-210 to inhibit the TCA cycle and oxidative phosphorylation. By regulating miR-210, HIF-1α mediates a new mechanism of adaptation to hypoxia through the downregulation of mitochondrial oxygen consumption via iron-sulfur cluster metabolism and free radical generation (Favaro et al. 2010). Other mechanisms by which hypoxia can deregulate mitochondrial respiration have yet to be identified. For example, one study describes a newly characterized HIF-1α target gene that inhibits mitochondrial complex I activity (Tello et al. 2011). This gene encodes for a mitochondrial protein named NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2 (NDUFA4L2). NDUFA4L2 is important for cell survival in hypoxia because it inhibits complex I activity in both normal and tumor cells, and silencing of NDUFA4L2 also leads to increased ROS production and reduced cell proliferation (Tello et al. 2011). HIF-1α and HIF-2α have distinct roles in cancer, and these can vary among different tumor types. HIF-1α activation in breast cancer cells promotes a shift toward aerobic glycolysis (Chiavarina et al. 2012).HIF-2α regulates the mitochondrial matrix protein superoxide dismutase 2 (SOD2) and protects against oxidative stress (Oktay et al. 2007) and promotes mitochondrial activity and induces the expression of oncogenes such as *Myc* (Chiavarina et al. 2012). HIF-2α also enhances *Myc* transcriptional activity by binding MAX (Gordan et al. 2007).

Mutations in the mitochondrial enzymes SDH, FH, and IDH can have a direct regulatory effect on HIF-1α activity (Fig. 1.3). Inhibition of the tumor suppressors SDH and FH leads to the inhibition of PHDs because of the accumulation of the TCA cycle intermediates succinate and fumarate, respectively. PHDs belong to a large family of 2-oxoglutarate-dependent dioxygenases that includes the TET DNA hydroxymethylases and JmjC-containing histone demethylases. PHDs exploit one reaction of the TCA cycle – the oxidative decarboxylation of α-ketoglutarate – to transfer a hydroxyl group onto their substrates, including HIFs. This reaction is inhibited in presence of succinate or fumarate and thus leads to the accumulation of HIFs (Gottlieb and Tomlinson 2005). Thereby, mutations in *SDH* or *FH*, associated with certain form of hereditary renal cancer, and the accumulation of succinate or fumarate leading to HIF-1α stabilization could result in the development of cancer. It has recently been shown that renal cyst formation in Fh1-deficient mice is independent of HIF-1α or HIF-2α but involves activation of the nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway by fumarate. Fumarate represses the Kelch-like ECH-associated protein 1 (KEAP1), abrogating its ability to inhibit the NRF2-mediated antioxidant response pathway. This activation may also contribute to the development of FH-deficient cancers (Adam et al. 2011). PHDs can also be inhibited by 2-hydroglutarate (2HG) detected in IDH-mutant tumors. Mutations in *IDH1*

and *IDH2* result in the exclusive accumulation of the (R)-enantiomer of 2HG. (R)-2HG stimulates PHD activity in human astrocytes, leading to HIF-α proteasomal degradation and increased proliferation and transformation of astrocytes (Koivunen et al. 2012) (Fig. 1.3). In addition, oligodendrogliomas expressing an *IDH* mutation express low levels of HIF-1α and show lower induction of HIF target genes, suggesting that HIF-1α has a suppressive function in this type of tumor (Koivunen et al. 2012). A recent work implicates (R)-2HG as an oncometabolite that can promote leukemogenesis and suggests that inhibitors of (R)-2HG production or PHD activity might be effective in cancers with *IDH* mutations (Losman et al. 2013).

Taken together, these findings highlight two things: first, the essential role of HIFs in limiting oxygen consumption under hypoxic conditions through various independent processes, and second, the new understanding that mutations in mitochondrial enzymes can also affect the stability and function of HIFs.

## 1.4 Glutamine Metabolism

### *1.4.1 Glutaminolysis in Normal and Cancer Cells*

The high demand for energy and building block molecules in proliferating cells suggests that the use of alternative substrates other than glucose would provide a selective advantage for tumor cell growth and survival. Amino acids can act as an alternative energy source, and glutamine has been proven to be essential for cell proliferation (DeBerardinis et al. 2008). Glutamine is a nonessential amino acid synthesized by the cell, and it constitutes the most abundant amino acid in circulation. Compared with other amino acids, glutamine has several functions in metabolism, and tumor cells consume and use much more glutamine than any other amino acid (Daye and Wellen 2012). The metabolism of glutamine, also called glutaminolysis, includes steps in which glutamine is converted to glutamate in the cytosol by glutaminase 1 (GLS1) or in the mitochondria by glutaminase 2 (GLS2) (Fig. 1.1). Glutamate in the mitochondria can be further converted to α-ketoglutarate (a cofactor for dioxygenases) in the TCA cycle, a series of mitochondrial reactions involved in the breakdown and oxidation of carbohydrates, lipids, and proteins to generate energy. The glutamine carbon skeleton participates then to a mixed TCA cycle comprising carbon molecules derived from both glucose and glutamine (Fig. 1.1).

As previously mentioned, NADPH is derived mainly from the flux of glucose through the PPP. However, NADPH can also be generated via the glutamine flux though the conversion from malate to pyruvate driven by malic enzyme 1 (ME1) in the cytoplasm (Fig. 1.1). Pyruvate generated from glutaminolysis in the cytosol can be further converted, adding to the cellular pool of lactate. In particular, several steps in the metabolism of lipids involve the oxidation of the reduced form of NADPH to provide reducing power (Pike et al. 2010; Smeland et al. 1992). NADPH generated through glutaminolysis is sufficient to support fatty acid synthesis (DeBerardinis et al. 2007).

Apart from its role in modulating glycolysis, the oncogene *Myc* can also activate the expression of the glutamine transporter amino acid transporter type 2 (ASCT2) and GLS2 (Fig. 1.1) and thereby stimulate glutaminolysis (Wise et al. 2008; Wise and Thompson 2010). *Myc* also inhibits miR-23a/b to activate GLS2 (Gao et al. 2009). Glucose and $O_2$ deprivation in *Myc*-inducible P493 Burkitt lymphoma cells leads to an essential role for *Myc* in driving glutamine intermediates (fumarate, malate, and citrate) in the TCA cycle (Le et al. 2012). GLS2 is also a p53 target that regulates glutamine metabolism and intracellular levels of ROS (Hu et al. 2010; Suzuki et al. 2010) (Fig. 1.1). Thereby the activation of p53 leads to increased mitochondrial glutamine metabolism, mitochondrial respiration, and ATP generation. Taken together, these observations support the idea that glutamine metabolism is important for cancer cell survival under stress conditions.

### *1.4.2 Interplay Between Glycolysis and Glutaminolysis*

In glycolysis, G6P is isomerized to F6P before the pathway progresses. A small fraction of F6P (3–5 %) is diverted to the hexosamine biosynthesis pathway (HBP) (Fig. 1.1). Both glucose and glutamine are required for hexosamine biosynthesis (DeBerardinis and Cheng 2010). The formation of glucosamine-6-phosphate from glutamine and F6P is the first limiting step of this pathway and is driven by the enzyme glucosamine fructose-6-phosphate amidotransferase (GFAT). HBP results in the production of UDP-N-acetylglucosamine (UDP-GlcNAc) and other nucleotide hexosamines used for protein modifications. The flux of glucose into the HBP results in protein N-linked glycosylation and O-linked N-acetylglucosamine (O-GlcNAc) modification. O-GlcNAc modification is elevated in various types of tumor cells and is stimulated by oncogenic Myc (Caldwell et al. 2010; Gu et al. 2010; Morrish et al. 2009; Shi et al. 2010). Inhibited expression of the enzyme responsible for this modification, O-GlcNAc transferase (OGT), results in tumor growth suppression and decreased metastasis (Caldwell et al. 2010; Gu et al. 2010). A recent study showed that glucose flux through the hexosamine biosynthetic pathway regulates growth factor receptor glycosylation and enables glutamine transport and consumption (Wellen et al. 2010). By this method, cells ensure that anabolic metabolism pathways are reduced during nutrient or glucose deprivation.

Another essential metabolic pathway is dependent on the availability of both glucose and glutamine. The serine biosynthesis pathway has recently been shown to be essential for cancer cell survival. Serine biosynthesis starts with the glycolytic intermediate 3-phosphoglycerate (3-PG) (Fig. 1.1). 3-Phosphoglycerate is first converted to 3-phosphopyruvate. Next, 3-phosphopyruvate undergoes a transamination reaction with glutamate to form 3-phosphoserine and α-ketoglutarate. In the last step of the serine synthesis pathway, phosphoserine is converted to serine by phosphoserine phosphatase. Some breast cancer cells are dependent on increased serine pathway flux caused by the overexpression of phosphoglycerate dehydrogenase (PHGDH), the enzyme that catalyzes the first step in the serine biosynthesis pathway (Possemato et al. 2011). It also has been observed that glycolytic metabolism

is diverted into serine metabolism because of PHGDH amplification in human cancer and that PHGDH overexpression is essential for breast cancer cell proliferation (Locasale et al. 2011). Another study demonstrated high expression of other serine biosynthesis genes in breast cancer, and an essential function for serine stimulation in cancer cell proliferation (Pollari et al. 2011). It has recently been shown that PKM2 promotes serine synthesis and that serine can bind and activate PKM2 to support cell proliferation (Chaneton et al. 2012; Ye et al. 2012). Serine deprivation also induces *p53* to protect cancer cells under this condition (Maddocks et al. 2013).

### *1.4.3 Hypoxia and Glutaminolysis*

Glutamine is a precursor of glutathione, and reduced proliferation due to glutamine deprivation during hypoxia can be restored by adding exogenous glutathione (Izaki et al. 2008). While hypoxia diverts glucose/pyruvate toward lactate production and the mitochondria is less active, glutamine metabolism through the TCA cycle is not affected, and glutamine can become the main source of carbon for lipid synthesis. Under glucose deprivation, the TCA cycle could be driven exclusively by glutamine carbon building blocks and sustains cell proliferation and survival (Le et al. 2012). In addition, deprivation of glutamine or glucose, but not pyruvate, suppresses the elevation of HIF-1α during hypoxia in prostate cancer cell lines (Kwon and Lee 2005). Hypoxia upregulates glutamine transport in a neuroblastoma cell line (Soh et al. 2007), whereas chronic hypoxia has been shown to induce changes in enzymes related to glutamate metabolism and transport, a phenomenon that is consistent with a decrease in glutamine levels (Kobayashi and Millhorn 2001).

Several recent studies have shown that there is a switch from pyruvate oxidation to reductive glutamine metabolism for de novo lipid synthesis in cancer cells under hypoxic conditions (Metallo et al. 2011; Wise et al. 2011) or with defective mitochondria (Mullen et al. 2011). This pathway is dependent on IDH1 for cytosolic citrate production. In hypoxic conditions, cells depend almost exclusively on the reductive carboxylation of glutamine-derived α-ketoglutarate for de novo lipogenesis, thus maximizing the use of glutamine for lipid synthesis (Metallo et al. 2011; Mullen et al. 2011; Wise et al. 2011). As the oncogene *Myc* stimulates glutaminolysis and HIF-2α activates *Myc*, this finding may explain why the interaction between HIF-2α and *Myc* is beneficial to tumor cell proliferation under conditions of oxygen deprivation.

## 1.5 Lipid Metabolism

### *1.5.1 Lipid Metabolism in Normal Cells*

Lipids consist of triglycerides (TGs), phosphoglycerides, sterols, and sphingolipids. TGs comprise a glycerol bound to three fatty acids and are mainly used for energy storage. The other types of lipids are mainly incorporated in membranes.

De novo synthesis of LCFAs starts with pyruvate, the end product of the glycolytic pathway (Fig. 1.1). Once transported into mitochondria, pyruvate is converted to acetyl-CoA by PDH. The condensation of acetyl-CoA with oxaloacetate (OAA) by citrate synthase leads then to the formation of citrate in the TCA cycle. Citrate can be exported by a translocase from mitochondria into the cytoplasm and then converted back to acetyl-coA (and oxaloacetate) by ATP citrate lyase (ACLY) for cholesterol and fatty acid de novo synthesis (Fig. 1.1). This last step is required because acetyl-CoA cannot cross the mitochondrial membrane. Afterward, acetyl-CoA carboxylase (ACC), the key control enzyme in lipogenesis, drives the committed step of the fatty acid synthesis pathway. ACC catalyzes the two-step reaction by which acetyl-CoA is carboxylated to form malonyl-CoA. ACC can be regulated allosterically by phosphorylation and dephosphorylation events at serine residues, as well as at the transcription level. LCFA synthesis, primarily the saturated fatty acid palmitate from acetyl-CoA and malonyl-CoA, occurs in a series of steps that are catalyzed by fatty acid synthase (FASN) (Fig. 1.1). NADPH, produced by the PPP and glutaminolysis, serves as electron donor for these reactions. In summary, 7 molecules of ATP and 14 molecules of NADPH are required for the synthesis of a single molecule of palmitate. Fatty acid synthesis from acetyl-CoA can be represented as follows: acetyl-CoA + 7 $CO_2$ + 7 ATP → 7 malonyl-CoA + 7 ADP + 7 Pi + 7 $H^+$ and acetyl-CoA + 7 malonyl-CoA + 14 NADPH + 14 $H^+$ → palmitate + 7 $CO_2$ + 8 CoA + 14 $NADP^+$ + 6 $H_2O$.

The next step in lipogenesis takes place at the cytoplasmic face of the endoplasmic reticulum, where palmitate (C-16) is elongated to form stearate (C-18) and very-long-chain fatty acids by very-long-chain fatty acid proteins (ELOVL1 to 7). Oxygen-dependent desaturation by stearoyl-CoA desaturase 1 (SCD1), or other desaturases, induces the unsaturation of palmitate and stearate to generate monounsaturated fatty acids. After all of these modifications, fatty acids can be used for TGs, sphingolipids, glycolipids, or phospholipids synthesis, or they can be transported from the liver as very-low-density lipoproteins (VLDL). Lipogenesis is mainly regulated by two transcription factors: carbohydrate response element-binding protein (ChERBP) and sterol response element binding protein 1 (SREBP-1). SREBP-2 upregulates the transcription of genes involved in cholesterol synthesis, an important biosynthetic pathway linked to lipid metabolism that also uses acetyl-CoA as the first precursor. In the presence of a cholesterol signal, SREBP-1 is sequestered by SREBP cleavage-activating protein (SCAP) in the endoplasmic reticulum. In the absence of cholesterol, SREBP-1 undergoes proteolysis that leads to the activation of specific target genes. SREBP-1 induces the expression of ACLY, ACC, FASN, and SCD1 (Fig. 1.4). ChREBP is regulated by glucose and induces ACC and FASN. Both SREBP-1 and SREBP-2 are overexpressed in cancer cells (Menendez and Lupu 2007).

Besides de novo synthesis from acetyl-CoA, fatty acids can also be obtained from the diet. Most normal tissues, except liver and adipose tissues, possess little capacity for de novo fatty acid synthesis and depend on fatty acid uptake for their needs (Swinnen et al. 2006). Cells can take up free fatty acids from the circulation to support their macromolecular needs for energy production via fatty acid

oxidation or membrane synthesis. LCFAs can diffuse across the plasma membrane, but there is evidence that LCFA uptake is facilitated by numerous cytosolic and membrane-associated proteins (Su and Abumrad 2009). Fatty acid translocation occurs via fatty acid transporters such as fatty acid transport proteins (FATPs), fatty acid translocase (FAT/CD36), and fatty acid binding proteins (FABPs).

Accumulated lipids are stored as cytoplasmic lipid droplets (LDs). LDs are dynamic lipid storage organelles found in most eukaryotic cells and are formed from the endoplasmic reticulum. LDs form and degrade, move inside cells, and can undergo fusion. A lipid droplet consists of a monolayer of polar lipids (phospholipids and cholesterols) that surrounds a core of neutral lipids (TGs and cholesterol esters). Several proteins (Hypoxia-inducible protein-2, perilipin, adipophilin, and Tip47), located at the surface of LDs, are essential for the integrity of LD membranes (Bozza and Viola 2010; Farese and Walther 2009). Hypoxia-inducible protein-2 (HIG2) has been shown to be involved in the deposition of neutral lipids into LDs (Gimm et al. 2010).

The lipolysis of TGs contained in LDs is induced to generate free fatty acids when cells need to use lipids to generate ATP via fatty acid oxidation (also called β-oxidation) or to synthesize membranes. TGs can be degraded into free fatty acids through the serial actions of various lipases, such as hormone-sensitive lipase (HSL), adipose TGs lipase (ATGL), and monoacylglycerol lipase (MAGL) (Fig. 1.1). The latter is highly expressed in aggressive human cancer cells and primary tumors and regulates a fatty acid network that promotes tumorigenesis (Nomura et al. 2010). An alternate pathway for degradation of TG stores, called lipophagy, has been recently described. In conditions of nutrient deprivation, TGs are taken up by autophagosomes and delivered to lysosomes for degradation by lysosomal enzymes. Free fatty acids generated by lipophagy can then be oxidized in the mitochondria to generate ATP via fatty acid oxidation (Liu and Czaja 2013; Singh and Cuervo 2012; Singh et al. 2009).

The first step in the use of fatty acids for energy production is the conversion of a fatty acids to a CoA molecule. This process occurs in two steps catalyzed by fatty acyl-CoA synthetases (ACSLs). The transport of fatty acyl-CoA into the mitochondria is accomplished via an acyl-carnitine intermediate, which itself is generated by the action of carnitine palmitoyltransferase 1 (CPT1), an enzyme that resides in the outer mitochondrial membrane. Then the oxidation of fatty acyl-CoA occurs in the mitochondria, leading to the production of ATP using the mitochondrial respiratory chain. In total, 129 molecules of ATP are formed from the completed oxidation of palmitate.

Lipogenesis and fatty acid oxidation are mutually exclusive. The activity of CPT1 is inhibited by malonyl-CoA, the product of ACC, during lipogenesis. Also, 5′ adenosine monophosphate-activated protein kinase (AMPK) inhibits ACC and lipid synthesis, resulting in the induction of fatty acid oxidation (Hardie et al. 2012).

### *1.5.2 Lipid Metabolism in Cancer Cells*

In adult, normal, nonadipose or nonliver tissues, the majority of fatty acids are acquired from diet via the circulation. As a result, de novo lipogenesis and expression of lipogenic enzymes are usually low in most adult tissues. In contrast, cancer

cells synthesize higher levels of fatty acids, even in the presence of high levels of lipids in the circulation. Several enzymes involved in de novo fatty acid biosynthesis, such as FASN, ACC, or ACLY, are either upregulated or activated in tumors, and de novo fatty acid synthesis is active (Menendez and Lupu 2007). Overexpression of SCD1 also has been observed in cancer cells (Li et al. 1994; Scaglia et al. 2005), and elongation of very-LCFA protein 7 (ELOVL7) has been shown to be overexpressed in prostate cancer and to participate in the growth of prostate cancer cells (Tamura et al. 2009). Another study has shown an accumulation of LDs in cancer cells compared to normal cells (Accioly et al. 2008). Numerous studies have shown that increased β-oxidation is essential for tumorigenesis. Fatty acid oxidation is a dominant bioenergenetic pathway in prostate cancer (Liu 2006).

The most evident explanation for lipid synthesis in cancer cells is that fatty acids can function as building blocks for membrane synthesis and repair, which are required for both cell growth and proliferation. In addition, lipid synthesis in cancer may act as an acid sump in highly glycolytic cancer cells to sequester excessive pyruvate and avoid accumulation of lactate, and thus an excessive acidification of the tumor microenvironment. Lipogenesis can also contribute to the generation of redox power: $NADP^+$ generated during lipid synthesis leads to increased $NAD^+$, which is required to maintain glycolysis. During β-oxidation, acetyl-CoA enters the TCA cycle where it is broken down to $CO_2$, producing the reducing equivalents NADH, which fuels mitochondrial respiration, or NADPH. It has been reported that inhibition of β-oxidation by etomoxir, a specific inhibitor of CPT1, decreases NADPH levels and increases ROS levels (Pike et al. 2010). Fatty acids can be modulators of cellular production of ROS (Schonfeld and Wojtczak 2008). Increased intracellular saturated fatty acids, especially palmitate, induce intracellular ROS accumulation leading to lipotoxicity and cell death through a mechanism that still is not fully understood (Brookheart et al. 2009). TGs accumulating in LDs can protect against this free fatty acid–induced lipotoxicity (Listenberger et al. 2003). Treatment of cancer cells with novel LDs binding thalidomide analogs results in the induction of ROS and cell death (Puskas et al. 2010). It also has been observed that de novo lipogenesis can protect cancer cells from ROS and pharmacological drugs by promoting membrane lipid saturation (Rysman et al. 2010). After degradation to free fatty acids, lipids can also provide cells with energy via β-oxidation, especially to compensate for the low oxygen concentration observed under hypoxic conditions. TG degradation into free fatty acids can also lead to the activation of various signaling pathways (Santos and Schulze 2012).

### *1.5.3 Hypoxia and Lipid Metabolism*

Despite the fact that HIF-1α diverts pyruvate to lactate and therefore prevents glucose-derived de novo lipid synthesis, it has been shown that in breast cancer cell lines the *FASN* gene is upregulated by hypoxia through Akt and SREBP-1 pathways

(Furuta et al. 2008). In addition, cancer cells express high levels of cytosolic acetyl-CoA synthetase 2 (ACSS2) under hypoxic conditions. Therefore, (ACSS2) can catalyze the formation of acetyl-CoA from acetate and plays a significant role in fatty acid synthesis and tumor cell survival in low $O_2$ conditions (Yoshii et al. 2009).

Another interesting work showed that hypoxia could lead to decreased de novo fatty acid synthesis. Specific loss of HIF-1α in mouse liver is related to hepatic lipid accumulation associated with the activation of SREBP-1 and its target gene *ACC*, and these transcriptional modifications are inversely correlated with the expression of the HIF-1α target gene differentiated embryo chondrocyte 1 (*DEC1*), a transcriptional repressor of SREBP-1 (Nishiyama et al. 2012) (Fig. 1.4). As mentioned earlier, glutamine can contribute to lipid metabolism through the reprogramming of the TCA cycle via the reductive metabolism of α-ketoglutarate to synthesize acetyl-coA for lipid synthesis. It is important to note that, despite the important role of glutamine in de novo fatty acid synthesis, tumor cells exhibit overall decreased de novo lipogenesis in hypoxic compared to normoxic conditions (Metallo et al. 2011).

Another study showed that the reprogramming of cellular metabolism by HIF-1α involves a switch toward increased glycolysis as well as uptake of free fatty acids. HIF-1α induces the uptake of free fatty acids and the synthesis of triglycerides in liver and adipose tissue through the induction of peroxisome proliferator-activated receptor ϒ (PPARϒ) at the transcriptional level (Krishnan et al. 2009) (Fig. 1.4). This is further supported by the fact that adipophilin expression selectively stimulates the uptake of LCFAs and that downregulation of adipophilin reduces the uptake of fatty acids (Faleck et al. 2010; Gao and Serrero 1999). It is interesting to note that it also has been reported that the expression of another member of the PPAR protein family, PPARα, is inhibited by HIF-1α during hypoxia in some cell types (Narravula and Colgan 2001). PPARα is a transcription factor involved in the regulation of lipid metabolism in the liver. Activation of PPARα induces the uptake, usage, and catabolism of fatty acids by the upregulation of genes involved in fatty acid transport and β-oxidation.

It has long been recognized by light microscopy that LDs accumulate in hypoxic cells (Zoula et al. 2003), and several recent studies have shown increased lipid metabolism under hypoxic conditions (Laurenti et al. 2011; Shen et al. 2010). Hypoxia causes TG accumulation in LDs by a mechanism involving HIF-1α-dependent stimulation of lipin 1 expression (Mylonis et al. 2012). Both HIG2 and adipophilin (also called PLIN-2) also are induced by HIF-1α during hypoxia (Gimm et al. 2010; Saarikoski et al. 2002) (Fig. 1.4).

Fatty acid oxidation is an oxygen-dependent mechanism; therefore free fatty acids produced from TG stores probably do not take the β-oxidation route in hypoxic conditions. HIF-2α, rather than HIF-1α, has been shown to be important for the regulation of fatty acid oxidation. HIF-2α is an important regulator of hepatic lipid metabolism, and activation of HIF-2α in hypoxia impairs fatty acid β-oxidation, decreases lipogenic gene expression, and increases lipid storage capacity (Rankin et al. 2009). These authors showed that constitutive activation of HIF-2α in *VHL*-deficient mice results in the development of severe hepatic lipid accumulation associated with impaired fatty acid β-oxidation, decreased lipogenic gene expression, and increased lipid storage capacity. This observation is further supported by

another recent work demonstrating that the combined loss of PHD2 and PHD3 in a mouse knockout model resulted in the development of severe hepatic steatosis in an HIF-2α-dependent fashion (Minamishima et al. 2009). Nevertheless, this particular research field is controversial because HIF-2α-deficient mice show dysregulated fatty acid oxidation but also hepatic steatosis (Scortegagna et al. 2003). In addition, constitutively active HIF-1α, but not HIF-2α, in mouse liver leads to the accumulation of lipids (Kim et al. 2006b). In ischemia, it has been shown that fatty acid oxidation represents the limiting step of fatty acid metabolism in the heart as the rate of β-oxidation is limited by high levels of NADH and FADH2 secondary to the reduced supply of $O_2$ (Whitmer et al. 1978). In addition, the transport of free fatty acids in the mitochondria can also be altered by hypoxia. CPT1C can be induced by hypoxia or glucose deprivation and promotes cell survival and tumor growth under these conditions. Finally, the inhibition of β-oxidation sensitizes human leukemia cells to cell death (Samudio et al. 2010; Zaugg et al. 2011).

These collective data strongly suggest that HIF-1α inhibits de novo fatty acid synthesis and stimulates fatty acid uptake and lipid storage, whereas HIF-2α may negatively regulate genes responsible for β-oxidation during limited $O_2$ availability. The key point that $O_2$ is required for SCD1 desaturase activity could explain the function of HIF-1α in lipid metabolism. Under low oxygen conditions, fatty acid desaturation might be suppressed and uptake of desaturated fatty acids from the circulation might be increased. HIF-mediated metabolic alterations of lipid metabolism seem to be essential for limiting $O_2$ consumption by regulating fatty acid synthesis and β-oxidation and for limiting the use of ATP needed for de novo fatty acid synthesis.

## 1.6 New Therapeutic Opportunities

The dependence of tumor cells on survival mechanisms that are coupled to metabolic reprogramming suggests several prospective therapeutic advantages of targeting metabolism in cancer cells. The biggest challenge would be to specifically target the metabolism of cancer cells without affecting the growth and survival of nontransformed cells or noncancerous tissues. The metabolic changes in cancer cells associated with hypoxia may render them more susceptible to metabolic targeting and need to be explored as targets for anticancer therapy.

### *1.6.1 Targeting Hypoxia*

Studies have provided evidence that HIF-1α mediates resistance to chemotherapy. Anti-angiogenic therapies have been demonstrated to induce hypoxia within tumors, resulting in both increased local invasion and distant metastatic spread (Azam et al. 2010). Thus, understanding the biology of hypoxia induced by anti-angiogenic therapy and its role in metabolic reprogramming in cancer could contribute to improvements in existing therapy and the discovery of new targets to overcome resistance to

this therapy (Bridges and Harris 2011). As a consequence, inhibition of HIF-1α activity, or specific metabolic pathways induced by HIF-1α, could represent a crucial constituent in discovering efficient anticancer therapies.

### *1.6.2 Targeting Glucose Metabolism*

Highly glycolytic cancer cells may be particularly sensitive to the inhibition of glycolysis when glucose and $O_2$ are limited in their environment. Studies have already been performed with glycolytic inhibitors (Ko et al. 2004; Pelicano et al. 2006) or inhibitors of specific glycolytic enzymes such as phosphoglycerate mutase, hexokinase 2, or LDHA (Engel et al. 2004; Fantin et al. 2006; Ganapathy-Kanniappan et al. 2010; Kondoh et al. 2005). Inhibition of LDHA by a small-molecule inhibitor reduces glycolysis by reducing the available pool of $NAD^+$ and induces oxidative stress and cell death through inappropriate mitochondrial respiration (Le et al. 2010). Nevertheless, strategies to target high glycolytic activity in cancer have not been successful so far as anticancer treatment (Porporato et al. 2011; Vander Heiden 2011). Numerous metabolic enzymes involved in glucose metabolism, such as PFKFB4, PKM2, or PHGDH, are essential for the survival of various cancer cell types and have been targeted by inhibitory drugs in several studies. The PFKFB3 and PFKFB4 enzymes are overexpressed in tumors and are induced by hypoxia and oncogenes. These direct HIF-1α targets that produce fructose-2,6-bisphosphate (F2,6BP) and enhance glycolysis would be of interest for targeted therapy. A small-molecule inhibitor of PFKFB3 named 3-PO (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one) has already been shown to inhibit glycolysis in vitro and impair xenograft tumor growth in vivo (Clem et al. 2008). Another strategy would be to reactivate PKM2 in cancer cells using specific drug activators. One of these specific small-molecule PKM2 activators inhibits the growth of non-small-cell lung cancer xenograft tumors (Anastasiou et al. 2012). Cancer cells are highly dependent on ROS metabolism, mainly to change their overall metabolism. Targeting the antioxidant machinery could provide an effective treatment strategy. Serine biosynthesis is involved in the generation of cysteine for the production of glutathione, which has an essential role in the removal of intracellular ROS. Inhibition of PHGDH in breast cancer cell lines with high PHGDH expression, but not in those without, causes a reduction in serine (and cysteine) synthesis and a strong decrease in cell proliferation (Possemato et al. 2011). Moreover, drugs that block the antioxidant machinery, such as molecules blocking the PPP and NADPH production, could be combined with molecules that induce oxidative stress.

### *1.6.3 Targeting Glutamine Metabolism*

Increasing evidence suggests that metabolic reprogramming of cancer cells under hypoxic conditions renders them addicted to certain nutrients, such as glutamine, in a way that nontransformed cells are not. The essential role of specific metabolic

enzymes or pathways for cell survival and proliferation under hypoxia thereby may render cancer cells more susceptible to inhibitors and hence could be exploited for cancer therapy. Targeting glutamine metabolism could be an efficient way to decrease cancer cell survival (DeBerardinis et al. 2007). Numerous cancer cell lines are highly sensitive to glutamine starvation, and targeting GLS2 activity with a small-molecule inhibitor suppresses oncogenic transformation without affecting normal cells (Wang et al. 2010a). The glutamine analog acivicin, in association with glutaminase, synergistically inhibits the proliferation and invasion of MCF-7 and OAW-42 cancer cells (Roy et al. 2008). Reprogramming cancer cell metabolism by *IDH* mutations is also able to be exploited for therapy. The development of small-molecule inhibitors of the production of 2HG by *IDH* mutations may restore normal PHD function and normalize both HIF-1α levels and chromatin structure. Furthermore, cancer cells with *IDH* mutations are addicted to glutamine as a source of 2HG. Inhibition of GLS1 by a small-molecule inhibitor slows the growth of glioblastoma cells expressing mutant *IDH1* compared to those expressing wild-type *IDH1* (Seltzer et al. 2010). These studies suggest that glutamine addiction in cancer cells can be targeted.

### *1.6.4 Targeting Mitochondrial Function*

Drugs inducing the reactivation of metabolic pathways suppressed in cancer cells, such as the reactivation of mitochondrial function, could be an advantage for anticancer therapy. The HIF-1α target PDK1 is inhibited by the drug dichloroacetate (DCA), which induces cancer cell death, decreases proliferation, and inhibits tumor growth (Bonnet et al. 2007). The clinical effects of DCA have been assessed in patients with glioblastomas; DCA was shown to reactivate mitochondrial function and generate ROS (Michelakis et al. 2010).

### *1.6.5 Targeting Lipid Metabolism*

Several aspects of tumor biology can explain the increased lipid metabolism in cancer cells. The importance of lipogenesis in cell growth and proliferation has been highlighted by various studies showing the effect of lipid synthesis inhibition in cancer cells using pharmacological drugs or small interfering RNA against FASN or ACC (Pandey et al. 2012; Wang et al. 2010b). Inhibition of *SCD1* expression impairs the proliferation of cancer cells by blocking cell cycle progression and inducing cell death (Fritz et al. 2010; Hess et al. 2010; Scaglia et al. 2009). Inhibition of β-oxidation with pharmacological drugs induces cell death in human leukemia and glioblastoma cells (Pike et al. 2010; Samudio et al. 2010). FASN inhibitors are promising anticancer agents that have been shown to be effective in vitro and in

xenograft models (Menendez and Lupu 2007). Nevertheless, a specific FASN inhibitory molecule called C75 decreased food intake and body weight in mice (Mera et al. 2009). Therefore, a better understanding of the alterations of lipid metabolism at both the cellular and organism levels is required before considering the targeting of lipid metabolism as a therapeutic strategy for cancer treatment.

### *1.6.6 Metabolic Synthetic Lethality*

Alteration of a single route of the metabolic network may induce compensatory pathways to generate alternate sources for the limiting metabolites. Identifying new metabolic targets for therapies that specifically kill tumor cells while sparing normal tissue is the next major challenge in cancer research. Metabolic synthetic lethality occurs when the simultaneous mutation of two different metabolic genes is lethal but mutation of each individual gene is dispensable for normal growth. The simultaneous suppression of several synthetic lethal genes may open new avenues for anticancer treatment.

Metformin, an activator of AMPK activity, is a nonreversible inhibitor of complex I of the mitochondrial respiratory chain. Metformin has shown promising results in slowing the growth of tumor cells in vitro and in tumor xenograft experiments (Ben Sahra et al. 2008; Buzzai et al. 2007; Hirsch et al. 2009; Zakikhani et al. 2006). Diabetic patients treated with metformin showed a reduced risk of cancer compared with patients treated with other drugs (Evans et al. 2006). The combination treatment of metformin and 2-deoxyglucose, a specific glycolysis inhibitor, impaired tumor growth in mouse xenograft models for a larger range of tumor types (Cheong et al. 2011).

$NAD^+$ metabolism could also be an important target for cancer therapy. The conversion of pyruvate to lactate by the HIF-1α target LDHA leads to the production of the cofactor $NAD^+$, which is necessary for glycolysis. The combination of an LDHA inhibitor with the $NAD^+$ synthesis inhibitor FK866 drastically reduces $NAD^+$ cellular pool and leads to lymphoma regression (Le et al. 2010).

Synthetic lethality screens targeted at metabolic enzymes could be useful tools to add to the number of metabolic targets for anticancer therapy (Folger et al. 2011; Locasale and Cantley 2011). The enzyme glycine decarboxylase (GLDC) is one example of a novel metabolic target. GLDC is implicated in glycine synthesis via serine metabolism and has recently been found to promote cellular transformation and tumorigenesis (Zhang et al. 2012). Synthetic lethality could be exploited to overcome drug resistance to conventional chemotherapy, such as bevacizumab treatment. PYGL is a potential target that showed increased expression following bevacizumab treatment in a xenograft model in vivo (Favaro et al. 2012). Combination treatment with a PYGL inhibitor and/or an inhibitor of other HIF-dependent metabolic enzymes essential for survival in hypoxic conditions and an anti-angiogenic drug could be of a great interest for metabolic cancer therapy.

## 1.7 Conclusion

Otto Warburg was the first contributor to our early knowledge about cancer metabolism. Following his findings, aerobic glycolysis was long thought to be the main trigger for the development of cancer. Ever since, the field of cancer metabolism has been constantly evolving, and our understanding of tumor metabolism has greatly improved in recent years. New biological and computational approaches, such as the use of synthetic lethality screens and systems biology tools, will most certainly enable the identification of key tumor metabolic pathways. New metabolic pathways are still emerging today, such as the discovery of an alternative glycolytic pathway (Vander Heiden et al. 2010). In the future, the focus might not only be on glucose metabolism because it is becoming clearer that tumor cells use a large range of other nutrients, such as glutamine or fatty acids, that are also essential to support growth and proliferation. Hypoxia, which is a common event in cancer, could also be exploited to develop new therapies targeting cancer cell metabolism. Another center of attention and potential target for therapy might be the interrelationship between cancer and stroma cells, as the tumor metabolic microenvironment may influence its own growth. The recent concept of metabolic cooperation and symbiosis has been described as a two-compartment model of tumor metabolism. In this model, ROS generated by tumor cells induce autophagy in adjacent stromal cells, and lactate and other metabolites generated by the stroma can then fuel the growth and proliferation of the tumor cells (Salem et al. 2012; Sotgia et al. 2012). There is also a growing interest in the development of new molecule inhibitors of specific metabolic enzymes. Drugs targeting diverse metabolic routes induced in cancer could be used in combination to induce synthetic lethality in tumor cells while preserving the survival of normal proliferating cells. It is notable that there are more than 200 different types of cancer, and reliance on specific metabolic pathways may differ from one type of tumor to another; this may lead to the development of tumor-specific therapies. The next decade will be exciting as both basic and clinical research in the metabolic field may lead to drastic improvements in cancer diagnosis and therapy.

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## References

Accioly MT, Pacheco P, Maya-Monteiro CM, Carrossini N, Robbs BK, Oliveira SS, Kaufmann C, Morgado-Diaz JA, Bozza PT, Viola JP (2008) Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E2 synthesis in colon cancer cells. Cancer Res 68(6):1732–1740, doi:68/6/1732 [pii] 10.1158/0008-5472.CAN-07-1999

Adam J, Hatipoglu E, O'Flaherty L, Ternette N, Sahgal N, Lockstone H, Baban D, Nye E, Stamp GW, Wolhuter K, Stevens M, Fischer R, Carmeliet P, Maxwell PH, Pugh CW, Frizzell N,

Soga T, Kessler BM, El-Bahrawy M, Ratcliffe PJ, Pollard PJ (2011) Renal cyst formation in Fh1-deficient mice is independent of the Hif/Phd pathway: roles for fumarate in KEAP1 succination and Nrf2 signaling. Cancer Cell 20(4):524–537, doi:S1535-6108(11)00354-0 [pii] 10.1016/j.ccr.2011.09.006

Amary MF, Damato S, Halai D, Eskandarpour M, Berisha F, Bonar F, McCarthy S, Fantin VR, Straley KS, Lobo S, Aston W, Green CL, Gale RE, Tirabosco R, Futreal A, Campbell P, Presneau N, Flanagan AM (2011) Ollier disease and Maffucci syndrome are caused by somatic mosaic mutations of IDH1 and IDH2. Nat Genet 43(12):1262–1265, doi:ng.994 [pii] 10.1038/ng.994

Amith SR, Fliegel L (2013) Regulation of the Na+/H+Exchanger (NHE1) in breast cancer metastasis. Cancer Res 73(4):1259–1264, doi:0008-5472.CAN-12-4031 [pii] 10.1158/0008-5472.CAN-12-4031

Anastasiou D, Poulogiannis G, Asara JM, Boxer MB, Jiang JK, Shen M, Bellinger G, Sasaki AT, Locasale JW, Auld DS, Thomas CJ, Vander Heiden MG, Cantley LC (2011) Inhibition of pyruvate kinase M2 by reactive oxygen species contributes to cellular antioxidant responses. Science 334(6060):1278–1283, doi:science.1211485 [pii] 10.1126/science.1211485

Anastasiou D, Yu Y, Israelsen WJ, Jiang JK, Boxer MB, Hong BS, Tempel W, Dimov S, Shen M, Jha A, Yang H, Mattaini KR, Metallo CM, Fiske BP, Courtney KD, Malstrom S, Khan TM, Kung C, Skoumbourdis AP, Veith H, Southall N, Walsh MJ, Brimacombe KR, Leister W, Lunt SY, Johnson ZR, Yen KE, Kunii K, Davidson SM, Christofk HR, Austin CP, Inglese J, Harris MH, Asara JM, Stephanopoulos G, Salituro FG, Jin S, Dang L, Auld DS, Park HW, Cantley LC, Thomas CJ, Vander Heiden MG (2012) Pyruvate kinase M2 activators promote tetramer formation and suppress tumorigenesis. Nat Chem Biol 8(10):839–847, doi:nchembio.1060 [pii] 10.1038/nchembio.1060

Azam F, Mehta S, Harris AL (2010) Mechanisms of resistance to antiangiogenesis therapy. Eur J Cancer 46(8):1323–1332, doi:S0959-8049(10)00147-4 [pii] 10.1016/j.ejca.2010.02.020

Bardos JI, Ashcroft M (2004) Hypoxia-inducible factor-1 and oncogenic signalling. Bioessays 26(3):262–269. doi:10.1002/bies.20002

Bartrons R, Caro J (2007) Hypoxia, glucose metabolism and the Warburg's effect. J Bioenerg Biomembr 39(3):223–229. doi:10.1007/s10863-007-9080-3

Bell EL, Emerling BM, Ricoult SJ, Guarente L (2011) SirT3 suppresses hypoxia inducible factor 1alpha and tumor growth by inhibiting mitochondrial ROS production. Oncogene 30(26):2986–2996, doi:onc201137 [pii] 10.1038/onc.2011.37

Ben Sahra I, Laurent K, Loubat A, Giorgetti-Peraldi S, Colosetti P, Auberger P, Tanti JF, Le Marchand-Brustel Y, Bost F (2008) The antidiabetic drug metformin exerts an antitumoral effect in vitro and in vivo through a decrease of cyclin D1 level. Oncogene 27(25):3576–3586, doi:1211024 [pii] 10.1038/sj.onc.1211024

Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH (2006) TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 126(1):107–120, doi:S0092-8674(06)00762-8 [pii] 10.1016/j.cell.2006.05.036

Bensaad K, Cheung EC, Vousden KH (2009) Modulation of intracellular ROS levels by TIGAR controls autophagy. EMBO J 28(19):3015–3026, doi:emboj2009242 [pii] 10.1038/emboj.2009.242

Bertout JA, Patel SA, Simon MC (2008) The impact of $O_2$ availability on human cancer. Nat Rev Cancer 8(12):967–975, doi:nrc2540 [pii] 10.1038/nrc2540

Bluemlein K, Gruning NM, Feichtinger RG, Lehrach H, Kofler B, Ralser M (2011) No evidence for a shift in pyruvate kinase PKM1 to PKM2 expression during tumorigenesis. Oncotarget 2(5):393–400, doi:278 [pii]

Bobarykina AY, Minchenko DO, Opentanova IL, Moenner M, Caro J, Esumi H, Minchenko OH (2006) Hypoxic regulation of PFKFB-3 and PFKFB-4 gene expression in gastric and pancreatic cancer cell lines and expression of PFKFB genes in gastric cancers. Acta Biochim Pol 53(4):789–799, doi:20061343 [pii]

Bonnet S, Archer SL, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R, Lee CT, Lopaschuk GD, Puttagunta L, Harry G, Hashimoto K, Porter CJ, Andrade MA, Thebaud B, Michelakis ED

(2007) A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. Cancer Cell 11(1):37–51, doi:S1535-6108(06)00372-2 [pii] 10.1016/j.ccr.2006.10.020

Bozza PT, Viola JP (2010) Lipid droplets in inflammation and cancer. Prostaglandins Leukot Essent Fatty Acids 82(4–6):243–250, doi:S0952-3278(10)00049-9 [pii] 10.1016/j.plefa.2010.02.005

Bridges EM, Harris AL (2011) The angiogenic process as a therapeutic target in cancer. Biochem Pharmacol 81(10):1183–1191, doi:S0006-2952(11)00120-1 [pii] 10.1016/j.bcp.2011.02.0160

Brookheart RT, Michel CI, Schaffer JE (2009) As a matter of fat. Cell Metab 10(1):9–12, doi:S1550-4131(09)00089-8 [pii] 10.1016/j.cmet.2009.03.011

Buzzai M, Jones RG, Amaravadi RK, Lum JJ, DeBerardinis RJ, Zhao F, Viollet B, Thompson CB (2007) Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. Cancer Res 67(14):6745–6752, doi:67/14/6745 [pii] 10.1158/0008-5472.CAN-06-4447

Cairns RA, Harris IS, Mak TW (2011) Regulation of cancer cell metabolism. Nat Rev Cancer 11(2):85–95, doi:nrc2981 [pii] 10.1038/nrc2981

Caldwell SA, Jackson SR, Shahriari KS, Lynch TP, Sethi G, Walker S, Vosseller K, Reginato MJ (2010) Nutrient sensor O-GlcNAc transferase regulates breast cancer tumorigenesis through targeting of the oncogenic transcription factor FoxM1. Oncogene 29(19):2831–2842, doi:onc201041 [pii] 10.1038/onc.2010.41

Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem 275(33):25130–25138. doi:10.1074/jbc.M001914200 M001914200 [pii]

Chaneton B, Hillmann P, Zheng L, Martin AC, Maddocks OD, Chokkathukalam A, Coyle JE, Jankevics A, Holding FP, Vousden KH, Frezza C, O'Reilly M, Gottlieb E (2012) Serine is a natural ligand and allosteric activator of pyruvate kinase M2. Nature 491(7424):458–462, doi:nature11540 [pii] 10.1038/nature11540

Chen L, Endler A, Shibasaki F (2009) Hypoxia and angiogenesis: regulation of hypoxia-inducible factors via novel binding factors. Exp Mol Med 41(12):849–857. doi:10.3858/emm.2009.41.12.103

Chen H, Wang L, Beretov J, Hao J, Xiao W, Li Y (2010) Co-expression of CD147/EMMPRIN with monocarboxylate transporters and multiple drug resistance proteins is associated with epithelial ovarian cancer progression. Clin Exp Metastasis 27(8):557–569. doi:10.1007/s10585-010-9345-9

Cheong JH, Park ES, Liang J, Dennison JB, Tsavachidou D, Nguyen-Charles C, Wa Cheng K, Hall H, Zhang D, Lu Y, Ravoori M, Kundra V, Ajani J, Lee JS, Ki Hong W, Mills GB (2011) Dual inhibition of tumor energy pathway by 2-deoxyglucose and metformin is effective against a broad spectrum of preclinical cancer models. Mol Cancer Ther 10(12):2350–2362, doi:1535-7163.MCT-11-0497 [pii] 10.1158/1535-7163.MCT-11-0497

Chiavarina B, Martinez-Outschoorn UE, Whitaker-Menezes D, Howell A, Tanowitz HB, Pestell RG, Sotgia F, Lisanti MP (2012) Metabolic reprogramming and two-compartment tumor metabolism: opposing role(s) of HIF1alpha and HIF2alpha in tumor-associated fibroblasts and human breast cancer cells. Cell Cycle 11(17):3280–3289, doi:21643 [pii] 10.4161/cc.21643

Chiche J, Brahimi-Horn MC, Pouyssegur J (2010) Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer. J Cell Mol Med 14(4):771–794, doi:JCMM994 [pii] 10.1111/j.1582-4934.2009.00994.x

Christofk HR, Vander Heiden MG, Harris MH, Ramanathan A, Gerszten RE, Wei R, Fleming MD, Schreiber SL, Cantley LC (2008a) The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. Nature 452(7184):230–233, doi:nature06734 [pii] 10.1038/nature06734

Christofk HR, Vander Heiden MG, Wu N, Asara JM, Cantley LC (2008b) Pyruvate kinase M2 is a phosphotyrosine-binding protein. Nature 452(7184):181–186, doi:nature06667 [pii] 10.1038/nature06667

Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A, Rasku MA, Arumugam S, Dean WL, Eaton J, Lane A, Trent JO, Chesney J (2008) Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. Mol Cancer Ther 7(1):110–120, doi:7/1/110 [pii] 10.1158/1535-7163.MCT-07-0482

Dang CV (2012) Links between metabolism and cancer. Genes Dev 26(9):877–890, doi:26/9/877 [pii] 10.1101/gad.189365.112

Dang CV, Kim JW, Gao P, Yustein J (2008) The interplay between MYC and HIF in cancer. Nat Rev Cancer 8(1):51–56, doi:nrc2274 [pii] 10.1038/nrc2274

Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM (2009) Cancer-associated IDH1 mutations produce 2-hydroxyglutarate. Nature 462(7274):739–744, doi:nature08617 [pii] 10.1038/nature08617

Daye D, Wellen KE (2012) Metabolic reprogramming in cancer: unraveling the role of glutamine in tumorigenesis. Semin Cell Dev Biol 23(4):362–369, doi:S1084-9521(12)00034-1 [pii] 10.1016/j.semcdb.2012.02.002

DeBerardinis RJ, Cheng T (2010) Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. Oncogene 29(3):313–324, doi:onc2009358 [pii] 10.1038/onc.2009.358

DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB (2007) Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A 104(49):19345–19350, doi:0709747104 [pii] 10.1073/pnas.0709747104

DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 7(1):11–20, doi:S1550-4131(07)00295-1 [pii] 10.1016/j.cmet.2007.10.002

Denko NC (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 8(9):705–713. doi:10.1038/nrc2468

Diers AR, Broniowska KA, Chang CF, Hogg N (2012) Pyruvate fuels mitochondrial respiration and proliferation of breast cancer cells: effect of monocarboxylate transporter inhibition. Biochem J 444(3):561–571, doi:BJ20120294 [pii] 10.1042/BJ20120294

Dioum EM, Chen R, Alexander MS, Zhang Q, Hogg RT, Gerard RD, Garcia JA (2009) Regulation of hypoxia-inducible factor 2alpha signaling by the stress-responsive deacetylase sirtuin 1. Science 324(5932):1289–1293, doi:324/5932/1289 [pii] 10.1126/science.1169956

Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, Thompson CB (2004) Akt stimulates aerobic glycolysis in cancer cells. Cancer Res 64(11):3892–3899, 10.1158/0008-5472.CAN-03-2904 64/11/3892 [pii]

Engel M, Mazurek S, Eigenbrodt E, Welter C (2004) Phosphoglycerate mutase-derived polypeptide inhibits glycolytic flux and induces cell growth arrest in tumor cell lines. J Biol Chem 279(34):35803–35812, doi:10.1074/jbc.M402768200 M402768200 [pii]

Evans JM, Ogston SA, Emslie-Smith A, Morris AD (2006) Risk of mortality and adverse cardiovascular outcomes in type 2 diabetes: a comparison of patients treated with sulfonylureas and metformin. Diabetologia 49(5):930–936. doi:10.1007/s00125-006-0176-9

Faleck DM, Ali K, Roat R, Graham MJ, Crooke RM, Battisti R, Garcia E, Ahima RS, Imai Y (2010) Adipose differentiation-related protein regulates lipids and insulin in pancreatic islets. Am J Physiol Endocrinol Metab 299(2):E249–E257, doi:ajpendo.00646.2009 [pii] 10.1152/ajpendo.00646.2009

Fantin VR, St-Pierre J, Leder P (2006) Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9(6):425–434, doi:S1535-6108(06)00145-0 [pii] 10.1016/j.ccr.2006.04.023

Farese RV Jr, Walther TC (2009) Lipid droplets finally get a little R-E-S-P-E-C-T. Cell 139(5):855–860, doi:S0092-8674(09)01417-2 [pii] 10.1016/j.cell.2009.11.005

Favaro E, Ramachandran A, McCormick R, Gee H, Blancher C, Crosby M, Devlin C, Blick C, Buffa F, Li JL, Vojnovic B, Piresdas Neves R, Glazer P, Iborra F, Ivan M, Ragoussis J, Harris AL (2010) MicroRNA-210 regulates mitochondrial free radical response to hypoxia and Krebs

cycle in cancer cells by targeting iron sulfur cluster protein ISCU. PLoS One 5(4):e10345. doi:10.1371/journal.pone.0010345

Favaro E, Bensaad K, Chong MG, Tennant DA, Ferguson DJ, Snell C, Steers G, Turley H, Li JL, Gunther UL, Buffa FM, McIntyre A, Harris AL (2012) Glucose utilization via glycogen phosphorylase sustains proliferation and prevents premature senescence in cancer cells. Cell Metab. doi:S1550-4131(12)00451-2 [pii] 10.1016/j.cmet.2012.10.017

Fico A, Paglialunga F, Cigliano L, Abrescia P, Verde P, Martini G, Iaccarino I, Filosa S (2004) Glucose-6-phosphate dehydrogenase plays a crucial role in protection from redox-stress-induced apoptosis. Cell Death Differ 11(8):823–831. doi:10.1038/sj.cdd.4401420, 4401420 [pii]

Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, Wolniak K, Peeters JK, Liu W, Choe SE, Fantin VR, Paietta E, Lowenberg B, Licht JD, Godley LA, Delwel R, Valk PJ, Thompson CB, Levine RL, Melnick A (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 18(6):553–567, doi:S1535-6108(10)00483-6 [pii] 10.1016/j.ccr.2010.11.015

Folger O, Jerby L, Frezza C, Gottlieb E, Ruppin E, Shlomi T (2011) Predicting selective drug targets in cancer through metabolic networks. Mol Syst Biol 7:501, doi:msb201135 [pii] 10.1038/msb.2011.35

Fritz V, Benfodda Z, Rodier G, Henriquet C, Iborra F, Avances C, Allory Y, de la Taille A, Culine S, Blancou H, Cristol JP, Michel F, Sardet C, Fajas L (2010) Abrogation of de novo lipogenesis by stearoyl-CoA desaturase 1 inhibition interferes with oncogenic signaling and blocks prostate cancer progression in mice. Mol Cancer Ther 9(6):1740–1754, doi:1535-7163.MCT-09-1064 [pii] 10.1158/1535-7163.MCT-09-1064

Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL (2007) HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. Cell 129(1):111–122, doi:S0092-8674(07)00307-8 [pii] 10.1016/j.cell.2007.01.047

Furuta E, Pai SK, Zhan R, Bandyopadhyay S, Watabe M, Mo YY, Hirota S, Hosobe S, Tsukada T, Miura K, Kamada S, Saito K, Iiizumi M, Liu W, Ericsson J, Watabe K (2008) Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1. Cancer Res 68(4):1003–1011, doi:68/4/1003 [pii] 10.1158/0008-5472.CAN-07-2489

Ganapathy-Kanniappan S, Vali M, Kunjithapatham R, Buijs M, Syed LH, Rao PP, Ota S, Kwak BK, Loffroy R, Geschwind JF (2010) 3-bromopyruvate: a new targeted antiglycolytic agent and a promise for cancer therapy. Curr Pharm Biotechnol 11(5):510–517, doi:BSP/CPB/E-Pub/0078-11-5 [pii]

Gao J, Serrero G (1999) Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. J Biol Chem 274(24):16825–16830

Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 458(7239):762–765, doi:nature07823 [pii] 10.1038/nature07823

Gimm T, Wiese M, Teschemacher B, Deggerich A, Schodel J, Knaup KX, Hackenbeck T, Hellerbrand C, Amann K, Wiesener MS, Honing S, Eckardt KU, Warnecke C (2010) Hypoxia-inducible protein 2 is a novel lipid droplet protein and a specific target gene of hypoxia-inducible factor-1. FASEB J 24(11):4443–4458, doi:fj.10-159806 [pii] 10.1096/fj.10-159806

Gordan JD, Bertout JA, Hu CJ, Diehl JA, Simon MC (2007) HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell 11(4):335–347, doi:S1535-6108(07)00059-1 [pii] 10.1016/j.ccr.2007.02.006

Gordan JD, Lal P, Dondeti VR, Letrero R, Parekh KN, Oquendo CE, Greenberg RA, Flaherty KT, Rathmell WK, Keith B, Simon MC, Nathanson KL (2008) HIF-alpha effects on c-Myc distinguish two subtypes of sporadic VHL-deficient clear cell renal carcinoma. Cancer Cell 14(6):435–446, doi:S1535-6108(08)00366-8 [pii] 10.1016/j.ccr.2008.10.016

Gottlieb E, Tomlinson IP (2005) Mitochondrial tumour suppressors: a genetic and biochemical update. Nat Rev Cancer 5(11):857–866, doi:nrc1737 [pii] 10.1038/nrc1737

Gu Y, Mi W, Ge Y, Liu H, Fan Q, Han C, Yang J, Han F, Lu X, Yu W (2010) GlcNAcylation plays an essential role in breast cancer metastasis. Cancer Res 70(15):6344–6351, doi:0008-5472.CAN-09-1887 [pii] 10.1158/0008-5472.CAN-09-1887

Gupta SC, Hevia D, Patchva S, Park B, Koh W, Aggarwal BB (2012) Upsides and downsides of reactive oxygen species for cancer: the roles of reactive oxygen species in tumorigenesis, prevention, and therapy. Antioxid Redox Signal 16(11):1295–1322. doi:10.1089/ars.2011.4414

Guzy RD, Schumacker PT (2006) Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol 91(5):807–819, doi:expphysiol.2006.033506 [pii] 10.1113/expphysiol.2006.033506

Guzy RD, Hoyos B, Robin E, Chen H, Liu L, Mansfield KD, Simon MC, Hammerling U, Schumacker PT (2005) Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab 1(6):401–408, doi:S1550-4131(05)00139-7 [pii] 10.1016/j.cmet.2005.05.001

Guzy RD, Mack MM, Schumacker PT (2007) Mitochondrial complex III is required for hypoxia-induced ROS production and gene transcription in yeast. Antioxid Redox Signal 9(9):1317–1328. doi:10.1089/ars.2007.1708

Haigis MC, Yankner BA (2010) The aging stress response. Mol Cell 40(2):333–344, doi:S1097-2765(10)00778-1 [pii] 10.1016/j.molcel.2010.10.002

Halestrap AP, Wilson MC (2012) The monocarboxylate transporter family–role and regulation. IUBMB Life 64(2):109–119. doi:10.1002/iub.572

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100(1):57–70, doi:S0092-8674(00)81683-9 [pii]

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674, doi:S0092-8674(11)00127-9 [pii] 10.1016/j.cell.2011.02.013

Hardie DG, Ross FA, Hawley SA (2012) AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat Rev Mol Cell Biol 13(4):251–262, doi:nrm3311 [pii] 10.1038/nrm3311

Harris AL (2002) Hypoxia–a key regulatory factor in tumour growth. Nat Rev Cancer 2(1):38–47. doi:10.1038/nrc704

Hess D, Chisholm JW, Igal RA (2010) Inhibition of stearoylCoA desaturase activity blocks cell cycle progression and induces programmed cell death in lung cancer cells. PLoS One 5(6):e11394. doi:10.1371/journal.pone.0011394

Hickey MM, Simon MC (2006) Regulation of angiogenesis by hypoxia and hypoxia-inducible factors. Curr Top Dev Biol 76:217–257, doi:S0070-2153(06)76007-0 [pii] 10.1016/S0070-2153(06)76007-0

Hirsch HA, Iliopoulos D, Tsichlis PN, Struhl K (2009) Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res 69(19):7507–7511, doi:0008-5472.CAN-09-2994 [pii] 10.1158/0008-5472.CAN-09-2994

Hu W, Zhang C, Wu R, Sun Y, Levine A, Feng Z (2010) Glutaminase 2, a novel p53 target gene regulating energy metabolism and antioxidant function. Proc Natl Acad Sci U S A 107(16):7455–7460, doi:1001006107 [pii] 10.1073/pnas.1001006107

Izaki S, Goto H, Yokota S (2008) Increased chemosensitivity and elevated reactive oxygen species are mediated by glutathione reduction in glutamine deprived neuroblastoma cells. J Cancer Res Clin Oncol 134(7):761–768. doi:10.1007/s00432-007-0338-2

Jensen KS, Binderup T, Jensen KT, Therkelsen I, Borup R, Nilsson E, Multhaupt H, Bouchard C, Quistorff B, Kjaer A, Landberg G, Staller P (2011) FoxO3A promotes metabolic adaptation to hypoxia by antagonizing Myc function. EMBO J 30(22):4554–4570, doi:emboj2011323 [pii] 10.1038/emboj.2011.323

Jiang P, Du W, Wang X, Mancuso A, Gao X, Wu M, Yang X (2011) p53 regulates biosynthesis through direct inactivation of glucose-6-phosphate dehydrogenase. Nat Cell Biol 13(3):310–316, doi:ncb2172 [pii] 10.1038/ncb2172

Kilburn DG, Lilly MD, Webb FC (1969) The energetics of mammalian cell growth. J Cell Sci 4(3):645–654

Kim W, Liau LM (2012) IDH mutations in human glioma. Neurosurg Clin N Am 23(3):471–480, doi:S1042-3680(12)00048-4 [pii] 10.1016/j.nec.2012.04.009

Kim JW, Tchernyshyov I, Semenza GL, Dang CV (2006a) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 3(3):177–185, doi:S1550-4131(06)00062-3 [pii] 10.1016/j.cmet.2006.02.002

Kim WY, Safran M, Buckley MR, Ebert BL, Glickman J, Bosenberg M, Regan M, Kaelin WG Jr (2006b) Failure to prolyl hydroxylate hypoxia-inducible factor alpha phenocopies VHL inactivation in vivo. EMBO J 25(19):4650–4662, doi:7601300 [pii] 10.1038/sj.emboj.7601300

Kletzien RF, Harris PK, Foellmi LA (1994) Glucose-6-phosphate dehydrogenase: a "housekeeping" enzyme subject to tissue-specific regulation by hormones, nutrients, and oxidant stress. FASEB J 8(2):174–181

Ko YH, Smith BL, Wang Y, Pomper MG, Rini DA, Torbenson MS, Hullihen J, Pedersen PL (2004) Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP. Biochem Biophys Res Commun 324(1):269–275, doi:S0006-291X(04)02062-5 [pii] 10.1016/j.bbrc.2004.09.047

Kobayashi S, Millhorn DE (2001) Hypoxia regulates glutamate metabolism and membrane transport in rat PC12 cells. J Neurochem 76(6):1935–1948

Koivunen P, Lee S, Duncan CG, Lopez G, Lu G, Ramkissoon S, Losman JA, Joensuu P, Bergmann U, Gross S, Travins J, Weiss S, Looper R, Ligon KL, Verhaak RG, Yan H, Kaelin WG Jr (2012) Transformation by the (R)-enantiomer of 2-hydroxyglutarate linked to EGLN activation. Nature 483(7390):484–488, doi:nature10898 [pii] 10.1038/nature10898

Kondoh H, Lleonart ME, Gil J, Wang J, Degan P, Peters G, Martinez D, Carnero A, Beach D (2005) Glycolytic enzymes can modulate cellular life span. Cancer Res 65(1):177–185, doi:65/1/177 [pii]

Koppenol WH, Bounds PL, Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer 11(5):325–337, doi:nrc3038 [pii] 10.1038/nrc3038

Krishnan J, Suter M, Windak R, Krebs T, Felley A, Montessuit C, Tokarska-Schlattner M, Aasum E, Bogdanova A, Perriard E, Perriard JC, Larsen T, Pedrazzini T, Krek W (2009) Activation of a HIF1alpha-PPARgamma axis underlies the integration of glycolytic and lipid anabolic pathways in pathologic cardiac hypertrophy. Cell Metab 9(6):512–524, doi:S1550-4131(09)00139-9 [pii] 10.1016/j.cmet.2009.05.005

Kwon SJ, Lee YJ (2005) Effect of low glutamine/glucose on hypoxia-induced elevation of hypoxia-inducible factor-1alpha in human pancreatic cancer MiaPaCa-2 and human prostatic cancer DU-145 cells. Clin Cancer Res 11(13):4694–4700, doi:11/13/4694 [pii] 10.1158/1078-0432.CCR-04-2530

Laemmle A, Lechleiter A, Roh V, Schwarz C, Portmann S, Furer C, Keogh A, Tschan MP, Candinas D, Vorburger SA, Stroka D (2012) Inhibition of SIRT1 impairs the accumulation and transcriptional activity of HIF-1alpha protein under hypoxic conditions. PLoS One 7(3):e33433, doi:10.1371/journal.pone.0033433 PONE-D-12-00867 [pii]

Laurenti G, Benedetti E, D'Angelo B, Cristiano L, Cinque B, Raysi S, Alecci M, Ceru MP, Cifone MG, Galzio R, Giordano A, Cimini A (2011) Hypoxia induces peroxisome proliferator-activated receptor alpha (PPARalpha) and lipid metabolism peroxisomal enzymes in human glioblastoma cells. J Cell Biochem 112(12):3891–3901. doi:10.1002/jcb.23323

Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, Royer RE, Vander Jagt DL, Semenza GL, Dang CV (2010) Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc Natl Acad Sci U S A 107(5):2037–2042, doi:0914433107 [pii] 10.1073/pnas.0914433107

Le A, Lane AN, Hamaker M, Bose S, Gouw A, Barbi J, Tsukamoto T, Rojas CJ, Slusher BS, Zhang H, Zimmerman LJ, Liebler DC, Slebos RJ, Lorkiewicz PK, Higashi RM, Fan TW, Dang CV (2012) Glucose-independent glutamine metabolism via TCA cycling for proliferation and survival in B cells. Cell Metab 15(1):110–121, doi:S1550-4131(11)00468-2 [pii] 10.1016/j.cmet.2011.12.009

Levine AJ, Puzio-Kuter AM (2010) The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. Science 330(6009):1340–1344, doi:330/6009/1340 [pii] 10.1126/science.1193494

Li J, Ding SF, Habib NA, Fermor BF, Wood CB, Gilmour RS (1994) Partial characterization of a cDNA for human stearoyl-CoA desaturase and changes in its mRNA expression in some normal and malignant tissues. Int J Cancer 57(3):348–352

Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, Kim JW, Yustein JT, Lee LA, Dang CV (2005) Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. Mol Cell Biol 25(14):6225–6234, doi:25/14/6225 [pii] 10.1128/MCB.25.14.6225-6234.2005

Lim JH, Lee YM, Chun YS, Chen J, Kim JE, Park JW (2010) Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. Mol Cell 38(6):864–878, doi:S1097-2765(10)00385-0 [pii] 10.1016/j.molcel.2010.05.023

Listenberger LL, Han X, Lewis SE, Cases S, Farese RV Jr, Ory DS, Schaffer JE (2003) Triglyceride accumulation protects against fatty acid-induced lipotoxicity. Proc Natl Acad Sci U S A 100(6):3077–3082, doi:10.1073/pnas.0630588100 0630588100 [pii]

Liu Y (2006) Fatty acid oxidation is a dominant bioenergetic pathway in prostate cancer. Prostate Cancer Prostatic Dis 9(3):230–234, doi:4500879 [pii] 10.1038/sj.pcan.4500879

Liu K, Czaja MJ (2013) Regulation of lipid stores and metabolism by lipophagy. Cell Death Differ 20(1):3–11, doi:cdd201263 [pii] 10.1038/cdd.2012.63

Locasale JW, Cantley LC (2011) Metabolic flux and the regulation of mammalian cell growth. Cell Metab 14(4):443–451, doi:S1550-4131(11)00345-7 [pii] 10.1016/j.cmet.2011.07.014

Locasale JW, Grassian AR, Melman T, Lyssiotis CA, Mattaini KR, Bass AJ, Heffron G, Metallo CM, Muranen T, Sharfi H, Sasaki AT, Anastasiou D, Mullarky E, Vokes NI, Sasaki M, Beroukhim R, Stephanopoulos G, Ligon AH, Meyerson M, Richardson AL, Chin L, Wagner G, Asara JM, Brugge JS, Cantley LC, Vander Heiden MG (2011) Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. Nat Genet 43(9):869–874, doi:ng.890 [pii] 10.1038/ng.890

Loo SY, Chang MK, Chua CS, Kumar AP, Pervaiz S, Clement MV (2012) NHE-1: a promising target for novel anti-cancer therapeutics. Curr Pharm Des 18(10):1372–1382, doi:CPD-EPUB-20120223-002 [pii]

Losman JA, Looper R, Koivunen P, Lee S, Schneider RK, McMahon C, Cowley G, Root D, Ebert BL, Kaelin WG Jr (2013) (R)-2-hydroxyglutarate is sufficient to promote leukemogenesis and its effects are reversible. Science. doi:doi:science.1231677 [pii] 10.1126/science.1231677

Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O, Edwards CR, Khanin R, Figueroa ME, Melnick A, Wellen KE, O'Rourke DM, Berger SL, Chan TA, Levine RL, Mellinghoff IK, Thompson CB (2012) IDH mutation impairs histone demethylation and results in a block to cell differentiation. Nature 483(7390):474–478, doi:nature10860 [pii] 10.1038/nature10860

Luo W, Semenza GL (2011) Pyruvate kinase M2 regulates glucose metabolism by functioning as a coactivator for hypoxia-inducible factor 1 in cancer cells. Oncotarget 2(7):551–556, doi:299 [pii]

Luo W, Hu H, Chang R, Zhong J, Knabel M, O'Meally R, Cole RN, Pandey A, Semenza GL (2011) Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell 145(5):732–744, doi:S0092-8674(11)00436-3 [pii] 10.1016/j.cell.2011.03.054

Maddocks OD, Vousden KH (2011) Metabolic regulation by p53. J Mol Med (Berl) 89(3):237–245. doi:10.1007/s00109-011-0735-5

Maddocks OD, Berkers CR, Mason SM, Zheng L, Blyth K, Gottlieb E, Vousden KH (2013) Serine starvation induces stress and p53-dependent metabolic remodelling in cancer cells. Nature 493(7433):542–546, doi:nature11743 [pii] 10.1038/nature11743

Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurley PJ, Bunz F, Hwang PM (2006) p53 regulates mitochondrial respiration. Science 312(5780):1650–1653, doi:1126863 [pii] 10.1126/science.1126863

Menendez JA, Lupu R (2007) Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer 7(10):763–777, doi:nrc2222 [pii] 10.1038/nrc2222

Mera P, Bentebibel A, Lopez-Vinas E, Cordente AG, Gurunathan C, Sebastian D, Vazquez I, Herrero L, Ariza X, Gomez-Puertas P, Asins G, Serra D, Garcia J, Hegardt FG (2009) C75 is

converted to C75-CoA in the hypothalamus, where it inhibits carnitine palmitoyltransferase 1 and decreases food intake and body weight. Biochem Pharmacol 77(6):1084–1095, doi:S0006-2952(08)00855-1 [pii] 10.1016/j.bcp.2008.11.020

Metallo CM, Gameiro PA, Bell EL, Mattaini KR, Yang J, Hiller K, Jewell CM, Johnson ZR, Irvine DJ, Guarente L, Kelleher JK, Vander Heiden MG, Iliopoulos O, Stephanopoulos G (2011) Reductive glutamine metabolism by IDH1 mediates lipogenesis under hypoxia. Nature 481(7381):380–384, doi:nature10602 [pii] 10.1038/nature10602

Michelakis ED, Sutendra G, Dromparis P, Webster L, Haromy A, Niven E, Maguire C, Gammer TL, Mackey JR, Fulton D, Abdulkarim B, McMurtry MS, Petruk KC (2010) Metabolic modulation of glioblastoma with dichloroacetate. Sci Transl Med 2(31):31ra34, doi:2/31/31ra34 [pii] 10.1126/scitranslmed.3000677

Minamishima YA, Moslehi J, Padera RF, Bronson RT, Liao R, Kaelin WG Jr (2009) A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo. Mol Cell Biol 29(21):5729–5741, doi:MCB.00331-09 [pii] 10.1128/MCB.00331-09

Minchenko OH, Opentanova IL, Ogura T, Minchenko DO, Komisarenko SV, Caro J, Esumi H (2005) Expression and hypoxia-responsiveness of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 4 in mammary gland malignant cell lines. Acta Biochim Pol 52(4):881–888, doi:20051025 [pii]

Morrish F, Isern N, Sadilek M, Jeffrey M, Hockenbery DM (2009) c-Myc activates multiple metabolic networks to generate substrates for cell-cycle entry. Oncogene 28(27):2485–2491, doi:onc2009112 [pii] 10.1038/onc.2009.112

Mullen AR, Wheaton WW, Jin ES, Chen PH, Sullivan LB, Cheng T, Yang Y, Linehan WM, Chandel NS, DeBerardinis RJ (2011) Reductive carboxylation supports growth in tumour cells with defective mitochondria. Nature 481(7381):385–388, doi:nature10642 [pii] 10.1038/nature10642

Murdoch C, Muthana M, Lewis CE (2005) Hypoxia regulates macrophage functions in inflammation. J Immunol 175(10):6257–6263, doi:175/10/6257 [pii]

Mylonis I, Sembongi H, Befani C, Liakos P, Siniossoglou S, Simos G (2012) Hypoxia causes triglyceride accumulation by HIF-1-mediated stimulation of lipin 1 expression. J Cell Sci 125(Pt 14):3485–3493, doi:jcs.106682 [pii] 10.1242/jcs.106682

Narravula S, Colgan SP (2001) Hypoxia-inducible factor 1-mediated inhibition of peroxisome proliferator-activated receptor alpha expression during hypoxia. J Immunol 166(12):7543–7548

Nishiyama Y, Goda N, Kanai M, Niwa D, Osanai K, Yamamoto Y, Senoo-Matsuda N, Johnson RS, Miura S, Kabe Y, Suematsu M (2012) HIF-1alpha induction suppresses excessive lipid accumulation in alcoholic fatty liver in mice. J Hepatol 56(2):441–447, doi:S0168-8278(11)00659-3 [pii] 10.1016/j.jhep.2011.07.024

Nomura DK, Long JZ, Niessen S, Hoover HS, Ng SW, Cravatt BF (2010) Monoacylglycerol lipase regulates a fatty acid network that promotes cancer pathogenesis. Cell 140(1):49–61, doi:S0092-8674(09)01439-1 [pii] 10.1016/j.cell.2009.11.027

Obach M, Navarro-Sabate A, Caro J, Kong X, Duran J, Gomez M, Perales JC, Ventura F, Rosa JL, Bartrons R (2004) 6-Phosphofructo-2-kinase (pfkfb3) gene promoter contains hypoxia-inducible factor-1 binding sites necessary for transactivation in response to hypoxia. J Biol Chem 279(51):53562–53570, doi:M406096200 [pii] 10.1074/jbc.M406096200

Oktay Y, Dioum E, Matsuzaki S, Ding K, Yan LJ, Haller RG, Szweda LI, Garcia JA (2007) Hypoxia-inducible factor 2alpha regulates expression of the mitochondrial aconitase chaperone protein frataxin. J Biol Chem 282(16):11750–11756, doi:M611133200 [pii] 10.1074/jbc.M611133200

Pan Y, Mansfield KD, Bertozzi CC, Rudenko V, Chan DA, Giaccia AJ, Simon MC (2007) Multiple factors affecting cellular redox status and energy metabolism modulate hypoxia-inducible factor prolyl hydroxylase activity in vivo and in vitro. Mol Cell Biol 27(3):912–925, doi:MCB.01223-06 [pii] 10.1128/MCB.01223-06

Pandey PR, Liu W, Xing F, Fukuda K, Watabe K (2012) Anti-cancer drugs targeting fatty acid synthase (FAS). Recent Pat Anticancer Drug Discov 7(2):185–197, doi:PRA-EPUB-20120209-002 [pii]

Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC (2006) HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 3(3):187–197, doi:S1550-4131(06)00060-X [pii] 10.1016/j.cmet.2006.01.012

Patel SA, Simon MC (2008) Biology of hypoxia-inducible factor-2 alpha in development and disease. Cell Death Differ 15(4):628–634, doi:cdd200817 [pii] 10.1038/cdd.2008.17

Pelicano H, Martin DS, Xu RH, Huang P (2006) Glycolysis inhibition for anticancer treatment. Oncogene 25(34):4633–4646, doi:1209597 [pii] 10.1038/sj.onc.1209597

Pike LS, Smift AL, Croteau NJ, Ferrick DA, Wu M (2010) Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. Biochim Biophys Acta, doi:S0005-2728(10)00740-1 [pii] 10.1016/j.bbabio.2010.10.022

Pinheiro C, Longatto-Filho A, Simoes K, Jacob CE, Bresciani CJ, Zilberstein B, Cecconello I, Alves VA, Schmitt F, Baltazar F (2009) The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer. Eur J Cancer 45(13):2418–2424, doi:S0959-8049(09)00485-7 [pii] 10.1016/j.ejca.2009.06.018

Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, Schmitt F, Baltazar F (2010) Monocarboxylate transporter 1 is up-regulated in basal-like breast carcinoma. Histopathology 56(7):860–867, doi:HIS3560 [pii] 10.1111/j.1365-2559.2010.03560.x

Pollari S, Kakonen SM, Edgren H, Wolf M, Kohonen P, Sara H, Guise T, Nees M, Kallioniemi O (2011) Enhanced serine production by bone metastatic breast cancer cells stimulates osteoclastogenesis. Breast Cancer Res Treat 125(2):421–430. doi:10.1007/s10549-010-0848-5

Porporato PE, Dhup S, Dadhich RK, Copetti T, Sonveaux P (2011) Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. Front Pharmacol 2:49. doi:10.3389/fphar.2011.00049

Possemato R, Marks KM, Shaul YD, Pacold ME, Kim D, Birsoy K, Sethumadhavan S, Woo HK, Jang HG, Jha AK, Chen WW, Barrett FG, Stransky N, Tsun ZY, Cowley GS, Barretina J, Kalaany NY, Hsu PP, Ottina K, Chan AM, Yuan B, Garraway LA, Root DE, Mino-Kenudson M, Brachtel EF, Driggers EM, Sabatini DM (2011) Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. Nature 476(7360):346–350, doi:nature10350 [pii] 10.1038/nature10350

Prabhakar NR, Kumar GK, Nanduri J (2010) Intermittent hypoxia augments acute hypoxic sensing via HIF-mediated ROS. Respir Physiol Neurobiol 174(3):230–234, doi:S1569-9048(10)00326-5 [pii] 10.1016/j.resp.2010.08.022

Puskas LG, Feher LZ, Vizler C, Ayaydin F, Raso E, Molnar E, Magyary I, Kanizsai I, Gyuris M, Madacsi R, Fabian G, Farkas K, Hegyi P, Baska F, Ozsvari B, Kitajka K (2010) Polyunsaturated fatty acids synergize with lipid droplet binding thalidomide analogs to induce oxidative stress in cancer cells. Lipids Health Dis 9:56, doi:1476-511X-9-56 [pii] 10.1186/1476-511X-9-56

Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D (2011) RAS oncogenes: weaving a tumorigenic web. Nat Rev Cancer 11(11):761–774, doi:nrc3106 [pii] 10.1038/nrc3106

Qing G, Skuli N, Mayes PA, Pawel B, Martinez D, Maris JM, Simon MC (2010) Combinatorial regulation of neuroblastoma tumor progression by N-Myc and hypoxia inducible factor HIF-1alpha. Cancer Res 70(24):10351–10361, doi:0008-5472.CAN-10-0740 [pii] 10.1158/0008-5472.CAN-10-0740

Rademakers SE, Lok J, van der Kogel AJ, Bussink J, Kaanders JH (2011) Metabolic markers in relation to hypoxia; staining patterns and colocalization of pimonidazole, HIF-1alpha, CAIX, LDH-5, GLUT-1, MCT1 and MCT4. BMC Cancer 11:167, doi:1471-2407-11-167 [pii] 10.1186/1471-2407-11-167

Rakheja D, Konoplev S, Medeiros LJ, Chen W (2012) IDH mutations in acute myeloid leukemia. Hum Pathol 43(10):1541–1551, doi:S0046-8177(12)00165-7 [pii] 10.1016/j.humpath.2012.05.003

Rankin EB, Rha J, Selak MA, Unger TL, Keith B, Liu Q, Haase VH (2009) Hypoxia-inducible factor 2 regulates hepatic lipid metabolism. Mol Cell Biol 29(16):4527–4538, doi:MCB.00200-09 [pii] 10.1128/MCB.00200-09

Rey S, Semenza GL (2010) Hypoxia-inducible factor-1-dependent mechanisms of vascularization and vascular remodelling. Cardiovasc Res 86(2):236–242, doi:cvq045 [pii] 10.1093/cvr/cvq045

Rios EJ, Fallon M, Wang J, Shimoda LA (2005) Chronic hypoxia elevates intracellular pH and activates Na+/H+ exchange in pulmonary arterial smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 289(5):L867–L874, doi:00455.2004 [pii] 10.1152/ajplung.00455.2004

Robey RB, Hay N (2009) Is Akt the "Warburg kinase"?-Akt-energy metabolism interactions and oncogenesis. Semin Cancer Biol 19(1):25–31, doi:S1044-579X(08)00105-3 [pii] 10.1016/j.semcancer.2008.11.010

Roy S, Ghosh S, Mallick P, Maity P (2008) Acivicin with glutaminase regulates proliferation and invasion of human MCF-7 and OAW-42 cells–an in vitro study. Indian J Exp Biol 46(1):22–26

Rysman E, Brusselmans K, Scheys K, Timmermans L, Derua R, Munck S, Van Veldhoven PP, Waltregny D, Daniels VW, Machiels J, Vanderhoydonc F, Smans K, Waelkens E, Verhoeven G, Swinnen JV (2010) De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. Cancer Res 70(20):8117–8126, doi:0008-5472.CAN-09-3871 [pii] 10.1158/0008-5472.CAN-09-3871

Saarikoski ST, Rivera SP, Hankinson O (2002) Mitogen-inducible gene 6 (MIG-6), adipophilin and tuftelin are inducible by hypoxia. FEBS Lett 530(1–3):186–190, doi:S0014579302034750 [pii]

Salem AF, Whitaker-Menezes D, Lin Z, Martinez-Outschoorn UE, Tanowitz HB, Al-Zoubi MS, Howell A, Pestell RG, Sotgia F, Lisanti MP (2012) Two-compartment tumor metabolism: autophagy in the tumor microenvironment and oxidative mitochondrial metabolism (OXPHOS) in cancer cells. Cell Cycle 11(13):2545–2556, doi:20920 [pii] 10.4161/cc.20920

Samudio I, Harmancey R, Fiegl M, Kantarjian H, Konopleva M, Korchin B, Kaluarachchi K, Bornmann W, Duvvuri S, Taegtmeyer H, Andreeff M (2010) Pharmacologic inhibition of fatty acid oxidation sensitizes human leukemia cells to apoptosis induction. J Clin Invest 120(1):142–156, doi:38942 [pii] 10.1172/JCI38942

Santos CR, Schulze A (2012) Lipid metabolism in cancer. FEBS J 279(15):2610–2623. doi:10.1111/j.1742-4658.2012.08644.x

Sasaki M, Knobbe CB, Munger JC, Lind EF, Brenner D, Brustle A, Harris IS, Holmes R, Wakeham A, Haight J, You-Ten A, Li WY, Schalm S, Su SM, Virtanen C, Reifenberger G, Ohashi PS, Barber DL, Figueroa ME, Melnick A, Zuniga-Pflucker JC, Mak TW (2012) IDH1(R132H) mutation increases murine haematopoietic progenitors and alters epigenetics. Nature 488(7413):656–659, doi:nature11323 [pii] 10.1038/nature11323

Scaglia N, Caviglia JM, Igal RA (2005) High stearoyl-CoA desaturase protein and activity levels in simian virus 40 transformed-human lung fibroblasts. Biochim Biophys Acta 1687(1–3):141–151, doi:S1388-1981(04)00199-4 [pii] 10.1016/j.bbalip.2004.11.015

Scaglia N, Chisholm JW, Igal RA (2009) Inhibition of stearoylCoA desaturase-1 inactivates acetyl-CoA carboxylase and impairs proliferation in cancer cells: role of AMPK. PLoS One 4(8):e6812. doi:10.1371/journal.pone.0006812

Schonfeld P, Wojtczak L (2008) Fatty acids as modulators of the cellular production of reactive oxygen species. Free Radic Biol Med 45(3):231–241, doi:S0891-5849(08)00250-5 [pii] 10.1016/j.freeradbiomed.2008.04.029

Schug TT, Li X (2011) Sirtuin 1 in lipid metabolism and obesity. Ann Med 43(3):198–211, 10.3109/07853890.2010.547211

Schwartzenberg-Bar-Yoseph F, Armoni M, Karnieli E (2004) The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. Cancer Res 64(7): 2627–2633

Scortegagna M, Ding K, Oktay Y, Gaur A, Thurmond F, Yan LJ, Marck BT, Matsumoto AM, Shelton JM, Richardson JA, Bennett MJ, Garcia JA (2003) Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet 35(4):331–340, doi:10.1038/ng1266ng1266 [pii]

Sears R, Leone G, DeGregori J, Nevins JR (1999) Ras enhances Myc protein stability. Mol Cell 3(2):169–179, doi:S1097-2765(00)80308-1 [pii]

Seltzer MJ, Bennett BD, Joshi AD, Gao P, Thomas AG, Ferraris DV, Tsukamoto T, Rojas CJ, Slusher BS, Rabinowitz JD, Dang CV, Riggins GJ (2010) Inhibition of glutaminase preferentially slows growth of glioma cells with mutant IDH1. Cancer Res 70(22):8981–8987, doi:0008-5472.CAN-10-1666 [pii] 10.1158/0008-5472.CAN-10-1666

Semenza GL (2010) Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29(5):625–634, doi:onc2009441 [pii] 10.1038/onc.2009.441

Semenza GL (2012) Hypoxia-inducible factors: mediators of cancer progression and targets for cancer therapy. Trends Pharmacol Sci 33(4):207–214, doi:S0165-6147(12)00017-X [pii] 10.1016/j.tips.2012.01.005

Semenza GL, Roth PH, Fang HM, Wang GL (1994) Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 269(38):23757–23763

Shen GM, Zhao YZ, Chen MT, Zhang FL, Liu XL, Wang Y, Liu CZ, Yu J, Zhang JW (2010) Hypoxia-inducible factor-1 (HIF-1) promotes LDL and VLDL uptake through inducing VLDLR under hypoxia. Biochem J 441(2):675–683, doi:BJ20111377 [pii] 10.1042/BJ20111377

Shi Y, Tomic J, Wen F, Shaha S, Bahlo A, Harrison R, Dennis JW, Williams R, Gross BJ, Walker S, Zuccolo J, Deans JP, Hart GW, Spaner DE (2010) Aberrant O-GlcNAcylation characterizes chronic lymphocytic leukemia. Leukemia 24(9):1588–1598, doi:leu2010152 [pii] 10.1038/leu.2010.152

Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV (1997) c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci U S A 94(13):6658–6663

Shimoda LA, Fallon M, Pisarcik S, Wang J, Semenza GL (2006) HIF-1 regulates hypoxic induction of NHE1 expression and alkalinization of intracellular pH in pulmonary arterial myocytes. Am J Physiol Lung Cell Mol Physiol 291(5):L941–L949, doi:00528.2005 [pii] 10.1152/ajplung.00528.2005

Singh R, Cuervo AM (2012) Lipophagy: connecting autophagy and lipid metabolism. Int J Cell Biol 2012:282041. doi:10.1155/2012/282041

Singh R, Kaushik S, Wang Y, Xiang Y, Novak I, Komatsu M, Tanaka K, Cuervo AM, Czaja MJ (2009) Autophagy regulates lipid metabolism. Nature 458(7242):1131–1135, doi:nature07976 [pii] 10.1038/nature07976

Smeland TE, Nada M, Cuebas D, Schulz H (1992) NADPH-dependent beta-oxidation of unsaturated fatty acids with double bonds extending from odd-numbered carbon atoms. Proc Natl Acad Sci U S A 89(15):6673–6677

Soh H, Wasa M, Fukuzawa M (2007) Hypoxia upregulates amino acid transport in a human neuroblastoma cell line. J Pediatr Surg 42(4):608–612, doi:S0022-3468(06)00940-7 [pii] 10.1016/j.jpedsurg.2006.12.010

Sotgia F, Martinez-Outschoorn UE, Howell A, Pestell RG, Pavlides S, Lisanti MP (2012) Caveolin-1 and cancer metabolism in the tumor microenvironment: markers, models, and mechanisms. Annu Rev Pathol 7:423–467. doi:10.1146/annurev-pathol-011811-120856

Stambolsky P, Weisz L, Shats I, Klein Y, Goldfinger N, Oren M, Rotter V (2006) Regulation of AIF expression by p53. Cell Death Differ 13(12):2140–2149, doi:4401965 [pii] 10.1038/sj.cdd.4401965

Su X, Abumrad NA (2009) Cellular fatty acid uptake: a pathway under construction. Trends Endocrinol Metab 20(2):72–77, doi:S1043-2760(09)00003-4 [pii] 10.1016/j.tem.2008.11.001

Suzuki S, Tanaka T, Poyurovsky MV, Nagano H, Mayama T, Ohkubo S, Lokshin M, Hosokawa H, Nakayama T, Suzuki Y, Sugano S, Sato E, Nagao T, Yokote K, Tatsuno I, Prives C (2010) Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. Proc Natl Acad Sci U S A 107(16):7461–7466, doi:1002459107 [pii] 10.1073/pnas.1002459107

Swinnen JV, Brusselmans K, Verhoeven G (2006) Increased lipogenesis in cancer cells: new players, novel targets. Curr Opin Clin Nutr Metab Care 9(4):358–365, doi:10.1097/01.mco.0000232894.28674.30, 00075197-200607000-00005 [pii]

Tamura K, Makino A, Hullin-Matsuda F, Kobayashi T, Furihata M, Chung S, Ashida S, Miki T, Fujioka T, Shuin T, Nakamura Y, Nakagawa H (2009) Novel lipogenic enzyme ELOVL7 is involved in prostate cancer growth through saturated long-chain fatty acid metabolism. Cancer Res 69(20):8133–8140, doi:0008-5472.CAN-09-0775 [pii] 10.1158/0008-5472.CAN-09-0775

Tan EY, Yan M, Campo L, Han C, Takano E, Turley H, Candiloro I, Pezzella F, Gatter KC, Millar EK, O'Toole SA, McNeil CM, Crea P, Segara D, Sutherland RL, Harris AL, Fox SB (2009) The key hypoxia regulated gene CAIX is upregulated in basal-like breast tumours and is associated

with resistance to chemotherapy. Br J Cancer 100(2):405–411, doi:6604844 [pii] 10.1038/sj.bjc.6604844

Tello D, Balsa E, Acosta-Iborra B, Fuertes-Yebra E, Elorza A, Ordonez A, Corral-Escariz M, Soro I, Lopez-Bernardo E, Perales-Clemente E, Martinez-Ruiz A, Enriquez JA, Aragones J, Cadenas S, Landazuri MO (2011) Induction of the mitochondrial NDUFA4L2 protein by HIF-1alpha decreases oxygen consumption by inhibiting complex I activity. Cell Metab 14(6):768–779, doi:S1550-4131(11)00394-9 [pii] 10.1016/j.cmet.2011.10.008

Tian WN, Braunstein LD, Apse K, Pang J, Rose M, Tian X, Stanton RC (1999) Importance of glucose-6-phosphate dehydrogenase activity in cell death. Am J Physiol 276(5 Pt 1):C1121–C1131

Ullah MS, Davies AJ, Halestrap AP (2006) The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem 281(14):9030–9037, doi:M511397200 [pii] 10.1074/jbc.M511397200

Vander Heiden MG (2011) Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov 10(9):671–684, doi:nrd3504 [pii] 10.1038/nrd3504

Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324(5930):1029–1033, doi:324/5930/1029 [pii] 10.1126/science.1160809

Vander Heiden MG, Locasale JW, Swanson KD, Sharfi H, Heffron GJ, Amador-Noguez D, Christofk HR, Wagner G, Rabinowitz JD, Asara JM, Cantley LC (2010) Evidence for an alternative glycolytic pathway in rapidly proliferating cells. Science 329(5998):1492–1499, doi:329/5998/1492 [pii] 10.1126/science.1188015

Vousden KH, Ryan KM (2009) p53 and metabolism. Nat Rev Cancer 9(10):691–700, doi:nrc2715 [pii] 10.1038/nrc2715

Wang JB, Erickson JW, Fuji R, Ramachandran S, Gao P, Dinavahi R, Wilson KF, Ambrosio AL, Dias SM, Dang CV, Cerione RA (2010a) Targeting mitochondrial glutaminase activity inhibits oncogenic transformation. Cancer Cell 18(3):207–219, doi:S1535-6108(10)00306-5 [pii] 10.1016/j.ccr.2010.08.009

Wang C, Rajput S, Watabe K, Liao DF, Cao D (2010b) Acetyl-CoA carboxylase-a as a novel target for cancer therapy. Front Biosci (Schol Ed) 2:515–526, doi:82 [pii]

Warburg O (1956) On the origin of cancer cells. Science 123(3191):309–314

Ward PS, Patel J, Wise DR, Abdel-Wahab O, Bennett BD, Coller HA, Cross JR, Fantin VR, Hedvat CV, Perl AE, Rabinowitz JD, Carroll M, Su SM, Sharp KA, Levine RL, Thompson CB (2010) The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. Cancer Cell 17(3):225–234, doi:S1535-6108(10)00036-X [pii] 10.1016/j.ccr.2010.01.020

Wellen KE, Lu C, Mancuso A, Lemons JM, Ryczko M, Dennis JW, Rabinowitz JD, Coller HA, Thompson CB (2010) The hexosamine biosynthetic pathway couples growth factor-induced glutamine uptake to glucose metabolism. Genes Dev 24(24):2784–2799, doi:gad.1985910 [pii] 10.1101/gad.1985910

Whitmer JT, Idell-Wenger JA, Rovetto MJ, Neely JR (1978) Control of fatty acid metabolism in ischemic and hypoxic hearts. J Biol Chem 253(12):4305–4309

Wise DR, Thompson CB (2010) Glutamine addiction: a new therapeutic target in cancer. Trends Biochem Sci 35(8):427–433, doi:S0968-0004(10)00091-5 [pii] 10.1016/j.tibs.2010.05.003

Wise DR, DeBerardinis RJ, Mancuso A, Sayed N, Zhang XY, Pfeiffer HK, Nissim I, Daikhin E, Yudkoff M, McMahon SB, Thompson CB (2008) Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. Proc Natl Acad Sci U S A 105(48):18782–18787, doi:0810199105 [pii] 10.1073/pnas.0810199105

Wise DR, Ward PS, Shay JE, Cross JR, Gruber JJ, Sachdeva UM, Platt JM, DeMatteo RG, Simon MC, Thompson CB (2011) Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. Proc Natl Acad Sci U S A 108(49):19611–19616, doi:1117773108 [pii] 10.1073/pnas.1117773108

Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL (2000) Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60(24):7075–7083

Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Xiao MT, Liu LX, Jiang WQ, Liu J, Zhang JY, Wang B, Frye S, Zhang Y, Xu YH, Lei QY, Guan KL, Zhao SM, Xiong Y (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. Cancer Cell 19(1):17–30, doi:S1535-6108(10)00527-1 [pii] 10.1016/j.ccr.2010.12.014

Yalcin A, Telang S, Clem B, Chesney J (2009) Regulation of glucose metabolism by 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases in cancer. Exp Mol Pathol 86(3):174–179, doi:S0014-4800(09)00008-2 [pii] 10.1016/j.yexmp.2009.01.003

Ye J, Mancuso A, Tong X, Ward PS, Fan J, Rabinowitz JD, Thompson CB (2012) Pyruvate kinase M2 promotes de novo serine synthesis to sustain mTORC1 activity and cell proliferation. Proc Natl Acad Sci U S A 109(18):6904–6909, doi:1204176109 [pii] 10.1073/pnas.1204176109

Yoshii Y, Furukawa T, Yoshii H, Mori T, Kiyono Y, Waki A, Kobayashi M, Tsujikawa T, Kudo T, Okazawa H, Yonekura Y, Fujibayashi Y (2009) Cytosolic acetyl-CoA synthetase affected tumor cell survival under hypoxia: the possible function in tumor acetyl-CoA/acetate metabolism. Cancer Sci 100(5):821–827

Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M (2006) Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res 66(21):10269–10273, doi:0008-5472.CAN-06-1500 [pii] 10.1158/0008-5472.CAN-06-1500

Zaugg K, Yao Y, Reilly PT, Kannan K, Kiarash R, Mason J, Huang P, Sawyer SK, Fuerth B, Faubert B, Kalliomaki T, Elia A, Luo X, Nadeem V, Bungard D, Yalavarthi S, Growney JD, Wakeham A, Moolani Y, Silvester J, Ten AY, Bakker W, Tsuchihara K, Berger SL, Hill RP, Jones RG, Tsao M, Robinson MO, Thompson CB, Pan G, Mak TW (2011) Carnitine palmitoyltransferase 1C promotes cell survival and tumor growth under conditions of metabolic stress. Genes Dev 25(10):1041–1051, doi:25/10/1041 [pii] 10.1101/gad.1987211

Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, Dang CV, Semenza GL (2007) HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer Cell 11(5):407–420, doi:S1535-6108(07)00115-8 [pii] 10.1016/j.ccr.2007.04.001

Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB, Gonzalez FJ, Semenza GL (2008) Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. J Biol Chem 283(16):10892–10903, doi:M800102200 [pii] 10.1074/jbc.M800102200

Zhang WC, Shyh-Chang N, Yang H, Rai A, Umashankar S, Ma S, Soh BS, Sun LL, Tai BC, Nga ME, Bhakoo KK, Jayapal SR, Nichane M, Yu Q, Ahmed DA, Tan C, Sing WP, Tam J, Thirugananam A, Noghabi MS, Pang YH, Ang HS, Mitchell W, Robson P, Kaldis P, Soo RA, Swarup S, Lim EH, Lim B (2012) Glycine decarboxylase activity drives non-small cell lung cancer tumor-initiating cells and tumorigenesis. Cell 148(1–2):259–272, doi:S0092-8674(11)01444-9 [pii] 10.1016/j.cell.2011.11.050

Zhao F, Mancuso A, Bui TV, Tong X, Gruber JJ, Swider CR, Sanchez PV, Lum JJ, Sayed N, Melo JV, Perl AE, Carroll M, Tuttle SW, Thompson CB (2010) Imatinib resistance associated with BCR-ABL upregulation is dependent on HIF-1alpha-induced metabolic reprograming. Oncogene 29(20):2962–2972, doi:onc201067 [pii] 10.1038/onc.2010.67

Zoula S, Rijken PF, Peters JP, Farion R, Van der Sanden BP, Van der Kogel AJ, Decorps M, Remy C (2003) Pimonidazole binding in C6 rat brain glioma: relation with lipid droplet detection. Br J Cancer 88(9):1439–1444, doi:10.1038/sj.bjc.6600837 6600837 [pii]

# Chapter 2
# Hypoxia and Regulation of Cancer Cell Stemness

**Zhong Yun and Qun Lin**

**Abstract** Spontaneous tumors often contain heterogeneous populations of tumor cells with different tumor-initiating potentials or cancer cell "stemness." Clonal heterogeneity can be traced to specific locations inside a tumor where clones with different metastatic capabilities are identified, suggesting that the tumor microenvironment can exert a significant effect on the evolution of different clonal populations. Hypoxia is a common feature of tumor microenvironments and has the potential to facilitate malignant progression. This chapter provides a synopsis of hypoxia-regulated pathways implicated in the maintenance of cancer stem cells.

**Keywords** Cancer stem cells • Differentiation • Hypoxia • Progenitor cells • Tumor microenvironment

## 2.1 Introduction

In primary tumors, there are often functional and phenotypical heterogeneities among tumor cell populations (Marusyk et al. 2012) although they share the same clonal origin. As was elegantly shown by Yachida et al. (2010), clonal populations with variable metastatic potentials are found in distinct regions within the primary carcinoma of patients with pancreatic cancer, although these clones are genetically evolved from the original parental, nonmetastatic clone. The mechanisms underlying clonal heterogeneity, however, remain to be investigated. Nonetheless, it is highly possible that the heterogeneous nature of tumor microenvironments plays a critical role in the evolution and selection of aggressive clones.

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One of the most commonly recognized features of tumor microenvironment is hypoxia, that is, insufficient oxygenation to meet the metabolic demands of viable tumor cells (Vaupel and Mayer 2007). Hypoxic (oxygen partial pressure $pO_2 < 10$ mmHg) regions have been directly detected using a needle-based, polarographic $pO_2$ electrode in many types of human cancers (Vaupel et al. 2007). The presence of tumor hypoxia also has been indirectly analyzed by immunohistochemistry using hypoxia-activated compounds (Evans and Koch 2003), such as EF5 and pimonidazole, or endogenous hypoxia-induced molecules (Moon et al. 2007), such as hypoxia-inducible factor (HIF)-1α, glucose transporter 1, and carbonic anhydrase 9. However, the staining patterns of different hypoxia markers are not identical (Li et al. 2007b; Vukovic et al. 2001), likely because of their different modes of activation and/or regulation by hypoxia. Nevertheless, it has been shown that hypoxic regions are randomly distributed within the tumor proper (Horsman et al. 2012), suggesting that hypoxia may play a critical role in the clonal evolution of tumor cells in different tumor microenvironments.

Tumor hypoxia is, clinically, an independent prognostic factor for poor patient survival (Nordsmark and Overgaard 2004; Brizel et al. 1996, 1999; Hockel et al. 1996; Young et al. 1988). Hypoxic tumor cells seem to be more aggressive, with reduced apoptosis (Graeber et al. 1996), increased drug-resistance (Wartenberg et al. 2003; Comerford et al. 2002), and increased metastatic potential (Rofstad 2000; Subarsky and Hill 2003). Hypoxia can also increase genomic instability by downregulating the expression of DNA repair genes (Koshiji et al. 2005; Mihaylova et al. 2003; Bindra et al. 2004, 2005). These observations strongly suggest that hypoxia exerts a powerful selection pressure for the emergence of aggressive tumor clones.

The malignant progression of a benign growth is a slow process. It often takes more than a decade for metastatic clones to emerge in spontaneous human tumors (Luebeck 2010; Yachida et al. 2010; Jones et al. 2008; Beerenwinkel et al. 2007). Malignant progression often results from cumulative genetic mutations in oncogenes and tumor suppressor genes, as well as epigenetic changes (Hanahan and Weinberg 2000; Vogelstein and Kinzler 2004). It is imperative to note that these seemingly random and independent mutational events must take place in a single tumor cell originating from the initial oncogenic transformation and that this tumor cell must be able to copy itself so that previously acquired mutations can be inherited in the subsequent daughter cell stages. Therefore, only a stem cell–like cancer cell can complete this protracted journey of change from a benign cell to a metastatic tumor cell.

A number of recent studies have shown that hypoxia can inhibit differentiation of embryonic stem cells and progenitor cells (Ezashi et al. 2005; Gustafsson et al. 2005; Lin et al. 2006; Yun et al. 2002). Hypoxic tumor cells seem to be poorly differentiated and express stem cell markers (Das et al. 2008; Jogi et al. 2002). Under hypoxic conditions, tumor cells show increased clonogenic potential (Desplat et al. 2002; Kim et al. 2009; Schmaltz et al. 1998). Exposure to hypoxia in vitro also results in enhanced tumorigenic potential in vivo (Jogi et al. 2002). These interesting observations lead to a new paradigm that tumor hypoxia may facilitate the emergence of malignant clones by maintaining cancer stem cells in their undifferentiated stem cell state, which permits self-renewal and uninterrupted accumulation of genetic and epigenetic changes over a protracted period of time. This chapter briefly reviews the current advances in

the understanding of hypoxia and its role in stem cell maintenance. The field of cancer stem cell research is witnessing an explosive expansion. We apologize to those authors whose work is not cited because of space limitations.

## 2.2 Mechanisms of Hypoxia-Dependent Stemness Regulation

### *2.2.1 Hypoxia-Inducible Factors and Cancer Cell Stemness*

In addition to being an essential molecule for oxidative phosphorylation in mitochondria, oxygen ($O_2$) also functions as an important signaling molecule and regulates a wide range of biological processes, including erythropoiesis, angiogenesis, and cellular differentiation. $O_2$ cannot be stored in cells and needs to be constantly supplied to support cellular functions and maintain cell viability. Because of the limited supply, specific $O_2$-sensing pathways have evolved in higher-order organisms, especially mammals, to deal with potential $O_2$ deficiency.

The most prominent and best understood hypoxia-induced signaling pathways currently are anchored by HIF-1 and HIF-2, heterodimeric transcription factors consisting of an $O_2$-regulated alpha subunit (HIF-1α or -2α), and the $O_2$-insensitive HIF-1β (Semenza 2003). Although they share similar structures and functions, HIF-1α is ubiquitously expressed, whereas HIF-2α has relatively limited tissue distribution; they also each have nonoverlapping functions (Hu et al. 2006). Furthermore, the expression of HIF-1α and HIF-2α is differentially regulated under conditions of acute and chronic hypoxia, respectively (Lin and Yun 2010).

The $O_2$-sensing ability of HIF-α subunits is realized via $O_2$-dependent hydroxylation of two proline residues located in the $O_2$-dependent degradation domain (Ivan et al. 2001; Jaakkola et al. 2001). The hydroxylated HIF-α interacts with the von Hippel-Lindau (VHL) protein in the E3 ligase complex for ubiquitination and proteasome-mediated degradation (Maxwell et al. 1999; Ohh et al. 2000). Under hypoxia (generally at $pO_2$ levels < 2 %), proline hydroxylation is impaired and the unhydroxylated HIF-α translocates into the nucleus, where it dimerizes with the $O_2$-insensitive HIF-1β. The enhancer regions of hypoxia-induced genes typically contain one or more of the consensus sequence 5′-ACGTG-3′, dubbed the hypoxia-responsive enhancer element (HRE), which is directly bound by HIF-1 or -2 (Semenza 2000; Harris 2002).

In general, increased HIF accumulation and activity facilitate tumor development, which is perhaps best illustrated by renal cell carcinomas (RCCs). Genetic mutations of the *VHL* tumor suppressor gene result in loss of function of the VHL tumor suppressor protein and consequent activation of the HIF pathway under normoxia, which promotes RCC development (Ohh et al. 2000; Maxwell et al. 1999). However, it should be noted that *Vhl* deletion in murine renal proximal tubule cells does not lead to the development of renal cancers (Rankin et al. 2006), suggesting that additional pathways also are required for RCC development. Nevertheless, solid tumors often show elevated levels of the HIF-1α protein compared to adjacent

normal tissues (Harris 2002; Semenza 2003; Vaupel and Mayer 2007). Elevated levels of HIF-1α protein (Aebersold et al. 2001; Burri et al. 2003) or HIF-2α protein (Holmquist-Mengelbier et al. 2006) are significantly correlated with poor patient survival. Furthermore, studies have shown that HIF-1α and HIF-2α can synergize with different proto-oncogenes, such as *Akt* and *c-Myc*, to facilitate tumor cell survival and growth (Bedogni et al. 2005; Gordan et al. 2007).

However, it is worth noting that, under certain circumstances, increased HIF expression or activity seems to have a negative effect on tumor growth. Teratomas derived from HIF-1α-deficient murine embryonic stem (ES) cells grow faster than those from the wild-type ES cells, in part because apoptosis occurs less often in HIF-1α-deficient, ES-derived tumors (Carmeliet et al. 1998). On the other hand, overexpression of HIF-2α in rat glioma tumors increases tumor cell apoptosis and reduces the growth of these tumors, despite enhanced angiogenesis (Acker et al. 2005). These inconsistent observations suggest that the dichotomous functions of HIFs may depend on interactions between the HIF and other pathways in different tumor cell types, their microenvironments, or both.

Several lines of evidence have demonstrated that HIF activation is associated with an undifferentiated phenotype. In primary pancreatic cancers, nuclear accumulation of the HIF-1α protein is primarily found in poorly differentiated tumor cells (Couvelard et al. 2005). Increased levels of HIF-1α and HIF-2α have been found in the stem cell–like populations of neuroblastomas (Pietras et al. 2008, 2009) and gliomas (Li et al. 2009). Downregulation of HIF-1α or HIF-2α by RNA interference results in reduced growth of the tumor sphere, an in vitro assay of self-renewal, and survival of glioma stem cells (Li et al. 2009). It is interesting to note that HIF-2α expression can be easily detected in glioma stem cells under hypoxic conditions (Li et al. 2009). In a similar way, HIF-2α is also preferentially expressed in immature neural crest-like neuroblastoma cells in vivo and seems to be required for maintenance of the undifferentiated neuroblastoma cells (Pietras et al. 2008, 2009). These data strongly support a role of HIF-1 and/or HIF-2 in the maintenance of cancer stem cells. These studies also point out the functional differences between HIF-1 and HIF-2 in the maintenance of undifferentiated cancer stem cell phenotypes.

### 2.2.2 *Hypoxia-Inducible Factors and Stem Cell Gene Expression*

Studies have shown that the HIF pathway is involved in upregulating the expression of several stem cell genes. The pluripotency gene *POU5F1* (Oct3/4) is one of the four or five critical genes that collectively transform adult somatic cells into pluripotent stem cells (Meissner et al. 2007; Takahashi et al. 2007; Yu et al. 2007). In transgenic mice with doxycycline-inducible expression of *POU5F1*, induced *POU5F1* expression results in inhibition of cellular differentiation and dysplastic growths in epithelial tissues (Hochedlinger et al. 2005), thus demonstrating a direct role of *POU5F1* in tumorigenesis. Consistent with this notion, it has been found that germ cell cancers and several types of somatic cancers – including human cervical

carcinomas, breast carcinomas, and pancreatic cancers – express elevated levels of *POU5F1* (Cheng 2004; Gidekel et al. 2003; Jones et al. 2004; Tai et al. 2005).

Using a genetic "knock-in" mouse model, Covello et al. (2006) replaced the endogenous *Hif1a* gene locus with the *Hif2a* locus. The increased *Hif2a* gene dosage and the absence of *Hif1a* resulted in increased expression of HIF-2α-specific genes including *POU5F1* in mouse embryonic tissues (Covello et al. 2006). HIF-2α, but not HIF-1α, directly binds to the *POU5F1* promoter/enhancer. Loss of HIF-2α reduces the number of embryonic primordial germ cells that require *POU5F1* for survival and maintenance. Furthermore, the loss of *POU5F1* results in decreased growth of mouse ES cell–derived teratomas (Covello et al. 2006).Reduced HIF-2α expression similarly results in decreased expression of *POU5F1* and other stem cell genes in human ES cells cultured at 5 % $O_2$ (Forristal et al. 2010). These observations strongly suggest that HIF-2α plays a significant role in stem cell maintenance. It will be interesting to see whether hypoxia increases *POU5F1* expression in common types of tumors.

Delta-like 1 homolog (*Drosophila*), or DLK1, is a type I transmembrane protein with abundant expression in embryonic tissues and immature cells, but not in differentiated adult tissues (Floridon et al. 2000), suggesting a role for DLK1 in the regulation of stem cells and progenitor cells. Elevated expression of DLK1 has been reported in several tumor types (Jensen et al. 1994; Tornehave et al. 1996; Yin et al. 2006; Sakajiri et al. 2005; Van Limpt et al. 2003; Li et al. 2005). Studies have shown that DLK1 is robustly expressed in undifferentiated, but not differentiated, neuroblastoma cells (Begum et al. 2012; Kim et al. 2009). Downregulation of DLK1 by RNA interference sensitizes neuroblastoma cells to spontaneous neuronal differentiation, decreases clonogenicity or colony-forming potential, and suppresses tumorigenicity (Begum et al. 2012; Kim et al. 2009). Overexpression of DLK1, on the other hand, inhibits differentiation, enhances clonogenicity, and increases tumorigenicity (Kim et al. 2009). The DLK1 cytoplasmic domain, especially tyrosine-339 and serine-355, is required for maintaining both clonogenicity and tumorigenicity (Kim et al. 2009). The HIF pathway directly regulates *DLK1* transcription as both HIF-1α and HIF-2α can bind to the HRE in the upstream *DLK1* promoter/enhancer region under hypoxic conditions (Kim et al. 2009). In neuroblastoma xenografts, the DLK1-positve neuroblastoma cells seem to be preferentially localized in the pimonidazole-positive hypoxic region (Begum et al. 2012). These observations demonstrate that the HIF-DLK1 pathway has the potential to maintain cancer stem cells in the hypoxic tumor microenvironment.

The pentaspan transmembrane glycoprotein prominin-1 (CD133), a widely used marker for isolating perspective cancer stem cells from a variety of tumors (Visvader and Lindeman 2008), experiences increased expression in hypoxia -treated (1 % $O_2$) human glioma cells and can promote the expansion of the $CD133^+$ tumor cell population (Griguer et al. 2008; Seidel et al. 2010; Soeda et al. 2009). Both HIF-1α and HIF-2α seem to be involved in the hypoxia-dependent induction of *CD133* expression because knocking down either HIF-1α (Soeda et al. 2009) or HIF-2α (Seidel et al. 2010) reduces the hypoxia-induced *CD133* expression in glioma cells. However, it remains to be determined how HIF enhances *CD133* transcription. On the other hand, severe hypoxia (0.1 % $O_2$) seems to downregulate *CD133*

expression in several gastric, colorectal, and lung cancer cell lines (Matsumoto et al. 2009). These seemingly contradictory findings nonetheless suggest that CD133 may be more involved in cancer stem cell maintenance under moderate (1 % $O_2$) rather than severe (0.1 % $O_2$) hypoxia. Investigation of the transcriptional regulation of *CD133* expression by HIF at different $pO_2$ levels may provide mechanistic insights into the $O_2$ concentration–dependent regulation of *CD133* expression.

The $CD44^+/CD24^{-/low}$ signature has been used to identify breast cancer stem cells (Al-Hajj et al. 2003). As shown by global gene expression and genetic profiles, $CD24^+$ and $CD44^+$ breast cancer cells from the same tumor are clonally related but genetically different (Shipitsin et al. 2007). Elevated CD24 levels have been found to significantly – but counterintuitively – correlate with advanced disease stages in several types of human epithelial cancers, including breast cancer, ovarian cancer, and prostate cancer (Kristiansen et al. 2004). Large-scale immunohistochemical analyses of CD24 and CD44 protein levels in human breast cancer tumor samples have found that the combined $CD44^+/CD24^-$ phenotype is associated with the most favorable prognosis, whereas the $CD44^-/CD24^+$ phenotype predicts the worst outcome (Mylona et al. 2008; Ahmed et al. 2012). In addition, $CD24^+$ tumor-initiating populations also have been found in pancreatic cancers (Ishizawa et al. 2010; Li et al. 2007a), liver cancers (Lee et al. 2011), and colorectal cancers (Vermeulen et al. 2008; Ke et al. 2012). An interesting recent study has shown that CD24 expression is strongly induced by hypoxia in a human bladder cancer cell line (Thomas et al. 2012). Promoter analysis has demonstrated that an HRE in the upstream promoter/enhancer region is required for both hypoxia-induced and HIF-1α-dependent CD24 expression (Thomas et al. 2012). Combined $HIF\text{-}1\alpha^+$ and $CD24^+$ immunostaining in a cohort of 101 human urothelial cancer samples showed a statistically significant association with reduced overall survival (Thomas et al. 2012). These data suggest that HIF and/or hypoxia may play an important role in the clonal maintenance or evolution of the aggressive $CD24^+$ tumor stem populations in the tumor microenvironment.

### *2.2.3 Other Hypoxia-Regulated Genes and Cancer Stemness*

Structures of chromosomes dynamically change during DNA replication and gene transcription and are accompanied by posttranslational modifications of histones, including acetylation of lysine residues and methylation of lysine or arginine residues. Histone demethylases are members of the JmjC domain-containing 2-oxoglutarate oxygenases and catalyze the removal of Nε-methyl groups from lysine residues via $O_2$-dependent hydroxylation (Loenarz and Schofield 2011). They play an important role in both normal embryonal development and cancer (Yamane et al. 2007; Klose et al. 2007; Lan et al. 2007; Iwase et al. 2007). Using the histone 3 lysine 4 (H3K4) demethylase JARID1B (KDM5B/PLU-1/RBP2-H1) as a biomarker, a small subpopulation of slow-cycling melanoma cells that are essential for continuous tumor growth has been identified in patients with advanced tumors (Roesch et al. 2008, 2010). It is interesting that *JARID1B* expression in melanoma cells increases rapidly under hypoxia (1 % $pO_2$) and gradually returns to normal

levels after extended culture under atmospheric conditions (Roesch et al. 2010). However, it is not yet clear how *JARID1B* expression and its enzymatic activity are regulated under hypoxic conditions. Nonetheless, because melanoma cells can easily transition between JARID1B$^+$ and JARID1B$^-$ states, these data suggest that the hypoxic microenvironment may play a significant role in maintaining a population of melanoma cells with long-term repopulating potential, at least in part by augmenting *JARID1B* expression.

Krieg et al. (2010) also have reported that histone demethylase genes *JMJD1A*, *JMJD2B*, and *JARID1B* are induced by hypoxia in human RCCs. Interestingly, their hypoxia-dependent expression is abolished in HIF-1α-knockout mouse embryonic fibroblasts, suggesting that HIF-1 is necessary for hypoxic induction. Furthermore, downregulation of *JMJD1A* reduces xenograft tumor growth in vivo (Krieg et al. 2010). These data indicate that hypoxia can facilitate tumor growth via histone demethylase–mediated chromatin remodeling.

The histone methyltransferase mixed-lineage leukemia 1 (MLL1), also known as human trithorax or acute lymphocytic leukemia-1, is a member of the trithorax family of global transcription activators. MLL1 is preferentially expressed in glioma stem cells and is necessary for maintaining their self-renewal (Heddleston et al. 2012). Hypoxia significantly increases MLL1 expression in both stem and non–stem cell populations. Both HIF-1α and HIF-2α seem to be involved in the regulation of MLL1 expression (Heddleston et al. 2012), although the mechanism of regulation remains to be determined. It is interesting to note that inhibition of MLL1 expression decreases the expression of HIF-2α as well as that of hypoxia-induced genes (Heddleston et al. 2012). These data suggest a positive feedback between HIF-2α and MLL1 for the induction and maintenance of glioma stem cells.

## 2.3 Summary

As discussed earlier, hypoxia clearly has the potential to exert significant effect on the maintenance and evolution of cancer stem cells via both HIF-dependent transcriptions and chromatin remodeling in cancer cells (Fig. 2.1). Hypoxia also inhibits differentiation of mesenchymal stem/progenitor cells (Lin et al. 2006, 2008, 2009; Yun et al. 2002, 2005), thus creating a niche wherein cancer stem cells could be arrested in an undifferentiated state via interactions with their surrounding immature stromal cells (Lin and Yun 2010). However, it is worth noting that because of the plasticity of stemness and the expression of a heterogeneous array of stem cell markers (Magee et al. 2012; Shipitsin et al. 2007; Visvader and Lindeman 2012), cancer stem cells that are localized in or emerge from a hypoxic microenvironment may exist in a different stem cell state or express different sets of stem cell markers compared to developmentally similar cancer stem cells localized in nonhypoxic regions. Nonetheless, the hypoxia-stemness paradigm offers a new perspective on the role of hypoxia in facilitating malignant progression and therapy resistance. Since hypoxic regions are heterogeneously located throughout the tumor proper (Horsman et al. 2012), it is highly probable that hypoxic cancer stem cell niches may contribute to

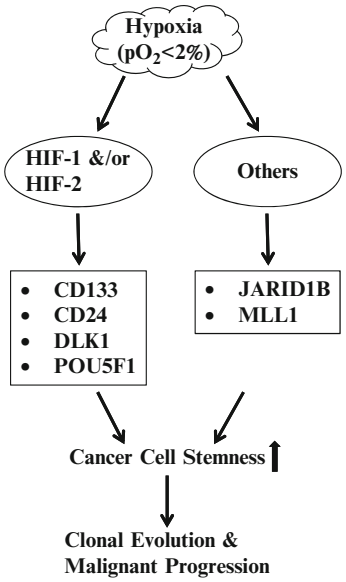


**Fig. 2.1** Hypoxia-activated pathways leading to cancer stem cell maintenance. Multiple stem cell–related genes encoding cell surface proteins, transcription factors, or chromatin-modifying enzymes are upregulated under hypoxic conditions either directly by the HIF transcription factor pathway or by other mechanisms that are yet unknown. These different pathways may function either synergistically or additively to maintain cancer stem cells by enhancing their self-renewal and blocking their differentiation. Increased lifespan of cancer stem cells allows inheritable accumulation of multiple genetic mutations and epigenetic changes that are crucial for clonal evolution and malignant progression

the microenvironment-specific emergence of metastatic clones (Yachida et al. 2010). Therefore, targeting the hypoxic cancer stem cell niche would be highly effective for controlling tumor growth, as well as for preventing metastasis.

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## References

Acker T, Diez-Juan A, Aragones J, Tjwa M, Brusselmans K, Moons L, Fukumura D, Moreno-Murciano MP, Herbert JM, Burger A, Riedel J, Elvert G, Flamme I, Maxwell PH, Collen D, Dewerchin M, Jain RK, Plate KH, Carmeliet P (2005) Genetic evidence for a tumor suppressor role of HIF-2α. Cancer Cell 8:131–141

Aebersold DM, Burri P, Beer KT, Laissue J, Djonov V, Greiner RH, Semenza GL (2001) Expression of hypoxia-inducible factor-1α: a novel predictive and prognostic parameter in the radiotherapy of oropharyngeal cancer. Cancer Res 61:2911–2916

Ahmed MA, Aleskandarany MA, Rakha EA, Moustafa RZ, Benhasouna A, Nolan C, Green AR, Ilyas M, Ellis IO (2012) A CD44(−)/CD24(+) phenotype is a poor prognostic marker in early invasive breast cancer. Breast Cancer Res Treat 133:979–995

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100:3983–3988

Bedogni B, Welford SM, Cassarino DS, Nickoloff BJ, Giaccia AJ, Powell MB (2005) The hypoxic microenvironment of the skin contributes to Akt-mediated melanocyte transformation. Cancer Cell 8:443–454

Beerenwinkel N, Antal T, Dingli D, Traulsen A, Kinzler KW, Velculescu VE, Vogelstein B, Nowak MA (2007) Genetic progression and the waiting time to cancer. PLoS Comput Biol 3:e225

Begum A, Kim Y, Lin Q, Yun Z (2012) DLK1, delta-like 1 homolog (Drosophila), regulates tumor cell differentiation in vivo. Cancer Lett 318:26–33

Bindra RS, Schaffer PJ, Meng A, Woo J, Maseide K, Roth ME, Lizardi P, Hedley DW, Bristow RG, Glazer PM (2004) Down-regulation of Rad51 and decreased homologous recombination in hypoxic cancer cells. Mol Cell Biol 24:8504–8518

Bindra RS, Gibson SL, Meng A, Westermark U, Jasin M, Pierce AJ, Bristow RG, Classon MK, Glazer PM (2005) Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. Cancer Res 65:11597–11604

Brizel DM, Scully SP, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, Dewhirst MW (1996) Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 56:941–943

Brizel DM, Dodge RK, Clough RW, Dewhirst MW (1999) Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. Radiother Oncol 53:113–117

Burri P, Djonov V, Aebersold DM, Lindel K, Studer U, Altermatt HJ, Mazzucchelli L, Greiner RH, Gruber G (2003) Significant correlation of hypoxia-inducible factor-1α with treatment outcome in cervical cancer treated with radical radiotherapy. Int J Radiat Oncol Biol Phys 56:494–501

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshert E (1998) Role of HIF-1α in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature 394:485–490

Cheng L (2004) Establishing a germ cell origin for metastatic tumors using OCT4 immunohistochemistry. Cancer 101:2006–2010

Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP (2002) Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res 62:3387–3394

Couvelard A, O'Toole D, Turley H, Leek R, Sauvanet A, Degott C, Ruszniewski P, Belghiti J, Harris AL, Gatter K, Pezzella F (2005) Microvascular density and hypoxia-inducible factor pathway in pancreatic endocrine tumours: negative correlation of microvascular density and VEGF expression with tumour progression. Br J Cancer 92:94–101

Covello KL, Kehler J, Yu H, Gordan JD, Arsham AM, Hu CJ, Labosky PA, Simon MC, Keith B (2006) HIF-2α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev 20:557–570

Das B, Tsuchida R, Malkin D, Koren G, Baruchel S, Yeger H (2008) Hypoxia enhances tumor stemness by increasing the invasive and tumorigenic side population fraction. Stem Cells 26:1818–1830

Desplat V, Faucher JL, Mahon FX, Dello Sbarba P, Praloran V, Ivanovic Z (2002) Hypoxia modifies proliferation and differentiation of CD34(+) CML cells. Stem Cells 20:347–354

Evans SM, Koch CJ (2003) Prognostic significance of tumor oxygenation in humans. Cancer Lett 195:1–16

Ezashi T, Das P, Roberts RM (2005) Low $O_2$ tensions and the prevention of differentiation of hES cells. Proc Natl Acad Sci U S A 102:4783–4788

Floridon C, Jensen CH, Thorsen P, Nielsen O, Sunde L, Westergaard JG, Thomsen SG, Teisner B (2000) Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. Differentiation 66:49–59

Forristal CE, Wright KL, Hanley NA, Oreffo RO, Houghton FD (2010) Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. Reproduction 139:85–97

Gidekel S, Pizov G, Bergman Y, Pikarsky E (2003) Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell 4:361–370

Gordan JD, Bertout JA, Hu CJ, Diehl JA, Simon MC (2007) HIF-2α promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell 11:335–347

Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379:88–91

Griguer CE, Oliva CR, Gobin E, Marcorelles P, Benos DJ, Lancaster JR Jr, Gillespie GY (2008) CD133 is a marker of bioenergetic stress in human glioma. PLoS One 3:e3655

Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, Ruas JL, Poellinger L, Lendahl U, Bondesson M (2005) Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell 9:617–628

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Harris AL (2002) Hypoxia – a key regulatory factor in tumour growth. Nat Rev Cancer 2:38–47

Heddleston JM, Wu Q, Rivera M, Minhas S, Lathia JD, Sloan AE, Iliopoulos O, Hjelmeland AB, Rich JN (2012) Hypoxia-induced mixed-lineage leukemia 1 regulates glioma stem cell tumorigenic potential. Cell Death Differ 19:428–439

Hochedlinger K, Yamada Y, Beard C, Jaenisch R (2005) Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. Cell 121:465–477

Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P (1996) Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56:4509–4515

Holmquist-Mengelbier L, Fredlund E, Lofstedt T, Noguera R, Navarro S, Nilsson H, Pietras A, Vallon-Christersson J, Borg A, Gradin K, Poellinger L, Pahlman S (2006) Recruitment of HIF-1α and HIF-2α to common target genes is differentially regulated in neuroblastoma: HIF-2α promotes an aggressive phenotype. Cancer Cell 10:413–423

Horsman MR, Mortensen LS, Petersen JB, Busk M, Overgaard J (2012) Imaging hypoxia to improve radiotherapy outcome. Nat Rev Clin Oncol 9:674–687

Hu CJ, Iyer S, Sataur A, Covello KL, Chodosh LA, Simon MC (2006) Differential regulation of the transcriptional activities of hypoxia-inducible factor 1α (HIF-1α) and HIF-2α in stem cells. Mol Cell Biol 26:3514–3526

Ishizawa K, Rasheed ZA, Karisch R, Wang Q, Kowalski J, Susky E, Pereira K, Karamboulas C, Moghal N, Rajeshkumar NV, Hidalgo M, Tsao M, Ailles L, Waddell TK, Maitra A, Neel BG, Matsui W (2010) Tumor-initiating cells are rare in many human tumors. Cell Stem Cell 7:279–282

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG Jr (2001) HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for $O_2$ sensing. Science 292:464–468

Iwase S, Lan F, Bayliss P, de la Torre-Ubieta L, Huarte M, Qi HH, Whetstine JR, Bonni A, Roberts TM, Shi Y (2007) The X-linked mental retardation gene SMCX/JARID1C defines a family of histone H3 lysine 4 demethylases. Cell 128:1077–1088

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ (2001) Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by $O_2$-regulated prolyl hydroxylation. Science 292:468–472

Jensen CH, Krogh TN, Hojrup P, Clausen PP, Skjodt K, Larsson LI, Enghild JJ, Teisner B (1994) Protein structure of fetal antigen 1 (FA1). A novel circulating human epidermal-growth-factor-like protein expressed in neuroendocrine tumors and its relation to the gene products of dlk and pG2. Eur J Biochem 225:83–92

Jogi A, Ora I, Nilsson H, Lindeheim A, Makino Y, Poellinger L, Axelson H, Pahlman S (2002) Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci U S A 99:7021–7026

Jones TD, Ulbright TM, Eble JN, Cheng L (2004) OCT4: a sensitive and specific biomarker for intratubular germ cell neoplasia of the testis. Clin Cancer Res 10:8544–8547

Jones S, Chen WD, Parmigiani G, Diehl F, Beerenwinkel N, Antal T, Traulsen A, Nowak MA, Siegel C, Velculescu VE, Kinzler KW, Vogelstein B, Willis J, Markowitz SD (2008) Comparative lesion sequencing provides insights into tumor evolution. Proc Natl Acad Sci U S A 105:4283–4288

Ke J, Wu X, Wu X, He X, Lian L, Zou Y, He X, Wang H, Luo Y, Wang L, Lan P (2012) A subpopulation of CD24(+) cells in colon cancer cell lines possess stem cell characteristics. Neoplasma 59:282–288

Kim Y, Lin Q, Zelterman D, Yun Z (2009) Hypoxia-regulated delta-like 1 homologue enhances cancer cell stemness and tumorigenicity. Cancer Res 69:9271–9280

Klose RJ, Yan Q, Tothova Z, Yamane K, Erdjument-Bromage H, Tempst P, Gilliland DG, Zhang Y, Kaelin WG Jr (2007) The retinoblastoma binding protein RBP2 is an H3K4 demethylase. Cell 128:889–900

Koshiji M, To KK, Hammer S, Kumamoto K, Harris AL, Modrich P, Huang LE (2005) HIF-1α induces genetic instability by transcriptionally downregulating MutSα expression. Mol Cell 17:793–803

Krieg AJ, Rankin EB, Chan D, Razorenova O, Fernandez S, Giaccia AJ (2010) Regulation of the histone demethylase JMJD1A by hypoxia-inducible factor 1α enhances hypoxic gene expression and tumor growth. Mol Cell Biol 30:344–353

Kristiansen G, Sammar M, Altevogt P (2004) Tumour biological aspects of CD24, a mucin-like adhesion molecule. J Mol Histol 35:255–262

Lan F, Bayliss PE, Rinn JL, Whetstine JR, Wang JK, Chen S, Iwase S, Alpatov R, Issaeva I, Canaani E, Roberts TM, Chang HY, Shi Y (2007) A histone H3 lysine 27 demethylase regulates animal posterior development. Nature 449:689–694

Lee TK, Castilho A, Cheung VC, Tang KH, Ma S, Ng IO (2011) CD24(+) liver tumor-initiating cells drive self-renewal and tumor initiation through STAT3-mediated NANOG regulation. Cell Stem Cell 9:50–63

Li L, Forman SJ, Bhatia R (2005) Expression of DLK1 in hematopoietic cells results in inhibition of differentiation and proliferation. Oncogene 24:4472–4476

Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM (2007a) Identification of pancreatic cancer stem cells. Cancer Res 67:1030–1037

Li XF, Carlin S, Urano M, Russell J, Ling CC, O'Donoghue JA (2007b) Visualization of hypoxia in microscopic tumors by immunofluorescent microscopy. Cancer Res 67:7646–7653

Li Z, Bao S, Wu Q, Wang H, Eyler C, Sathornsumetee S, Shi Q, Cao Y, Lathia J, Mclendon RE, Hjelmeland AB, Rich JN (2009) Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell 15:501–513

Lin Q, Yun Z (2010) Impact of the hypoxic tumor microenvironment on the regulation of cancer stem cell characteristics. Cancer Biol Ther 9:949–956

Lin Q, Lee YJ, Yun Z (2006) Differentiation arrest by hypoxia. J Biol Chem 281:30678–30683

Lin Q, Kim Y, Alarcon RM, Yun Z (2008) Oxygen and cell fate decisions. Gene Regul Syst Biol 2:1–9

Lin Q, Gao Z, Alarcon RM, Ye J, Yun Z (2009) A role of miR-27 in the regulation of adipogenesis. FEBS J 276:2348–2358

Loenarz C, Schofield CJ (2011) Physiological and biochemical aspects of hydroxylations and demethylations catalyzed by human 2-oxoglutarate oxygenases. Trends Biochem Sci 36:7–18

Luebeck EG (2010) Cancer: genomic evolution of metastasis. Nature 467:1053–1055

Magee JA, Piskounova E, Morrison SJ (2012) Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer Cell 21:283–296

Marusyk A, Almendro V, Polyak K (2012) Intra-tumour heterogeneity: a looking glass for cancer? Nat Rev Cancer 12:323–334

Matsumoto K, Arao T, Tanaka K, kaneda H, Kudo K, Fujita Y, Tamura D, Aomatsu K, Tamura T, Yamada Y, Saijo N, Nishio K (2009) mTOR signal and hypoxia-inducible factor-1α regulate CD133 expression in cancer cells. Cancer Res 69:7160–7164

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ (1999) The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275

Meissner A, Wernig M, Jaenisch R (2007) Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells. Nat Biotechnol 25:1177–1181

Mihaylova VT, Bindra RS, Yuan J, Campisi D, Narayanan L, Jensen R, Giordano F, Johnson RS, Rockwell S, Glazer PM (2003) Decreased expression of the DNA mismatch repair gene Mlh1 under hypoxic stress in mammalian cells. Mol Cell Biol 23:3265–3273

Moon EJ, Brizel DM, Chi JT, Dewhirst MW (2007) The potential role of intrinsic hypoxia markers as prognostic variables in cancer. Antioxid Redox Signal 9:1237–1294

Mylona E, Giannopoulou I, Fasomytakis E, Nomikos A, Magkou C, Bakarakos P, Nakopoulou L (2008) The clinicopathologic and prognostic significance of CD44+/CD24(−/low) and CD44-/CD24+ tumor cells in invasive breast carcinomas. Hum Pathol 39:1096–1102

Nordsmark M, Overgaard J (2004) Tumor hypoxia is independent of hemoglobin and prognostic for loco-regional tumor control after primary radiotherapy in advanced head and neck cancer. Acta Oncol 43:396–403

Ohh M, Park CW, Ivan M, Hoffman MA, Kim TY, Huang LE, Pavletich N, Chau V, Kaelin WG (2000) Ubiquitination of hypoxia-inducible factor requires direct binding to the β-domain of the von Hippel-Lindau protein. Nat Cell Biol 2:423–427

Pietras A, Gisselsson D, Ora I, Noguera R, Beckman S, Navarro S, Pahlman S (2008) High levels of HIF-2α highlight an immature neural crest-like neuroblastoma cell cohort located in a perivascular niche. J Pathol 214:482–488

Pietras A, Hansford LM, Johnsson AS, Bridges E, Sjolund J, Gisselsson D, Rehn M, Beckman S, Noguera R, Navarro S, Cammenga J, Fredlund E, Kaplan DR, Pahlman S (2009) HIF-2α maintains an undifferentiated state in neural crest-like human neuroblastoma tumor-initiating cells. Proc Natl Acad Sci U S A 106:16805–16810

Rankin EB, Tomaszewski JE, Haase VH (2006) Renal cyst development in mice with conditional inactivation of the von Hippel-Lindau tumor suppressor. Cancer Res 66:2576–2583

Roesch A, Mueller AM, Stempfl T, Moehle C, Landthaler M, Vogt T (2008) RBP2-H1/JARID1B is a transcriptional regulator with a tumor suppressive potential in melanoma cells. Int J Cancer 122:1047–1057

Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, Vultur A, Basu D, Gimotty P, Vogt T, Herlyn M (2010) A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell 141:583–594

Rofstad EK (2000) Microenvironment-induced cancer metastasis. Int J Radiat Biol 76:589–605

Sakajiri S, O'Kelly J, Yin D, Miller CW, Hofmann WK, Oshimi K, Shih LY, Kim KH, Sul HS, Jensen CH, Teisner B, Kawamata N, Koeffler HP (2005) Dlk1 in normal and abnormal hematopoiesis. Leukemia 19:1404–1410

Schmaltz C, Hardenbergh PH, Wells A, Fisher DE (1998) Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol Cell Biol 18:2845–2854

Seidel S, Garvalov BK, Wirta V, Von Stechow L, Schanzer A, Meletis K, Wolter M, Sommerlad D, Henze AT, Nister M, Reifenberger G, Lundeberg J, Frisen J, Acker T (2010) A hypoxic niche regulates glioblastoma stem cells through hypoxia inducible factor 2α. Brain 133:983–995

Semenza GL (2000) HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 88:1474–1480

Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3:721–732

Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, Nikolskaya T, Serebryiskaya T, Beroukhim R, Hu M, Halushka MK, Sukumar S, Parker LM, Anderson KS, Harris LN, Garber JE, Richardson AL, Schnitt SJ, Nikolsky Y, Gelman RS, Polyak K (2007) Molecular definition of breast tumor heterogeneity. Cancer Cell 11:259–273

Soeda A, Park M, Lee D, Mintz A, Androutsellis-Theotokis A, Mckay RD, Engh J, Iwama T, Kunisada T, Kassam AB, Pollack IF, Park DM (2009) Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1α. Oncogene 28:3949–3959

Subarsky P, Hill RP (2003) The hypoxic tumour microenvironment and metastatic progression. Clin Exp Metastasis 20:237–250

Tai MH, Chang CC, Kiupel M, Webster JD, Olson LK, Trosko JE (2005) Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis 26:495–502

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Thomas S, Harding MA, Smith SC, Overdevest JB, Nitz MD, Frierson HF, Tomlins SA, Kristiansen G, Theodorescu D (2012) CD24 is an effector of HIF-1-driven primary tumor growth and metastasis. Cancer Res 72:5600–5612

Tornehave D, Jensen CH, Teisner B, Larsson LI (1996) FA1 immunoreactivity in endocrine tumours and during development of the human fetal pancreas; negative correlation with glucagon expression. Histochem Cell Biol 106:535–542

Van Limpt VA, Chan AJ, Van Sluis PG, Caron HN, Van Noesel CJ, Versteeg R (2003) High delta-like 1 expression in a subset of neuroblastoma cell lines corresponds to a differentiated chromaffin cell type. Int J Cancer 105:61–69

Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26:225–239

Vaupel P, Hockel M, Mayer A (2007) Detection and characterization of tumor hypoxia using $pO_2$ histography. Antioxid Redox Signal 9:1221–1235

Vermeulen L, Todaro M, De Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, Richel DJ, Stassi G, Medema JP (2008) Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. Proc Natl Acad Sci U S A 105:13427–13432

Visvader JE, Lindeman GJ (2008) Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. Nat Rev Cancer 8:755–768

Visvader JE, Lindeman GJ (2012) Cancer stem cells: current status and evolving complexities. Cell Stem Cell 10:717–728

Vogelstein B, Kinzler KW (2004) Cancer genes and the pathways they control. Nat Med 10:789–799

Vukovic V, Haugland HK, Nicklee T, Morrison AJ, Hedley DW (2001) Hypoxia-inducible factor-1α is an intrinsic marker for hypoxia in cervical cancer xenografts. Cancer Res 61:7394–7398

Wartenberg M, Ling FC, Muschen M, Klein F, Acker H, Gassmann M, Petrat K, Putz V, Hescheler J, Sauer H (2003) Regulation of the multidrug resistance transporter P-glycoprotein in multicellular tumor spheroids by hypoxia-inducible factor (HIF-1) and reactive oxygen species. FASEB J 17:503–505

Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, Kamiyama M, Hruban RH, Eshleman JR, Nowak MA, Velculescu VE, Kinzler KW, Vogelstein B, Iacobuzio-Donahue CA (2010) Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature 467:1114–1117

Yamane K, Tateishi K, Klose RJ, Fang J, Fabrizio LA, Erdjument-Bromage H, Taylor-Papadimitriou J, Tempst P, Zhang Y (2007) PLU-1 is an H3K4 demethylase involved in transcriptional repression and breast cancer cell proliferation. Mol Cell 25:801–812

Yin D, Xie D, Sakajiri S, Miller CW, Zhu H, Popoviciu ML, Said JW, Black KL, Koeffler HP (2006) DLK1: increased expression in gliomas and associated with oncogenic activities. Oncogene 25:1852–1861

Young SD, Marshall RS, Hill RP (1988) Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci U S A 85:9533–9537

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920

Yun Z, Maecker HL, Johnson RS, Giaccia AJ (2002) Inhibition of PPARγ2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. Dev Cell 2:331–341

Yun Z, Lin Q, Giaccia AJ (2005) Adaptive myogenesis under hypoxia. Mol Cell Biol 25:3040–3055

# Chapter 3
# Hypoxia-Mediated Metastasis

**Joan Chang and Janine Erler**

**Abstract** Metastasis is responsible for more than 90 % of deaths among cancer patient. It is a highly complex process that involves the interplay between cancer cells, the tumor microenvironment, and even noncancerous host cells. Metastasis can be seen as a step-wise process: acquisition of malignant phenotype, invasion into surrounding tissue, intravasation into blood vessels, survival in circulation, extravasation to distant sites, and colonization of new organs. Before the actual metastatic process, the secondary site is also prepared for the arrival of the cancer cells through formation of "premetastatic niches." Hypoxia (low oxygen tension) is commonly found in solid tumors more than a few millimeters cubed and often is associated with a poor prognosis. Hypoxia increases angiogenesis, cancer cell survival, and metastasis. This chapter described how hypoxia regulates each step of the metastatic process and how blocking hypoxia-driven metastasis through targeting hypoxia-inducible factor 1, or downstream effector molecules such as the lysyl oxidase family may represent highly effective preventive strategies against metastasis in cancer patients.

**Keywords** Hypoxia • Metastasis • Extracellular matrix (ECM) • Epithelial-mesenchymal transition (EMT) • Microenvironment • Angiogenesis

## 3.1 Metastasis

Solid tumors, regardless of organ type and cell of origin, can be described as either benign or malignant. Benign tumors remain localized and lack the ability to escape the primary tumor site, whereas malignant tumors can spread through invasion and

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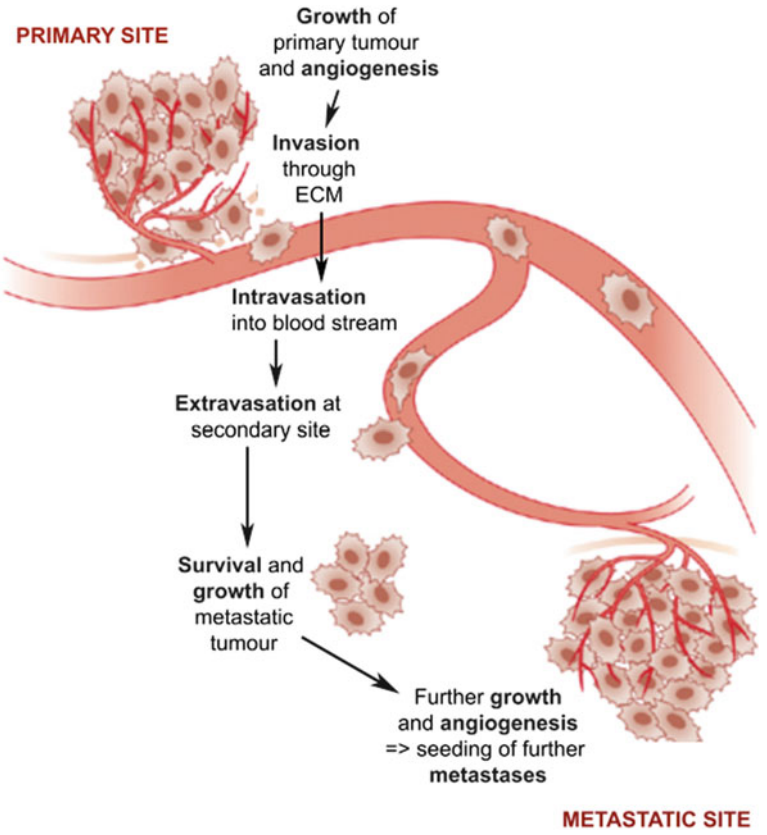



**Fig. 3.1** The multistep process of metastasis. The metastatic process consists of a series of distinct, sequential steps, each of which must be achieved for successful metastasis. Adapted from Oncology News Volume 6 Issue 4, 2011. http://www.oncologynews.biz/pdf/sep_oct_11/128-131_ONSO11_feature%20art.pdf

metastasis of the cancer cells. Initial cancer research mostly focused on investigating the molecular basis of oncogenic transformation, which gives rise to (primary) tumors; relatively less is known about the process by which tumor cells become metastatic and colonize distant organs. However, focus has recently shifted to understanding the metastatic process because metastasis remains the cause of more than 90 % of deaths among cancer patients with solid tumors (Gupta and Massague 2006).

Metastasis is considered one of the original six acquired hallmarks of cancer (Hanahan and Weinberg 2000, 2011). It is generally believed to be a multistep process (Fig. 3.1) consisting of discrete biological processes. To escape from the primary tumor, cancer cells first disrupt the integrity of the basement membrane (BM), then invade the surrounding interstitial extracellular matrix (ECM), and intravasate into the circulatory system. The cancer cells then must survive the fluctuating environment in transit as circulating tumor cells (CTCs), and extravasate to distant sites as disseminated tumor cells (DTCs) that invade into and colonize new organs within

the body. The final step of metastasis is acquiring vasculature to support the growth of the metastases; in some cases, these metastases can repeat the whole process to give rise to new metastases.

## 3.2 Hypoxia and Metastasis

Hypoxia has been shown to decrease the efficacy of radiation therapy (Overgaard and Horsman 1996), and because of the poor vasculature support, the efficiency of drug delivery to hypoxic tumor cells is greatly reduced (Chaudary and Hill 2007). In addition, tumor cells in regions of hypoxia undergo slower cell division and have decreased apoptotic potential – thus chemotherapies are less effective (Erler et al. 2004; Finger and Giaccia 2010). However, the clinical effect of hypoxia on cancer biology extends beyond its effects on therapeutic efficacy.

The presence of tumor hypoxia is associated with poor survival and increased metastatic incidence and burden in patients with various cancer types, including head and neck, cervical, and breast (Hockel and Vaupel 2001; Harris 2002; Cairns et al. 2003; Pouyssegur et al. 2006). In one of the earliest clinical studies, a computerized polarographic electrode system was used to investigate the tumor oxygenation in locally advanced cancer of the uterine cervix over a period of 8 years, where tissue $pO_2$ partial pressure of oxygen in the blood was measured in patients with cervical tumors ≥3 cm in diameter (Hockel et al. 1996). The study showed that 52 patients with hypoxic tumors (median $pO_2$<10 mmHg) had worse disease-free and overall survival, and in patients in whom the primary tumor was surgically removed there was greater incidence of distant metastases if the primary tumor was hypoxic (Hockel et al. 1996). A more recent study showed hypoxia to be a prognostic marker of distant disease recurrence in 106 node-negative patients with cervical cancer and that tumor hypoxia ($pO_2$<5 mmHg in this case) can be used to predict progression-free survival of these patients (Fyles et al. 2002). Moreover, patients with hypoxic tumors also had a significant increase in the incidence of distant metastases when compared to patients with more oxygenated tumors (Fyles et al. 2002).

Hypoxia plays a dual role in cancer progression: on the one hand it limits the primary tumor growth because cancer cells require oxygen for fundamental cellular processes; on the other hand hypoxia selects for more invasive cells and thus promotes malignant progression – as one can imagine the tumor cells would want to physically move toward an oxygen-rich environment. It is interesting to note that hypoxic tumor cells may be found not only toward the center of a primary tumor mass but also at the invasive front, highlighting the dynamic nature of tumor hypoxia (Buchler et al. 2004).

The cellular response to hypoxia is predominantly mediated by the helix-loop-helix transcription factor hypoxia-inducible factor (HIF)-1. HIF-1, a heterodimer composed of one of three alpha subunits (HIF-1α, HIF-2α, HIF-3α) and one beta subunit (HIF-1β), is active under hypoxia by the stabilized expression of the alpha subunit. The HIF-1α and HIF-2α subunits in particular are overexpressed and

associated with poor prognosis in many cancer types (Semenza 2003; Qing and Simon 2009). Moreover, HIF-1α is expressed at a higher fraction (69 %) in metastases compared with primary tumors (29 %) (Zhong et al. 1999), and patients with higher proportions of hypoxic cells have decreased disease-free and overall survival rates after surgical resection of the primary tumor due to the recurrence of metastatic disease (Hockel et al. 1996; Vergis et al. 2008). These two observations strongly link hypoxia with the metastatic progression of cancer. Studies of hypoxia-regulated genes have revealed upregulation of genes involved in multiple biological functions that strongly influence metastatic progression, such cell proliferation, angiogenesis, and ECM remodeling (Table 3.1) (Le et al. 2004; Rankin and Giaccia 2008).

## 3.3 Rise of the Metastatic Population

Primary tumor cells may become metastatic in various ways; however, we focus on three main possibilities. The first possibility suggests that Darwinian evolution selection pressures may have been present in the primary tumor environment, selecting for cancer cells that have acquired aggressive phenotypic traits through genetic or epigenetic changes, thus allowing for clonal expansion of this "fitter" cell type. The fitter populations are eventually (as well as inevitably) able to disseminate to secondary sites. Metastatic events in this case rely on the "nature" of the primary tumor cells. Hypoxia is known to select for this type of fitter population. Paradoxically, while hypoxia is usually lethal for most normal cell types, hypoxia selects for cancer cells with low apoptotic potential (Graeber et al. 1996; Erler et al. 2004) and increases genomic instability, which in turn allows cancer cells to rapidly mutate and adapt to the microenvironment and more quickly acquire aggressive traits (Young et al. 1988; Reynolds et al. 1996). In addition, hypoxia can also induce cancer cells to secrete various growth factors and proteases to alter their immediate microenvironment, thereby permitting invasion and promoting angiogenesis.

The second possibility suggests that the primary tumor cells have enhanced survival and proliferative abilities but have not yet acquired the aggressive traits that allow for invasion and metastasis. In this case, the metastatic events occur because of the tumor cells responding to contextual signals provided by the tumor microenvironment. In normal cellular microenvironments, malignant cell growth is suppressed; the tumor microenvironment (including hypoxia), however, promotes invasion and metastasis of the cancer cells.

The third possibility in a way unites the first two possibilities, suggesting the existence of metastatic cancer stem cells that are the fitter population, as described in the first possibility. These are thought either to be present right from the beginning or are cancer stem cells (CSCs) modulated by the tumor microenvironment in such a way that makes them metastatic, as described in the second possibility. Recent studies revealed that within a tumor, populations of cells are organized in a hierarchy, recapitulating the scheme of self-renewing stem cells, progenitor cells, and fully differential cells found in normal tissues (Bonnet and Dick 1997; Al-Hajj et al. 2003; Ailles and Weissman 2007) and suggesting the presence of CSCs that

**Table 3.1** Overview of reported hypoxia-regulated genes and their respective roles in cancer progression

**GLUCOSE TRANSPORTATION/ METABOLISM**
Acetoacetyl CoA thiolase
Adenylate kinase-3
Aldolase A,C
Aminopeptidase A
Cabonic anhydrase-IX, -XII
Ceruloplasmin
Enolase-1
Erythropoietin
Ferritin light chain
Fructose-2,6-bisphosphatase-3
GLUT-1, -3
Glyceraldehyde-3-phosphate dehydrogenase
Glycogen-branching enzyme
Heme oxygenase
Hexokinase-1, -2
Lactate dehydrogenase A, B
Max interactor-1
Phosphofructokinase L
Phosphoglycerate kinase-1
Phosphoribosyl pyrophosphate synthetase
Pyruvate dehydrogenase kinase-1
Pyruvate kinase-M
Solute carrier family
Spermidine N1-acetyltransferase
Transferrin and receptor
Transglutaminase-2
Triose phosphate isomerase
Tyrosine dehydroxylase
6-Phosphofructo-2 kinase

**PROLIFERATION/ DIFFERENTIATION**
Adipophilin
B-cell translocation gene-1
Cyclin-dependent kinase inhibitor-1B (p27, kip1)
Cyclin D1, G2
Cyclin-dependent kinase-1
Cyclin-dependent kinase inhibitor-1 (p21)
Deleted in esophageal cancer-1 (DEC1)
Erythropoietin
Inhibitor of DNA binding-2
IGF-2
IGF-binding protein-2
Mitogen-inducible gene-6
N-myc downstream regulated gene-1 (Cap43)
Stimulated by retinoic acid-13 (stra13)
TGF-α

**ANGIOGENESIS**
Adrenomedullin
Angiopoietin-1, -2
Angiopoietin-1 receptor (TIE-2)
Cyclooxygenase-1, -2
Endothelin-1, -2
Ephrin A1
Fibroblast growth factor-3
Hepatocyte growth factor
Matrix metalloproteinase-2, -9
Nitric oxide synthase
Placental growth factor
Plasminogen activator inhibitor-1
PDGF-B
Thrombospondin-1, -2
TGF-α, -β1, -β3
VEGF-A, -B, -C, -D
VEGF receptor 1 (FLT-1)
VEGF receptor 2 (FLK-1)

**GROWTH FACTORS/ CYTOKINES**
IGF-2
Interleukin-6, -8
Intestinal trefoil factor
Macrophage migration inhibitory factor
PDGF-B
Stanniocalcin-2
TGF-α

**APOPTOSIS**
BCL-w like
BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3)
BNIP3-like
Hepatic fibrinogen/angiopoietin-related protein
IGF-binding protein-1, -3, -5
Proto-oncogene serine/threonine-protein kinase (PIM)-1, -2
RTP801 (REDD1)

**STRESS-RESPONSE**
Growth arrest and DNA damage inducible gene (GADD)-153
Heat shock factors
Heat shock proteins
Huntington-associated protein-1
Hypoxia upregulated protein 1 (ORP150)
Thioredoxin

**TISSUE REMODELING**
c-MET
CD99
CXCR type-4
Collagen-5a
Galectin-1
Integrin-5a
Ku70
Low-density lipoprotein receptor-related protein
LOX
LOX-like 2
Lysyl hydroxylase-2 (PLOD2)
MMP-7, -13
Mucin 1
Osteopontin
Plasminogen activator inhibitor-1
Prolyl-4-hydroxylase
Tissue factor
UPAR
Vimentin

**GENE EXPRESSION**
Early growth response 1
P35srj
ETS-1
Mxi-1
Annexin V
BCL-interacting killer
FOS
Jun
Lipocortin
Nuclear factor κB
NIX
NR3C1 glucocorticoid receptor-α
Nuclear factor IL-3

**INVASION/METASTASIS**
Connective tissue growth factor
CXCR type-4
E-cadherin
LOX
LOXL2
PAI-1
Stromal-derived factor-1
UPAR

*CoA* coenzyme A; *CXCR* C-X-C chemokine receptor; *GLUT* glucose transporter; *IGF* insulin-like growth factor; *LOX* lysyl oxidase; *TGF* transforming growth factor; *PDGF* platelet-derived growth factor; *UPAR* urokinase plasminogen activator receptor; *VEGF* vascular endothelial growth factor

have self-renewal properties and enhanced tumor-initiating potential. The definition of a CSC in research is that the cancer cell has the capacity to self-propagate to form new tumors when experimentally implanted into animal hosts. This is, in theory, similar to tumor initiation at secondary sites by DTCs; both scenarios require the "seeder" to have the ability to self-renew and initiate colonization by producing progenies. As such, each metastatic cell of origin is thought to be from a DTC with CSC properties. Thus, CSC phenotype–enhancing mechanisms should increase the efficiency of metastasis. Hypoxic regulation of stem cells is covered in Chap. 2.

Regardless of how the metastatic population arises, it is known that hypoxia greatly increases the invasive and metastatic potential of cancer cells. Hypoxia is thus considered a potent driving force in the prometastatic microenvironment, and it influences each stage of the metastatic process, as detailed in the following sections.

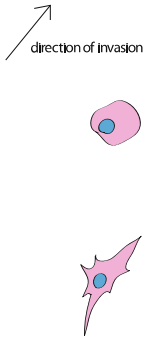
## 3.4 Cancer Cell Invasion

Invasion is the first step of metastasis in which the cells invade various biological barriers to get into blood vessels for dissemination. In addition to breaking down physical barriers, the invasive cells can also acquire changes in their cell-cell and cell-matrix adhesion interactions.

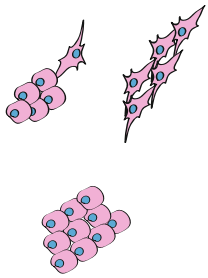
### *3.4.1 Hypoxia and the Epithelial-Mesenchymal Transition*

Investigations into cancer cell movement (migration) have revealed that tumor cells can migrate individually or collectively as a group (Table 3.2). Single-cell migration takes the form of either amoeboid, leukocyte-like or mesenchymal migration (Friedl and Gilmour 2009; Madsen and Sahai 2010). Amoeboid-like migration allows the cancer cells to migrate through the stroma without the need to proteolytically remodel the matrix around them, often "hitching a ride" along collagen fibers in the ECM (Condeelis and Segall 2003). Mesenchymal migration, on the other hand, is thought to require cancer cells to undergo epithelial-mesenchymal transition (EMT). Cancer cells undergoing EMT lose their cell-cell adherent properties and polarity, acquire an invasive mesenchymal phenotype, and become resistant to apoptosis and senescence (Thiery et al. 2009; Yilmaz and Christofori 2010). EMT cancer cells can migrate either individually or collectively with cells that have undergone EMT at the front, clearing out a track through which follower cells can move (Erler and Giaccia 2006; Friedl and Wolf 2008). This requires proteolytic degradation of the various components of the biological barriers, which involves various protein families such as the matrix metalloproteinase (MMP) family, the adamalysin-related membrane proteinases, and tissue serine proteinases (Andreasen et al. 2000; Sternlicht and Werb 2001). Interestingly, collectively migrating cancer cells may still retain epithelial characteristics by either hitching a ride with the invasive EMT cells or following migrating host stromal fibroblasts (cancer-associated fibroblasts, CAFs)/tumor-associated

**Table 3.2** Mechanisms of cancer cell migration




| INDIVIDUAL | COLLECTIVE |
|---|---|
| **Ameboid** (e.g. lymphoma, small cell lung cancer, leukemia) | **Clusters** (e.g. epithelial cancers, melanoma) |
| **Mesenchymal** (e.g. fibrosarcoma, glioblastoma, anaplastic tumours) | **Sheets** (e.g. epithelial cancers, vascular tumours) |



macrophages (TAMs) (Condeelis and Pollard 2006; Gaggioli et al. 2007; Joyce and Pollard 2009), which are described in more detail in the next section.

Nonetheless, the clinical relevance of EMT is unknown and it is still unclear how much tumors depend on EMT for metastatic progression. However, experimental studies clearly show the benefit of EMT in cancer progression (Iwatsuki et al. 2010; Tsai et al. 2012). EMT is typically represented by a loss of the epithelial cell marker E-cadherin, which facilitates cell-cell adhesion, and induction of the mesenchymal cell marker N-cadherin, which facilitates cell-matrix adhesion (Lee et al. 2006). It is well established that hypoxia can directly induce EMT through HIF-1, upregulating the expression of various EMT-activating transcription factors: Twist-related protein 1 (Twist 1, also known as Twist), zinc finger protein Snai1 (SNAI1), zinc finger E-box-binding homeobox 1/2, and transcription factor 3 (Imai et al. 2003; Krishnamachary et al. 2006; Yang et al. 2008).

It is interesting to note that another hypoxia-induced ECM protein family – the lysyl oxidase (LOX) family (Fig. 3.2) – has been shown to play a key role in the EMT process, in particular the members LOX and LOX-like 2 (LOXL2). The *LOX* and *LOXL2* genes are targets of HIF-1 and may play a role in EMT through both their reported intracellular roles and extracellular roles (Schietke et al. 2010). The enzymatic function of extracellular LOX has been shown to stimulate Twist transcription, thereby mediating the EMT of cancer cells (El-Haibi et al. 2012). LOXL2, on the other hand, is involved in the regulation of epithelial cell motility through HIF1 and hypoxia (Higgins et al. 2007), and intracellular LOXL2 has been shown to interact with and stabilize SNAI1, thus inducing EMT (Peinado et al. 2005). However, it also has been shown that while LOXL2 influences SNAI1-dependent invasive properties in cancer cells, LOXL2 also has specific SNAI1-independent functions in cancer progression (Peinado et al. 2005, 2008). Nonetheless, the LOXL2 enzymatic function in particular has been shown to modulate the EMT-like phenotype in cancer cells (Barry-Hamilton et al. 2010).

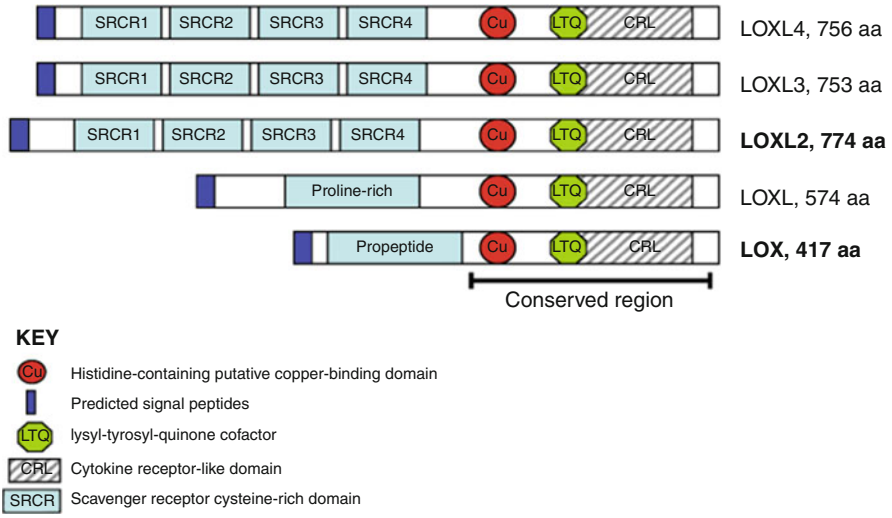



**Fig. 3.2** The lysyl oxidase (*LOX*) family. The LOX family of proteins has a highly conserved C-terminal region that contains a copper-binding motif (depicted in *red*), a lysyl-tyrosyl-quinone (*LTQ*) cofactor (depicted in *green*), and a cytokine receptor-like domain (*shaded*). The N-terminal region is highly variable. LOXL2, 3, and 4 all have four scavenger receptor cysteine-rich regions (*SRCR*s; *light blue*), which is replaced by a proline-rich domain in LOXL (also known as *LOXL1*) and a propeptide region in LOX. *Purple boxes* depict predicted signal peptides

### *3.4.2 Hypoxic Regulation of Invasion*

While EMT may strongly influence invasion, there are also other factors contributing to tumor cell invasion. As mentioned above, hypoxia-induced proteins such as LOX and LOXL2 also have extracellular roles that facilitate cancer cell invasion. LOX cross-links collagens, which increases matrix stiffness, thereby activating integrins that enhance cell-to-matrix adhesion, invasion, proliferation, and malignant transformation (Barker et al. 2012). The enzymatic function of hypoxia-induced LOX increases focal adhesion kinase activity, and proto-oncogene tyrosine-protein kinase Src activity, thereby mediating cell migration/invasion and metastasis in various cancer types (Erler et al. 2006; Baker et al. 2011, 2012). LOX also plays a role in the formation of premetastatic niches, which will be discussed in detail later.

Like LOX, both intracellular and secreted LOXL2 have been shown to be involved in focal adhesion kinase /Src activation in various types of cancers, mediating the invasive/migratory properties and thus metastasis of these cells (Peng et al. 2009; Moreno-Bueno et al. 2011). In addition, the enzymatic function of LOXL2 was involved in the invasion and metastasis of cancer cells through tissue remodeling, during which it regulated the expression and activity of tissue inhibitor of metalloproteinase-1 and MMP-9 (Barker et al. 2011).

Mobilized tumor cells first have to overcome the BM before they can successfully move through the ECM to intravasate into the circulatory system. The BM serves as a physical barrier between the cancer cells and the interstitial ECM and comprises collagen IV, entactin, laminin, glycoproteins, and proteoglycans. It is not normally permeable to cells, but tumor cells can alter their cell surface receptors, such as integrins, to allow contact with BM components and invade through this layer (Nicolson 1989; Liotta and Stetler-Stevenson 1991). Cancer cells also secrete enzymes to degrade the BM to allow easier penetration, such as cathepsin D, urokinase-type plasminogen-activator receptor, and MMP-2. These proteins all are upregulated by hypoxia. Hypoxia also induces fibronectin production, which facilitates cell motility through activation of integrins on the surface of tumor cells (Krishnamachary et al. 2003).

Another HIF-1 target, and thus one regulated by hypoxia, is the *MET* proto-oncogene (Pennacchietti et al. 2003). MET is a receptor tyrosine kinase that also interacts with integrins to activate downstream signaling events, leading to increased invasion/metastasis. Tumor cells overexpress *MET* under hypoxia, responding to the MET-ligand hepatocyte growth factor (HGF); CAFs have been known to secrete HGF under hypoxia (Ide et al. 2006). In this context hypoxia increases tumor cell motility both intrinsically (through *MET* overexpression on tumor cells) and externally (through HGF produced by CAFs). The tumor-secreted cytokine, autocrine motility factor, is another HIF-1 target that can also be induced by vascular endothelial growth factor (VEGF), another well-known hypoxia-induced growth factor. The autocrine motility factor enhances tumor cell proliferation and migration, and can act in either an autocrine or paracrine manner (Funasaka and Raz 2007).

### *3.4.3 Intravasation*

Intravasation is the entry of tumor cells into the circulatory system, and it is most likely different to the exit of tumor cells from the circulation into distant metastatic sites (extravasation). Intravasation occurs at the tumor blood vessels, which often are malformed and irregular, thus allowing relatively easy access of tumor cells into the circulation. Extravasation, on the other hand, occurs at a distant organ site where the blood vessels are usually well formed and mature and thus present a tougher barrier for tumor cells to pass through. Nonetheless, tumor cells may still remodel the vessels to allow entry by secreting HIF-1α-regulated metalloproteinases MMP1 and MMP2 (Shyu et al. 2007), which have been shown to be synergistic mediators of vascular permeability and intravasation (Gupta et al. 2007). The MMP inhibitory protein RECK is also implicated in intravasation through HIF-1 upregulation of microRNA miR-372/373 (Loayza-Puch et al. 2010). Such observations open the possibility that other ECM proteins, such as LOXL2, could also play a role in the intravasation stage of metastasis; however, further investigations are required to elucidate the mechanisms of intravasation.

Hypoxia-induced VEGF, although often recognized as a angiogenic factor (detailed below), can also facilitate microvascular permeability and increase interstitial fluid pressure, thereby increasing the chances of intravasation by cancer cells (Sullivan and Graham 2007). It is interesting that the EMT-inducing and hypoxia-regulated transcription factor Twist, mentioned in the previous section, also has been found to increase the ability of tumor cells to intravasate (Yang et al. 2004), highlighting the complexity of the metastatic process.

## 3.5 The Influence of Hypoxia on Stromal Cells to Promote Tumor Cell Invasion

The tumor stroma consists of a noncellular component (the ECM), as described above, and a cellular component comprising a diverse range of nontumor "normal" cell types (Fig. 3.3). These stromal cells include fibroblasts, endothelial cells, perivascular cells, and inflammatory cells, and these all have been shown to play a significant role in cancer progression by mediating angiogenesis, desmoplasia, lymphangiogenesis, and inflammation (Finger and Giaccia 2010). In particular, CAFs and TAMs have gained much attention for their roles in promoting metastasis.

CAFs are myofibroblast-like cells that promote tumor growth by inducing desmoplastic reactive stroma around tumor cells (Kalluri and Zeisberg 2006). CAFs can induce tumor-promoting inflammation, enhance vascularization of primary tumor growth, and recruit immune cells that promote tumor progression. It also has been shown that normal fibroblasts can be educated by carcinoma cells to become proinflammatory and thus promote tumor progression (Erez et al. 2010). It has recently been demonstrated that hypoxia alone is sufficient to induce degradation of caveolin-1, a hallmark of CAFs that promotes higher tumor aggressiveness in patients with breast cancer, which can predict lymph node metastasis and chemoresistance. Moreover, the loss of stromal caveolin-1 also protects adjacent cancer cells against apoptosis and autophagy (Martinez-Outschoorn et al. 2010).

TAMs are recruited to areas of hypoxia, and their presence correlates with poor outcome in cancer patients (Bingle et al. 2002; Murdoch et al. 2004). It has been shown that TAMs form clusters within the primary tumors; these clusters are correlated with angiogenesis and elevated VEGF levels (Goede et al. 1999; Salvesen and Akslen 1999); however, this remains in dispute because other researchers did not find correlation between microvessel density and accumulation of macrophages in invasive carcinomas (Davidson et al. 1999). Host cells such as TAMs have been demonstrated to directly assist tumor cell intravasation without the need for local angiogenesis (Wyckoff et al. 2007), and it has been shown that TAMs accumulate in hypoxic areas because of hypoxia-induced secretion of macrophage chemoattractants such as endothelin 2 and VEGF by both tumor and stromal cells (Murdoch et al. 2004). It is intriguing that hypoxia can induce changes in the expression of a range of genes in normal macrophages (Table 3.3), including a range of proangiogenic factors (see the section "Hypoxia and Angiogenesis").

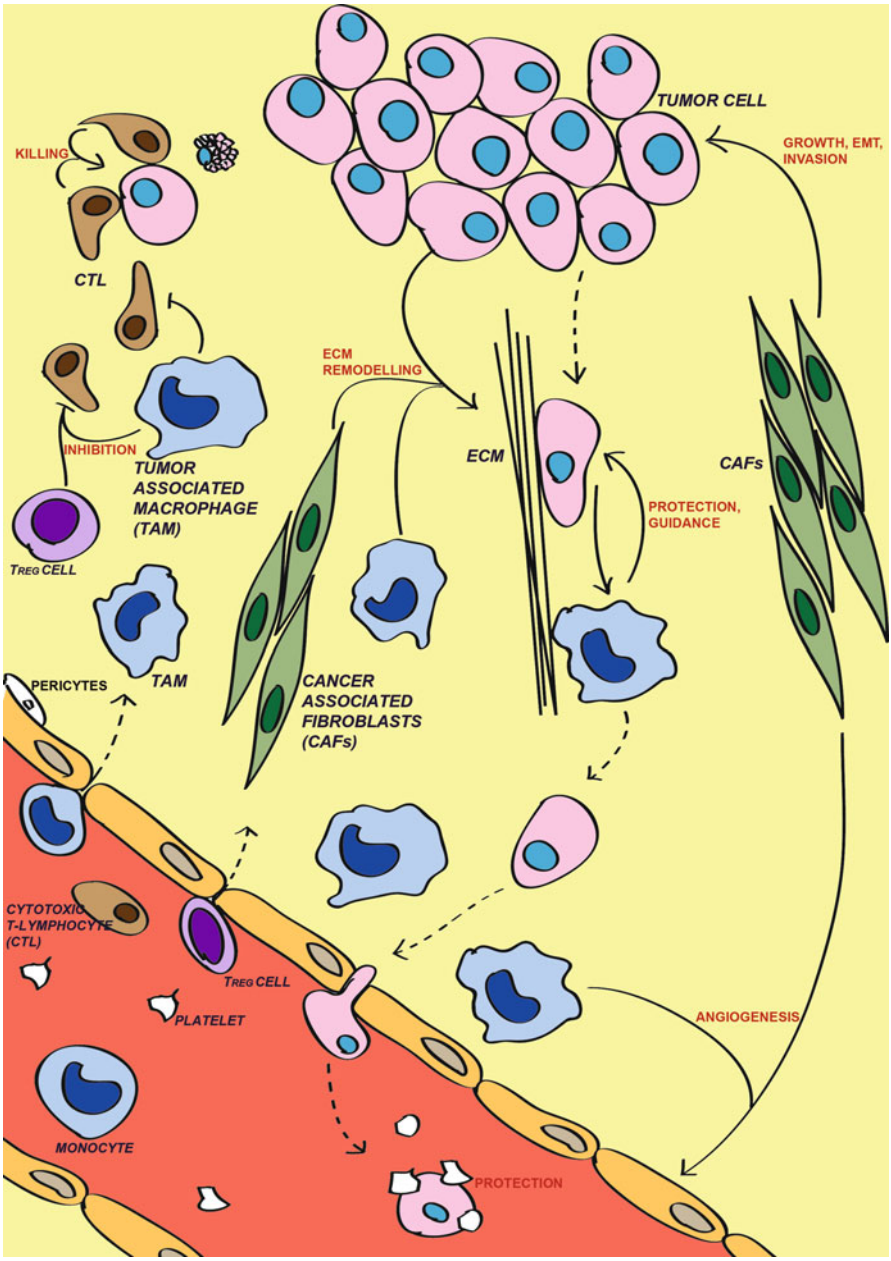



**Fig. 3.3** The various stromal cells involved in tumor cell invasion. Host-derived stromal cells present in the tumor stroma can be involved in the invasion process of tumor cells and become cancer-associated stromal cells. For example, monocytes develop into tumor-associated macrophages (*TAMs*), and fibroblasts become cancer-associated fibroblasts (*CAFs*)

**Table 3.3** Gene expression changes induced by hypoxia in normal macrophages

| Factor | Function | Up-/downregulation |
|---|---|---|
| Glucose transporter-1 | Survival | Up |
| Vascular endothelial growth factor | Proangiogenic | Up |
| Fibroblast growth factor-2 | Proangiogenic | Up |
| PIGF | Proangiogenic | Up |
| Cyclooxygenase-2 | Proangiogenic<br>Prostanoid synthesis | Up |
| Leptin | Proangiogenic | Up |
| Platelet-derived growth factor-β | Proangiogenic | Up |
| Hepatocyte growth factor | Proangiogenic | Up |
| Fibronectin | Proangiogenic | Up |
| Angiopoietin-1 | Proangiogenic | Up |
| Matrix metalloproteinase-1, -7 | Proangiogenic<br>Prometastatic | Up |
| Tissue factor | Proangiogenic<br>Promigratory<br>Prothrombotic | Up |
| Inducible nitric oxide synthase | Proangiogenic, proinflammatory | Up |
| Tumor necrosis factor-α | Proangiogenic<br>Proinflammatory<br>Cytotoxic | Up |
| IL-1 | Proinflammatory | Up |
| Prostaglandin $E_2$ | Immunosuppressive | Up |
| IL-10 | Immunosuppressive | Up |
| CCL-2, -3 | Chemokine | Down, up |
| C-X-C chemokine receptor type 4 | Proangiogenic | Up |
| Interferon-γ | Immunostimulatory | Up |
| CXCL-8 | Proangiogenic<br>Chemokine | Up |
| CXCL-1 | Chemokine | Up |
| NMB-R | Unknown | Up |
| CCR-5 | Chemokine receptor | Down |
| CD80 | Antigen presentation | Down |
| Robo4 | Slit receptor<br>Anti-angiogenic | Up |
| MIF | Antimigratory<br>Prometastatic | Up |
| Very-low-density lipoprotein receptor | Proatherosclerotic | Up |
| ORP150 | Proatherosclerotic | Up |
| IL-6 | Proinflammatory | Up |
| Arginase | Proinflammatory | Up |

*IL* interleukin

Adapted from Murdoch C, Muthana M, Lewis CE. Hypoxia regulates macrophage functions in inflammation. J Immunol 2005;175(10):6257–6263

## 3.6 Survival in Circulation

Tumor cells that have successfully entered the circulation (CTCs) can now travel to most organs in the body, provided that they first survive the harsh environment of the circulatory system. In particular, CTCs are vulnerable to anoikis, a form of apoptosis induced by a loss of adhesion that was first described by Frisch and Francis in 1994. CTCs also are subjected to the sheer forces exerted by blood flow as well as attacks from host immune cells (Gupta and Massague 2006). CTCs can increase their chance of survival by binding to platelets or lymphocytes for protection (Fidler and Bucana 1977; Gasic 1984; Nash et al. 2002) or by forming emboli by binding to coagulation factors such as thrombin, fibrinogen, and fibrin (Zhan et al. 2004). These emboli, also called heterotypic clumps because of the population of different cells, have a higher metastatic potential than aggregates of tumor cells alone (i.e., homotypic clumps) presumably because of the presence of host cells, and thus they are better shielded from immune cells. Nonetheless, the formation of aggregates, be it homotypic or heterotypic, probably also confers resistance to apoptosis by anoikis. From a diagnostic point of view, it has been demonstrated that the detection of CTCs in blood circulation has prognostic value in many carcinomas, suggesting a new noninvasive strategy of determining treatments for cancer patients (Gorges and Pantel 2013).

Hypoxia may seem to do little to assist CTCs because hypoxia is not present in circulating blood. However, the duration between intravasation and extravasation may only be a few hours, as demonstrated by in vivo videomicroscopy (Chambers et al. 1995); thus the hypoxia-induced response that occurs in the invasive cells in the primary tumor may act long enough to affect the survival and extravasation of CTCs, allowing them to successfully metastasize. It has been reported that hypoxia confers resistance to anoikis through suppression of α5 integrin, a sensitizer toward anoikis (Rohwer et al. 2008). Hypoxia also induces suppression of *Bim* and *Bmf*, thus inhibiting anoikis (Whelan et al. 2010). Another potential mediator is the hypoxia-responsive factor TrkB, which is a suppressor of anoikis (Martens et al. 2007).

## 3.7 Homing (Extravasation) and Metastatic Colonization

The first step for CTCs to successfully colonize a secondary site (and thus become DTCs) is to stop circulating and exit the blood vessel (extravasation) (Fig. 3.4). CTCs lodge in the capillary beds at secondary sites and may either extravasate and invade the foreign parenchyma as single cells or proliferate intraluminally and eventually rupture the wall of the microvessels (due to the size of the metastatic lesion), allowing the CTCs to enter the surrounding tissue (Al-Mehdi et al. 2000; Wong et al. 2002). Individual CTCs may become arrested mechanically because of their large size in comparison to the capillary lumen (Naumov et al. 1999; Ito et al. 2001) or through direct interaction with the surface molecules of endothelial cells (Nicolson 1988; Arap et al. 1998; Pasqualini et al. 2000). These processes may be facilitated by the endothelial cell P- and E-selectins (Mannori et al. 1997; Kim et al. 1998) as well as integrins and CD44 on the tumor cells (Birch et al. 1991; Ruoslahti 1994;

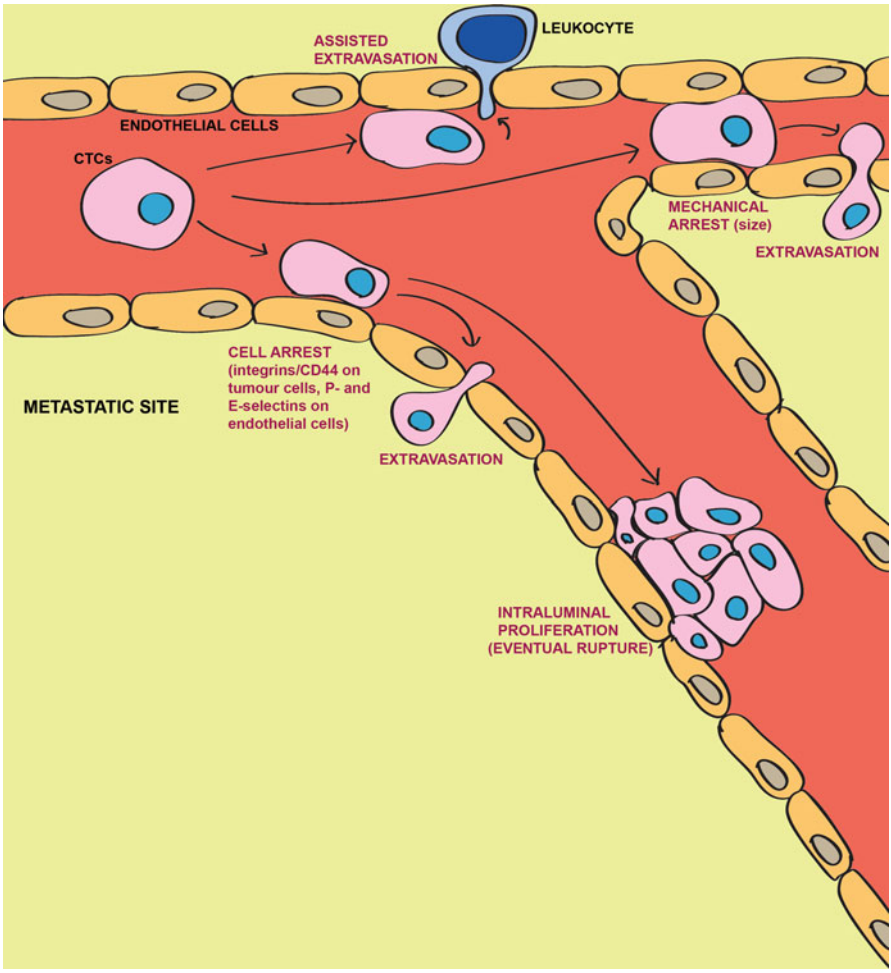



**Fig. 3.4** The colonization process of circulating tumor cells. As a first step toward colonization of a distant site, the circulating tumor cells need to stop and undergo extravasation to leave the blood vessels. This can be achieved by various mechanisms: cell arrest extravasation, assisted extravasation, mechanical arrest extravasation, or intraluminal proliferation followed by rupture and spilling into the secondary site. Other cell types may be involved in this process (Adapted from http://www.nature.com/nrc/journal/v7/n10/pdf/nrc2229.pdf Figure 1)

Friedrichs et al. 1995; Wang et al. 2004). Because endothelial cells are constantly shed from blood vessels, in some cases CTCs may interact with the exposed BM directly through integrins such as α3β1 (Weiss et al. 1988; el-Sabban and Pauli 1994; Wang et al. 2004). This type of arrest can be enhanced by platelets aggregating with the CTCs, and ECM components such as fibronectin and laminin can also enhance tumor cell arrest (Terranova et al. 1984). Indeed, host cells such as leukocytes may also be recruited following CTCs arrest and lead the extravasation process, with the CTCs following them (Wood 1958; Sahai 2007).

### 3.7.1 Hypoxia and Extravasation

As mentioned earlier, the time between intravasation and extravasation may be only a few hours; thus the effects of hypoxia on CTCs may be maintained until after extravasation has occurred. This is supported by the observation that transient hypoxic treatment of tumor cells in vitro before intravenous injection into mice can increase colonization (Young et al. 1988) and that acute hypoxic treatment of the primary tumor (during tumor growth tumor-bearing mice were subjected to low oxygen conditions for 10 min 12 times a day) increases spontaneous metastasis (Cairns et al. 2001). Despite the fact that both intravasation and extravasation involve crossing the blood barrier, they are two different processes: tumor-associated blood vessels are usually highly permeable, while normal tissue vasculature at the metastatic site has a higher integrity and thus acts as a more effective barrier. Nevertheless, factors that contribute to intravasation may also be involved in extravasation. One such example is VEGF; it was shown that direct inhibition of VEGF can suppress extravasation and metastasis to the lungs in breast cancer (Lee et al. 2003).

### 3.7.2 Hypoxia and the Selection of Metastatic Sites

It has long been established that each type of cancer has favored metastatic sites, and this may be due to physical attributions of the various organs. One such attribution could be the structural differences of the capillaries in the organs, such as the sinusoid capillaries in bone marrow. This type of capillary has only a single layer of endothelial cells and no supporting structures, thus allowing easy trafficking of hematopoietic cells in and out of the bone marrow. This in turn presents an easy route along which cancer cells can extravasate, which may explain why bone marrow is the favored target organ for the metastases of a wide range of cancers (Alix-Panabieres et al. 2008). Another determining factor is the pattern of circulation within the body. For example, colorectal cancers have a strong preference for metastasizing to the liver (Schluter et al. 2006). In this case the blood circulation drains from the colon directly into the liver, bringing with it an extremely high number of CTCs (Chaffer and Weinberg 2011); thus even if the probability of a colorectal cancer cell being able to colonize the liver microenvironment is low, liver metastasis will still occur because of the sheer number of colorectal CTCs entering the liver.

The process of homing described above is likely to be a passive process; however, it is apparent that cancer cells actively seek out preferred organs to which they can metastasize, as the anatomical distribution of the metastases do not conform to the blood circulation pattern alone or the type of capillaries present. In fact, the idea was first put forward by Stephen Paget in 1989 as the "seed and soil" hypothesis: he proposed that metastatic spread is a combined result of properties intrinsic of the "seed," that is, cancer cells, and the properties of the secondary site – the "soil" – such that the compatibility of the tumor cells with the microenvironment is a predominant determinant of successful metastasis.

One such intrinsic property of cancer cells is the hypoxia-induced expression of the C-X-C chemokine receptor type-4 (CXCR4) (Murdoch 2000). CXCR4 allows CTCs to home in to tissues expressing high levels of the CXCR4-specific ligand stromal cell-derived factor-1 (SDF-1, also known as CXCL12) and has been shown to be important in various cancers such as renal cell carcinoma (Staller et al. 2003), ovarian cancer (Scotton et al. 2001, 2002), breast cancer (Lu et al. 2010), lung cancer (Liu et al. 2006), and neuroblastoma (Geminder et al. 2001).

Hypoxia has recently been demonstrated to affect the gene signatures of lung- and bone-specific metastases using different mechanisms. Hypoxia-induced angiogenesis genes are associated with lung metastasis but not bone metastasis, and hypoxia enhances a significant number of lung metastasis gene signatures, whereas only a few bone metastasis genes, such as the previously mentioned CXCR4 and dual specificity protein phosphatase 1, are induced by hypoxia (Lu et al. 2010).

Bone metastasis can be classified into two types: osteoblastic and osteolytic (Mundy 2002; Teicher and Fricker 2010). Osteoblastic metastases are commonly found in prostate cancer, whereas osteolytic metastases are commonly found in breast cancer and multiple myeloma. Regardless of the type of bone metastasis, osteoclast proliferation/activation and bone hypertrophy are commonly observed (Halvorson et al. 2006). Several known hypoxia-regulated proteins have been shown to drive osteolytic bone metastases. Connective tissue growth factor (Higgins et al. 2004) is involved in osteoclastogenesis and bone resorption, liberating tumor-promoting factors from the bone matrix (Kang et al. 2003; Nozawa et al. 2009). These tumor-promoting factors include bone morphogenic proteins and transforming growth factor-β, which also are upregulated by hypoxia signaling (Falanga et al. 1991; Maegdefrau et al. 2009). Hypoxia-induced osteopontin interacts with osteoclasts that express αvβ3, thus promoting bone metastasis (Engleman et al. 1997). The cytokines interleukin-6 and -8 are upregulated by hypoxia and have multiple functions that could promote bone metastasis: they induce angiogenesis, migration, and osteolysis (Bendre et al. 2005; Ara and Declerck 2010). VEGF also attracts VEGF receptor–positive tumor cells to the metastatic site.

It is widely accepted that hypoxia probably regulates various organ-specific metastases in different ways; however, it has been shown in animal models that inhibiting HIF-1α significantly reduced metastases to both lungs and bones, highlighting the importance of HIF-1α as a potential therapeutic target for multiple organotypic metastases (Lu et al. 2010).

### *3.7.3 Hypoxia and the Premetastatic Niche*

In recent years the concept of the premetastatic niche has become increasing important, whereby factors secreted by tumor cells stimulate the preparation of distant sites of future metastasis, recruiting clusters of host bone marrow–derived cells (BMDCs) to home in and modify the microenvironment, thereby preparing the secondary sites to aid cancer cell colonization and growth (Psaila et al. 2006; Peinado

et al. 2011). The presence of these premetastatic niches greatly enhances tumor cell colonization and growth at the secondary site, and can influence the route of metastatic spread (Kaplan et al. 2006).

It was first demonstrated in 2002 that MMP-9 is induced in premetastatic lung endothelial cells by distant primary tumors through VEGF receptor 1 and is involved in lung-specific metastasis (Hiratsuka et al. 2002). The term *premetastatic niche*, however, was not coined until 2005, when it was demonstrated that host BDMCs expressing VEGF receptor 1 travel to premetastatic sites and form cellular clusters before tumor cells arrive, creating a permissive niche for incoming tumor cells. These events were shown to be influenced by factors secreted by the tumor cells. Exciting research showed that by introducing secreted factors of different tumors with distinct metastatic preferences, one could transform the metastatic profile and redirect organ colonization, indicating that premetastatic niches also guide CTCs to specific organs (Kaplan et al. 2005). It has since been shown that inflammatory chemoattractants affect both the primary tumor invasion and recruitment of myeloid cells to the lungs in the formation of the premetastatic niche (Hiratsuka et al. 2006).

Hypoxia also plays an important role in the formation of premetastatic niches. Hypoxic tumor cells secrete the aforementioned HIF-1 target LOX, which then accumulates at the premetastatic sites. The presence of LOX is essential for the recruitment of $CD11b^+$ myeloid cells to the premetastatic sites through matrix remodeling, allowing the $CD11b^+$ cells adhere to the ECM and secrete MMP-2. MMP-2 in turn cleaves collagens and in mouse models of breast cancer enhances further recruitment of BMDCs and invasion of CTCs (Erler et al. 2009). Furthermore, HIF-1 has been shown to be critical in the formation of the premetastatic niche in a breast cancer model through the induction of various members of the LOX family, including LOX, LOXL2, and LOX-like 4 (LOXL4). These LOX family members catalyze cross-linking of collagens at the site of premetastatic niche, promoting BDMC recruitment and thus enhancing lung colonization. It is interesting that each LOX family member is involved in the formation of the premetastatic niche of different subsets of breast cancer, highlighting the complexity of the cellular and molecular effects of LOX, LOXL2, and LOXL4, as well as the highly heterogeneous nature of responses to hypoxia (Wong et al. 2011). Of note, we recently showed that LOX mediates collagen cross-linking in normal lungs and livers in response to fibrotic signals, that this modified matrix is responsible for fibrosis-enhanced metastasis, and that altering collagen cross-linking alone was sufficient to significantly increase tumor cell proliferation (Cox et al. 2013). These findings suggest a key role for matrix remodeling mediated by hypoxia-regulated proteins at metastatic sites in enhancing metastasis. Exciting research showed that the HIF-1 inhibitors digoxin and acriflavine block the formation of premetastatic niches in breast cancer metastasis by inhibiting the hypoxia-induced expression of the LOX family members (Wong et al. 2012). In addition, inhibition of LOXL2 enzymatic activity modifies the tumor microenvironment by reducing the secretion of growth factors that are instrumental in invasion and metastasis by the cancer cells (Barry-Hamilton et al. 2010). This presents a therapeutic angle whereby breast cancer patients with high levels of HIF1 may benefit from inhibitors against HIF1 or against the LOX family.

### *3.7.4 Hypoxia and Secondary Tumor Growth*

Metastatic tumor cells that have successfully disseminated into the metastatic site (i.e., DTCs) may become dormant as micrometastases for long periods of time before they can colonize efficiently and become macrometastases (Morris et al. 1994; Chambers et al. 1995; Pantel and Alix-Panabieres 2010). This dormancy may be due to the DTCs entering quiescence, because cell proliferation and cell death is balanced as a result of immune surveillance, or because of the lack of vascular support. It has been demonstrated in mice that the presence of hypoxia in a primary tumor is correlated with metastatic tumor growth (Buchler et al. 2004). Hypoxia-responsive genes such as *GPR56*, *KISS1*, and *CD82* (*KAI1*) can prevent DTCs from proliferating at the secondary sites (Horak et al. 2008; Nguyen et al. 2009). The transition of micrometastases to macrometastases requires vessel co-option or new blood vessel formation (angiogenesis) (Moserle et al. 2009; Kienast et al. 2010), and hypoxia is known to induce angiogenesis (Fraisl et al. 2009). Another hypoxia-inducible transcription regulator inhibitor of DNA-binding 1 (Id-1) can be upregulated by recruited BDMCs to promote progression of micro- to macrometastases (Gao et al. 2008). It is interesting that VEGF can also be involved in the progress of micro- to macrometastases by promoting vascularisation (see the next section).

## 3.8 Hypoxia and Angiogenesis

Angiogenesis is defined as the formation of new blood vessels. The process of angiogenic sprouting involves several steps and is regulated by the balance between angiogenic and anti-angiogenic factors present in the tissue. Angiogenesis is a critical step in cancer progression because it limits primary and metastatic tumor growth. It provides nutrients and oxygen to promote tumor growth and provides escape routes for cancer cells, allowing them to metastasize further.

Hypoxia induces the secretion of proangiogenic factors such as VEGF, angiopoietin 2, platelet-derived growth factor, fibroblast growth factor (Hanahan and Folkman 1996; Ruan et al. 2009) and decreases angiogenic inhibitors such as thrombospondin – all through HIF-1 (Laderoute et al. 2000). Of these, VEGF is characterized best. Tumor cells secrete VEGF, which stimulates endothelial cells and recruits endothelial progenitor cells. VEGF also stimulates outgrowth of pericytes and increases vascular permeability, as mentioned earlier (Senger et al. 1983; Leung et al. 1989). A mouse model of liver metastasis demonstrated that when micrometastases start to grow and become hypoxic, hepatic stellate cells are recruited to the hypoxic sites and secrete VEGF, which then recruits endothelial cells and promotes formation of the vasculature, thereby enabling macrometastases to develop (Olaso et al. 2003). The presence of the secondary vasculature can provide a route through which further metastases can occur. Of note, we recently showed that LOX regulates VEGF expression through activation of PDGFRβ,

resulting in *Akt* activation, which then upregulates the VEGF messenger RNA and protein (Baker et al. 2012). This demonstrates the involvement of ECM proteins such as LOX in angiogenesis.

The recruitment of circulating bone marrow–derived endothelial cells also increases angiogenesis (Nolan et al. 2007). In particular, the hypoxia-regulated SDF-1/CXCR4 pathway involved in the homing of CTCs is heavily involved in angiogenesis, as CXCR4 is also expressed by hematopoietic and endothelial cells, making this signaling pathway an attractive therapeutic target (Petit et al. 2007).

Angiogenesis is clearly important in enabling tumor progression at both primary and metastatic sites, and therefore it is understandable that much focus has been put on anti-angiogenic therapy in treating cancer. VEGFR inhibitors have been approved for treatment of patients with late-stage metastatic colorectal cancer, metastatic breast cancer, renal cell carcinoma, non-small-cell lung cancer, hepatocellular carcinoma, glioblastoma multiforme, medullary thyroid, and gastrointestinal stromal tumors (Crawford and Ferrara 2009), but they have generally yielded disappointing results. However, anti-angiogenic therapies also produce a paradox: inhibiting angiogenesis may cause hypoxia in the tumor, which in turn may promote metastasis, as described in this chapter. In preclinical models, VEGF receptor inhibitors such as sunitinib have been shown to have opposite effects on metastasis, depending on the type of cancer and treatment (Osusky et al. 2004; Ebos et al. 2009; Paez-Ribes et al. 2009; Zhang et al. 2009), underscoring the complexity of targeting angiogenesis. This was demonstrated in a recent study in which two murine carcinoma models (mammary carcinoma and renal adenocarcinoma) treated with various doses of sunitinib showed varying effects of the drug – not just on the metastases but also on myeloid cell recruitment and survival (Welti et al. 2012). Nonetheless, large-scale clinical studies have reported that blocking VEGF does not aggravate the clinical outcome of patients with advanced-stage metastatic disease; in fact it prolongs progression-free survival or overall survival of these patients (De Bock et al. 2011). However, rapid tumor regrowth has been reported in some cancer patients after anti-angiogenic therapy is withdrawn (Burstein et al. 2008). This suggests that anti-angiogenic therapy may not be optimal on its own, and coupling it with therapy targeting HIF1 may actually be of benefit to eliminate potential hypoxic promotion of cancer progression caused by inhibition of angiogenesis.

## 3.9 Conclusions

Metastasis is a highly complex, multistep process and is responsible for the majority of deaths among cancer patient. Hypoxia is correlated with treatment failure, metastasis, and poor patient survival. However, there is increasing evidence demonstrating that hypoxia potently influences every step of the metastatic process, driving cancer progression. Thus, targeting hypoxia may successfully reduce or even prevent cancer metastasis. It should be remembered that the heterogeneity of hypoxia within the tumor indicates complex hypoxia-mediated metastasis, and most studies

to date have focused on individual proteins and thus lack perspective on how these are interconnected. This highlights the need for further research into hypoxia-regulated metastasis, taking a more systems-biology approach to investigate the dynamics of the molecular networks involved and determine how best to target these. Nonetheless, it is clear that targeting hypoxia may be beneficial as an adjuvant to existing cancer therapies.

## References

Ailles LE, Weissman IL (2007) Cancer stem cells in solid tumors. Curr Opin Biotechnol 18:460–466

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100:3983–3988

Alix-Panabieres C, Riethdorf S, Pantel K (2008) Circulating tumor cells and bone marrow micrometastasis. Clin Cancer Res: Off J Am Assoc Cancer Res 14:5013–5021

Al-Mehdi AB, Tozawa K, Fisher AB, Shientag L, Lee A, Muschel RJ (2000) Intravascular origin of metastasis from the proliferation of endothelium-attached tumor cells: a new model for metastasis. Nat Med 6:100–102

Andreasen PA, Egelund R, Petersen HH (2000) The plasminogen activation system in tumor growth, invasion, and metastasis. Cell Mol Life Sci 57:25–40

Ara T, Declerck YA (2010) Interleukin-6 in bone metastasis and cancer progression. Eur J Cancer 46:1223–1231

Arap W, Pasqualini R, Ruoslahti E (1998) Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 279:377–380

Baker AM, Cox TR, Bird D, Lang G, Murray GI, Sun XF et al (2011) The role of lysyl oxidase in SRC-dependent proliferation and metastasis of colorectal cancer. J Natl Cancer Inst 103:407–424

Baker AM, Bird D, Welti JC, Gourlaouen M, Lang G, Murray GI et al (2012) Lysyl oxidase plays a critical role in endothelial cell stimulation to drive tumor angiogenesis. Cancer Res 73(2):583–594

Barker HE, Chang J, Cox TR, Lang G, Bird D, Nicolau M et al (2011) LOXL2-mediated matrix remodeling in metastasis and mammary gland involution. Cancer Res 71:1561–1572

Barker HE, Cox TR, Erler JT (2012) The rationale for targeting the LOX family in cancer. Nat Rev Cancer 12:540–552

Barry-Hamilton V, Spangler R, Marshall D, McCauley S, Rodriguez HM, Oyasu M et al (2010) Allosteric inhibition of lysyl oxidase-like-2 impedes the development of a pathologic microenvironment. Nat Med 16:1009–1017

Bendre MS, Margulies AG, Walser B, Akel NS, Bhattacharrya S, Skinner RA et al (2005) Tumor-derived interleukin-8 stimulates osteolysis independent of the receptor activator of nuclear factor-kappaB ligand pathway. Cancer Res 65:11001–11009

Bingle L, Brown NJ, Lewis CE (2002) The role of tumor-associated macrophages in tumor progression: implications for new anticancer therapies. J Pathol 196:254–265

Birch M, Mitchell S, Hart IR (1991) Isolation and characterization of human melanoma cell variants expressing high and low levels of CD44. Cancer Res 51:6660–6667

Bonnet D, Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med 3:730–737

Buchler P, Reber HA, Lavey RS, Tomlinson J, Buchler MW, Friess H et al (2004) Tumor hypoxia correlates with metastatic tumor growth of pancreatic cancer in an orthotopic murine model. J Surg Res 120:295–303

Burstein HJ, Elias AD, Rugo HS, Cobleigh MA, Wolff AC, Eisenberg PD et al (2008) Phase II study of sunitinib malate, an oral multitargeted tyrosine kinase inhibitor, in patients with metastatic breast cancer previously treated with an anthracycline and a taxane. J Clin Oncol: Off J Am Soc Clin Oncol 26:1810–1816

Cairns RA, Kalliomaki T, Hill RP (2001) Acute (cyclic) hypoxia enhances spontaneous metastasis of KHT murine tumors. Cancer Res 61:8903–8908

Cairns RA, Khokha R, Hill RP (2003) Molecular mechanisms of tumor invasion and metastasis: an integrated view. Curr Mol Med 3:659–671

Chaffer CL, Weinberg RA (2011) A perspective on cancer cell metastasis. Science 331:1559–1564

Chambers AF, MacDonald IC, Schmidt EE, Koop S, Morris VL, Khokha R et al (1995) Steps in tumor metastasis: new concepts from intravital videomicroscopy. Cancer Metastasis Rev 14:279–301

Chaudary N, Hill RP (2007) Hypoxia and metastasis. Clin Cancer Res 13:1947–1949

Condeelis J, Pollard JW (2006) Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell 124:263–266

Condeelis J, Segall JE (2003) Intravital imaging of cell movement in tumors. Nat Rev Cancer 3:921–930

Cox TR, Bird D, Baker AM, Barker HE, Ho MW, Lang G et al (2013) LOX-mediated collagen crosslinking is responsible for fibrosis-enhanced metastasis. Cancer Res 73(6):1721–1732

Crawford Y, Ferrara N (2009) VEGF inhibition: insights from preclinical and clinical studies. Cell Tissue Res 335:261–269

Davidson B, Goldberg I, Gotlieb WH, Lerner-Geva L, Ben-Baruch G, Agulansky L et al (1999) Macrophage infiltration and angiogenesis in cervical squamous cell carcinoma–clinicopathologic correlation. Acta Obstet Gynecol Scand 78:240–244

De Bock K, Mazzone M, Carmeliet P (2011) Antiangiogenic therapy, hypoxia, and metastasis: risky liaisons, or not? Nature reviews. Clin Oncol 8:393–404

Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS (2009) Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 15:232–239

El-Haibi CP, Bell GW, Zhang J, Collmann AY, Wood D, Scherber CM et al (2012) Critical role for lysyl oxidase in mesenchymal stem cell-driven breast cancer malignancy. Proc Natl Acad Sci U S A 109:17460–17465

el-Sabban ME, Pauli BU (1994) Adhesion-mediated gap junctional communication between lung-metastatatic cancer cells and endothelium. Invasion Metast 14:164–176

Engleman VW, Nickols GA, Ross FP, Horton MA, Griggs DW, Settle SL et al (1997) A peptidomimetic antagonist of the alpha(v)beta3 integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo. J Clin Investig 99:2284–2292

Erez N, Truitt M, Olson P, Arron ST, Hanahan D (2010) Cancer-associated fibroblasts are activated in incipient neoplasia to orchestrate tumor-promoting inflammation in an NF-kappaB-dependent manner. Cancer Cell 17:135–147

Erler JT, Giaccia AJ (2006) Lysyl oxidase mediates hypoxic control of metastasis. Cancer Res 66:10238–10241

Erler JT, Cawthorne CJ, Williams KJ, Koritzinsky M, Wouters BG, Wilson C et al (2004) Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. Mol Cell Biol 24:2875–2889

Erler JT, Bennewith KL, Nicolau M, Dornhofer N, Kong C, Le QT et al (2006) Lysyl oxidase is essential for hypoxia-induced metastasis. Nature 440:1222–1226

Erler JT, Bennewith KL, Cox TR, Lang G, Bird D, Koong A et al (2009) Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. Cancer Cell 15:35–44

Falanga V, Qian SW, Danielpour D, Katz MH, Roberts AB, Sporn MB (1991) Hypoxia upregulates the synthesis of TGF-beta 1 by human dermal fibroblasts. J Investig Dermatol 97:634–637
Fidler IJ, Bucana C (1977) Mechanism of tumor cell resistance to lysis by syngeneic lymphocytes. Cancer Res 37:3945–3956
Finger EC, Giaccia AJ (2010) Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. Cancer Metastasis Rev 29:285–293
Fraisl P, Mazzone M, Schmidt T, Carmeliet P (2009) Regulation of angiogenesis by oxygen and metabolism. Dev Cell 16:167–179
Friedl P, Gilmour D (2009) Collective cell migration in morphogenesis, regeneration and cancer. Nat Rev Mol Cell Biol 10:445–457
Friedl P, Wolf K (2008) Tube travel: the role of proteases in individual and collective cancer cell invasion. Cancer Res 68:7247–7249
Friedrichs K, Franke F, Lisboa BW, Kugler G, Gille I, Terpe HJ et al (1995) CD44 isoforms correlate with cellular differentiation but not with prognosis in human breast cancer. Cancer Res 55:5424–5433
Frisch SM, Francis H (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 124:619–626
Funasaka T, Raz A (2007) The role of autocrine motility factor in tumor and tumor microenvironment. Cancer Metastasis Rev 26:725–735
Fyles A, Milosevic M, Hedley D, Pintilie M, Levin W, Manchul L et al (2002) Tumor hypoxia has independent predictor impact only in patients with node-negative cervix cancer. J Clin Oncol: Off J Am Soc Clin Oncol 20:680–687
Gaggioli C, Hooper S, Hidalgo-Carcedo C, Grosse R, Marshall JF, Harrington K et al (2007) Fibroblast-led collective invasion of carcinoma cells with differing roles for RhoGTPases in leading and following cells. Nat Cell Biol 9:1392–1400
Gao D, Nolan DJ, Mellick AS, Bambino K, McDonnell K, Mittal V (2008) Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. Science 319:195–198
Gasic GJ (1984) Role of plasma, platelets, and endothelial cells in tumor metastasis. Cancer Metastasis Rev 3:99–114
Geminder H, Sagi-Assif O, Goldberg L, Meshel T, Rechavi G, Witz IP et al (2001) A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. J Immunol 167:4747–4757
Goede V, Brogelli L, Ziche M, Augustin HG (1999) Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. Int J Cancer 82:765–770
Gorges TM, Pantel K (2013) Circulating tumor cells as therapy-related biomarkers in cancer patients. Cancer Immunol Immunother. 2013 May;62(5):931–9
Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW et al (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. Nature 379:88–91
Gupta GP, Massague J (2006) Cancer metastasis: building a framework. Cell 127:679–695
Gupta GP, Nguyen DX, Chiang AC, Bos PD, Kim JY, Nadal C et al (2007) Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. Nature 446:765–770
Halvorson KG, Sevcik MA, Ghilardi JR, Rosol TJ, Mantyh PW (2006) Similarities and differences in tumor growth, skeletal remodeling and pain in an osteolytic and osteoblastic model of bone cancer. Clin J Pain 22:587–600
Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364
Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70
Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674
Harris AL (2002) Hypoxia–a key regulatory factor in tumor growth. Nat Rev Cancer 2:38–47
Higgins DF, Biju MP, Akai Y, Wutz A, Johnson RS, Haase VH (2004) Hypoxic induction of Ctgf is directly mediated by Hif-1. Am J Physiol Ren Physiol 287:F1223–F1232
Higgins DF, Kimura K, Bernhardt WM, Shrimanker N, Akai Y, Hohenstein B et al (2007) Hypoxia promotes fibrogenesis in vivo via HIF-1 stimulation of epithelial-to-mesenchymal transition. J Clin Investig 117:3810–3820

Hiratsuka S, Nakamura K, Iwai S, Murakami M, Itoh T, Kijima H et al (2002) MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. Cancer Cell 2:289–300

Hiratsuka S, Watanabe A, Aburatani H, Maru Y (2006) Tumor-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. Nat Cell Biol 8:1369–1375

Hockel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93:266–276

Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P (1996) Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56:4509–4515

Horak CE, Lee JH, Marshall JC, Shreeve SM, Steeg PS (2008) The role of metastasis suppressor genes in metastatic dormancy. APMIS 116:586–601

Ide T, Kitajima Y, Miyoshi A, Ohtsuka T, Mitsuno M, Ohtaka K et al (2006) Tumor-stromal cell interaction under hypoxia increases the invasiveness of pancreatic cancer cells through the hepatocyte growth factor/c-Met pathway. Int J Cancer 119:2750–2759

Imai T, Horiuchi A, Wang C, Oka K, Ohira S, Nikaido T et al (2003) Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. Am J Pathol 163:1437–1447

Ito S, Nakanishi H, Ikehara Y, Kato T, Kasai Y, Ito K et al (2001) Real-time observation of micrometastasis formation in the living mouse liver using a green fluorescent protein gene-tagged rat tongue carcinoma cell line. Int J Cancer 93:212–217

Iwatsuki M, Mimori K, Yokobori T, Ishi H, Beppu T, Nakamori S et al (2010) Epithelial-mesenchymal transition in cancer development and its clinical significance. Cancer Sci 101:293–299

Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9:239–252

Kalluri R, Zeisberg M (2006) Fibroblasts in cancer. Nat Rev Cancer 6:392–401

Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C et al (2003) A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 3:537–549

Kaplan RN, Riba RD, Zacharoulis S, Bramley AH, Vincent L, Costa C et al (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. Nature 438:820–827

Kaplan RN, Rafii S, Lyden D (2006) Preparing the "soil": the premetastatic niche. Cancer Res 66:11089–11093

Kienast Y, von Baumgarten L, Fuhrmann M, Klinkert WE, Goldbrunner R, Herms J et al (2010) Real-time imaging reveals the single steps of brain metastasis formation. Nat Med 16:116–122

Kim YJ, Borsig L, Varki NM, Varki A (1998) P-selectin deficiency attenuates tumor growth and metastasis. Proc Natl Acad Sci U S A 95:9325–9330

Krishnamachary B, Berg-Dixon S, Kelly B, Agani F, Feldser D, Ferreira G et al (2003) Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res 63:1138–1143

Krishnamachary B, Zagzag D, Nagasawa H, Rainey K, Okuyama H, Baek JH et al (2006) Hypoxia-inducible factor-1-dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. Cancer Res 66:2725–2731

Laderoute KR, Alarcon RM, Brody MD, Calaoagan JM, Chen EY, Knapp AM et al (2000) Opposing effects of hypoxia on expression of the angiogenic inhibitor thrombospondin 1 and the angiogenic inducer vascular endothelial growth factor. Clin Cancer Res 6:2941–2950

Le QT, Denko NC, Giaccia AJ (2004) Hypoxic gene expression and metastasis. Cancer Metastasis Rev 23:293–310

Lee TH, Avraham HK, Jiang S, Avraham S (2003) Vascular endothelial growth factor modulates the transendothelial migration of MDA-MB-231 breast cancer cells through regulation of brain microvascular endothelial cell permeability. J Biol Chem 278:5277–5284

Lee JM, Dedhar S, Kalluri R, Thompson EW (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. J Cell Biol 172:973–981

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309

Liotta LA, Stetler-Stevenson WG (1991) Tumor invasion and metastasis: an imbalance of positive and negative regulation. Cancer Res 51:5054s–5059s

Liu YL, Yu JM, Song XR, Wang XW, Xing LG, Gao BB (2006) Regulation of the chemokine receptor CXCR4 and metastasis by hypoxia-inducible factor in non small cell lung cancer cell lines. Cancer Biol Ther 5:1320–1326

Loayza-Puch F, Yoshida Y, Matsuzaki T, Takahashi C, Kitayama H, Noda M (2010) Hypoxia and RAS-signaling pathways converge on, and cooperatively downregulate, the RECK tumor-suppressor protein through microRNAs. Oncogene 29:2638–2648

Lu X, Yan CH, Yuan M, Wei Y, Hu G, Kang Y (2010) In vivo dynamics and distinct functions of hypoxia in primary tumor growth and organotropic metastasis of breast cancer. Cancer Res 70:3905–3914

Madsen CD, Sahai E (2010) Cancer dissemination–lessons from leukocytes. Dev Cell 19:13–26

Maegdefrau U, Amann T, Winklmeier A, Braig S, Schubert T, Weiss TS et al (2009) Bone morphogenetic protein 4 is induced in hepatocellular carcinoma by hypoxia and promotes tumor progression. J Pathol 218:520–529

Mannori G, Santoro D, Carter L, Corless C, Nelson RM, Bevilacqua MP (1997) Inhibition of colon carcinoma cell lung colony formation by a soluble form of E-selectin. Am J Pathol 151:233–243

Martens LK, Kirschner KM, Warnecke C, Scholz H (2007) Hypoxia-inducible factor-1 (HIF-1) is a transcriptional activator of the TrkB neurotrophin receptor gene. J Biol Chem 282:14379–14388

Martinez-Outschoorn UE, Trimmer C, Lin Z, Whitaker-Menezes D, Chiavarina B, Zhou J et al (2010) Autophagy in cancer associated fibroblasts promotes tumor cell survival: role of hypoxia, HIF1 induction and NFkappaB activation in the tumor stromal microenvironment. Cell Cycle 9:3515–3533

Moreno-Bueno G, Salvador F, Martin A, Floristan A, Cuevas EP, Santos V et al (2011) Lysyl oxidase-like 2 (LOXL2), a new regulator of cell polarity required for metastatic dissemination of basal-like breast carcinomas. EMBO Mol Med 3:528–544

Morris VL, Koop S, MacDonald IC, Schmidt EE, Grattan M, Percy D et al (1994) Mammary carcinoma cell lines of high and low metastatic potential differ not in extravasation but in subsequent migration and growth. Clin Exp Metastasis 12:357–367

Moserle L, Amadori A, Indraccolo S (2009) The angiogenic switch: implications in the regulation of tumor dormancy. Curr Mol Med 9:935–941

Mundy GR (2002) Metastasis to bone: causes, consequences and therapeutic opportunities. Nat Rev Cancer 2:584–593

Murdoch C (2000) CXCR4: chemokine receptor extraordinaire. Immunol Rev 177:175–184

Murdoch C, Giannoudis A, Lewis CE (2004) Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. Blood 104:2224–2234

Nash GF, Turner LF, Scully MF, Kakkar AK (2002) Platelets and cancer. Lancet Oncol 3:425–430

Naumov GN, Wilson SM, MacDonald IC, Schmidt EE, Morris VL, Groom AC et al (1999) Cellular expression of green fluorescent protein, coupled with high-resolution in vivo videomicroscopy, to monitor steps in tumor metastasis. J Cell Sci 112(Pt 12):1835–1842

Nguyen DX, Bos PD, Massague J (2009) Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer 9:274–284

Nicolson GL (1988) Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. Biochim Biophys Acta 948:175–224

Nicolson GL (1989) Metastatic tumor cell interactions with endothelium, basement membrane and tissue. Curr Opin Cell Biol 1:1009–1019

Nolan DJ, Ciarrocchi A, Mellick AS, Jaggi JS, Bambino K, Gupta S et al (2007) Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. Genes Dev 21:1546–1558

Nozawa K, Fujishiro M, Kawasaki M, Kaneko H, Iwabuchi K, Yanagida M et al (2009) Connective tissue growth factor promotes articular damage by increased osteoclastogenesis in patients with rheumatoid arthritis. Arthr Res Ther 11:R174

Olaso E, Salado C, Egilegor E, Gutierrez V, Santisteban A, Sancho-Bru P et al (2003) Proangiogenic role of tumor-activated hepatic stellate cells in experimental melanoma metastasis. Hepatology 37:674–685

Osusky KL, Hallahan DE, Fu A, Ye F, Shyr Y, Geng L (2004) The receptor tyrosine kinase inhibitor SU11248 impedes endothelial cell migration, tubule formation, and blood vessel formation in vivo, but has little effect on existing tumor vessels. Angiogenesis 7:225–233

Overgaard J, Horsman MR (1996) Modification of hypoxia-induced radioresistance in tumors by the use of oxygen and sensitizers. Semin Radiat Oncol 6:10–21

Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F et al (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 15:220–231

Paget S (1989) The distribution of secondary growths in cancer of the breast. 1889. Cancer Metastasis Rev 8:98–101

Pantel K, Alix-Panabieres C (2010) Circulating tumor cells in cancer patients: challenges and perspectives. Trends Mol Med 16:398–406

Pasqualini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A et al (2000) Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer Res 60:722–727

Peinado H, Del Carmen Iglesias-de la Cruz M, Olmeda D, Csiszar K, Fong KS, Vega S et al (2005) A molecular role for lysyl oxidase-like 2 enzyme in snail regulation and tumor progression. EMBO J 24:3446–3458

Peinado H, Moreno-Bueno G, Hardisson D, Perez-Gomez E, Santos V, Mendiola M et al (2008) Lysyl oxidase-like 2 as a new poor prognosis marker of squamous cell carcinomas. Cancer Res 68:4541–4550

Peinado H, Lavotshkin S, Lyden D (2011) The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. Semin Cancer Biol 21:139–146

Peng L, Ran YL, Hu H, Yu L, Liu Q, Zhou Z et al (2009) Secreted LOXL2 is a novel therapeutic target that promotes gastric cancer metastasis via the Src/FAK pathway. Carcinogenesis 30:1660–1669

Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM (2003) Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3:347–361

Petit I, Jin D, Rafii S (2007) The SDF-1-CXCR4 signaling pathway: a molecular hub modulating neo-angiogenesis. Trends Immunol 28:299–307

Pouyssegur J, Dayan F, Mazure NM (2006) Hypoxia signalling in cancer and approaches to enforce tumor regression. Nature 441:437–443

Psaila B, Kaplan RN, Port ER, Lyden D (2006) Priming the 'soil' for breast cancer metastasis: the pre-metastatic niche. Breast Dis 26:65–74

Qing G, Simon MC (2009) Hypoxia inducible factor-2alpha: a critical mediator of aggressive tumor phenotypes. Curr Opin Genet Dev 19:60–66

Rankin EB, Giaccia AJ (2008) The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ 15:678–685

Reynolds TY, Rockwell S, Glazer PM (1996) Genetic instability induced by the tumor microenvironment. Cancer Res 56:5754–5757

Rohwer N, Welzel M, Daskalow K, Pfander D, Wiedenmann B, Detjen K et al (2008) Hypoxia-inducible factor 1alpha mediates anoikis resistance via suppression of alpha5 integrin. Cancer Res 68:10113–10120

Ruan K, Song G, Ouyang G (2009) Role of hypoxia in the hallmarks of human cancer. J Cell Biochem 107:1053–1062

Ruoslahti E (1994) Fibronectin and its alpha 5 beta 1 integrin receptor in malignancy. Invasion Metastasis 14:87–97

Sahai E (2007) Illuminating the metastatic process. Nat Rev Cancer 7:737–749
Salvesen HB, Akslen LA (1999) Significance of tumor-associated macrophages, vascular endothelial growth factor and thrombospondin-1 expression for tumor angiogenesis and prognosis in endometrial carcinomas. Int J Cancer 84:538–543
Schietke R, Warnecke C, Wacker I, Schodel J, Mole DR, Campean V et al (2010) The lysyl oxidases LOX and LOXL2 are necessary and sufficient to repress E-cadherin in hypoxia: insights into cellular transformation processes mediated by HIF-1. J Biol Chem 285:6658–6669
Schluter K, Gassmann P, Enns A, Korb T, Hemping-Bovenkerk A, Holzen J et al (2006) Organ-specific metastatic tumor cell adhesion and extravasation of colon carcinoma cells with different metastatic potential. Am J Pathol 169:1064–1073
Scotton CJ, Wilson JL, Milliken D, Stamp G, Balkwill FR (2001) Epithelial cancer cell migration: a role for chemokine receptors? Cancer Res 61:4961–4965
Scotton CJ, Wilson JL, Scott K, Stamp G, Wilbanks GD, Fricker S et al (2002) Multiple actions of the chemokine CXCL12 on epithelial tumor cells in human ovarian cancer. Cancer Res 62:5930–5938
Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3:721–732
Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985
Shyu KG, Hsu FL, Wang MJ, Wang BW, Lin S (2007) Hypoxia-inducible factor 1alpha regulates lung adenocarcinoma cell invasion. Exp Cell Res 313:1181–1191
Staller P, Sulitkova J, Lisztwan J, Moch H, Oakeley EJ, Krek W (2003) Chemokine receptor CXCR4 downregulated by von Hippel-Lindau tumor suppressor pVHL. Nature 425:307–311
Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516
Sullivan R, Graham CH (2007) Hypoxia-driven selection of the metastatic phenotype. Cancer Metastasis Rev 26:319–331
Teicher BA, Fricker SP (2010) CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res: Off J Am Assoc Cancer Res 16:2927–2931
Terranova VP, Williams JE, Liotta LA, Martin GR (1984) Modulation of the metastatic activity of melanoma cells by laminin and fibronectin. Science 226:982–985
Thiery JP, Acloque H, Huang RY, Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139:871–890
Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J (2012) Spatiotemporal regulation of epithelial-mesenchymal transition is essential for squamous cell carcinoma metastasis. Cancer Cell 22:725–736
Vergis R, Corbishley CM, Norman AR, Bartlett J, Jhavar S, Borre M et al (2008) Intrinsic markers of tumor hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. Lancet Oncol 9:342–351
Wang H, Fu W, Im JH, Zhou Z, Santoro SA, Iyer V et al (2004) Tumor cell alpha3beta1 integrin and vascular laminin-5 mediate pulmonary arrest and metastasis. J Cell Biol 164:935–941
Weiss L, Orr FW, Honn KV (1988) Interactions of cancer cells with the microvasculature during metastasis. FASEB J: Off Publ Fed Am Soc Exp Biol 2:12–21
Welti JC, Powles T, Foo S, Gourlaouen M, Preece N, Foster J et al (2012) Contrasting effects of sunitinib within in vivo models of metastasis. Angiogenesis 15:623–641
Whelan KA, Caldwell SA, Shahriari KS, Jackson SR, Franchetti LD, Johannes GJ et al (2010) Hypoxia suppression of Bim and Bmf blocks anoikis and luminal clearing during mammary morphogenesis. Mol Biol Cell 21:3829–3837
Wong CW, Song C, Grimes MM, Fu W, Dewhirst MW, Muschel RJ et al (2002) Intravascular location of breast cancer cells after spontaneous metastasis to the lung. Am J Pathol 161:749–753
Wong CC, Gilkes DM, Zhang H, Chen J, Wei H, Chaturvedi P et al (2011) Hypoxia-inducible factor 1 is a master regulator of breast cancer metastatic niche formation. Proc Natl Acad Sci U S A 108:16369–16374

Wong CC, Zhang H, Gilkes DM, Chen J, Wei H, Chaturvedi P et al (2012) Inhibitors of hypoxia-inducible factor 1 block breast cancer metastatic niche formation and lung metastasis. J Mol Med 90:803–815

Wood S Jr (1958) Pathogenesis of metastasis formation observed in vivo in the rabbit ear chamber. AMA Arch Pathol 66:550–568

Wyckoff JB, Wang Y, Lin EY, Li JF, Goswami S, Stanley ER et al (2007) Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. Cancer Res 67:2649–2656

Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C et al (2004) Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell 117:927–939

Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, Liu CJ et al (2008) Direct regulation of TWIST by HIF-1alpha promotes metastasis. Nat Cell Biol 10:295–305

Yilmaz M, Christofori G (2010) Mechanisms of motility in metastasizing cells. Mol Cancer Res 8:629–642

Young SD, Marshall RS, Hill RP (1988) Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. Proc Natl Acad Sci U S A 85:9533–9537

Zhan M, Zhao H, Han ZC (2004) Signalling mechanisms of anoikis. Histol Histopathol 19:973–983

Zhang L, Smith KM, Chong AL, Stempak D, Yeger H, Marrano P et al (2009) In vivo antitumor and antimetastatic activity of sunitinib in preclinical neuroblastoma mouse model. Neoplasia 11:426–435

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D et al (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59:5830–5835

# Chapter 4
# Escape Mechanisms from Antiangiogenic Therapy: An Immune Cell's Perspective

**Lee Rivera, Melissa Pandika, and Gabriele Bergers**

**Abstract** Neovascularization, the formation of new blood vessels, has become a well-established hallmark of cancer. Its functional importance for the manifestation and progression of tumors has been validated further by the beneficial therapeutic effects of angiogenesis inhibitors, most notably those targeting vascular endothelial growth factor signaling pathways. However, with the transient and short-lived nature of patient response, it has become evident that tumors have the ability to adapt to the pressures of vascular growth restriction. Observations made both in the clinic and at the bench suggest the existence of several escape mechanisms that either reestablish neovascularization in tumors or change tumor behavior to enable propagation and progression without obligate neovascularization. Some of these bypass mechanisms are regulated by low oxygen conditions (hypoxia) caused by therapy-induced vessel regression. Induction of hypoxia and hypoxia-inducible factors regulate a wide range of tumor-promoting pathways, including those of neovascularization, that can

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upregulate additional proangiogenic factors and drive the recruitment of various bone marrow–derived cells that have the capacity to express proangiogenic factors or directly contribute to neovasculature.

**Keywords** Cancer • Antiangiogenic therapy • Resistance • Innate immune cells • Macrophages • Myeloid-derived suppressor cells (MDSCs) • Neovascularization

## 4.1 Introduction

Pathologists examining the histology of human cancers first suggested the significance of blood vessel formation in tumor growth more than a century ago when they observed that tumor growth could be accompanied by increased vascularity (Ferrara 2002). Nearly three decades later, the development of a transparent chamber through which microscopic observations of living tissues could be made pushed the field further. In 1945, a group at the National Cancer Institute led by Glenn Algire used transparent chambers in mice, noting that transplanted tumors, but not normal tissues, caused a dramatic increase in vascularity and that vascular growth preceded a phase of rapid growth in all tumor types examined. These findings led them to hypothesize that blood vessel formation is a crucial step in tumor progression. Then, in 1971, Judah Folkman described the isolation of a soluble tumor angiogenesis factor from human and animal tumors that stimulated endothelial cell proliferation and capillary formation in vivo (Folkman et al. 1971).

Since then, researchers have identified a variety of proangiogenic molecules that are produced or released in the tumor microenvironment. In 1983, Beth Israel Hospital investigators led by Donald Senger and Harold Dvorak, identified and purified a protein they named vascular permeability factor because of its ability to induce vascular leakage (Senger et al. 1983). In 1989, Napoleone Ferrara and others at Genentech isolated and later cloned a protein they named vascular endothelial growth factor (VEGF) because of its mitogenicity toward only endothelial cells (Leung et al. 1989). Further tests revealed vascular permeability factor and VEGF were the same protein. VEGF is one of the most common proangiogenic factors, as evidenced by its upregulation in nearly every tumor type, and one of the most fundamental: it is implicated in almost all processes of vessel formation. VEGF endorses endothelial cell proliferation, migration, and survival (Ferrara 2004), increases vessel permeability (Bates et al. 2002; Senger et al. 1983), and causes vasodilation (Ku et al. 1993). Moreover, VEGF inhibits vessel maturation through its interaction with platelet-derived growth factor (PDGF) subunit B, which induces the formation of a VEGF receptor (VEGFR) 2–PDGF receptor β complex (Greenberg et al. 2008), thereby disrupting PDGF receptor β signaling in pericytes. The pleiotropic effects of VEGF on the vasculature have made VEGF and its receptors the most common targets of antiangiogenic therapy. Congruent with this, drugs targeting the VEGF signaling pathway have shown efficacy in numerous animal tumor models, in which they increase intratumoral endothelial cell apoptosis and vessel pruning. Such drugs also decrease vascular permeability and endorse

increased pericyte coverage of tumor blood vessels, leading to a reduction in both hemorrhage formation and edema and thus increasing the efficiency of blood flow and chemotherapeutic drug delivery (Jain 2005b).

These findings supported the hopeful belief that targeting VEGF would have tremendous efficacy in treating human cancer. Indeed, clinical studies of VEGF pathway inhibitors demonstrated improvements in relapse-free survival in patients with metastatic colorectal cancer, advanced non-small-cell lung cancer, renal cell carcinoma, hepatocellular carcinoma, gastrointestinal stromal tumors, and glioblastoma (Ebos and Kerbel 2011). These results led to US Food and Drug Administration approval of bevacizumab (Avastin; Genentech/Roche), a ligand-trapping monoclonal antibody, as well as sorafenib (Nexavar; Bayer) and sunitinib (Sutent; Pfizer), kinase inhibitors that target the VEGFR tyrosine kinases – primarily VEGFR2 – as well as other receptor tyrosine kinases. Since March 2008, bevacizumab has been an approved treatment as an adjunct to chemotherapy for late-stage colon cancer and non-small-cell lung cancer and as a single regimen for recurrent glioblastoma. Sorafenib and sunitinib both have been approved for treating renal carcinoma, a highly vascularized (and angiogenic) tumor type. In addition, sunitinib has been approved for treating gastrointestinal stromal tumors and pancreatic endocrine tumors, and sorafenib has been approved to treat hepatocellular carcinomas (Ferrara et al. 2005; Folkman 2007; Jain 2005a; Milan and Yeo 2012; Smith et al. 2004). The favorable effects of these inhibitors, ranging from improvement in quality of life to survival benefits in some patients, initially generated much enthusiasm within the scientific community, which hoped that inhibiting angiogenesis might potentially starve tumors to death. However, antiangiogenic therapy has ultimately been found to have rather transient beneficial effects, which improved progression-free survival and quality of life but only modestly influenced overall survival in patients with various cancer. The truth, however, is that all cancer drugs developed to date exhibit only temporary efficacy followed inevitably by tumor resistance and regrowth.

From a positive perspective, although drugs targeting the VEGF pathway did not fulfill initial expectations, their development has been a seminal first step in targeting tumor vessels to restrict tumor growth, providing pivotal information about how tumors react to vessel growth restriction. What has emerged so far from these studies is the critical observation that, unlike tumor cells, which become resistant to targeted therapy by acquiring mutations in the gene encoding a drug target or by adjusting drug uptake and efflux, endothelial cells show sustained inhibition of VEGFR signaling (Gorre and Sawyers 2002; O'Connor et al. 2007). Resistance stems from a tumor's ability to induce distinct, alternative pathways to bypass and overcome VEGF-dependent restrictions on vessel growth. The identification of evasive resistance can also be interpreted as good news because it opens new avenues for the assessment of combinatorial treatment modalities aimed at targets within these evasive pathways. Therefore, substantial effort has been given to studying the underlying mechanisms that contribute to tumor relapse during the course of anti-VEGF therapy; testing the blockade and efficacy of alternate vascular pathways (Kuhnert et al. 2011); and identifying new approaches to targeting the communication between endothelial cells and tumor cells in an effort to restrict tumor growth without destroying the tumor vasculature (Butler et al. 2010).

## 4.2 Patterns of Resistance to Antiangiogenic Therapy

Current experimental evidence suggests that there are two major relapse patterns: tumors either reinstate growth by neovascularization or alter their growth behavior without obligate neovascularization (Fig. 4.1). Adaptive mechanisms of the latter resistance pattern include activation and enhancement of invasion and potentially metastasis (it has been shown in only some preclinical tumor models), as well as increased pericyte coverage of the tumor vasculature to support its integrity and attenuate the necessity of VEGF-mediated survival signaling (Bergers and Song 2005; Ebos et al. 2009; Jain 2005b; Paez-Ribes et al. 2009). Reneovascularization can theoretically be activated by any of the mechanisms that induce physiological and pathological angiogenesis (Potente et al. 2011). For example, signals that initiate blood vessel sprouting can be conveyed by growth factors other than VEGF (Fig. 4.2a), including fibroblast growth factor 2 (Hanahan and Folkman 1996). Indeed, an increase in the level of alternative proangiogenic factors, such as *Fgf-2*, *Sdf-1*, *Vegf-C*, and *PlGF*, during antiangiogenic therapy has been described in various preclinical mouse tumor models and are upregulated in part by therapy-induced hypoxia (Casanovas et al. 2005; Fischer et al. 2007). In addition to driving angiogenic sprouting, alternative proangiogenic factors may also facilitate the

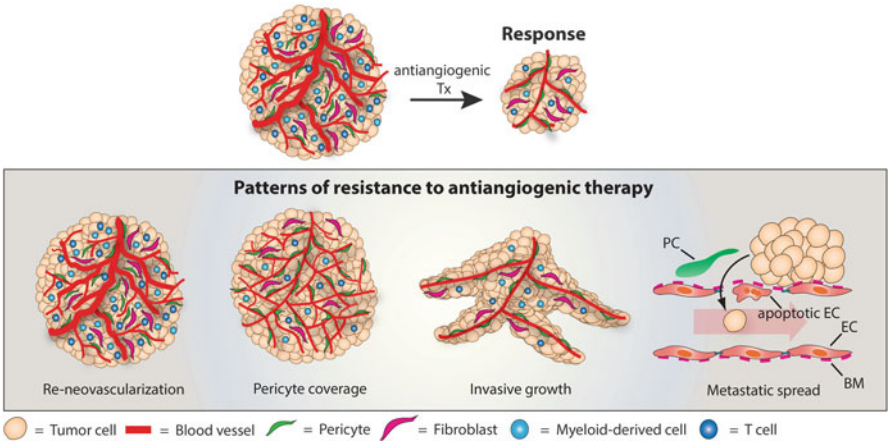


**Fig. 4.1** Adaptive resistance to antiangiogenic therapy (*Tx*). Tumor growth is initially blocked by antiangiogenic therapy, but tumors eventually adapt to this pressure and thrive. This can occur by reinstating the angiogenic cascade to induce reneovascularization, either by expressing alternate angiogenic factors directly or by recruiting angiogenic myeloid-derived cells. As an alternative, pericyte coverage protects a subset of blood vessels from the effects of therapy on endothelial cell survival. Tumors exploit these remaining vessels to survive and grow in the face of therapy. Tumors may also co-opt vessels that resist therapy, thus exhibiting an invasive phenotype. These vessels are grossly similar in architecture to normal vasculature. Combined targeting of endothelial cells and pericytes can critically compromise the residual vasculature such that it is characterized by relatively few (but detached) pericytes as well as disrupted basement membranes, conditions that facilitate tumor escape from the primary location, a prerequisite for metastatic spread

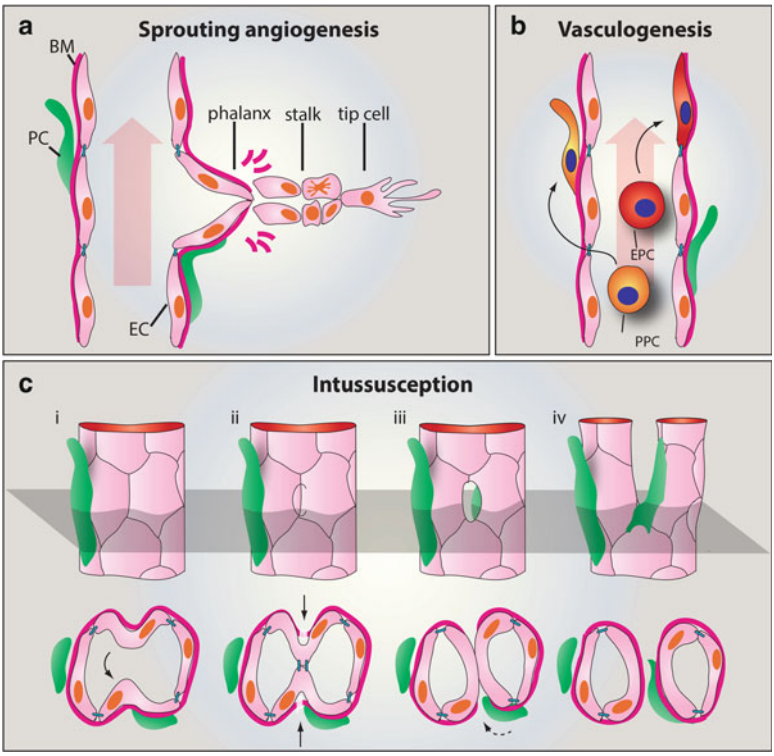


**Fig. 4.2** Mechanisms of reneovascularization. Several distinct mechanisms contribute to the generation of new blood vessels within tumors. (**a**) New vessel formation via sprouting angiogenesis involves the activation and mobilization of existing capillary endothelial cells and pericytes. Angiogenic stimuli induce secretion of endothelial cell (*EC*)–derived proteases that degrade the surrounding basement membrane, after which tip cells extend filopodia into the neighboring parenchyme. Sprouts consist of three functionally distinct parts: the phalanx, which joins the newly formed vessel to the existing vessel; the stalk, which contains proliferating endothelial cells that follow the tip cell; and the tip cell itself, which migrates in response to angiogenic cues. (**b**) Reneovascularization also occurs via the recruitment of bone marrow–derived vascular progenitor cells, including both endothelial progenitor cells (*EPC*) and pericyte progenitor cells (*PPC*). These cells become incorporated into an existing vessel, thus facilitating vessel formation. (**c**) New vessels can also be formed via the division of an existing vessel through intussusception. This process occurs through a series of steps involving (*i*) luminal indentation of opposing endothelial cells; (*ii*) formation of a transluminal pillar by contact with these endothelial cells (perforation of the endothelial plasma membranes and proteolysis of the basement membrane (*BM*) occurs here, as indicated by the *arrows*); (*iii*) migration of mural cells (indicated by the *dotted arrow*) into the area between the newly formed vessels; and (*iv*) deposition of basement membrane proteins by mural cells. The *broad arrows* (**a** and **b**) represent luminal blood flow. *PC* pericytes

recruitment of circulating endothelial cells (Gao et al. 2008; Lyden et al. 2001; Peters et al. 2005) and pericyte progenitor cells (Song et al. 2005) to the tumor endothelium, where they are incorporated into existing vessels (Fig. 4.2b). Such activity has been shown for stromal cell-derived factor-1, whose signaling through

its cognate receptor C-X-C chemokine receptor type-4 (CXCR4) on circulating vascular progenitor cells promoted the recruitment of these cells to tumors in a preclinical model of glioblastoma (Du et al. 2008a).

Although most of the current evidence implicates vascular sprouting as the primary mechanism of new blood vessel formation in relapsing tumors, splitting of blood vessels, also known as intussusception, has been proposed as another alternate mechanism for maintaining functional properties of tumor vasculature during VEGF ablation and has been demonstrated in various tumors (Hlushchuk et al. 2011) (Fig. 4.2c). The induction of such a mechanism seems to be conceivable because intussusception allows for a vast increase in the number of vessels without an increase in the number of endothelial cells (Hlushchuk et al. 2011). To date its activation has been described only during antiangiogenic treatment with the receptor tyrosine kinase inhibitor vatalanib (PTK/ZK) in mouse xenograft tumor models (Hillen and Griffioen 2007; Hlushchuk et al. 2008).

Of note is the capacity of tumor and tumor stem cells to contribute to the tumor vasculature in one of two ways: (1) by posing as a functional vasculature by forming channels through which blood can flow, known as vascular mimicry (Maniotis et al. 1999; Sharma et al. 2002; Shirakawa et al. 2002; Sun et al. 2004); or (2) by differentiating into endothelial cells and incorporating into the existing endothelium (Ricci-Vitiani et al. 2010; Soda et al. 2011; Wang et al. 2010). One can imagine that, in response to antiangiogenic stress, tumor cells directly facilitate blood flow as an alternate means of supplying oxygen and nutrients to the bulk of the tumor. In support of this notion, antiangiogenic therapy with the VEGFR2 small-molecule inhibitor AG28262 increased the number of glioblastoma-derived endothelial cells associated with the tumor vasculature in a glioblastoma mouse model (Soda et al. 2011). Although there is still controversy about the ability of tumor stem cells to differentiate into functional endothelial cells, these results suggest that tumor cells could potentially adapt to antiangiogenic therapy to functionally reconstitute the depleted vasculature via adaptive reneovascularization.

By causing vessel pruning and reduction, antiangiogenic therapy should generate or enhance intratumoral hypoxia; the relapse mechanisms responsible for tumor relapse have been thought to be greatly attributed to induction of hypoxia and hypoxia-inducible factors (HIFs). Indeed, results from recent studies suggest that hypoxia can promote reneovascularization as well as adaptive tumor growth without neovascularization. For example, increased metastasis and pro-invasive growth during antiangiogenic therapy in preclinical tumor models have been correlated with increased hypoxia (Cooke et al. 2012; Paez-Ribes et al. 2009). However, hypoxia is unlikely to be the sole mechanism accounting for not only metastasis but also invasive growth because invading tumor cells originate from the tumor rim, which, unlike the tumor core, does not face severe therapy-induced hypoxia. In fact, a recent study revealed a hypoxia-independent mechanism by which VEGF blockade enhanced c-Met phosphorylation and subsequent invasion in murine and human glioblastomas (Lu et al. 2012). Glioblastoma cells moved predominantly along blood vessels away from the main tumor mass and deep into the brain parenchyma when tumor cells were unable to initiate VEGF-dependent angiogenesis through

either genetic ablation of key angiogenic factors (HIF-1α, VEGF, matrix metalloproteinase [MMP]-9, MMP-2) (Blouw et al. 2003; Du et al. 2008a, b) or pharmacologic targeting of VEGF signaling (Ebos et al. 2009; Paez-Ribes et al. 2009). These studies led to an unexpected link between hepatocyte growth factor and VEGF signaling in which VEGF directly and negatively modulated the activity of c-Met through the interaction of a novel c-Met–VEGFR2 heterocomplex on tumor cells (Lu et al. 2012). In this scenario, VEGF-dependent regulation of c-Met activity occurred independent of hypoxia, as total c-Met expression levels did not change despite differences in c-Met phosphorylation (Lu et al. 2012).

On the other hand, there is extensive evidence that hypoxia-induced activation of HIF proteins and their targets promotes the upregulation of alternative proangiogenic factors, as described earlier, and facilitates the recruitment of specific proangiogenic innate immune cell populations that endorse VEGF-independent neovascularization. This review specifically focuses on discussion of innate immune cell populations, the molecular mechanisms driving their recruitment to tumors, and the mechanisms by which they confer resistance to antiangiogenic therapy. Myeloid-derived immune cells represent a widely heterogeneous cell population that is chemotactically recruited to the primary tumor. Because these cells facilitate the angiogenic switch in a variety of solid tumors, understanding their contribution to tumor resistance to antiangiogenic therapy is highly relevant (Bergers and Hanahan 2008; Zumsteg and Christofori 2009). Indeed, recent studies have identified significant roles for numerous innate immune cell subtypes in promoting resistance to therapy.

## 4.3 Innate Immune Cells Facilitate Reneovascularization and Resistance to Antiangiogenic Therapy

Macrophages are tissue-associated phagocytes that result from monocyte recruitment and differentiation. These cells are functionally defined as members of one of two general subtypes: either proinflammatory, classically activated M1 macrophages that arise after interferon-γ and lipopolysaccharide stimulation, or, alternatively activated, angiogenic M2 macrophages that arise in response to interleukin (IL)-4, IL-13, or IL-10 signaling (Sica et al. 2008). The accepted view that macrophages contribute to tumor angiogenesis is supported by the apparent correlation between macrophage infiltration and tumor angiogenesis in clinical specimens (Leek et al. 1996; Li et al. 2002; Takanami et al. 1999). Moreover, there exist several experimental studies of various murine models demonstrating the relevance of macrophages to tumor angiogenesis (Lin et al. 2001, 2006; Zeisberger et al. 2006).

Emerging from such studies is the proposition that, along with promoting tumor angiogenesis during the natural progression of disease, macrophages possess the capacity to protect tumors from the deleterious effects of antiangiogenic therapy. Using a clodronate-mediated depletion strategy, macrophages were shown to reduce the sensitivity of subcutaneously growing CT26 colon carcinoma cells to the

anti-VEGFR2 antibody DC101, as well as to a soluble form of VEGFR2 (Fischer et al. 2007). Furthermore, this resistance was conferred at least in part by the proangiogenic function of these cells: macrophage depletion enhanced the negative effect of DC101 on blood vessel density. Although it lowered blood vessel density, DC101 did not reduce the number of intratumoral F4/80$^+$ macrophages, suggesting that tumor-associated macrophages become more angiogenic during the course of therapy. Congruent to this, DC101 increased tumor expression of *Sdf-1*, *Fgf-1*, *Fgf-2*, *VEGF*, *Plgf*, *Mmp9*, and *Cxcl1*.

It is interesting that the same study found that targeting the VEGFR1 ligand placental growth factor (PlGF) recapitulated the effect of macrophage depletion in both CT26 and Panc02 pancreatic tumors. In fact, tumors receiving such therapy failed to show increases in angiogenic growth factor expression and displayed blunted macrophage infiltration. These results suggest that PlGF secretion in the tumor microenvironment acts by both recruiting monocytic cells and promoting their differentiation toward an angiogenic subtype. Autocrine PlGF signaling in macrophages was recently discovered to promote an M2 phenotype, and attenuation of PlGF expression by histidine-rich glycoprotein (HRG) shifted the expression profile of tumor-associated macrophages toward a proinflammatory M1 phenotype (MRC1, ARG1, CCL2, IL-10 low, CXCL9 high) (Rolny et al. 2011). Skewing macrophage polarization from M2 to M1 by HRG correlated with reduced growth of T241 fibrosarcoma tumors. Together, these studies suggest that enhanced M2 macrophage polarization can allow for persistent tumor growth in the face of VEGF blockade. However, whether other molecules that evoke an M2 phenotype in macrophages can also promote resistance to therapy, or if such activity is specific to PlGF, still remains to be determined. These studies suggest the important idea that preventing or reversing M2 polarization may extend the efficacy of antiangiogenic therapy.

Tie2-expressing monocytes (TEMs) seem to represent a subclass of macrophages. These circulating monocytes typically exhibit a perivascular distribution within the tumor stroma (De Palma et al. 2007). TEMs are also found in a vast array of malignancies, and their recruitment to primary tumors is believed to be mediated in part by the angiopoietin 2 (Ang2)/Tie2 axis (Venneri et al. 2007). Their relevance to tumor angiogenesis was shown using mice in which circulating TEMs could be selectively ablated by virtue of Tie2 promoter-driven thymidine kinase expression (De Palma et al. 2005). TEMs were found to express higher levels of angiogenic factors compared with other tumor-associated myeloid cells and were necessary to initiate angiogenesis and subsequent growth of implanted mammary and brain tumors.

A recent study demonstrated that antibody-mediated neutralization of the Tie2 ligand, Ang2, in late-stage (13.5-week-old) Rip1Tag2 mice reduced the number of MCR1$^+$ Tie2$^+$ TEMs associated with tumor vasculature and significantly lowered vascular density within end-stage tumors (Mazzieri et al. 2011). This finding is particularly noteworthy because previous reports found that VEGF blockade in the same model evoked a transient response phase characterized by tumor hypoxia and reduced vessel density, followed by a relapse phase marked by considerable

neovascularization and heightened expression of the proangiogenic growth factors *Fgf1*, *Fgf2*, *Fgf7*, *Fgf8*, *Efna1*, *Efna2*, and *Angpt2* (Bergers et al. 1999; Casanovas et al. 2005). Together, these results suggest that VEGF blockade induces the expression and secretion of Ang2, which activates Tie2-mediated, VEGF-independent angiogenic activity of TEMs in a nonredundant fashion.

$Tie2^+$ $CXCR4^+$ TEMs were recently found to play a protective role in spontaneous MMTV-PyMT mammary tumors treated with the stilbenoid plant phenol combrestatin A4 phosphate (CA4P), a potent vascular-disrupting agent (Welford et al. 2011). In contrast to VEGF inhibitors, CA4P is cytotoxic because of its high-affinity interaction with the β-subunit of tubulin, which inhibits tubulin polymerization (Reddy et al. 2008). Compared with a control, CA4P treatment generated a greater influx of TEMs, as well as elevated levels of the CXCR4-ligand CXCL12. On the other hand, combining CA4P with the CXCR4 inhibitor AMD-3100 effectively blocked TEM accumulation, enhanced CA4P-induced tumor necrosis, and further reduced tumor burden. The protective function of TEMs was similarly demonstrated in an implant model of HER-2/neu mammary carcinoma. These studies suggest that TEMs are highly attuned to the state of the tumor vasculature. As a mechanism of adaptive resistance, they rapidly mobilize to reinstate growth of the tumor vasculature when it becomes compromised either in response to VEGF blockade (via Ang2/Tie2) or vascular disruption (via CXCL12/CXCR4).

Monocyte recruitment to tumors is mediated to a great extent by colony-stimulating factor (CSF)-1. Deletion of the *Csf1* gene in mice is sufficient to block $F4/80^+$ macrophage accumulation in MMTV-PyMT mammary tumors (Lin et al. 2006). A recent study evaluating the effects of the CSF-1 receptor small-molecule inhibitor GW2580 found that DC101 treatment of subcutaneously implanted Lewis lung carcinoma resulted in an increase in tumor-associated $F4/80^+$ macrophages (Priceman et al. 2010). This effect was reversed in tumors treated with a combination of DC101 and GW2580. Furthermore, DC101 and GW2580 synergized to more effectively control tumor growth than either treatment alone. These results support the notion that VEGF blockade activates adaptive macrophages that confer resistance in the tumor microenvironment. It is interesting that inhibition of the CSF-1 receptor had similar effects on another angiogenic class of innate immune cells characterized by the expression of CD11b and Gr1.

$Gr1^+$ $CD11b^+$ cells encompass a heterogeneous population neutrophils, dendritic cells, and myeloid-derived immunosuppressive cells (MDSCs) (Chung et al. 2010; Murdoch et al. 2008). MDSCs can be divided into at least two subtypes: monocytic MDSCs, which are $Ly6G^{low}Ly6C^{high}$ ($Gr1^{low}$), and immature neutrophil-like MDSCs, which are $Ly6G^{high}Ly6C^{low}$ ($Gr1^{high}$) (Youn et al. 2008). These cells differentially suppress immune cell function, a feature that further supports their classification into distinct subclasses (Movahedi et al. 2008). This immunosuppressive role relies primarily on their ability to suppress human $CD3^+$ and mouse $CD4^+$ or $CD8^+$ T cells (Ostrand-Rosenberg and Sinha 2009). MDSCs have been found in human patients with cancer and correlate with advanced disease (Diaz-Montero et al. 2009; Filipazzi et al. 2007; Mirza et al. 2006; Pandit et al. 2000; Srivastava et al. 2008). However, humans lack Gr1, and MDSCs are defined as

$CD11b^+$ $CD33^+$ $CD34^+$ $CD14^-HLA^-DR^-$. Besides suppressing immune cell function, tumor-associated MDSCs are highly angiogenic and have been shown to promote blood vessel growth in tumors experimental models (Kujawski et al. 2008; Pan et al. 2008; Schmid et al. 2011; Yang et al. 2004, 2008).

In a series of experiments, $Gr1^+$ $CD11b^+$ myeloid cells were identified as the predominant bone marrow–derived cell type that conferred resistance to EL4 lymphoma and Lewis lung carcinoma implants refractory to treatment with the anti-VEGF monoclonal antibody G6.23 (Shojaei et al. 2007a). First, refractory tumors were shown to recruit $Gr1^+$ $CD11b^+$ cells from the bone marrow in the absence or presence of VEGF blockade. This intriguing research showed that the bone marrow of refractory tumor-bearing mice also exhibited a rise in the number of $CD11b^+$ $Gr1^+$ cells, suggesting that certain tumors may specifically induce $CD11b^+$ $Gr1^+$ cell expansion and recruitment. $Gr1^+$ $CD11b^+$ cells then were isolated from either the bone marrow or primary tumors of refractory tumor-bearing mice and, based on admix studies, were found to be sufficient to attenuate the effect of VEGF blockade on G6.23-sensitive B16F1 melanoma growth. Finally, blockade of $Gr1^+$ cell recruitment in refractory tumors using an anti-Gr1 antibody sustained response to anti-VEGF therapy to a certain extent (Shojaei et al. 2007a). The tumor-protective capacity of $Gr1^+$ $CD11b^+$ cells stemmed in part from their proangiogenic nature, as demonstrated by the reduction of intratumoral vessel density produced by anti-Gr1 therapy. Profiling of refractory tumor-derived $Gr1^+$ $CD11b^+$ cells revealed elevated expression of a variety of genes involved in angiogenesis, including *Fgf13*, *Hgf*, and *Angptl6*. These cells also were immunosuppressive, as shown by decreased expression of *CD83*, *CD48*, *Crea7*, and *Dectin-1*. A complementary study revealed that refractory EL4 and Lewis lung carcinoma cells secrete granulocyte CSF, which activates expression of the angiogenic factor Bv8 in $Gr1^+$ $CD11b^+$ cells (Shojaei et al. 2009). Bv8 then elicits tumor angiogenesis, allowing for tumor growth, even during VEGF blockade (Shojaei et al. 2007b). In another study, the number of circulating $Gr1^+$ $CD11b^+$ cells increased in treated spontaneous Rip1Tag2 insulinomas (Shojaei et al. 2008). Since these tumors are sensitive to VEGF blockade, this finding suggests an additional role for $Gr1^+$ $CD11b^+$ cells in adaptive responses (Casanovas et al. 2005; Song et al. 2005). Although future studies are needed to delineate which of the various $Gr1^+$ $CD11b^+$ cell subtypes are relevant to tumor resistance, the current body of experimental evidence suggests the existence of $Gr1^+$ $CD11b^+$ cell effector molecules and recruitment factors that likely drive resistance to antiangiogenic therapy and that can be targeted.

Although there is emerging evidence that tumors can inherently produce myeloid cell–recruiting factors that confer intrinsic resistance to antiangiogenic therapy, the molecular network(s) that directly respond to therapy and promote myeloid cell–mediated adaptive growth have not yet been completely described. Such pathways are likely activated by hypoxia. HIFs mediate the transcriptional responses elicited by hypoxia in cells, including the expression of *VEGF*, *PlGF*, *Bv8*, and *Ang2* (Forsythe et al. 1996; Keith et al. 2011; Kelly et al. 2003; LeCouter et al. 2001; Simon et al. 2008). In an orthotopic model of glioblastoma multiforme, transformed primary astrocytes develop into tumors characterized by a hypoxic response

(Blouw et al. 2003). Deletion of *Hif1α* in these cells resulted in reduced vascular remodeling, corroborating the idea that *Hif1α* mediates activation of tumor-associated vasculature by hypoxia. In a subsequent study using the same model, tumors were shown to recruit several myeloid-derived cell types via stromal cell-derived factor-1, including $CD11b^+$, $VEGFR1^+$, $Tie2^+$, and $F4/80^+$ subsets (Du et al. 2008a). Deletion of tumor *Hif1α* resulted in a striking reduction in the number of all recruited myeloid cells, which correlated with a lack of tumor neovascularization. Furthermore, blood vessel formation in *Hif1α*-expressing tumors was found to rely, to some degree, on myeloid-derived MMP-9, thus illustrating that *Hif1α*-mobilized myeloid cells can indeed evoke angiogenesis. *Hif1α* can therefore presumably mediate myeloid cell–dependent adaptive responses to anti-VEGF therapy.

The studies described in this chapter underscore the significance of the expansion, recruitment, and activation of myeloid cells in tumor recurrence during anti-VEGF therapy and tumor refractoriness. One can envision that such cells are attracted to and become associated with specific cancers, providing them with distinct yet overlapping repertoires of molecules that can foster blood vessel growth in the face of VEGF blockade. In a similar way, one might imagine that the overall result of hypoxia-induced changes in gene expression depends on context, with specific cancers adapting to VEGF blockade by recruiting specific subtypes of myeloid cells. This possibility may explain the transitory nature of the therapeutic response exhibited by most tumors (Bergers and Hanahan 2008). These studies also highlight the therapeutic potential of myeloid cell inhibition in several cancers, including those of the breast, brain, pancreas, and colon, and strongly reiterate the contribution of VEGF-independent pathways to mechanisms of resistance.

## 4.4 Conclusion

Although antiangiogenic therapy has been demonstrated to provide transient control of tumor growth, relapse associated with reneovascularization, in which the tumor sidesteps VEGF inhibition by activating alternate angiogenic pathways, still upholds the original hypothesis put forward by Folkman et al. (1971) of the necessity of angiogenesis for tumor progression. We can hypothetically target these alternative pathways to reinstate the inhibitory effects of VEGF blockade on the tumor endothelium. The recruitment and activation of proangiogenic myeloid cells in response to therapy likely represents a paradigm consisting of multiple targetable nodes (i.e., recruitment, expansion, polarization, expression of angiogenic factors) that collectively drive resistance (Fig. 4.3), thereby creating a roadmap for developing agents that increase the efficacy of anti-VEGF therapy. Further elucidation of how these cells interact with each other and respond to therapeutic pressures may reveal novel targetable factors and treatment modalities that are highly specific and capable of sustaining response to anti-VEGF therapy while minimizing the occurrence of side effects associated with targeting entire populations of myeloid cells. Therefore, in the road ahead there is promise for anti-VEGF therapy as a cancer treatment.

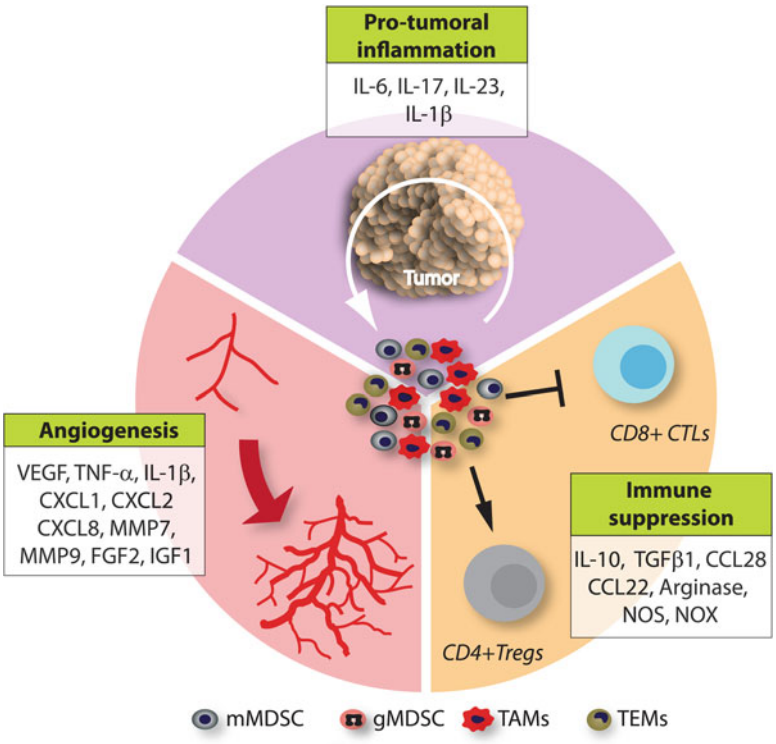


**Fig. 4.3** Innate immune cells promote tumor growth by inducing angiogenesis, suppressing anti-cancer immunity, and contributing to protumoral inflammation. Innate immune cells stimulate angiogenesis by secreting angiogenic growth factors or proteases that increase angiogenic growth factor availability within the tumor microenvironment. These cells also inhibit cancer killing by inhibiting cytotoxic T-cell (*CTL*) activation, proliferation, and recruitment, while at the same time inducing differentiation and recruitment of immunosuppressive regulatory T cells (*Tregs*). Furthermore, cytokines secreted by innate immune cells act both in autocrine anti-inflammatory feed-forward loops and on tumor cells, where they stimulate proliferation and survival pathways as well as secretion of anti-inflammatory cytokines (e.g., via activation of signal transducer and activator of transcription 3)

## References

Bates DO, Hillman NJ, Williams B, Neal CR, Pocock TM (2002) Regulation of microvascular permeability by vascular endothelial growth factors. J Anat 200:581–597

Bergers G, Hanahan D (2008) Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 8:592–603

Bergers G, Song S (2005) The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol 7:452–464

Bergers G, Javaherian K, Lo KM, Folkman J, Hanahan D (1999) Effects of angiogenesis inhibitors on multistage carcinogenesis in mice. Science 284:808–812

Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G (2003) The hypoxic response of tumors is dependent on their microenvironment. Cancer Cell 4:133–146

Butler JM, Kobayashi H, Rafii S (2010) Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. Nat Rev Cancer 10:138–146

Casanovas O, Hicklin DJ, Bergers G, Hanahan D (2005) Drug resistance by evasion of antiangiogenic targeting of VEGF signaling in late-stage pancreatic islet tumors. Cancer Cell 8: 299–309

Chung AS, Lee J, Ferrara N (2010) Targeting the tumour vasculature: insights from physiological angiogenesis. Nat Rev Cancer 10:505–514

Cooke VG, Lebleu VS, Keskin D, Khan Z, O'Connell JT, Teng Y, Duncan MB, Xie L, Maeda G, Vong S et al (2012) Pericyte depletion results in hypoxia-associated epithelial-to-mesenchymal transition and metastasis mediated by met signaling pathway. Cancer Cell 21:66–81

De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, Naldini L (2005) Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 8:211–226

De Palma M, Murdoch C, Venneri MA, Naldini L, Lewis CE (2007) Tie2-expressing monocytes: regulation of tumor angiogenesis and therapeutic implications. Trends Immunol 28:519–524

Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ, Montero AJ (2009) Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother 58:49–59

Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, Song H, Vandenberg S, Johnson RS, Werb Z et al (2008a) HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 13:206–220

Du R, Petritsch C, Lu K, Liu P, Haller A, Ganss R, Song H, Vandenberg S, Bergers G (2008b) Matrix metalloproteinase-2 regulates vascular patterning and growth affecting tumor cell survival and invasion in GBM. Neuro-oncology 10:254–264

Ebos JM, Kerbel RS (2011) Antiangiogenic therapy: impact on invasion, disease progression, and metastasis. Nat Rev Clin Oncol 8:210–221

Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS (2009) Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell 15:232–239

Ferrara N (2002) VEGF and the quest for tumour angiogenesis factors. Nat Rev Cancer 2:795–803

Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25:581–611

Ferrara N, Hillan KJ, Novotny W (2005) Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 333:328–335

Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M, Castelli C, Mariani L, Parmiani G, Rivoltini L (2007) Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J Clin Oncol Off J Am Soc Clin Oncol 25:2546–2553

Fischer C, Jonckx B, Mazzone M, Zacchigna S, Loges S, Pattarini L, Chorianopoulos E, Liesenborghs L, Koch M, De Mol M et al (2007) Anti-PlGF inhibits growth of VEGF(R)-inhibitor-resistant tumors without affecting healthy vessels. Cell 131:463–475

Folkman J (2007) Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6:273–286

Folkman J, Merler E, Abernathy C, Williams G (1971) Isolation of a tumor factor responsible for angiogenesis. J Exp Med 133:275–288

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol 16:4604–4613

Gao D, Nolan DJ, Mellick AS, Bambino K, McDonnell K, Mittal V (2008) Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. Science 319:195–198

Gorre ME, Sawyers CL (2002) Molecular mechanisms of resistance to STI571 in chronic myeloid leukemia. Curr Opin Hematol 9:303–307
Greenberg JI, Shields DJ, Barillas SG, Acevedo LM, Murphy E, Huang J, Scheppke L, Stockmann C, Johnson RS, Angle N et al (2008) A role for VEGF as a negative regulator of pericyte function and vessel maturation. Nature 456:809–813
Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364
Hillen F, Griffioen AW (2007) Tumour vascularization: sprouting angiogenesis and beyond. Cancer Metastasis Rev 26:489–502
Hlushchuk R, Riesterer O, Baum O, Wood J, Gruber G, Pruschy M, Djonov V (2008) Tumor recovery by angiogenic switch from sprouting to intussusceptive angiogenesis after treatment with PTK787/ZK222584 or ionizing radiation. Am J Pathol 173:1173–1185
Hlushchuk R, Makanya AN, Djonov V (2011) Escape mechanisms after antiangiogenic treatment, or why are the tumors growing again? Int J Dev Biol 55:563–567
Jain RK (2005a) Antiangiogenic therapy for cancer: current and emerging concepts. Oncology 19:7–16
Jain RK (2005b) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62
Keith B, Johnson RS, Simon MC (2011) HIF1alpha and HIF2alpha: sibling rivalry in hypoxic tumour growth and progression. Nat Rev Cancer 12:9–22
Kelly BD, Hackett SF, Hirota K, Oshima Y, Cai Z, Berg-Dixon S, Rowan A, Yan Z, Campochiaro PA, Semenza GL (2003) Cell type-specific regulation of angiogenic growth factor gene expression and induction of angiogenesis in nonischemic tissue by a constitutively active form of hypoxia-inducible factor 1. Circ Res 93:1074–1081
Ku DD, Zaleski JK, Liu S, Brock TA (1993) Vascular endothelial growth factor induces EDRF-dependent relaxation in coronary arteries. Am J Physiol 265:H586–H592
Kuhnert F, Kirshner JR, Thurston G (2011) Dll4-Notch signaling as a therapeutic target in tumor angiogenesis. Vas Cell 3:20
Kujawski M, Kortylewski M, Lee H, Herrmann A, Kay H, Yu H (2008) Stat3 mediates myeloid cell-dependent tumor angiogenesis in mice. J Clin Invest 118:3367–3377
LeCouter J, Kowalski J, Foster J, Hass P, Zhang Z, Dillard-Telm L, Frantz G, Rangell L, DeGuzman L, Keller GA et al (2001) Identification of an angiogenic mitogen selective for endocrine gland endothelium. Nature 412:877–884
Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J, Harris AL (1996) Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. Cancer Res 56:4625–4629
Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309
Li C, Shintani S, Terakado N, Nakashiro K, Hamakawa H (2002) Infiltration of tumor-associated macrophages in human oral squamous cell carcinoma. Oncol Rep 9:1219–1223
Lin EY, Nguyen AV, Russell RG, Pollard JW (2001) Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 193:727–740
Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue XN, Pollard JW (2006) Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res 66:11238–11246
Lu KV, Chang JP, Parachoniak CA, Pandika MM, Aghi MK, Meyronet D, Isachenko N, Fouse SD, Phillips JJ, Cheresh DA et al (2012) VEGF inhibits tumor cell invasion and mesenchymal transition through a MET/VEGFR2 complex. Cancer Cell 22:21–35
Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L et al (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 7:1194–1201
Maniotis AJ, Folberg R, Hess A, Seftor EA, Gardner LM, Pe'er J, Trent JM, Meltzer PS, Hendrix MJ (1999) Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. Am J Pathol 155:739–752

Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, Politi LS, Gentner B, Brown JL, Naldini L et al (2011) Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. Cancer Cell 19:512–526

Milan SA, Yeo CJ (2012) Neuroendocrine tumors of the pancreas. Curr Opin Oncol 24:46–55

Mirza N, Fishman M, Fricke I, Dunn M, Neuger AM, Frost TJ, Lush RM, Antonia S, Gabrilovich DI (2006) All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. Cancer Res 66:9299–9307

Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A, De Baetselier P, Van Ginderachter JA (2008) Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cell-suppressive activity. Blood 111:4233–4244

Murdoch C, Muthana M, Coffelt SB, Lewis CE (2008) The role of myeloid cells in the promotion of tumour angiogenesis. Nat Rev Cancer 8:618–631

O'Connor R, Clynes M, Dowling P, O'Donovan N, O'Driscoll L (2007) Drug resistance in cancer – searching for mechanisms, markers and therapeutic agents. Expert Opin Drug Metab Toxicol 3:805–817

Ostrand-Rosenberg S, Sinha P (2009) Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 182:4499–4506

Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, Inoue M, Bergers G, Hanahan D, Casanovas O (2009) Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell 15:220–231

Pan PY, Wang GX, Yin B, Ozao J, Ku T, Divino CM, Chen SH (2008) Reversion of immune tolerance in advanced malignancy: modulation of myeloid-derived suppressor cell development by blockade of stem-cell factor function. Blood 111:219–228

Pandit R, Lathers DM, Beal NM, Garrity T, Young MR (2000) CD34+ immune suppressive cells in the peripheral blood of patients with head and neck cancer. Ann Otol Rhinol Laryngol 109:749–754

Peters BA, Diaz LA, Polyak K, Meszler L, Romans K, Guinan EC, Antin JH, Myerson D, Hamilton SR, Vogelstein B et al (2005) Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 11:261–262

Potente M, Gerhardt H, Carmeliet P (2011) Basic and therapeutic aspects of angiogenesis. Cell 146:873–887

Priceman SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, Moughon DL, Johnson M, Lusis AJ, Cohen DA, Iruela-Arispe ML et al (2010) Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of antiangiogenic therapy. Blood 115:1461–1471

Reddy GR, Kuo CC, Tan UK, Coumar MS, Chang CY, Chiang YK, Lai MJ, Yeh JY, Wu SY, Chang JY et al (2008) Synthesis and structure-activity relationships of 2-amino-1-aroylnaphthalene and 2-hydroxy-1-aroylnaphthalenes as potent antitubulin agents. J Med Chem 51: 8163–8167

Ricci-Vitiani L, Pallini R, Biffoni M, Todaro M, Invernici G, Cenci T, Maira G, Parati EA, Stassi G, Larocca LM et al (2010) Tumour vascularization via endothelial differentiation of glioblastoma stem-like cells. Nature 468:824–828

Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, Squadrito ML, Segura I, Li X, Knevels E et al (2011) HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer Cell 19:31–44

Schmid MC, Avraamides CJ, Dippold HC, Franco I, Foubert P, Ellies LG, Acevedo LM, Manglicmot JR, Song X, Wrasidlo W et al (2011) Receptor tyrosine kinases and TLR/IL1Rs unexpectedly activate myeloid cell PI3kgamma, a single convergent point promoting tumor inflammation and progression. Cancer Cell 19:715–727

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219:983–985

Sharma N, Seftor RE, Seftor EA, Gruman LM, Heidger PM Jr, Cohen MB, Lubaroff DM, Hendrix MJ (2002) Prostatic tumor cell plasticity involves cooperative interactions of distinct phenotypic subpopulations: role in vasculogenic mimicry. Prostate 50:189–201

Shirakawa K, Kobayashi H, Heike Y, Kawamoto S, Brechbiel MW, Kasumi F, Iwanaga T, Konishi F, Terada M, Wakasugi H (2002) Hemodynamics in vasculogenic mimicry and angiogenesis of inflammatory breast cancer xenograft. Cancer Res 62:560–566

Shojaei F, Wu X, Malik AK, Zhong C, Baldwin ME, Schanz S, Fuh G, Gerber HP, Ferrara N (2007a) Tumor refractoriness to anti-VEGF treatment is mediated by CD11b+Gr1+ myeloid cells. Nat Biotechnol 25:911–920

Shojaei F, Wu X, Zhong C, Yu L, Liang XH, Yao J, Blanchard D, Bais C, Peale FV, van Bruggen N et al (2007b) Bv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature 450:825–831

Shojaei F, Singh M, Thompson JD, Ferrara N (2008) Role of Bv8 in neutrophil-dependent angiogenesis in a transgenic model of cancer progression. Proc Natl Acad Sci USA 105:2640–2645

Shojaei F, Wu X, Qu X, Kowanetz M, Yu L, Tan M, Meng YG, Ferrara N (2009) G-CSF-initiated myeloid cell mobilization and angiogenesis mediate tumor refractoriness to anti-VEGF therapy in mouse models. Proc Natl Acad Sci USA 106:6742–6747

Sica A, Larghi P, Mancino A, Rubino L, Porta C, Totaro MG, Rimoldi M, Biswas SK, Allavena P, Mantovani A (2008) Macrophage polarization in tumour progression. Semin Cancer Biol 18:349–355

Simon MP, Tournaire R, Pouyssegur J (2008) The angiopoietin-2 gene of endothelial cells is up-regulated in hypoxia by a HIF binding site located in its first intron and by the central factors GATA-2 and Ets-1. J Cell Physiol 217:809–818

Smith JK, Mamoon NM, Duhe RJ (2004) Emerging roles of targeted small molecule protein-tyrosine kinase inhibitors in cancer therapy. Oncol Res 14:175–225

Soda Y, Marumoto T, Friedmann-Morvinski D, Soda M, Liu F, Michiue H, Pastorino S, Yang M, Hoffman RM, Kesari S et al (2011) Transdifferentiation of glioblastoma cells into vascular endothelial cells. Proc Natl Acad Sci USA 108:4274–4280

Song S, Ewald AJ, Stallcup W, Werb Z, Bergers G (2005) PDGFRbeta+ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. Nat Cell Biol 7:870–879

Srivastava MK, Bosch JJ, Thompson JA, Ksander BR, Edelman MJ, Ostrand-Rosenberg S (2008) Lung cancer patients' CD4(+) T cells are activated in vitro by MHC II cell-based vaccines despite the presence of myeloid-derived suppressor cells. Cancer Immunol Immunother 57:1493–1504

Sun B, Zhang S, Zhao X, Zhang W, Hao X (2004) Vasculogenic mimicry is associated with poor survival in patients with mesothelial sarcomas and alveolar rhabdomyosarcomas. Int J Oncol 25:1609–1614

Takanami I, Takeuchi K, Kodaira S (1999) Tumor-associated macrophage infiltration in pulmonary adenocarcinoma: association with angiogenesis and poor prognosis. Oncology 57:138–142

Venneri MA, De Palma M, Ponzoni M, Pucci F, Scielzo C, Zonari E, Mazzieri R, Doglioni C, Naldini L (2007) Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. Blood 109:5276–5285

Wang R, Chadalavada K, Wilshire J, Kowalik U, Hovinga KE, Geber A, Fligelman B, Leversha M, Brennan C, Tabar V (2010) Glioblastoma stem-like cells give rise to tumour endothelium. Nature 468:829–833

Welford AF, Biziato D, Coffelt SB, Nucera S, Fisher M, Pucci F, Di Serio C, Naldini L, De Palma M, Tozer GM et al (2011) TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. J Clin Invest 121:1969–1973

Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC (2004) Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell 6:409–421

Yang L, Huang J, Ren X, Gorska AE, Chytil A, Aakre M, Carbone DP, Matrisian LM, Richmond A, Lin PC et al (2008) Abrogation of TGF beta signaling in mammary carcinomas recruits Gr-1+CD11b+ myeloid cells that promote metastasis. Cancer Cell 13:23–35

Youn JI, Nagaraj S, Collazo M, Gabrilovich DI (2008) Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol 181:5791–5802

Zeisberger SM, Odermatt B, Marty C, Zehnder-Fjallman AH, Ballmer-Hofer K, Schwendener RA (2006) Clodronate-liposome-mediated depletion of tumour-associated macrophages: a new and highly effective antiangiogenic therapy approach. Brit J Cancer 95:272–281

Zumsteg A, Christofori G (2009) Corrupt policemen: inflammatory cells promote tumor angiogenesis. Curr Opin Oncol 21:60–70

# Chapter 5
# Hypoxic VDAC1: A Potential Mitochondrial Marker for Cancer Therapy

**M. Christiane Brahimi-Horn and N.M. Mazure**

**Abstract** Finding new therapeutic targets to fight cancer is an ongoing quest. Because of insufficiencies in tumor vasculature, cells often are exposed to a hostile microenvironment that is low in oxygen (hypoxic) and nutrients. Thus, tumor cells face the challenge of finding new sources of energy and defying apoptosis, which allow them to survive, grow, and colonize other tissues. Eradicating specifically these hypoxic cells is one of the many goals of anticancer therapies. The mitochondrial voltage-dependent anion channel (VDAC) is a protein at the crossroads of metabolic and survival pathways. As its name suggests, VDAC is involved in ion transport as well as adenosine triphosphate and $NAD^+$ transport. We recently reported the presence in tumor cells of a novel hypoxia-induced form of VDAC. This form, a C-terminal truncated protein (VDAC1-ΔC), was associated in some cancer cell lines with a high output of adenosine triphosphate and a strong resistance to chemotherapy-induced apoptosis. Furthermore, VDAC1-ΔC was detected in tissues of 50 % of 46 patients with lung cancer. This review examines the significance of this new form of VDAC1 for anticancer therapy.

**Keywords** Cancer cell • Chemoresistance • Hypoxia • Mitochondria • VDAC1

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## 5.1 Introduction

Mitochondria are derived from a distant ancestor: α-proteobacteria, an ancient endosymbiotic bacterium (Andersson et al. 2003). They have evolved over time in a symbiotic relationship in eukaryotic cells by promoting the consumption of oxygen by the electron transport chain to produce adenosine triphosphate (ATP), one of the most vital functions of mitochondria. Indeed, the mitochondrion is the powerhouse of the cell: it provides the energy and heat needed by cells to grow and expand. Any disruption in this "energetic" network causes disorders, such as diabetes, Alzheimer's disease, Parkinson's disease and cancer. The incidence of these diseases is increasing, particularly in industrialized countries (Fogg et al. 2011). While many people in the Western world are overfed and aged, it is possible that the mitochondrion is a common element in metabolic and neurodegenerative diseases.

The past decade has seen a revival of interest in cellular metabolism in cancer. A number of strategies to target (1) the function of mitochondria and/or (2) mitochondrial proteins involved in the development of cancer have been investigated (Berridge et al. 2009; Fogg et al. 2011; Wenner 2012). However, not all the recently developed strategies aimed at deregulating metabolism have been successful. Thus, it is important to further investigate as potential targets metabolic enzymes involved in processes such as glycolysis, oxidative phosphorylation (OXPHOS), the pentose phosphate pathway, glycogen metabolism, and glutaminolysis (Schulze and Harris 2012). This quest for new therapeutic targets will be difficult because the cell shows plasticity in the choice of the metabolic network used.

Mitochondria are not only producers of energy. They also are involved in different processes such as signaling, aging, cell proliferation, production of reactive oxygen species (ROS) and apoptosis (Galluzzi et al. 2012a). Finding the mitochondrial protein that enables the dual function of cell death while managing metabolism would be very promising. VDAC1, the voltage-dependent anion channel 1, might meet this expectation. Indeed, we demonstrated the production of a new form of VDAC1, VDAC1-ΔC, which appears only after treatment of cells with long-term hypoxia. These hypoxic cells produce more ATP and are more resistant to apoptosis induced by staurosporine or etoposide than normoxic (atmospheric levels of oxygen) cells (Brahimi-Horn et al. 2012). This review examines the possible function of this new potential target and integrates it into the full picture of cancer cell metabolism and apoptosis.

## 5.2 The Voltage-Dependent Anion Channel

VDACs, also called mitochondrial porins, exist as three isoforms: VDAC1, VDAC2, and VDAC3 (Shoshan-Barmatz and Mizrachi 2012). These proteins are similar (70 % identity) and are expressed ubiquitously in all tissues. However, their levels of expression are different, but they are frequently present in relative amounts as VDAC1 > VDAC2 > VDAC3. In addition, their physiological functions seem to be different. Mice lacking VDAC1 and VDAC3 are viable, although the mice lacking

VDAC3 show male infertility due in part to defects in the mitochondria, whereas VDAC2 deficiency in mice is embryogenically lethal. The three forms of VDAC may also be posttranslationally modified by phosphorylation and acetylation, but the role of these modifications in the activity of VDAC remains to be clearly defined.

The VDAC1 protein is localized in the outer mitochondrial membrane (OMM), although it has been reported to be expressed in extramitochondrial localizations such as the plasma membrane (Bathori et al. 2000; De Pinto et al. 2010) and the sarcoplasmic reticulum (Shoshan-Barmatz and Israelson 2005). It acts as an anchor and a point of convergence for the delivery of messages of both life and death. However, these two functions are extremely difficult to separate, as the interaction of VDAC1 with its various partners will fine-tune the equilibrium between life and death. The oligomerization of VDAC seems to be an integral part of its activity and thus cell destiny (Shoshan-Barmatz and Mizrachi 2012). Dimers, trimers, tetramers, and higher oligomeric forms have been observed in association with the induction of apoptosis.

VDAC1 is a permeable pore that, when in an open position, allows many uncharged or charged molecules to pass through. The ability to open or close the pore is connected to a voltage sensor. The N-terminus of VDAC1 plays the role of a lock in the passageway, ensuring safe transfer from one compartment to another. NADH, ATP/adenosine diphosphate, citrate, succinate, glutamate, pyruvate, and even glucose, as well $Mg^{2+}$, $Ca^{2+}$, $Cl^{-}$, $K^{+}$, and $Na^{+}$ ions pass through this active pore and participate in the dynamic process of cell growth. Any process that changes the structure of VDAC1 will change the flow of molecules through VDAC and thus change the dynamics of cell growth. The C-terminus of VDAC1 possesses $NAD^{+}$ and ATP binding sites that are essential substrates for glycolysis. Finally, one of the most important aspects of VDAC1 in metabolism is its binding to hexokinase (HK) I and II. This binding is probably the key to the role of VDAC1 in the passage from cell life and death. Indeed, HKII acts as a molecular sentinel, coordinating mitochondrial generation of ATP and cytoplasmic glycolytic flux, which ensures that the tumor proliferates rapidly (Mathupala et al. 2010).

Research also has focused on the role of VDAC1 in apoptosis (Zamzami and Kroemer 2001; Shoshan-Barmatz and Mizrachi 2012). It has been shown that anti-VADC1 antibodies reduce or block the apoptotic response. VDAC1 can act as an anchor for different pro-and anti-apoptotic proteins (Shoshan-Barmatz and Mizrachi 2012), which might influence interaction with HKI or HKII. This association might also prevent release of cytochrome C and thus limit apoptosis. In fact, small interfering RNA against VDAC1 completely blocked apoptosis induced by cisplatin. In contrast, overexpression resulted in an increase in cell death.

## 5.3 Mitochondrial Phenotype and VDAC

Mitochondria are dynamic organelles, the phenotype of which is continually modified by two opposing processes: fusion and fission. Two key proteins, the dynamin-related GTPases mitofusins (MFNs) and optic atrophy, regulate fusion in humans. We recently reported that some cell lines derived from tumors exposed to long-term

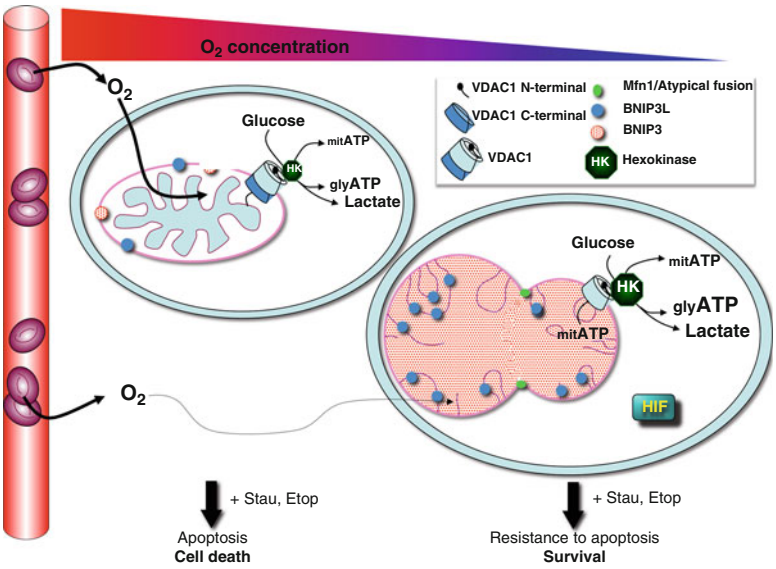


**Fig. 5.1** Cancer cells show either tubular mitochondria (in normoxia) or enlarged mitochondria (in hypoxia). Cells with tubular mitochondria release cytochrome C and die when exposed to a chemotherapeutic agent (*Stau* staurosporin, *Etop* etoposide), whereas cells with enlarged mitochondria resist chemotherapeutic agents and survive. Cells with enlarged mitochondria contain a C-terminal truncated form of the voltage-dependent anion channel 1 (*VDAC1-ΔC*), a transporter of mitochondrial adenosine triphosphate (*ATP*) (*mitATP*). VDAC1-ΔC associates strongly with the enzyme hexokinase (*HK*), the first enzyme of the glycolytic pathway that consumes ATP. By converting glucose into lactate, glycolysis also produces ATP (*glyATP*). Hypoxia stabilizes the hypoxia-inducible factor (*HIF*) transcription factor that induces many gene products, including enzymes of the glycolytic pathway and Bcl2/E1B 19-kDa protein-interacting protein 3 (BNIP3 and BNIP3L). BNIP3 and BNIP3L – together with mitofusins (*MFNs*) – promote mitochondrial fusion

hypoxia (72 h) had a network of enlarged mitochondria with rearrangement of cristae (LS174, HeLa, and A549 cells), whereas others had a normal network of tubular mitochondria (PC3 and SkMel cells). This altered mitochondrial phenotype is the result of a balance in favor of fusion, which implicates overexpression of MFN1 and Bcl2/E1B 19-kDa protein-interacting protein 3 (BNIP3 andBNIP3L) (Chiche et al. 2010). In addition, these cells were resistant to apoptosis induced by chemotherapeutic agents, particularly staurosporine, a nonselective protein kinase C inhibitor, from which many derivatives and structural analogs have been synthesized as potential anticancer treatments, including 7-hydroxystaurosporine, and etoposide, a topoisomerase II inhibitor (Celltop, Vépéside) (Brahimi-Horn et al. 2012). Hypoxic cells containing VDAC1-ΔC were less sensitive to staurosporine and cell death induced by etoposide, and the invalidation of VDAC1-ΔC restored sensitivity to cell death. Since fusion/fission participates in mitochondrial apoptosis (Youle and van der Bliek 2012), we hypothesized that some mitochondrial proteins that are dependent on a hypoxia-inducible factor (HIF) could play a role in the resistance to apoptosis. HIF is the key transcription factor of cell response to hypoxia (Brahimi-Horn et al. 2007a). Formation of VDAC1-ΔC was dependent on HIF-1 and was associated with cell survival (Fig. 5.1).

## 5.4 Mitochondria, Metabolism, and Hypoxic VDAC1

Proliferation of tumor cells, at all costs, is a cell's quest for the Holy Grail. The Grail is none other than the fuel that will allow them to grow again and again. What is the source of energy so vital for survival? Mainly glucose, which is converted by glycolysis; glycolysis literally means "lysis of glucose" into energy. An increase in the uptake of glucose is one of the earliest events of malignant transformation. Once in the cell, glucose undergoes a cascade of transformation to finally produce pyruvate (Gatenby and Gillies 2004; Brahimi-Horn et al. 2007b). Conversion is an extremely important focal point for tumor cell growth. Three top scientists have studied cell proliferation and have given their names to three different metabolic effects. Warburg (1928) described the conversion of pyruvate to lactic acid even in the presence of oxygen. This form of aerobic glycolysis used by the tumor cell is called the Warburg effect. However, Warburg proposed that the increase in glycolysis was in fact due to a deficiency in mitochondrial respiration. Indeed, the conversion of pyruvate to lactic acid does not occur in the presence of oxygen in normal differentiated cells because they draw their energy from mitochondrial OXPHOS, the oxidization of pyruvate to carbon dioxide and water via the tricarboxylic acid cycle, also called the Krebs cycle. The low activity of glycolysis as a result of mitochondrial ATP production is called the Pasteur effect, after the work of Louis Pasteur in 1857 on yeasts: he observed that they grew better in the presence of oxygen. In contrast, the third effect, known as the Crabtree effect, describes the increase in aerobic glycolysis, which then inhibits the oxidative pathway. It is important to note that complete oxidation, via mitochondria, produces 38 ATP molecules (representing more than 90 % of the intracellular ATP required for cells to grow), whereas glucose metabolism to pyruvate (glycolysis) produces only 2 ATP molecules (Pedersen 2007; Vander Heiden et al. 2009). Since OXPHOS requires oxygen, this process is reduced in hypoxic cells. Thus, to produce more ATP, the malignant cell increases the rate of glycolysis, thereby producing more ATP, as well as more lactate and hydrogen ions. This mechanism is regulated through HIFs, which induce the expression of glucose transporters such as GLUT-1 and GLUT-3. HKI/II is also upregulated by HIFs, as are most of the enzymes of the glycolytic pathway. Pyruvate lies at the crossroads between glycolysis and OXPHOS, that is, lactate or carbon dioxide and water. To save pyruvate from making a difficult choice, HIFs have been programmed to favor glycolysis by inducing the expression of lactate dehydrogenase and thus favoring the conversion of pyruvate to lactate. On the other hand, by inducing the expression of pyruvate dehydrogenase kinase-1, a kinase that phosphorylates and inhibits pyruvate dehydrogenase, HIFs thereby block the entry of pyruvate into the Krebs cycle (Fantin et al. 2006). This also results in a reduction in oxygen consumption by the mitochondria.

However, this change does not alter either the ultrastructure or the membrane potential of the mitochondria and only modifies slightly the number of mitochondria. In addition, by blocking mitochondrial respiration, the cells reduce their production of ROS during hypoxia. Nevertheless, mitochondria are not completely dormant during hypoxia. Indeed, hypoxic mitochondria have developed another more efficient and more direct mechanism to control respiration. HIFs induce

expression of LON and cytochrome C oxidase (COX4/2). The COX4/2 subunit is more efficient in respiration than the COX4/1 subunit (Fukuda et al. 2007). Moreover, the function of LON, a mitochondrial protease, is to degrade the COX4/1 subunit. Thus, mitochondria change their function in response to hypoxia by altering the expression of proteins of the electron transport chain.

The cell in general, and the tumor cell in particular, always finds a way to use whatever is at its disposal to avoid death. Because mitochondrial respiration is also associated with increased generation of damaging ROS, cells have developed another mechanism to control mitochondrial metabolism by inducing mitophagy, a process whereby mitochondria are degraded to supply components for metabolism. In 2009 we showed that BNIP3, by disrupting the complex Beclin-Bcl-2, could activate autophagy, or degradation of cytoplasmic components, of tumor cells during hypoxia (Bellot et al. 2009). However, we were unable to detect mitophagy. Zhang et al. (2008), however, observed mitophagy in mouse embryonic fibroblast cells and found it to be dependent on HIF-1. These results strongly suggest that the loss of mitochondria will decrease ROS production and thereby promote cell survival, especially under conditions of long-term hypoxia. This is only one example in a long list of things mitochondria might or might not do to promote survival *via* metabolism. It is tempting to think that mitochondria have other cards to play that have not yet been identified. We believe that we have identified one of these uncharacterized cards: VDAC1-ΔC. We first demonstrated that VDAC1-ΔC had the same channel activity and voltage dependency as VDAC1 and was thus capable of regulating the import and export of mitochondrial $Ca^{2+}$ (Brahimi-Horn et al. 2012). Indeed, VDAC1-ΔC seems to control cell survival in hypoxia by regulating the export of ATP and probably NADH. Enlarged mitochondria result from hyperfusion (see the section "Mitochondrial Phenotype and VDAC"). Tondera et al. (2009) showed that stress-related hyperfusion was accompanied by an increase in mitochondrial production of ATP. We likewise observed an increase in ATP levels in cells during hypoxia when VDAC1-ΔC and enlarged mitochondria were present. In addition, VDAC1-ΔC interacted with anti-apoptotic proteins such as Bcl-xL and HKI/II. HK, the first enzyme of the glycolytic pathway, converts glucose into glucose-6-phosphate (G6P) through ATP hydrolysis. Therefore it is a major player in maintaining the highly malignant state of cancer cells (Mathupala et al. 2006). Its expression is strongly induced by hypoxia via HIF-1, and it associates with VDAC1. This association has been hypothesized to offer preferential access of HK to the ATP produced by oxidative phosphorylation (Pedersen 2008). Therefore, glycolysis is exacerbated in hypoxic cells, a condition that contributes to the Warburg effect in cancer cells. It has been proposed that the binding of HKI/II to VDAC1 in cancer cells would maintain the mitochondrial membrane potential by facilitating the reverse reaction catalyzed by HKI/II, the conversion of G6P into glucose. We showed that VDAC1-ΔC also interacts with HKI/II, which increases glycolysis and probably stabilizes the conversion of G6P. This new association between a hypoxia-induced VDAC1-ΔC and an overexpressed HKI/II in hypoxia should bring benefit to hypoxic cancer cells. This may suggest symbiosis between VDAC1-ΔC, which transports more ATP, and HKI/II, which exacerbates glycolysis and may even

stabilize VDAC1-ΔC to produce more ATP. So, VDAC1 maintains the balance between mitochondrial respiration and glycolysis to provide energy to the cell.

## 5.5 Mitochondria, Apoptosis, and Hypoxic VDAC1

The mitochondrion is a real war machine. When the mitochondrial membrane potential ($\Delta\psi m$) is lost, mitochondria lose the integrity of their outer membrane, ATP synthesis is stopped, and proteins such as cytochrome C, apoptosis-inducing factor, and Smac/Diablo activate a cascade of caspases, ensuring the certain death of the cell (Galluzzi et al. 2012b). Although extensively studied, some aspects of the mechanism of apoptosis are still very controversial. A model has been proposed where the formation of pores in the OMM consists of proapoptotic proteins Bax or and Bak, or a combination of the two, and the activity of the pore may be regulated by anti-apoptotic proteins Bcl-xL and Bcl-2 (Berridge et al. 2009). A second model suggests the existence of a mitochondrial megaspore, the permeability transition pore complex (PTPC), which, depending on its opening or closing, would render the outer membrane permeable. The PTPC has been suggested to be composed of VDAC at the OMM, adenine nucleotide translocase (ANT) in the inner mitochondrial membrane, and cyclophilin D in the mitochondrial matrix. However, VDAC and ANT may be more regulators of the PTPC than constituents. Indeed, the invalidation of VDAC or ANT did not prevent the opening of the PTPC. Finally, a third model – and the most recent – involves the formation of a pore comprising an assembly of homo-oligomers of VDAC1.

Again, the couple VDAC1-HK has an important role to play in terms of apoptosis (Shoshan-Barmatz and Mizrachi 2012). Indeed, the binding of HKII to VDAC1 was shown to inhibit cell apoptosis by blocking the binding of proapoptotic molecules such as Bax to VDAC. In addition, it has been shown that overexpression of HK in certain tumor cell lines protects from apoptosis induced by staurosporine. It even seems that the attachment of HK to VDAC inhibits apoptosis, whereas its detachment fulfills a proapoptotic function.

In addition, the Bcl-2 family of proteins can inhibit or induce mitochondrial dysfunction. Bcl-2 and Bcl-xL are the best-studied members of this family and inhibit the release of cytochrome C, thereby blocking apoptosis. These proteins share significant homology in four regions, called consensus BH1–4 domains; Bcl-xL interacts with VDAC through the BH4 domain. It has been shown that VDAC1 interacts with Bcl-xL and that this interaction is anti-apoptotic (Arbel et al. 2012).

Finally, there exists a complex crosstalk between mitochondrial dynamics (fusion and fission) and apoptosis. Bcl-2, on the one hand, and Bak and Bax, on the other, interact with proteins involved in mitochondrial fusion (mitofusins) and fission (dynamin-associated proteins) (Brooks and Dong 2007; Rolland and Conradt 2010). It has been demonstrated that Bax and Bak participate in mitochondrial fission as precursors of apoptosis. Bak is known to associate with proteins of the OMM to form well-defined complexes and was found to be associated with MFN1 and

MFN2, which are mediators of mitochondrial fusion. It has been proposed that, following the induction of apoptosis, Bak dissociates from MFN2 to preferentially associate with MFN1. In the absence of Bax and Bak, the distribution of MFN2 is altered and is no longer competent to provoke inter-OMM fusion. These results suggest that the expression of Bax/Bak affects the mobility of the membranous MFN2. Taken together, these data suggest a role for Bax/Bak in the regulation of mitochondrial fusion (Palmer et al. 2011).

The interaction between VDAC1-ΔC and HKI/II in hypoxia probably functions not only to promote metabolism but also to favor resistance to apoptosis (Brahimi-Horn et al. 2012). First, it is noteworthy that the invalidation of HKII significantly diminished the formation of VDAC1-ΔC in hypoxia, whereas the same invalidation performed in normoxia did not affect VDAC1. Second, clotrimazole and bifonazole, two antifungal compounds that cause detachment of HKI/II from mitochondria and from VDAC1 (Penso and Beitner 1998; Shoshan-Barmatz et al. 2010), increased cell mortality to a degree that was similar to that obtained by invalidation of VDAC1 (Brahimi-Horn et al. 2012). Mortality was significantly increased in cells incubated in the presence of these agents during hypoxia. Therefore there is probably a strong interaction between these two proteins (VDAC1-ΔC and HKI/II) under hypoxic conditions.

In hypoxia, we observed a small but reproducible increase in the expression of Bcl-xL and showed that Bcl-xL interacted with VDAC1-ΔC. Bax was not detected at the protein level in the cells we used, but Bak was overexpressed. We have shown that the enlarged phenotype of mitochondria results from hyperfusion. However, what about Bax? Is the fact that Bax is not expressed in the cell line of importance in hyperfusion? It seems that Bax and Bak can sometimes be interchangeable. Could overexpression of Bak compensate for a deficiency in Bax? The expression of MFN1 was indeed induced by hypoxia. In addition, the invalidation of the MFN1 resensitized tumor cells to staurosporine. VDAC1-ΔC probably does not act alone in promoting resistance to apoptosis. Exacerbated fusion created enlarged mitochondria with rearranged cristae undoubtedly contributes to resistance.

## 5.6 Conclusion

Although VDAC is considered to be an “old” protein, certain aspects of the regulation of its expression, activity, and function remain unclear. What is clear, however, is that VDAC represents an interesting therapeutic target in cancer. Moreover, we detected VDAC1-ΔC in tumor tissue of patients with lung cancer (50 %), and the frequency of positivity for VDAC1-ΔC was higher in late-stage tumors than in early-stage tumors. We believe that VDAC1-ΔC represents a product of tumor progression.

A number of groups have shown that by blocking the activity of VDAC it is possible to trigger apoptosis, and this has stimulated a search for specific inhibitors of VDAC (Shoshan-Barmatz and Mizrachi 2012). By modulating interaction with

HKI/II, for example, it is possible to modify (1) the structure of the VDAC pore; (2) the flow of ATP out of the mitochondria *via* VDAC; (3) the activity of HK, which uses mitochondrial ATP; (4) the flux of glycolysis; and, finally, (5) the triggering of apoptosis by allowing Bax or Bak to act as proapoptotic players. Avicins (trigger apoptosis), cisplatin (an anticancer drug), endostatin (interferes with pro-angiogenic factors), oblimersen (a phosphorothioate antisense oligonucleotide), erastin (an anticancer agent), and geldanamycin (an antibiotic) are among the various interacting compounds potentially acting on the activity of VDAC. In addition, VDAC1-based peptides that interact with Bcl-2 and Bcl-xL and prevent the anti-apoptotic activities of these proteins have been developed recently as anticancer therapies.

The discovery of a new form of VDAC1 induced by hypoxia and its association with hypoxia-induced HK and Bcl-xL represents a metabolic and anti-apoptotic defense mechanism that promotes resistance of tumor cells to death. Thus, the disruption of this defense mechanism could be used as a selective target for therapy against hypoxic cells, which are the most resistant to chemotherapy and radiotherapy. VDAC1 could represent the Achilles heel of the hypoxic cell.

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## References

Andersson SG, Karlberg O, Canback B, Kurland CG (2003) On the origin of mitochondria: a genomics perspective. Philos Trans R Soc Lond B Biol Sci 358:165–177, discussion 77–79

Arbel N, Ben-Hail D, Shoshan-Barmatz V (2012) Mediation of the antiapoptotic activity of Bcl-xL protein upon interaction with VDAC1 protein. J biol chem 287:23152–23161

Bathori G, Parolini I, Szabo I, Tombola F, Messina A, Oliva M et al (2000) Extramitochondrial porin: facts and hypotheses. J Bioenerg Biomembr 32:79–89

Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J et al (2009) Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol 29:2570–2581

Berridge MV, Herst PM, Lawen A (2009) Targeting mitochondrial permeability in cancer drug development. Mol Nutr Food Res 53:76–86

Brahimi-Horn MC, Laferrière J, Mazure N, Pouysségur J (2007a) Hypoxia and tumour progression. In: Marmé D, Fusenig N (eds) Tumor angiogenesis. Springer, Berlin/New York, pp 171–194

Brahimi-Horn MC, Chiche J, Pouyssegur J (2007b) Hypoxia signalling controls metabolic demand. Curr Opin Cell Biol 19:223–229

Brahimi-Horn MC, Ben-Hail D, Ilie M, Gounon P, Rouleau M, Hofman V et al (2012) Expression of a truncated active form of VDAC1 in lung cancer associates with hypoxic cell survival and correlates with progression to chemotherapy resistance. Cancer Res 72:2140–2150

Brooks C, Dong Z (2007) Regulation of mitochondrial morphological dynamics during apoptosis by Bcl-2 family proteins: a key in Bak? Cell Cycle 6:3043–3047

Chiche J, Rouleau M, Gounon P, Brahimi-Horn MC, Pouyssegur J, Mazure NM (2010) Hypoxic enlarged mitochondria protect cancer cells from apoptotic stimuli. J Cell Physiol 222:648–657

De Pinto V, Messina A, Lane DJ, Lawen A (2010) Voltage-dependent anion-selective channel (VDAC) in the plasma membrane. FEBS lett 584:1793–1799

Fantin VR, St-Pierre J, Leder P (2006) Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9:425–434

Fogg VC, Lanning NJ, Mackeigan JP (2011) Mitochondria in cancer: at the crossroads of life and death. Chin J Cancer 30:526–539

Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL (2007) HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. Cell 129:111–122

Galluzzi L, Kepp O, Kroemer G (2012a) Mitochondria: master regulators of danger signalling. Nat Rev Mol Cell Biol 13:780–788

Galluzzi L, Kepp O, Trojel-Hansen C, Kroemer G (2012b) Mitochondrial control of cellular life, stress, and death. Circ Res 111:1198–1207

Gatenby RA, Gillies RJ (2004) Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4:891–899

Mathupala SP, Ko YH, Pedersen PL (2006) Hexokinase II: cancer's double-edged sword acting as both facilitator and gatekeeper of malignancy when bound to mitochondria. Oncogene 25:4777–4786

Mathupala SP, Ko YH, Pedersen PL (2010) The pivotal roles of mitochondria in cancer: Warburg and beyond and encouraging prospects for effective therapies. Biochim Biophys Acta 1797:1225–1230

Palmer CS, Osellame LD, Stojanovski D, Ryan MT (2011) The regulation of mitochondrial morphology: intricate mechanisms and dynamic machinery. Cell Signal 23:1534–1545

Pedersen PL (2007) The cancer cell's "power plants" as promising therapeutic targets: an overview. J Bioenerg Biomembr 39:1–12

Pedersen PL (2008) Voltage dependent anion channels (VDACs): a brief introduction with a focus on the outer mitochondrial compartment's roles together with hexokinase-2 in the "Warburg effect" in cancer. J Bioenerg Biomembr 40:123–126

Penso J, Beitner R (1998) Clotrimazole and bifonazole detach hexokinase from mitochondria of melanoma cells. Eur J Pharmacol 342:113–117

Rolland SG, Conradt B (2010) New role of the BCL2 family of proteins in the regulation of mitochondrial dynamics. Curr Opin Cell Biol 22:852–858

Schulze A, Harris AL (2012) How cancer metabolism is tuned for proliferation and vulnerable to disruption. Nature 491:364–373

Shoshan-Barmatz V, Israelson A (2005) The voltage-dependent anion channel in endoplasmic/sarcoplasmic reticulum: characterization, modulation and possible function. J Membr Biol 204:57–66

Shoshan-Barmatz V, Mizrachi D (2012) VDAC1: from structure to cancer therapy. Front Oncol 2:164

Shoshan-Barmatz V, De Pinto V, Zweckstetter M, Raviv Z, Keinan N, Arbel N (2010) VDAC, a multi-functional mitochondrial protein regulating cell life and death. Mol Aspects Med 31:227–285

Tondera D, Grandemange S, Jourdain A, Karbowski M, Mattenberger Y, Herzig S et al (2009) SLP-2 is required for stress-induced mitochondrial hyperfusion. EMBO J 28:1589–1600

Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324:1029–1033

Warburg O (1928) The chemical constitution of respiration ferment. Science 68:437–443

Wenner CE (2012) Targeting mitochondria as a therapeutic target in cancer. J Cell Physiol 227: 450–456

Youle RJ, van der Bliek AM (2012) Mitochondrial fission, fusion, and stress. Science 337: 1062–1065

Zamzami N, Kroemer G (2001) The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol 2:67–71

Zhang H, Bosch-Marce M, Shimoda LA, Tan YS, Baek JH, Wesley JB et al (2008) Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. J Biol Chem 283: 10892–10903

# Chapter 6
# Hypoxia-Directed Drug Strategies to Target the Tumor Microenvironment

**Michael P. Hay, Kevin O. Hicks, and Jingli Wang**

**Abstract** Hypoxia is an important component of the tumor microenvironment and has been the target of drug discovery efforts for almost half a century. These efforts have evolved from offsetting the impact of hypoxia on radiotherapy with oxygen-mimetic radiosensitizers to using hypoxia as a means to selectively target tumors. The more recent description of hypoxia-inducible factors and their role in the hypoxia response network has revealed a host of new drug targets to selectively target tumors. We are developing hypoxia-directed drugs in each of the following areas: novel radiosensitizers for hypofractionated radiotherapy, a second-generation benzotriazine di-N-oxide hypoxia-activated prodrug, and a hypoxia-inducible factor-1–dependent cytotoxin that targets glucose transport. These projects are discussed in the context of hypoxia-directed drug discovery.

**Keywords** Hypoxia • Drug discovery • Nitroimidazole • Radiosensitizer • Hypoxia-activated prodrug • Biomarker • Tirapazamine • SN30000 • HIF-1α • Glucose transport

## 6.1 Introduction

### *6.1.1 Hypoxia as a Therapeutic Target*

Hypoxia initially arises as a consequence of oxygen consumption in small tumors or metastases. The cellular response to this hypoxia plays a significant role in the development of the tumor microenvironment and influences the expansion of tumor

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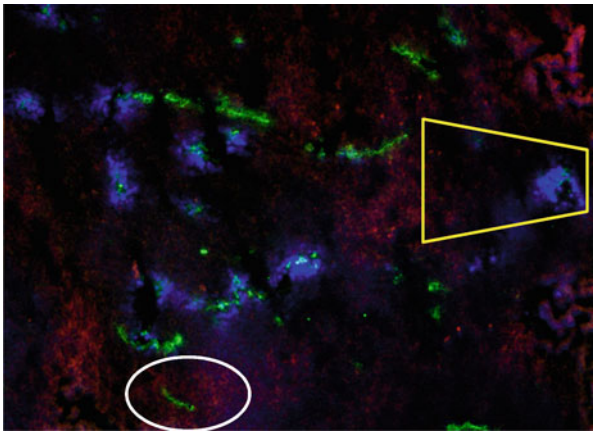

**Fig. 6.1** Detection of hypoxic cells in a human colon cancer xenograft HCT116 grown subcutaneously in nude mice. *Yellow box* shows diffusion-limited hypoxia. *White oval* shows perfusion-limited hypoxia. Hypoxic marker EF5 (60 mg/kg) was administrated intraperitoneally 1.5 h and blood vessel perfusion marker Hochest33342 (40 mg/kg) was administered intravenously 2 min before the mice were killed. The tumor was removed immediately and frozen in octanol. Frozen sections (8 **μ**m) were immunostained for the hypoxic marker (EF5, *red*), blood vessels (CD1, *green*), and perfused blood vessels (Hochest33342, *blue*). (Jingli Wang, unpublished data)

vasculature, resulting in a disorganized, inefficient tumor microvascular network that has irregular blood flow (Jain 2005; Pries et al. 2009). In turn, this exacerbates existing hypoxia and leads to considerable heterogeneity in oxygen concentrations that may fluctuate spatially and temporally (Dewhirst et al. 2008) (Fig. 6.1). The characterization of hypoxia accordingly depends on the techniques used to measure it. Whereas fine-needle oxygen electrode measurements provide a direct gauge of oxygen tension and have demonstrated a wide range of oxygen concentrations in human tumors (Vaupel et al. 2007), the use of exogenous molecular probes such as 2-nitroimidazoles or endogenous markers such as downstream products of genes regulated by hypoxia-inducible factors (HIFs) report different levels of hypoxia (Fig. 6.2). Nitroimidazole probes are typically activated at levels of less than 1 μM of oxygen, whereas HIF-1 is stabilized at higher oxygen concentrations (Tuttle et al. 2007).

The majority of clinical studies have shown that hypoxia results in compromised outcomes across a wide range of diseases and treatment modalities (Horsman et al. 2012; Nordsmark et al. 2005; Vaupel and Mayer 2007). Both chronic hypoxia (Gray et al. 1953; Thomlinson and Gray 1955) and intermittent, or cycling, hypoxia within solid tumors can limit radiotherapy (Brown 1979). Poor perfusion and significant diffusion gradients exist within tumors (Dewhirst et al. 2008) that, along with high interstitial pressures (Heldin et al. 2004), can limit the diffusion of chemotherapeutic agents into hypoxic regions (Minchinton and Tannock 2006). This, when combined with a slowing of proliferation in these areas, can cause resistance to commonly used antiproliferative agents. Identification of the role of HIF-1 in the hypoxia response network (Semenza 2003) has revealed how hypoxia influences survival processes such as increased angiogenesis (Gnarra et al. 1996) and

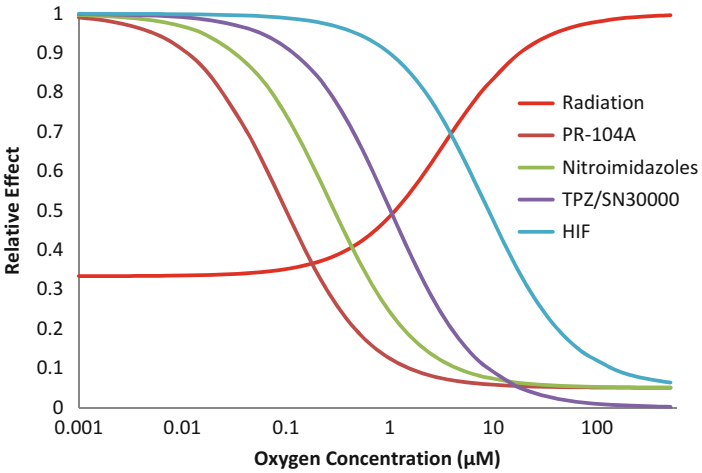


**Fig. 6.2** Illustration of oxygen dependence for cellular response. Radiation sensitivity of clonogenic cell killing increases with increasing oxygen concentration, reaching half-maximal at approximately 4–5 μM (Wouters and Brown 1997), whereas it decreases for hypoxia-activated prodrugs PR-104 (Hicks et al. 2007) and tirapazamine/SN30000 (Hicks et al. 2004, 2010). For nitroimidazoles, oxygen dependence of intracellular binding of EF5 (Tuttle et al. 2007) is similar to the oxygen dependence of the sensitizer enhancement ratio of misonidazole (Carlson et al. 2011). Stabilization of the hypoxia-inducible factor generally occurs under more moderate hypoxic conditions (Tuttle et al. 2007)

vasculogenesis (Kioi et al. 2010), resistance to cell death (Graeber et al. 1996), aerobic glycolysis (Semenza 2010), and genomic instability (Bindra et al. 2007; Huang et al. 2007). Furthermore, the contribution of tumor hypoxia to invasiveness and metastasis (Chang et al. 2011; Hill et al. 2009) potentially compromises a third treatment modality: surgery. The prevalence of tumor hypoxia, combined with its effect on tumor survival, progression, and resistance to therapy, marks hypoxia as a compelling target for current drug discovery efforts.

### *6.1.2 Drug Development*

In tandem with the growing understanding of the effect of hypoxia, an evolving series of drug discovery efforts have sought to overcome or leverage the effects of hypoxia for therapeutic gain (Denny 2010; Semenza 2007; Wilson and Hay 2011). The major drug discovery effort was centered on chemical radiosensitizers and spanned several decades (Wardman 2007), but minimal clinical success resulted in dwindling efforts in this field (Overgaard 2007).

A paradigm shift in hypoxia targeting occurred in the mid-1980s. Rather than minimizing the impact of hypoxia on radiotherapy, a new strategy sought to use hypoxia as a physiological target that could promote activation of prodrugs to kill tumor cells. Hypoxia-activated prodrugs (HAPs) have been extensively explored

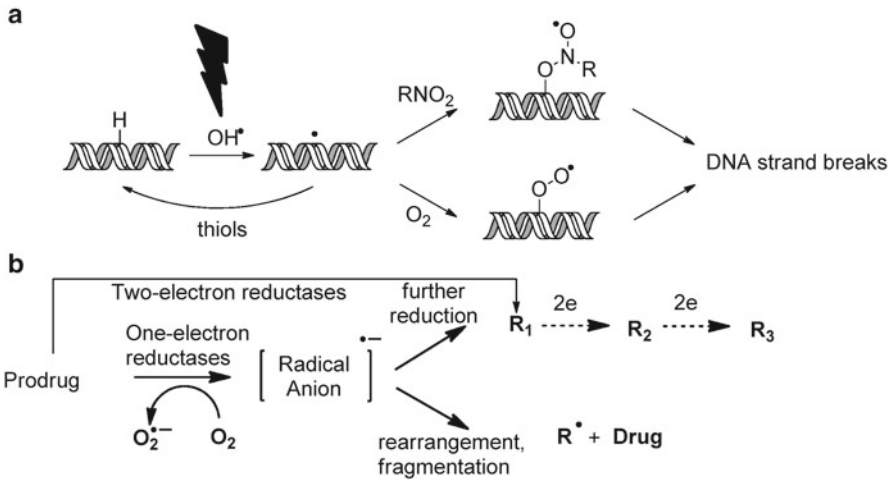


**Fig. 6.3** (**a**) Mechanism of oxygen-mimetic radiosensitization by electron-affinic nitroaryl radiosensitizers. (**b**) Mechanism of hypoxia-activated prodrugs

over several decades (Brown and Wilson 2004; Chen and Hu 2009; McKeown et al. 2007; Rockwell et al. 2009; Wilson and Hay 2011).

Identification of the HIF as the "master regulator" of hypoxic response and a key drug target (Giaccia et al. 2003; Semenza 2003) resulted in the discovery of a plethora of small molecules that could be potential HIF inhibitors (Xia et al. 2012). However, much of this work has had limited application. Many agents identified as HIF-1α inhibitors actually target upstream or downstream components in the HIF response network or have pleiotropic effects. In addition, these agents are not necessarily cytostatic or cytotoxic, nor are they necessarily selective for hypoxic cells.

Overall, few agents developed to target hypoxia have been registered. Tumor hypoxia has been a niche area, predominantly the preserve of academic groups, and only with the elaboration of the hypoxia response network has hypoxia received mainstream attention as a validated target for drug development.

### *6.1.3 Defining the Hypoxic "Target"*

Definition of the "target" depends on the approach directed against the hypoxic cells. For oxygen-mimetic radiosensitizers the target is a DNA radical generated by ionizing radiation (Fig. 6.3a), and selectivity results from competition between oxygen and the electron-affinic nitroimidazole group for this radical. Since oxygen is vastly more efficient at scavenging these radicals, nitroimidazoles effectively sensitize only hypoxic cells.

A more complex situation exists for HAPs (Fig. 6.3b). The prodrug is reduced by a one-electron reductase to form a radical anion. This radical anion may be "scavenged" by oxygen to reproduce the prodrug with production of superoxide. In the

absence of oxygen the radical anion may undergo a variety of transformations, depending on chemical class, leading to the activated drug. Reduction of the prodrug by two-electron reductases removes the potential for back-oxidation of the radical anion, leading to a loss of hypoxic selectivity. As a consequence, the target is the intersection of three elements: hypoxia, enzymes to activate the prodrug, and intrinsic sensitivity to the activated drug. The initial concept invoked tumor hypoxia as unique to tumor tissue and, essentially, a binary switch, suggesting it is an ideal drug target for HAPs (Denny et al. 1996). However, hypoxia exists in normal tissues, and tumor cells at intermediate oxygen tension are important for tumor progression (Wouters and Brown 1997), making hypoxia a more complex target. The second component requires the location of appropriate enzymes to activate the prodrug within the tumor. NADPH:cytochrome P450 oxidoreductase has been identified as a key one-electron reductase responsible for the activation of many HAPs (Guise et al. 2007; Meng et al. 2012; Patterson et al. 1998; Wang et al. 2012b), but contributions from other one-electron reductases (e.g., aldehyde oxidase, xanthine oxidase, nitric oxide synthases, thioredoxin reductase, NADH-dependent cytochrome b5 reductase, methionine synthase reductase, and NADPH-dependent diflavin oxidoreductase) also have been reported (Adams and Rickert 1995; Ask et al. 2003; Cenas et al. 2006; Chandor et al. 2008; Guise et al. 2012; Papadopoulou et al. 2003; Patterson et al. 1998; Tatsumi et al. 1986; Ueda et al. 2003). The expression of these enzymes and their relative contributions to HAP activity across human tumors is incompletely understood; however, significant variations between cell lines (Guise et al. 2012; Wang et al. 2012b) and individual human tumors (Evans et al. 2000; Patterson et al. 1997) has been demonstrated. The electron affinity of the prodrug seems to be the key determinant of activation (Wardman 2001), indicating little substrate specificity for most of the one-electron reductases. The third constituent of the HAP target is the intrinsic sensitivity of the target cells to the activated drug. Strategies using prodrugs that release cytotoxins that cross-link DNA will be dependent on DNA repair status and may cause normal tissue toxicity if activated inappropriately. Prodrugs delivering inhibitors of specific molecular targets, such as the human epidermal growth factor receptor, have been reported more recently (Patterson et al. 2009). For these agents, the relative expression of the molecular target in hypoxic and normal tissues contributes to the overall target. In the case of HIF inhibitors, the molecular targets are structurally diverse, and hypoxic selectivity is provided by the level of overexpression under hypoxic conditions relative to the levels of the target in normal tissue and the specificity of the inhibitor for the particular molecular target.

## 6.2 Radiosensitizers

### *6.2.1 Introduction*

Attempts to offset the negative effects of hypoxia on radiation therapy initially focused on manipulation of tumor oxygen status (e.g., fractionated radiation schedules to allow reoxygenation between fractions [Kallman and Dorie 1986]; hyperbaric

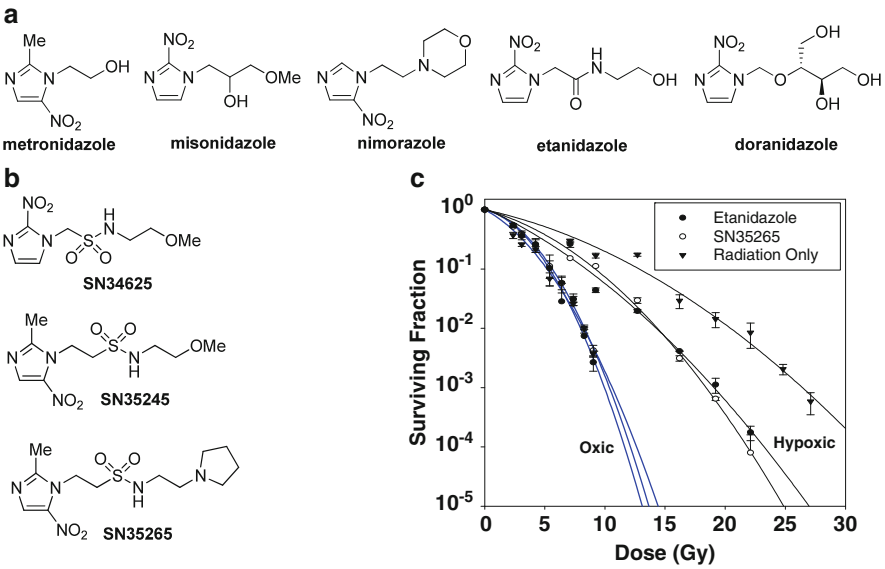


**Fig. 6.4** (**a**) Clinically investigated nitroimidazoles. (**b**) Representative novel 2- and 5-nitromidazole sulfonamides. (**c**) Clonogenic survival curves of HCT116 colorectal carcinoma cells after increasing dose of radiation in oxia (*blue*) and anoxia (*black*) in the presence of equitoxic doses of etanidazole (1 mM) and SN35265 (0.7 mmol)

oxygen treatment [Bennett et al. 2012; Overgaard and Horsman 1996], and nicotinamide in combination carbogen breathing with accelerated radiotherapy [ARCON] [Janssens et al. 2012; Kaanders et al. 2002]). Drug discovery efforts have been centered on the development of chemical radiosensitizers and, in particular, oxygen-mimetic sensitizers.

### *6.2.2 Nitroimidazole Oxygen Mimetics*

The concept of an electron-affinic nitroaryl molecule as a radiosensitizer in hypoxic tumor tissue has a long history (Dische 1985, 1991; Wardman 2007). The 5-nitroimidazole antibiotic metronidazole (Fig. 6.4) was identified as an effective radiosensitizer (Asquith et al. 1974) and displayed clinical benefit (Urtasun et al. 1976). More electron-affinic 2-nitroimidazoles were explored, and misonidazole was identified and advanced to clinical trials (Adams et al. 1976). Misonidazole underwent extensively trials with fractionated radiotherapy; despite indications of clinical benefit (Overgaard 1994), delayed peripheral neuropathy limited treatment (Grigsby et al. 1999; Saunders and Dische 1996). The electron affinity of the nitroimidazole group is the key parameter for radiosensitization and toxicity (Adams et al. 1979a, b). Thus, 5-nitroimidazoles with lower electron affinity had lower

toxicity and larger doses could be used to offset their weaker radiosensitization. This led to the identification of nimorazole as a radiosensitizer (Overgaard et al. 1982, 1983) that is well tolerated (Overgaard et al. 1998; Timothy et al. 1984) and used clinically, but only in Denmark. Nimorazole is currently undergoing a phase III clinical trial with accelerated radiotherapy (Overgaard 2012).

Attempts to design more polar analogs with reduced lipophilicity and increased systemic clearance to minimize the neurotoxicity observed with misonidazole led to the development of etanidazole (Brown et al. 1981) and doranidazole (Murata et al. 2008; Oya et al. 1995). This approach was only partially successful: Etanidazole had reduced neurotoxicity compared to misonidazole (Coleman et al. 1990) but failed to provide benefit in head and neck cancer (Eschwege et al. 1997; Lee et al. 1995). Doranidazole is currently under investigation for pancreatic cancer (Karasawa et al. 2008) and non-small-cell lung carcinoma (Nishimura et al. 2007).

It is salutary to note that although nitroimidazole radiosensitizers have been extensively investigated clinically and that hypoxic modification was shown to be effective in a meta-analysis (Overgaard 2011), only nimorazole is in clinical use. Two main factors have contributed to the limited clinical success of radiosensitizers. Their use with fractionated radiotherapy – where fractionation of the radiation dose is designed to allow tumor reoxygenation between radiation fractions – reduces the potential for radiosensitization (Hill 1986; Kallman 1972). Fractionated radiotherapy ideally requires a dose of radiosensitizer with each fraction of radiation, a schedule that was unachievable with early 2-nitroimidazoles because of cumulative peripheral neurotoxicity. Perhaps most significant is that many of the trials were small and were conducted without prospectively identifying patients with hypoxic tumors, despite considerable heterogeneity in the level and extent of tumor hypoxia among patients (Hoogsteen et al. 2009).

However, the development of stereotactic body radiotherapy (SBRT) may offer a new opportunity for this class. SBRT uses hypofractionated (one to five doses), high-dose (25–60 Gy in total dose) radiation to treat primary tumors and oligometastases. Initial clinical results of using SBRT to treat a variety of primary tumors suggest locoregional control and toxicity profiles that compare to or improve on those of fractionated radiotherapy (Lo et al. 2010). Prospective, randomized trials to confirm these results compared to standard care will drive increasing use of SBRT. In addition, reduced treatment time and fewer patient visits, combined with emerging potential to replace surgery in patients for whom an outpatient procedure presents risk, indicates potential economic health advantages for SBRT. However, SBRT may accentuate the role of hypoxia in radioresistance because of the reduced opportunity for tumor reoxygenation during therapy (Brown et al. 2010; Carlson et al. 2011). This would offer the possibility of a renaissance for nitroimidazole radiosensitizers in conjunction with SBRT. A recent small, phase III trial of doranidazole in conjunction with intraoperative radiotherapy (25 Gy) for pancreatic tumors demonstrated a survival advantage (Nishimura et al. 2007).

Nevertheless, several barriers exist in the development of radiosensitizers for use with SBRT. Limited (doranidazole) or expired (misonidazole, etanidazole, nimorazole) patent protection for clinically evaluated nitroimidazoles will limit their

application, while the wide range of analogs prepared across the field restricts discovery of novel, patentable nitroimidazoles. The other challenge for future development of such radiosensitizers is the use of a biomarker to prospectively identify hypoxia in patients (See Sect. 6.5).

In addressing these challenges, we have recently identified a new class of nitroimidazole with a sulfonamide side chain, providing chemical novelty (Bonnet et al. 2012). A series of 2- and 5-nitroimidazole analogs have been designed and synthesized, and preliminary results show that representative compounds (Fig. 6.4b) produce comparable in vitro radiosensitization to etanidazole at nontoxic concentrations in hypoxic HCT-116 human colorectal carcinoma cells (Fig. 6.4c). The electron affinity of these compounds, as measured by one-electron reduction potential, is higher than corresponding 2- and 5-nitroimidazoles because of the influence of the strong electron-withdrawing side chain and results in increased radiosensitization. Metabolism is also increased in the more electron-affinic examples, resulting in hypoxia-selective cytotoxicity. This novel series provides the opportunity to leverage 30 years of drug development around the class and develop a third-generation radiosensitizer while including extravascular transport (EVT) (See Sect. 6.3.5) and hypoxia biomarker studies (See Sect. 6.5) early in the drug design process.

### *6.2.3 Molecular Targets in DNA Repair as Radiosensitizers*

The targeting of DNA repair for radiosensitization using antimetabolites (Brown et al. 1971) is well established, although these agents work through multiple mechanisms (Shewach and Lawrence 2007). A range of histone deacetylation inhibitors also radiosensitize tumor cells through modulation of the DNA damage response (Camphausen and Tofilon 2007). Specific DNA repair proteins such as poly(ADP-ribose) polymerase (PARP) (Chalmers et al. 2010), ataxia telangiectasia mutated (ATM) pharmacokinetics (Sarkaria and Eshleman 2001), ATM- and Rad3-related (ATR) pharmacokinetics (Wang et al. 2004), and DNA-dependent pharmacokinetics (Blunt et al. 1995) are potential targets for radiosensitization (Helleday et al. 2008; Begg et al. 2011). PARP inhibitors can radiosensitize tumors (Albert et al. 2007; Calabrese et al. 2004), although some of their activity may be due to a vascular effect that results in reduced intermittent hypoxia (Senra et al. 2011). A range of PARP inhibitors are in clinical development and offer potential as radiosensitizers. Novel ATM inhibitors (KU55933 [Hickson et al. 2004] and KU60019 [Golding et al. 2009]) and ATR inhibitors (NU6027 [Peasland et al. 2011] and VE821 [Charrier et al. 2011; Reaper et al. 2011]) display radiosensitization in vitro (Pires et al. 2012). The selective DNA pharmacokinetics inhibitor NU7441 can radiosensitize tumor cells in vitro and in vivo (Zhao et al. 2006), whereas IC87361 (Kashishian et al. 2003) was reported to enhance radiation-induced delay in the growth of Lewis lung carcinomas (Shinohara et al. 2005). One concern about this approach is the potential for these agents to radiosensitize normal tissue within the radiation field. Although particular diseases may be

identified to provide synthetically lethal combinations (e.g., BRCA1 loss of function in combination with PARP inhibitors), another approach is to selectively target these agents to hypoxic tissues using a prodrug approach (Parveen et al. 1999; Cazares-Korner et al 2013).

## 6.3 Hypoxia-Activated Prodrugs

### *6.3.1 Introduction*

HAPs (also called bioreductive prodrugs or hypoxia-selective cytotoxins) can be grouped into six classes based on their activation chemistry (Fig. 6.5). Quinone prodrugs such as EO9, based on the reductive activation of mitomycin C, were the first class to be explored (Lin et al. 1972; Phillips et al. 2013). The observation that redox cycling could provide a basis for hypoxia-selective cytotoxicity of nitroaryl compounds (Mason and Holtzman 1975) was followed by observations that some nitroimidazole radiosensitizers were also selectively toxic to hypoxic tumor cells in culture (Hall and Roizin-Towle 1975; Mohindra and Rauth 1976). This led to extensive studies of nitroheterocycles as hypoxia-activated prodrugs (Jenkins et al. 1990; Naylor et al. 1990; Threadgill et al. 1991), culminating in the bifunctional prodrug RB-6145 (Naylor et al. 1993), in which an alkylating bromoethylamine side chain increased cytotoxic potency (Hill et al. 1986). Clinical development of RB-6145 and its R-enantiomer (CI-1010) (Cole et al. 1992) was halted because of retinal toxicity

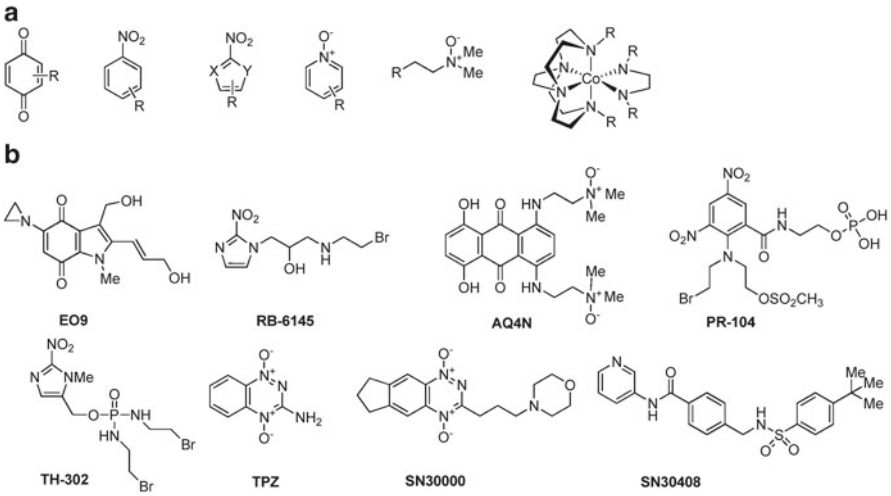


**Fig. 6.5** (**a**) Main chemical classes used for hypoxia-activated prodrugs (HAPs). (**b**) Examples of HAPs

in preclinical models, providing early evidence that hypoxia in normal tissues could result in dose-limiting toxicities (Breider et al. 1998; Lee and Wilson 2000).

Description of the principles of bioreductive activation of nitroaryl prodrugs of nitrogen mustard (Denny and Wilson 1986) laid the groundwork for the eventual discovery of PR-104 as a HAP (Patterson et al. 2007). The hypoxic selectivity of aromatic N-oxides based on the 1,2,4-benzotriazine system led to the identification of tirapazamine (TPZ) (Brown 1993). Aliphatic N-oxides were shown to compete with oxygen for reduction by two-electron reductases, providing a mechanism for masking the DNA binding of DNA intercalators (Patterson 1993; Wilson et al. 1992), such as AQ4N (banoxantrone). Stable transition metal complexes (e.g., Co[III] [Milbank et al. 2009; Ware et al. 1993; Yamamoto et al. 2012] and Cu[II] [Parker et al. 2004]) can undergo hypoxia-selective, one-electron reduction to relatively unstable complexes (e.g., Co[II] and Cu[I]), releasing a cytotoxic agent. A vast assortment of compounds from these classes has been explored in the laboratory but only a handful have been evaluated clinically. Several of these provide informative examples of the challenges facing HAP discovery and are briefly discussed below.

### *6.3.2 PR-104*

PR-104 arose from the structural optimization of simple nitroaryl nitrogen mustards (Denny and Wilson 1986) to selectively activated, diffusible mustard cytotoxins (Denny and Wilson 1993) and involved several design challenges. Elevation of the electron affinity of the 5-nitro group into a range suitable for bioreduction required additional electron-withdrawing substituents (e.g., a 3-$NO_2$ group) (Palmer et al. 1992). The relative arrangement of the four substituents provides the best combination of potency and hypoxic selectivity (Palmer et al. 1996). Addition of a carboxamide-linked solubilizing side chain (Palmer et al. 1994), combined with a phosphate prodrug approach, provides sufficient aqueous solubility.

The phosphate group is readily cleaved in plasma (Patel et al. 2007), and the nitro group then undergoes one-electron reduction to a nitro radical anion (Guise et al. 2007) (Fig. 6.6a), which is converted back to the prodrug in the presence of oxygen by redox cycling. Further reduction of the radical anion produces a nitrosobenzene that may undergo subsequent reduction to electron-donating hydroxylamine (PR-104H) and aminobenzene (PR-104M). These activated species cross-link DNA, forming cytotoxic lesions (Gu et al. 2009; Patterson et al. 2007; Singleton et al. 2009). PR-104 is activated under low oxygen concentrations (Hicks et al. 2007) (Fig. 6.2), but reduced species are sufficiently lipophilic and stable to diffuse from the cell of activation to surrounding tumor cells, known as the "bystander effect" (Foehrenbacher et al. 2013; Patterson et al. 2007; Wilson et al. 2007).

PR-104 displayed excellent in vitro hypoxic selectivity (6- to 160-fold), with single-agent activity and potentiation of radiation in SiHa, HT29, and H460 tumor xenografts (Patterson et al. 2007). PR-104 advanced to clinical trials (Jameson et al. 2010; McKeage et al. 2011), but normal tissue toxicity in humans prevented trials

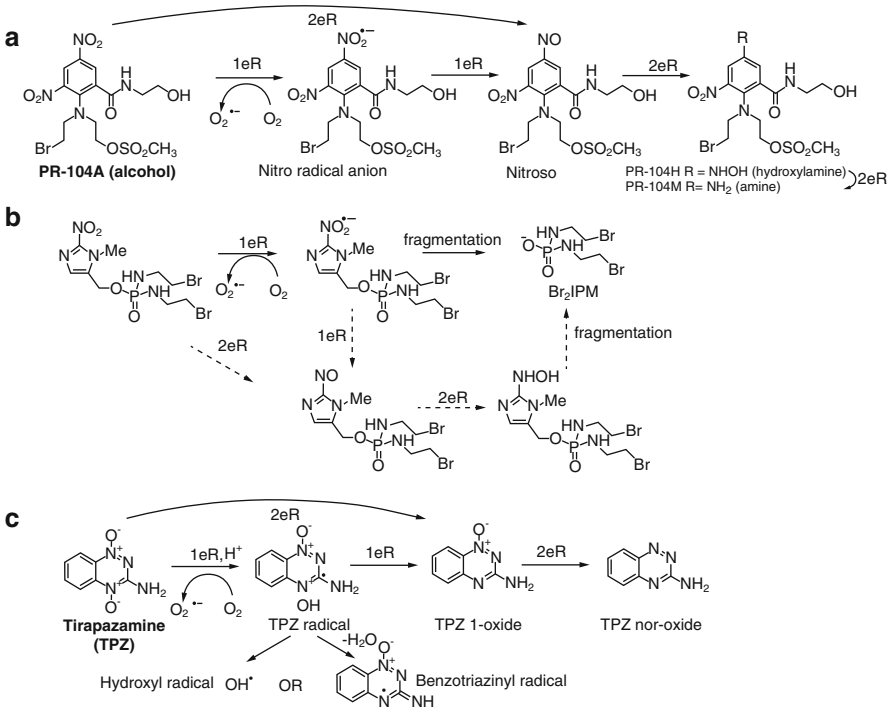


**Fig. 6.6** Mechanism of activation of leading hypoxia-activated prodrugs. (**a**) PR-104. One-electron reduction of PR-104A to the nitro radical anion is reversed in the presence of oxygen. Under hypoxia, further reduction of the radical anion leads sequentially to the deactivated nitroso and the activated hydroxylamine (PR-104H) and amine PR-104M. Two-electron reduction of PR-104A bypasses the nitro radical anion and is not hypoxia selective. (**b**) TH-302. One-electron reduction under hypoxia leads to a radical anion. Radiolytic studies have demonstrated direct fragmentation of the radical anion to release the bromo-phosphoramidate mustard ($Br_2$-IPM). An alternate, stepwise two-electron reduction to the 2-hydroxylamine and subsequent fragmentation has been previously proposed. (**c**) Tirapazamine. One-electron reduction gives an *N*-oxide radical that may be reoxidized by oxygen. Under hypoxia, protonation and then rearrangement produces a carbon-centered tirapazamine (TPZ) radical. This TPZ radical may then eliminate water to give a DNA-damaging benzotriazinyl nitrogen-centered radical or release a hydroxyl radical. Further reduction of the TPZ radical, or two-electron reduction of TPZ, leads to the relatively nontoxic 1-oxide and nor-oxide. An analogous activation mechanism has been proposed for the related benzotriazine dioxide SN30000

from reaching an efficacious dose (Patel et al. 2011). Activation of PR-104 by the oxygen-insensitive two-electron reductase aldo-ketoreductase AKR-1C3 (Guise et al. 2010), was subsequently suggested as a factor contributing to this toxicity. A new strategy to leverage the presence of both hypoxia and AKR-1C3 expression in particular tumor types, including advanced leukemia (Houghton et al. 2011; Benito et al. 2011), has led to subsequent clinical trials (www.ClinicalTrials.gov identifier NCT01037556).

### 6.3.3 TH-302

A versatile prodrug strategy based around the 2-nitroimidazole-5-methanol moiety was able to release enediynes (Hay et al. 1999), aspirin (Everett et al. 1999), and a PARP inhibitor (Parveen et al. 1999) in a hypoxia-selective manner; nitroheterocyclic prodrugs of phosphoramidate mustards were shown to release cytotoxins upon reduction (Borch et al. 2000, 2001). These studies were a precursor to the discovery of TH-302, a 2-nitroimidazole-5-methyl phosphoramidite, as a HAP with excellent hypoxic selectivity (Duan et al. 2008; Meng et al. 2012). Steady-state and pulse radiolysis methods showed that TH-302 undergoes one-electron reduction and fragmentation to release bromo-isophosphoramide mustard (Meng et al. 2012) but did not exclude the initially proposed stepwise reduction of 2-nitroimidazole prodrugs to hydroxylamine or amine and fragmentation via an iminomethide (Borch et al. 2001) (Fig. 6.6b). The increased toxicity observed in cells that overexpress bacterial nitroreductase provides evidence of the potential for oxygen-insensitive, two-electron reduction and release of bromo-isophosphoramide mustard (Meng et al. 2012). The released mustard generates DNA cross-links that are responsible for hypoxic cytotoxicity (Meng et al. 2012). Extensive preclinical studies have shown the antitumor activity of TH-302 – either as a single agent (Sun et al. 2012) or in combination with commonly used chemotherapeutic drugs (Liu et al. 2012) and radiation (Lohse et al. 2012) – in many animal xenograft models. The anticancer efficacy of TH-302 correlated well with the levels of xenograft tumor hypoxia, confirming the hypoxic specificity of drug action in vivo (Lohse et al. 2012; Sun et al. 2012). TH-302 is currently the most advanced HAP in clinical development. Promising outcomes from phase II clinical trials (Borad et al. 2012; Chawla et al. 2011) led to the commencement of two randomized, placebo-controlled, phase III trials: one with TH-302 in combination with doxorubicin for advanced soft tissue sarcoma and the other in combination with gemcitabine for advanced pancreatic cancer.

### 6.3.4 Tirapazamine

TPZ (tirazone) is the prototypic example of a heterocyclic N-oxide HAP and dominated the field for almost two decades (Brown 1993, 2010; Denny and Wilson 2000). TPZ shows highly selective killing in cell culture under hypoxic compared to aerobic conditions (Zeman et al. 1986) as a result of rapid bioreductive metabolism (Baker et al. 1988; Hicks et al. 2003; Siim et al. 1996). One-electron reduction by, for example, NADPH:cytochrome P450 oxidoreductase (Fitzsimmons et al. 1994; Patterson et al. 1997, 1998), inducible nitric oxide synthase (Chinje et al. 2003), or nuclear localized reductases (Evans et al. 1998) produces a N-centered radical (Baker et al. 1988; Laderoute et al. 1988) that is efficiently back-oxidized to TPZ by oxygen (Fig. 6.6c). In the absence of oxygen, protonation and rearrangement leads

to an oxidizing radical (Anderson et al. 2003; Shinde et al. 2009, 2010; Yin et al. 2012) or hydroxyl radical (Chowdhury et al. 2007; Daniels and Gates 1996), both of which have been proposed as the species that damages cytotoxic DNA. DNA damage measured by comet assay (Olive et al. 1996; Siim et al. 1996) or induction of γH2AX (Olive et al. 2004; Wang et al. 2012b) correlates with the rates of bioreduction and reductase expression (Wang et al. 2012b) and is repaired by multiple mechanisms, including homologous recombination repair of double-strand breaks (Evans et al. 2008; Hunter et al. 2012). The radical species are short-lived and do not contribute to the killing of surrounding cells. Despite the lack of the bystander effect, TPZ is able to kill cells at intermediate oxygen concentrations because of activation at relatively high oxygen concentrations, with K-values (oxygen concentration for half-maximal hypoxic potency) in the range 1–3 μM (Hicks et al. 2004, 2007; Koch 1993), resulting in good complementarity with radiation (Hicks et al. 2004, 2007; Koch 1993; Wouters and Brown 1997), (Fig. 6.2). In contrast to PR-104 and TH-302, the two- and four-electron reduction products are markedly less cytotoxic than the parent drug (Baker et al. 1988), but this unproductive metabolism reduces potency.

Xenograft studies demonstrated cell killing complementing that of single-dose (Zeman et al. 1988) and fractionated radiation (Brown and Lemmon 1990, 1991). TPZ also demonstrated synergy with cisplatin in preclinical tumor models (Dorie and Brown 1993, 1994), resulting from hypoxia-dependent inhibition of cisplatin DNA cross-link repair (Kovacs et al. 1999).

TPZ has been intensively studied in clinical trials in combination with radiation and chemotherapy in head and neck (Rischin et al. 2005, 2010b), non-small-cell lung (Sandler et al. 2000; Shepherd et al. 2000; von Pawel et al. 2000; Williamson et al. 2005) and cervical carcinomas (Aghajanian et al. 1997; Covens et al. 2006; Craighead et al. 2000; DiSilvestro et al. 2012; Maluf et al. 2006; Rischin et al. 2010a) and has been extensively reviewed (Ghatage and Sabagh 2012; McKeown et al. 2007; Reddy and Williamson 2009). TPZ was well tolerated in early phase trials at doses resulting in plasma drug concentrations in the therapeutic range (Johnson et al. 1997; Senan et al. 1997). Early trials produced signs of activity with the initial phase III trial of TPZ/cisplatin in advanced non-small-cell lung cancer, demonstrating increased overall survival relative to cisplatin and radiation alone (von Pawel et al. 2000). This indication of activity was not confirmed in larger, randomized phase III trials in head and neck (Rischin et al. 2010b) and cervical carcinomas (DiSilvestro et al. 2012), and further development of TPZ has been halted.

Several issues were identified as affecting the efficacy of TPZ as a HAP. TPZ demonstrated significant toxicities that limited the therapeutic ratio (Ghatage and Sabagh 2012; McKeown et al. 2007; Reddy and Williamson 2009). TPZ also has low solubility, which required long infusion times (Graham et al. 1997; Senan et al. 1997). In addition, preclinical studies demonstrated that TPZ is substantially less selective for hypoxic cells in three-dimensional (3D) culture (Durand and Olive 1992) or xenografts (Durand and Olive 1997) than in monolayer cell culture, a consequence of limited EVT (Hicks et al. 1998).

### 6.3.5 *Discovery of a Second-Generation Benzotriazine Dioxide (SN30000)*

With these issues in mind we embarked on the discovery of a second-generation benzotriazine dioxide (BTO) as a HAP. Our aim was to identify TPZ analogs with superior activity against hypoxic cells in tumors by improving the solubility-potency product, hypoxia selectivity, and EVT using the end point of improved therapeutic activity in preclinical xenograft models at equivalent toxicity. It also was necessary to identify chemically novel compounds to secure an intellectual property position to support development.

A limited number of TPZ analogs had been prepared and evaluated (Kelson et al. 1998; Minchinton et al. 1992; Zeman et al. 1989), and little information on structure-activity relationships (SARs) existed. We prepared an initial toolset of 42 compounds with a range of substituents to explore SARs and we confirmed the positive relationship between the one-electron reduction potential, E(1), and anoxic potency in both clonogenic and growth inhibition ($IC_{50}$) assays (Hay et al. 2003).

EVT was investigated using multicellular layers (MCLs), a model of the tumor extravascular compartment in which cells are grown on porous support membranes in culture inserts submerged in culture medium (Cowan et al. 1996; Minchinton et al. 1997) and form diffusion-limited structures with central hypoxia (Hicks et al. 1998). Anoxia reduced TPZ transport in MCLs (Hicks et al. 1998, 2003; Kyle and Minchinton 1999), and reaction diffusion modeling using measured TPZ diffusion coefficients and rate constants for anoxic metabolism predicted steep gradients of TPZ in hypoxic tumor tissue, resulting in reduced cell killing. A spatially resolved pharmacokinetic/pharmacodynamic model for HT29 MCLs incorporating cytotoxic potency measured in anoxic cell cultures predicted increased resistance to TPZ in anoxic MCLs compared to stirred suspensions (Hicks et al. 2003). This confirmed that multicellular resistance to TPZ in anoxic 3D culture was primarily a result of limited transport and was responsible for the reduced efficacy of TPZ in 3D models (Durand and Olive 1992, 1997). This model was extended to tumors by incorporating the measured oxygen dependence (K-curve) of TPZ metabolism (Hicks et al. 2004) and measured TPZ plasma pharmacokinetics to simulate TPZ transport in a mapped microvascular network (Hicks et al. 2006). The model predicted that cell killing by TPZ in the hypoxic region is reduced relative to that achievable with no EVT limitation. In addition, the model successfully predicted activity of TPZ and 15 analogs from the SAR toolset in HT29 xenografts using measured plasma pharmacokinetics, transport parameters, and anoxic cytotoxicity (Hicks et al. 2006).

We also used the molecular toolset to investigate the SAR for transport, demonstrating that diffusion coefficients in HT29 MCLs increased with increasing $logP_{7.4}$ and decreased with molecular weight, number of hydrogen bond donors, and acceptors (Pruijn et al. 2005, 2008).

After developing the tools to efficiently evaluate novel BTO analogs, we used the screening method guided by the pharmacokinetic/pharmacodynamic model (Fig. 6.7a)

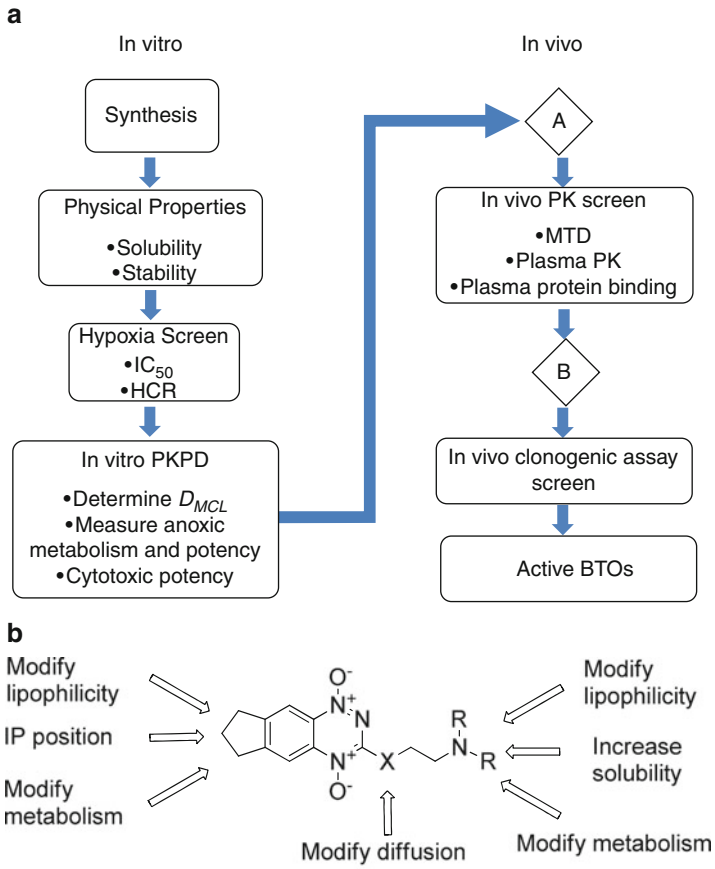


**Fig. 6.7** (**a**) A pharmacokinetic/pharmacodynamic (PKPD)-guided screening algorithm that incorporates drug penetration. After initial screening for physicochemical properties and hypoxia selectivity, parameters governing drug penetration (diffusion coefficient and rate of bioreductive metabolism) were measured in vitro or calculated and used in a spatially resolved PKPD model to calculate the drug exposure (AUC) required for 1 log of cell killing in addition to radiation alone. Compounds that demonstrated in vivo hypoxia selectivity at achievable AUC (*Prediction A*) were advanced to in vivo screening (*MTD*, *plasma PK*). The model was then run with measured plasma pharmacokinetics as input, and compounds predicted to add >0.3 log cell killing in addition to radiation alone (*Prediction B*) were advanced to in vivo clonogenic assay screens. (**b**) General structure of tricyclic benzotriazine dioxides, indicating drug design considerations

to specifically consider EVT at an early stage in the drug design process and to predict in vivo hypoxia selectivity resulting from changes in EVT. Increased bioreduction produces competing effects of increasing potency and decreasing EVT; thus designing improved analogs requires optimizing potency and EVT rather than simply

maximizing any individual parameter. A range of structural variations were explored in an effort to optimize these parameters (Fig. 6.7b) (Hay et al. 2007a, b, 2008). The confidence gained using the spatially resolved pharmacokinetic/pharmacodynamic validation allowed us to screen a large number of analogs in vitro and base our SAR on predicted in vivo hypoxic cell killing rather than conduct extensive in vivo testing. Diffusion coefficients and rates of reductive metabolism in the analog series varied by more than 100-fold (Hicks et al. 2010), and a high correlation between predicted and observed activity was found in initial HT29 xenograft screening. The addition of a third saturated ring to the benzotriazine core provided reduced hypoxic metabolism and increased lipophilicity, which increased EVT and created chemical novelty to substantiate an intellectual property position. The addition of a basic amine side chain increased aqueous solubility but reduced lipophilicity and affected hypoxic metabolism, reducing EVT. While the optimization of two SARs for EVT and metabolism provided analogs with superior in vivo hypoxic selectivity, their in vivo activity was influenced by a third SAR for host toxicity. This is exemplified by the variation in maximum tolerated doses, and consequently AUC, as a function of lipophilicity and amine pKa (Fig. 6.8). Whereas SN29143 was predicted to have substantially improved activity compared to TPZ, poor plasma AUC precluded in vivo activity. Attempts to improve the pharmacokinetics by modulating lipophilicity and amine pKa led to a high AUC and improved EVT but very low hypoxic potency, which compromises the activity of SN29434. Increasing both lipophilicity and pKa increased host toxicity and lowered AUC (SN29467). Replacing the strongly electron-donating 3-amino substituent with a weaker 3-alkyl substituent led to increased EVT from increased lipophilicity and increased hypoxic potency from higher rates of metabolism (SN30000). Substituents resulting in higher lipophilicity (SN30124) and higher pKa (SN30080, SN30081) resulted in a similar trend of increasing toxicity and poorer plasma AUC, as described above. SN30000 was predicted to be substantially more active than TPZ and SN29434 as a result of low toxicity and good plasma AUC, and this was demonstrated in the HT29 xenograft model.

SN30000 emerged as the lead tricyclic BTO from this program, with broadly improved activity relative to TPZ. Aqueous solubility is improved by almost an order of magnitude (Hicks et al. 2010). SN30000 demonstrates higher potency and hypoxic selectivity than TPZ in $IC_{50}$ assays across a panel of cell lines and in clonogenic assay in HT29 cells (Hicks et al. 2010). It is important to note that the measured K-value of SN30000 is not significantly different ($1.14 \pm 0.24$ μM oxygen) from TPZ ($1.21 \pm 0.09$ μM oxygen), indicating retention of the desirable property of activation at intermediate oxygen concentrations. Improved EVT for SN30000 was confirmed experimentally, with a threefold higher diffusion coefficient than TPZ in HT29 and SiHa MCLs (Hicks et al. 2010). SN30000 shows increased activity relative to TPZ against hypoxic cells in combination with single-dose or fractionated radiation in several tumor xenografts (HT29, SiHa, H460) by in vivo clonogenic assay and superior activity in SiHa xenografts with fractionated radiation by a delay in tumor regrowth (Hicks et al. 2010). SN30000 is currently in preclinical development with Cancer Research UK.

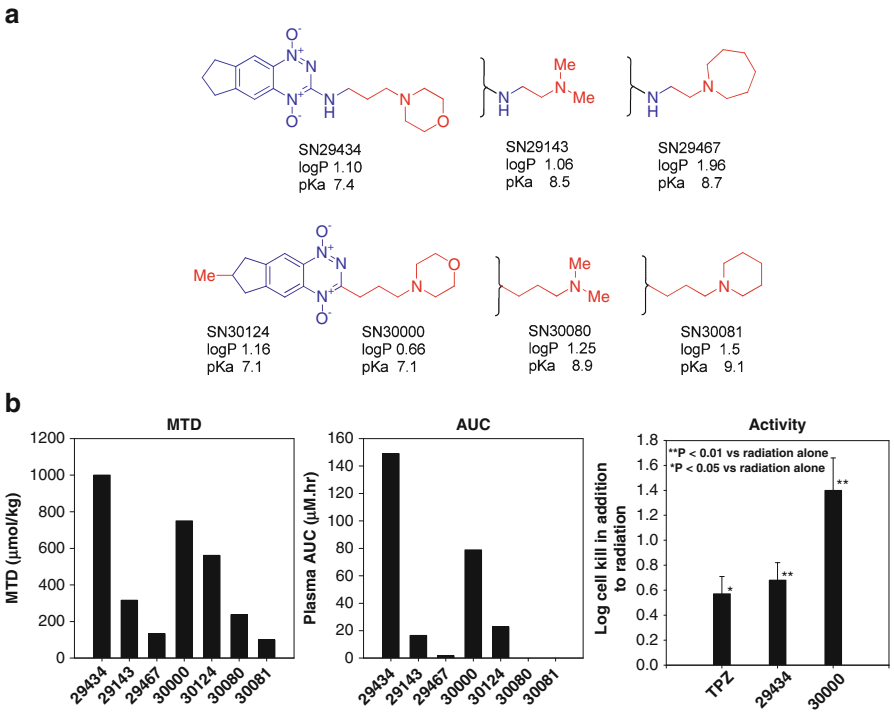


**Fig. 6.8** (**a**) Structure of an amine series of tricyclic benzotriazine dioxides, indicating the range of physicochemical parameters explored. (**b**) The maximum tolerated doses (*MTDs*), drug exposure (*AUC*), and hypoxic cell killing by compounds in combination with radiation (20 Gy) in HT29 xenografts by in vivo clonogenic survival assay

## 6.4 Targeting the Hypoxia Response Pathway

### *6.4.1 Introduction*

The HIF family of transcription factors is well established as the key mediator of the adaptive response to hypoxia, and their role in cancer has been extensively described (Poon et al. 2009; Semenza 2003, 2010). These transcription factors are the primary oxygen sensors and use oxygen and 2-ketoglutarate as substrates for the hydroxylation of specific proline residues on HIF-1α or HIF-2α by prolyl hydroxylase domain enzymes. This allows binding by the von Hippel-Lindau (VHL) factor and recruitment of an ubiquitin ligase complex that initiates ubiquitination and proteasomal degradation. In the absence of oxygen, HIF-1α is able to bind the constitutively expressed HIF-1β and coactivation partners, bind to hypoxia response elements (HREs), and activate transcription of a variety of genes involved in

angiogenesis, metabolic adaption, cell survival, and metastasis. However, HIF-1α activation may also be induced by other stimuli, including genetic changes to tumor suppressors (e.g., VHL [Kaelin 2008]) or tumor activators (e.g., Ras [Mazure et al. 1996]), growth factor stimulation (e.g., IGF-R [Ren et al. 2010]), and depletion of ascorbate (Kuiper et al. 2010). In addition, the differential expression and roles of HIF-1α and HIF-2α need to be considered (Carroll and Ashcroft 2006).

Inhibition of HIF-1α activity has been shown to slow angiogenesis and tumor growth in xenograft models (Maxwell et al. 1997), whereas inhibition of HIF-1α activity sensitizes hypoxic cells to conventional therapies (Moeller et al. 2004, 2005; Williams et al. 2005). The negative impact of HIF1α overexpression on treatment response and outcomes across a range of human tumors is also well described (Jubb et al. 2010; Semenza 2007). Multiple targets within the HIF-1α signaling pathway have been identified as a candidate drug targets (Giaccia et al. 2003; Semenza 2007). As a consequence, there has been a plethora of HIF-1 inhibitors that have been extensively reviewed (Poon et al. 2009; Semenza 2007; Xia et al. 2012). These inhibitors may be characterized as direct (interference with HIF-1α synthesis, stability, or binding to transcription partners and HREs) or indirect via the myriad of upstream or downstream participants in the hypoxia response network.

### *6.4.2 Direct HIF-1α Inhibitors*

Direct inhibition of HIF1α translation has been demonstrated by a wide range of agents through multiple mechanisms, with the topoisomerase-I inhibitor topotecan the best-described example. Topotecan was identified as an inhibitor of HIF-1α translation (Rapisarda et al. 2002) by a topoisomerase-I-dependent mechanism, but at concentrations below those necessary for DNA damage–mediated cytotoxicity (Rapisarda et al. 2004a). As well as inhibiting HIF1α protein expression and tumor growth in a glioma xenograft model (Rapisarda et al. 2004b), combination of daily low-dose topotecan with bevacizumab provided significantly increased tumor cell killing in U251-HRE xenografts compared to either agent alone (Rapisarda et al. 2009). Topotecan recently completed a phase I clinical trial exploring its effect on HIF-1α, and reduced HIF-1α expression was observed in some patients (Kummar et al. 2011). CPT-11 (EZN-2208), a more potent, soluble prodrug (Sapra et al. 2008), provides improved suppression of HIF-1α and downstream gene targets (Sapra et al. 2011) and is in a phase II trial as both a cytotoxin and an HIF-1α inhibitor in combination with bevacizumab (www.ClinicalTrials.gov identifier NCT01251926).

Many of the compounds reported as HIF-1α inhibitors are not specific for HIF-1α or have multiple mechanism of action. Examples of this are seen with the HSP90 inhibitors geldanamycin and 17AAG (Isaacs et al. 2002; Mabjeesh et al. 2002) and with inhibitors of thioredoxin-1, such as PX12, which inhibits assembly of the transcription complex but has other effects (Welsh et al. 2003). PX12 has completed a phase I clinical trial in which stable disease was seen in patients with elevated levels of thioredoxin-1 (Ramanathan et al. 2007).

### 6.4.3 Indirect HIF Inhibitors

Indirect approaches take advantage of the network of upstream stimulating factors (e.g., the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin pathway [Zhong et al. 2000] and the Ras/mitogen-activated pharmacokinetics pathway [Berra et al. 2000]) and downstream target genes (Semenza 2010) and may provide HIF-1α inhibition via multiple pathway interactions. The use of the multikinase inhibitor sorafenib in the treatment of advanced renal cell carcinoma (RCC) highlights this approach (Rini 2010). Advanced RCC is driven by HIF stabilization via the loss of functional VHL in a majority of cases and displays a highly angiogenic and invasive phenotype. Although sorafenib is primarily aimed at targeting downstream kinases involved directly in angiogenesis (vascular endothelial growth factor receptor-2 and -3 and platelet-derived growth factor receptor), its inhibition of upstream BRAF can also affect HIF-1 activity (Wilhelm et al. 2008).

### 6.4.4 Targeting Glucose Metabolism

Many of these downstream HIF targets are associated with the cellular reprogramming of metabolism from oxidative phosphorylation to aerobic glycolysis. This shift supports biosynthesis to maintain expansive tumor growth and presents a wide range of potential targets to disrupt tumor cell metabolism (Jones and Schulze 2012). Although regulated by other signaling factors such as p53 and Myc, HIF-1α plays an important role in the regulation of the glycolytic pathway (Cairns et al. 2011). Hypoxic cells are particularly vulnerable to reductions in the production of adenosine triphosphate, and so inhibition of glycolysis is potentially an effective strategy against hypoxic cells (Kurtoglu et al. 2007). This was first demonstrated for 2-deoxy-D-glucose (Song et al. 1976), which, after phosphorylation, inhibits hexokinases and their association with mitochondria. Although tolerated by patients in phase I/II trials, there is a dearth of published information on the efficacy of 2-deoxy-D-glucose in patients (Jones and Schulze 2012).

The glucose transporter GLUT-1 has been shown to be elevated in many tumor types and is a negative prognostic factor (Macheda et al. 2005). Although a variety of glucose transport inhibitors have been reported, many are not selective for GLUT-1 or have multiple mechanisms of action, making assessment of their value for targeting tumor metabolism difficult. For example, phloretin, a competitive inhibitor of GLUT-1, slows tumor growth (Kobori et al. 1997) and can sensitize tumor cells to chemotherapeutics under hypoxic conditions (Cao et al. 2007). However, it can also interact with the monocarboxylate lactate transporter MCT-4 (Dimmer et al. 2000).

A new strategy to identify agents that are selectively cytotoxic to cells overexpressing HIF-1α used a synthetic lethality approach (Kaelin 2005) based on VHL-deficient RCCs (Chan and Giaccia 2008; Sutphin et al. 2007). In this cell line, loss of functional VHL leads to constitutive expression of HIF-1α and mimics chronic

hypoxia. A high-throughput screen of small molecules with paired VHL-proficient/-deficient cell lines was used to identify compounds that selectively kill VHL-deficient cells (Sutphin et al. 2007). This approach furnished a series of compounds with diverse properties (Bonnet et al. 2011; Hay et al. 2010; Turcotte et al. 2008) (see Chap. 9). We conducted an SAR study around one class (3-pyridyl benzamidophenyl sulfonamides) and identified analogs with submicromolar cytotoxic potency and selectivity for von Hippel-Lindau negative (VHL-ve) RCC cells in excess of 100-fold in vitro (e.g., SN30408, also known as STF-31) (Sutphin et al. 2011). The presence of a 3-pyridyl carboxamide was key to this activity. Substituents on this ring or the central phenyl ring reduced activity. The methyl sulfonamide linker was required for activity, whereas a wide range of substituents were tolerated on the terminal ring. This SAR was used to design affinity chromatography reagents that selectively bind to GLUT-1 (Chan et al. 2011). Molecular modeling studies using a homology model of GLUT-1 (Salas-Burgos et al. 2004) predicted that SN30408 and related molecules could bind within the central solute channel and interact with ARG126 and TRP412, both key residues for glucose transport (Brockmann et al. 2001). SN30408 seems to occupy a similar binding location to fasentin (Wood et al. 2008) and a series of recently described thiazolidinedione inhibitors that inhibited glucose transport in LNCaP prostate carcinoma cells (Wang et al. 2012a). SN30408 was shown to bind to GLUT-1 and selectively inhibit glucose uptake into VHL-ve RCC cells that overexpress GLUT-1, resulting in necrotic cell death (Chan et al. 2011). A key concern with targeting glucose transport is the effect on normal tissues, such as in the case of GLUT-1, erythrocytes, and the blood-brain barrier. Although it reduced glucose uptake into erythrocytes, SN30408 did not cause hemolysis. This was further monitored in vivo, where $^{18}$F-2-fluorodeoxyglucose positron-emission tomography (PET) demonstrated that VHL-ve tumors had high uptake of glucose and that treatment with nontoxic doses of a more soluble analog (SN31154) consistently reduced this uptake while having a minimal effect on the use of glucose in the brain. Daily treatment with a nontoxic dose of SN31154 over 14 days inhibited tumor growth in vivo (Chan et al. 2011).

## 6.5 Identifying the Target in Patients

As targeted therapies move into the clinic, it becomes increasingly important to identify patients with susceptible tumor cell populations who may benefit clinically (Basu 2010; Mok 2011). To fully exploit hypoxia with targeted therapy, the use of biomarkers to select suitable patients and assess response to treatment will greatly aid clinical development.

While polarographic electrodes have demonstrated a wide range of oxygen tensions in solid tumors (Nordsmark et al. 2005) and hypoxia status has been related to outcome in a range of tumor types (Vaupel and Mayer 2007), this approach is limited to accessible tumors. Tumor oxygenation may also be evaluated using nuclear

magnetic resonance techniques with exogenous fluorocarbon markers for $^{19}F$ nuclear magnetic resonance or blood oxygen level–dependent magnetic resonance imaging (Tatum et al. 2006) (See Chap. 16).

Recent reports of hypoxic gene signatures in various cancer sites (Buffa et al. 2010; Chi et al. 2006; Jubb et al. 2010; Murat et al. 2009; Winter et al. 2007) have related clinical outcome following standard treatments. A signature of 15 hypoxic genes was developed, validated, and used to retrospectively analyze head and neck squamous cell carcinoma (HNSCC) samples from the DAHANCA5 trial (Toustrup et al. 2011). This analysis demonstrated that only patients with hypoxic tumors defined by the hypoxic gene signature benefited from nimorazole.

Exogenous nitroimidazole hypoxia probes such as pimonizadole or EF5, with immunostaining by antibodies to the reduced adducts (Evans et al. 2000; Raleigh et al. 1998), have been used clinically. In a substudy of the ARCON trial pimonidazole was used to measure tumor hypoxia in patients with laryngeal cancer. For patients with higher pimonidazole labeling, ARCON provided benefit in terms of local control and 5 years of disease-free survival (Janssens et al. 2012).

More convenient approaches using circulating surrogate hypoxic markers in blood, such as osteopontin (Le et al. 2003), hepatocyte growth factor, and interleukin-8 (Le et al. 2012) have provided equivocal results. In the DAHANCA5 trial, patients with high levels of plasma osteopontin were shown to benefit from the addition of nimorazole, while patients with intermediate or low osteopontin showed no benefit (Overgaard et al. 2005). However, osteopontin failed to show any correlation with adverse outcome or benefit from the addition of hypoxia-targeted therapy in the TROG 02.02 phase III trial, in which patients with stage III/IV HNSCC received chemoradiotherapy and TPZ (Lim et al. 2012). In the same trial, two other hypoxic markers – hepatocyte growth factor and interleukin-8 – gave some predictive indication (Le et al. 2012).

PET using 2-nitroimidazole–based markers such as $^{18}F$-misonidazole (Lee et al. 2009), $^{18}F$-EF5 (Koch et al. 2010; Komar et al. 2008), and $^{18}F$-HX4 (Dubois et al. 2011; van Loon et al. 2010) has been explored as a noninvasive method for measuring hypoxia (Horsman et al. 2012). In a phase II trial of patients with HNSCC who were treated with chemoradiotherapy with or without TPZ, patients with hypoxic tumors identified using $^{18}F$-fluoromisonidazole PET fared significantly better when treated with TPZ compared to standard chemoradiotherapy (Rischin et al. 2006). Despite this, PET was not used for patient selection in the subsequent phase III trial, which failed to demonstrate a benefit for the addition of TPZ to chemoradiotherapy (Ang 2010; Rischin et al. 2010b).

The clinical development of HAPs would benefit from biomarkers that interrogate multiple elements of their sensitivity. We recently demonstrated that the hypoxic activation of EF5 is highly correlated with activation of SN30000 (and TPZ) across a panel of human tumor cell lines (Wang et al. 2012b). This study suggests that PET imaging with [$^{18}F$]-EF5 will report on both hypoxia and the activity of the one-electron reductases for SN30000 in hypoxic regions of tumors, without having to identify all the contributors to activation.

## 6.6 Conclusions

Although there is clear evidence that hypoxia limits the response to therapy, extensive drug discovery efforts have delivered limited success in clinically targeting hypoxia. This failure may be attributed in part to difficulties faced by academic groups and small biotechnology companies advancing novel agents to clinical trial. It is beneficial to develop agents in combination with radiotherapy when hypoxia contributes greatly to resistance to therapy. An important issue is the failure to recognize hypoxia-directed drugs as targeted therapies, develop biomarkers to aid in the selection of patients for treatment, and monitor response. In each of three hypoxia-directed approaches under development in our laboratories, we are identifying appropriate biomarkers, while the radiosensitizer and SN30000 will be developed in conjunction with radiotherapy.

## References

Adams PC, Rickert DE (1995) Metabolism of [C-14]1,3-dinitrobenzene by rat small intestinal mucosa in vitro. Drug Metab Dispos 23:982–987

Adams GE, Dische S, Fowler JF et al (1976) Hypoxic cell sensitisers in radiotherapy. Lancet 1:186–188

Adams GE, Clarke ED, Flockhart IR et al (1979a) Structure-activity relationships in the development of hypoxic cell radiosensitizers. I. Sensitization efficiency. Int J Radiat Biol Relat Stud Phys Chem Med 35:133–150

Adams GE, Clarke ED, Gray P et al (1979b) Structure-activity relationships in the development of hypoxic cell radiosensitizers. II. Cytotoxicity and therapeutic ratio. Int J Radiat Biol Relat Stud Phys Chem Med 35:151–160

Aghajanian C, Brown C, O'flaherty C et al (1997) Phase I study of tirapazamine and cisplatin in patients with recurrent cervical cancer. Gynecol Oncol 67:127–130

Albert JM, Cao C, Kim KW et al (2007) Inhibition of poly(ADP-ribose) polymerase enhances cell death and improves tumor growth delay in irradiated lung cancer models. Clin Cancer Res 13:3033–3042

Anderson RF, Shinde SS, Hay MP et al (2003) Activation of 3-amino-1,2,4-benzotriazine 1,4-dioxide antitumor agents to oxidizing species following their one-electron reduction. J Am Chem Soc 125:748–756

Ang KK (2010) More lessons learned from the suffocation of hypoxia. J Clin Oncol 28:2941–2943

Ask K, Dijols S, Giroud C et al (2003) Reduction of nilutamide by NO synthases: implications for the adverse effects of this nitroaromatic antiandrogen drug. Chem Res Toxicol 16:1547–1554

Asquith JC, Foster JL, Willson RL et al (1974) Metronidazole ("Flagyl"). A radiosensitizer of hypoxic cells. Br J Radiol 47:474–481

Baker MA, Zeman EM, Hirst VK et al (1988) Metabolism of SR 4233 by Chinese hamster ovary cells: basis of selective hypoxic cytotoxicity. Cancer Res 48:5947–5952

Basu S (2010) Personalized versus evidence-based medicine with PET-based imaging. Nat Rev Clin Oncol 7:665–668

Begg AC, Stewart FA, Vens C (2011) Strategies to improve radiotherapy with targeted drugs. Nat Rev Cancer 11:239–253

Benito J, Shi Y, Szymanska B et al (2011) Pronounced hypoxia in models of murine and human leukemia: high efficacy of hypoxia-activated prodrug PR-104. PLoS One 6:e23108
Bennett MH, Feldmeier J, Smee R et al (2012) Hyperbaric oxygenation for tumor sensitisation to radiotherapy. Cochrane Database Syst Rev 4, CD005007. doi:10.1002/14651858.CD005007.pub3
Berra E, Pages G, Pouyssegur J (2000) MAP kinases and hypoxia in the control of VEGF expression. Cancer Metastasis Rev 19:139–145
Bindra RS, Crosby ME, Glazer PM (2007) Regulation of DNA repair in hypoxic cancer cells. Cancer Metastasis Rev 26:249–260
Blunt T, Finnie NJ, Taccioli GE et al (1995) Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine scid mutation. Cell 80:813–823
Bonnet M, Flanagan JU, Chan DA et al (2011) SAR studies of 4-pyridyl heterocyclic anilines that selectively induce autophagic cell death in von Hippel-Lindau-deficient renal cell carcinoma cells. Bioorg Med Chem 19:3347–3356
Bonnet M, Hay MP, Hicks KO et al (2012) Nitroimidazole compounds and their use in cancer therapy. New Zealand Patent
Borad MJ, Reddy R, Bahary N et al (2012) TH-302 plus gemcitabine vs. gemcitabine in patients with untreated advanced pancreatic adenocarcinoma. In: 37th European Society for Medical Oncology (ESMO) 2012 Congress, Vienna, 28 Sep–2 Oct 2012
Borch RF, Liu J, Schmidt JP et al (2000) Synthesis and evaluation of nitroheterocyclic phosphoramidates as hypoxia-selective alkylating agents. J Med Chem 43:2258–2265
Borch RF, Liu J, Joswig C et al (2001) Antitumor activity and toxicity of novel nitroheterocyclic phosphoramidates. J Med Chem 44:74–77
Breider MA, Ulloa HM, Pegg DG et al (1998) Nitro-Imidazole radiosensitizer-induced toxicity in cynomolgus monkeys. Toxicol Pathol 26:651–656
Brockmann K, Wang D, Korenke CG et al (2001) Autosomal dominant glut-1 deficiency syndrome and familial epilepsy. Ann Neurol 50:476–485
Brown JM (1979) Evidence for acutely hypoxic cells in mouse tumors, and a possible mechanism of reoxygenation. Br J Radiol 52:650–656
Brown JM (1993) SR 4233 (tirapazamine): a new anticancer drug exploiting hypoxia in solid tumors. Br J Cancer 67:1163–1170
Brown M (2010) Henry S. Kaplan Distinguished Scientist Award Lecture 2007. The remarkable yin and yang of tumor hypoxia. Int J Radiat Biol 86:907–917
Brown JM, Lemmon MJ (1990) Potentiation by the hypoxic cytotoxin SR 4233 of cell killing produced by fractionated irradiation of mouse tumors. Cancer Res 50:7745–7749
Brown JM, Lemmon MJ (1991) Tumor hypoxia can be exploited to preferentially sensitize tumors to fractionated irradiation. Int J Radiat Oncol Biol Phys 20:457–461
Brown JM, Wilson WR (2004) Exploiting tumor hypoxia in cancer treatment. Nat Rev Cancer 4:437–447
Brown JM, Goffinet DR, Cleaver JE et al (1971) Preferential radiosensitization of mouse sarcoma relative to normal skin by chronic intra-arterial infusion of halogenated pyrimidine analogs. J Natl Cancer Inst 47:75–89
Brown JM, Yu NY, Brown DM et al (1981) SR-2508: a 2-nitroimidazole amide which should be superior to misonidazole as a radiosensitizer for clinical use. Int J Radiat Oncol Biol Phys 7:695–703
Brown JM, Diehn M, Loo BW (2010) Stereotactic ablative radiotherapy should be combined with a hypoxic cell radiosensitizer. Int J Radiat Oncol Biol Phys 78:323–327
Buffa FM, Harris AL, West CM et al (2010) Large meta-analysis of multiple cancers reveals a common, compact and highly prognostic hypoxia metagene. Br J Cancer 102:428–435
Cairns RA, Harris IS, Mak TW (2011) Regulation of cancer cell metabolism. Nat Rev Cancer 11:85–95
Calabrese CR, Almassy R, Barton S et al (2004) Anticancer chemosensitization and radiosensitization by the novel poly(ADP-ribose) polymerase-1 inhibitor AG14361. J Natl Cancer Inst 96:56–67

Camphausen K, Tofilon PJ (2007) Inhibition of histone deacetylation: a strategy for tumor radiosensitization. J Clin Oncol 25:4051–4056

Cao X, Fang L, Gibbs S et al (2007) Glucose uptake inhibitor sensitizes cancer cells to daunorubicin and overcomes drug resistance in hypoxia. Cancer Chemother Pharmacol 59:495–505

Carlson DJ, Keall PJ, Loo BW Jr et al (2011) Hypofractionation results in reduced tumor cell kill compared to conventional fractionation for tumors with regions of hypoxia. Int J Radiat Oncol Biol Phys 79:1188–1195

Carroll VA, Ashcroft M (2006) Role of hypoxia-inducible factor (HIF)-1alpha versus HIF-2alpha in the regulation of HIF target genes in response to hypoxia, insulin-like growth factor-I, or loss of von Hippel-Lindau function: implications for targeting the HIF pathway. Cancer Res 66:6264–6270

Cazares-Korner C, Pires IM, Swallow ID et al (2013) CH-01 is a hypoxia-activated prodrug that sensitizes cells to hypoxia/reoxygenation through inhibition of Chk1 and Aurora A ACS. Med Chem Letts 8:1451−1459

Cenas N, Prast S, Nivinskas H et al (2006) Interactions of nitroaromatic compounds with the mammalian selenoprotein thioredoxin reductase and the relation to induction of apoptosis in human cancer cells. J Biol Chem 281:5593–5603

Chalmers AJ, Lakshman M, Chan N et al (2010) Poly(ADP-ribose) polymerase inhibition as a model for synthetic lethality in developing radiation oncology targets. Semin Radiat Oncol 20:274–281

Chan DA, Giaccia AJ (2008) Targeting cancer cells by synthetic lethality: autophagy and VHL in cancer therapeutics. Cell Cycle 7:2987–2990

Chan DA, Sutphin PD, Nguyen P et al (2011) Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. Sci Transl Med 3:94ra70

Chandor A, Dijols S, Ramassamy B et al (2008) Metabolic activation of the antitumor drug 5-(Aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) by NO synthases. Chem Res Toxicol 21:836–843

Chang Q, Jurisica I, Do T et al (2011) Hypoxia predicts aggressive growth and spontaneous metastasis formation from orthotopically grown primary xenografts of human pancreatic cancer. Cancer Res 71:3110–3120

Charrier JD, Durrant SJ, Golec JM et al (2011) Discovery of potent and selective inhibitors of ataxia telangiectasia mutated and Rad3 related (ATR) protein kinase as potential anticancer agents. J Med Chem 54:2320–2330

Chawla SP, Ganjoo KN, Adkins D et al (2011) A phase 2 study of TH-302 in combination with doxorubicin in advanced soft tissue sarcoma. In: Connective Tissue Oncology Society (CTOS) annual meeting, Chicago, 26–29 Oct 2011

Chen Y, Hu L (2009) Design of anticancer prodrugs for reductive activation. Med Res Rev 29:29–64

Chi JT, Wang Z, Nuyten DS et al (2006) Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. PLoS Med 3:e47

Chinje EC, Cowen RL, Feng J et al (2003) Non-nuclear localized human NOSII enhances the bioactivation and toxicity of tirapazamine (SR4233) in vitro. Mol Pharmacol 63:1248–1255

Chowdhury G, Junnotula V, Daniels JS et al (2007) DNA strand damage product analysis provides evidence that the tumor cell-specific cytotoxin tirapazamine produces hydroxyl radical and acts as a surrogate for O(2). J Am Chem Soc 129:12870–12877

Cole S, Stratford IJ, Fielden EM et al (1992) Dual function nitroimidazoles less toxic than RSU 1069: selection of candidate drugs for clinical trial (RB 6145 and/or PD 130908. Int J Radiat Oncol Biol Phys 22:545–548

Coleman CN, Wasserman TH, Urtasun RC et al (1990) Final report of the phase I trial of the hypoxic cell radiosensitizer SR 2508 (etanidazole) radiation therapy oncology group 83–03. Int J Radiat Oncol Biol Phys 18:389–393

Covens A, Blessing J, Bender D et al (2006) A phase II evaluation of tirapazamine plus cisplatin in the treatment of recurrent platinum-sensitive ovarian or primary peritoneal cancer: a Gynecologic Oncology Group study. Gynecol Oncol 100:586–590

Cowan DS, Hicks KO, Wilson WR (1996) Multicellular membranes as an in vitro model for extravascular diffusion in tumors. Br J Cancer Suppl 27:S28–S31

Craighead PS, Pearcey R, Stuart G (2000) A phase I/II evaluation of tirapazamine administered intravenously concurrent with cisplatin and radiotherapy in women with locally advanced cervical cancer. Int J Radiat Oncol Biol Phys 48:791–795

Daniels JS, Gates KS (1996) DNA cleavage by the antitumor agent 3-amino-1,2,4-benzotriazine 1,4-dioxide (SR4233): Evidence for involvement of hydroxyl radical. J Am Chem Soc 118:3380–3385

Denny WA (2010) Hypoxia-activated prodrugs in cancer therapy: progress to the clinic. Future Oncol 6:419–428

Denny WA, Wilson WR (1986) Considerations for the design of nitrophenyl mustards as agents with selective toxicity for hypoxic tumor cells. J Med Chem 29:879–887

Denny WA, Wilson WR (1993) Bioreducible mustards: a paradigm for hypoxia-selective prodrugs of diffusible cytotoxins (HPDCs). Cancer Metastasis Rev 12:135–151

Denny WA, Wilson WR (2000) Tirapazamine: a bioreductive anticancer drug that exploits tumor hypoxia. Expert Opin Investig Drugs 9:2889–2901

Denny WA, Wilson WR, Hay MP (1996) Recent developments in the design of bioreductive drugs. Br J Cancer 74(Suppl XXVII):S32–S38

Dewhirst MW, Cao Y, Moeller B (2008) Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. Nat Rev Cancer 8:425–437

Dimmer KS, Friedrich B, Lang F et al (2000) The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 350(Pt 1):219–227

Dische S (1985) Chemical sensitisers for hypoxic cells: a decade of experience in clinical radiotherapy. Radiother Oncol 3:97–115

Dische S (1991) A review of hypoxic cell radiosensitization. Int J Radiat Oncol Biol Phys 20:147–152

DiSilvestro P, Ali S, Peter C et al (2012) A Gynecologic Oncology Group phase III randomized trial of weekly cisplatin and radiation versus cisplatin and tirapazamine and radiation in stage IB2, IIA, IIIB and IVA cervical carcinoma limited to the pelvis. Gynecol Oncol 125(Suppl 1):S3

Dorie MJ, Brown JM (1993) Tumor-specific, schedule-dependent interaction between tirapazamine (SR 4233) and cisplatin. Cancer Res 53:4633–4636

Dorie MJ, Brown JM (1994) Potentiation of the anticancer effect of cisplatin by the hypoxic cytotoxin tirapazamine. In: Vaupel PW, Kelleher DK, Gunderoth M (eds) Tumor oxygenation. Fischer-Verlag, Stuttgart/New York, pp 125–135

Duan JX, Jiao H, Kaizerman J et al (2008) Potent and highly selective hypoxia-activated achiral phosphoramidate mustards as anticancer drugs. J Med Chem 51:2412–2420

Dubois LJ, Lieuwes NG, Janssen MH et al (2011) Preclinical evaluation and validation of [18F]HX4, a promising hypoxia marker for PET imaging. Proc Natl Acad Sci U S A 108:14620–14625

Durand RE, Olive PL (1992) Evaluation of bioreductive drugs in multicell spheroids. Int J Radiat Oncol Biol Phys 22:689–692

Durand RE, Olive PL (1997) Physiologic and cytotoxic effects of tirapazamine in tumor-bearing mice. Radiat Oncol Investig 5:213–219

Eschwege F, Sancho-Garnier H, Chassagne D et al (1997) Results of a European randomized trial of etanidazole combined with radiotherapy in head and neck carcinomas. Int J Radiat Oncol Biol Phys 39:275–281

Evans JW, Yudoh K, Delahoussaye YM et al (1998) Tirapazamine is metabolized to its DNA-damaging radical by intranuclear enzymes. Cancer Res 58:2098–2101

Evans SM, Hahn S, Pook DR et al (2000) Detection of hypoxia in human squamous cell carcinoma by EF5 binding. Cancer Res 60:2018–2024

Evans JW, Chernikova SB, Kachnic LA et al (2008) Homologous recombination is the principal pathway for the repair of DNA damage induced by tirapazamine in mammalian cells. Cancer Res 68:257–265

Everett SA, Naylor MA, Patel KB et al (1999) Bioreductively-activated prodrugs for targetting hypoxic tissues: elimination of aspirin from 2-nitroimidazole derivatives. Bioorg Med Chem Lett 9:1267–1272

Fitzsimmons SA, Lewis AD, Riley RJ et al (1994) Reduction of 3-amino-1,2,4-benzotriazine-1,4-di-N-oxide (tirapazamine, WIN 59075, SR 4233) to a DNA-damaging species: a direct role for NADPH:cytochrome P450 oxidoreductase. Carcinogenesis 15:1503–1510

Foehrenbacher A, Patel K, Abbattista M et al (2013) The role of bystander effects in the anti-tumor activity of the hypoxia-activated prodrug PR-104. Front Oncol 3:263. doi:10.3389/fonc.2013.00263

Ghatage P, Sabagh H (2012) Is there a role for tirapazamine in the treatment of cervical cancer? Expert Opin Drug Metab Toxicol 8:1589–1597

Giaccia A, Siim BG, Johnson RS (2003) HIF-1 as a target for drug development. Nat Rev Drug Discov 2:803–811

Gnarra JR, Zhou S, Merrill MJ et al (1996) Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. Proc Natl Acad Sci U S A 93:10589–10594

Golding SE, Rosenberg E, Valerie N et al (2009) Improved ATM kinase inhibitor KU-60019 radiosensitizes glioma cells, compromises insulin, AKT and ERK prosurvival signaling, and inhibits migration and invasion. Mol Cancer Ther 8:2894–2902

Graeber TG, Osmanian C, Jacks T et al (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. Nature 379:88–91

Graham MA, Senan S, Robin H Jr et al (1997) Pharmacokinetics of the hypoxic cell cytotoxic agent tirapazamine and its major bioreductive metabolites in mice and humans: retrospective analysis of a pharmacokinetically guided dose-escalation strategy in a phase I trial. Cancer Chemother Pharmacol 40:1–10

Gray LH, Conger AD, Ebert M et al (1953) Concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol 26:638–648

Grigsby PW, Winter K, Wasserman TH et al (1999) Irradiation with or without misonidazole for patients with stages IIIB and IVA carcinoma of the cervix: final results of RTOG 80–05. Radiation Therapy Oncology Group. Int J Radiat Oncol Biol Phys 44:513–517

Gu Y, Patterson AV, Atwell GJ et al (2009) Roles of DNA repair and reductase activity in the cytotoxicity of the hypoxia-activated dinitrobenzamide mustard PR-104A. Mol Cancer Ther 8:1714–1723

Guise CP, Wang A, Thiel A et al (2007) Identification of human reductases that activate the dinitrobenzamide mustard prodrug PR-104A: a role for NADPH:cytochrome P450 oxidoreductase under hypoxia. Biochem Pharmacol 74:810–820

Guise CP, Abbattista M, Singleton RS et al (2010) The bioreductive prodrug PR-104A is activated under aerobic conditions by human aldo-keto reductase 1C3. Cancer Res 70:1573–1584

Guise CP, Abbattista MR, Tipparaju SR et al (2012) Diflavin oxidoreductases activate the bioreductive prodrug PR-104A under hypoxia. Mol Pharmacol 81:31–40

Hall EJ, Roizin-Towle L (1975) Hypoxic sensitizers: radiobiological studies at the cellular level. Radiology 117:453–457

Hay MP, Wilson WR, Denny WA (1999) Nitrobenzyl carbamate prodrugs of enediynes for nitroreductase gene- directed enzyme prodrug therapy (GDEPT). Bioorg Med Chem Lett 9:3417–3422

Hay MP, Gamage SA, Kovacs MS et al (2003) Structure-activity relationships of 1,2,4-benzotriazine 1,4-dioxides as hypoxia-selective analogues of tirapazamine. J Med Chem 46:169–182

Hay MP, Hicks KO, Pruijn FB et al (2007a) Pharmacokinetic/pharmacodynamic model-guided identification of hypoxia-selective 1,2,4-benzotriazine 1,4-dioxides with antitumor activity: the role of extravascular transport. J Med Chem 50:6392–6404

Hay MP, Pchalek K, Pruijn FB et al (2007b) Hypoxia-selective 3-alkyl 1,2,4-benzotriazine 1,4-dioxides: the influence of hydrogen bond donors on extravascular transport and antitumor activity. J Med Chem 50:6654–6664

Hay MP, Hicks KO, Pchalek K et al (2008) Tricyclic [1,2,4]triazine 1,4-dioxides as hypoxia selective cytotoxins. J Med Chem 51:6853–6865
Hay MP, Turcotte S, Flanagan JU et al (2010) 4-Pyridylanilinothiazoles that selectively target von Hippel-Lindau deficient renal cell carcinoma cells by inducing autophagic cell death. J Med Chem 53:787–797
Heldin CH, Rubin K, Pietras K et al (2004) High interstitial fluid pressure – an obstacle in cancer therapy. Nat Rev Cancer 4:806–813
Helleday T, Petermann E, Lundin C et al (2008) DNA repair pathways as targets for cancer therapy. Nat Rev Cancer 8:193–204
Hicks KO, Fleming Y, Siim BG et al (1998) Extravascular diffusion of tirapazamine: effect of metabolic consumption assessed using the multicellular layer model. Int J Radiat Oncol Biol Phys 42:641–649
Hicks KO, Pruijn FB, Sturman JR et al (2003) Multicellular resistance to tirapazamine is due to restricted extravascular transport: a pharmacokinetic/pharmacodynamic study in HT29 multicellular layer cultures. Cancer Res 63:5970–5977
Hicks KO, Siim BG, Pruijn FB et al (2004) Oxygen dependence of the metabolic activation and cytotoxicity of tirapazamine: implications for extravascular transport and activity in tumors. Radiat Res 161:656–666
Hicks KO, Pruijn FB, Secomb TW et al (2006) Use of three-dimensional tissue cultures to model extravascular transport and predict in vivo activity of hypoxia-targeted anticancer drugs. J Natl Cancer Inst 98:1118–1128
Hicks KO, Myint H, Patterson AV et al (2007) Oxygen dependence and extravascular transport of hypoxia-activated prodrugs: comparison of the dinitrobenzamide mustard PR-104A and tirapazamine. Int J Radiat Oncol Biol Phys 69:560–571
Hicks KO, Siim BG, Jaiswal JK et al (2010) Pharmacokinetic/pharmacodynamic modeling identifies SN30000 and SN29751 as tirapazamine analogues with improved tissue penetration and hypoxic cell killing in tumors. Clin Cancer Res 16:4946–4957
Hickson I, Zhao Y, Richardson CJ et al (2004) Identification and characterization of a novel and specific inhibitor of the ataxia-telangiectasia mutated kinase ATM. Cancer Res 64:9152–9159
Hill RP (1986) Sensitizers and radiation dose fractionation: results and interpretations. Int J Radiat Oncol Biol Phys 12:1049–1054
Hill RP, Gulyas S, Whitmore GF (1986) Studies of the in vivo and in vitro cytotoxicity of the drug RSU-1069. Br J Cancer 53:743–751
Hill RP, Marie-Egyptienne DT, Hedley DW (2009) Cancer stem cells, hypoxia and metastasis. Semin Radiat Oncol 19:106–111
Hoogsteen IJ, Lok J, Marres HA et al (2009) Hypoxia in larynx carcinomas assessed by pimonidazole binding and the value of CA-IX and vascularity as surrogate markers of hypoxia. Eur J Cancer 45:2906–2914
Horsman MR, Mortensen LS, Petersen JB et al (2012) Imaging hypoxia to improve radiotherapy outcome. Nat Rev Clin Oncol 9:674–687
Houghton PJ, Lock R, Carol H et al (2011) Initial testing of the hypoxia activated prodrug PR-104 by the pediatric preclinical testing program. Pediatr Blood Cancer 57:443–453
Huang LE, Bindra RS, Glazer PM et al (2007) Hypoxia-induced genetic instability – a calculated mechanism underlying tumor progression. J Mol Med 85:139–148
Hunter FW, Wang J, Patel R et al (2012) Homologous recombination repair-dependent cytotoxicity of the benzotriazine di-N-oxide CEN-209: comparison with other hypoxia-activated prodrugs. Biochem Pharmacol 83:574–585
Isaacs JS, Jung YJ, Mimnaugh EG et al (2002) Hsp90 regulates a von Hippel Lindau-independent hypoxia-inducible factor-1 alpha-degradative pathway. J Biol Chem 277:29936–29944
Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307:58–62
Jameson MB, Rischin D, Pegram M et al (2010) A phase I trial of PR-104, a nitrogen mustard prodrug activated by both hypoxia and aldo-keto reductase 1C3, in patients with solid tumors. Cancer Chemother Pharmacol 65:791–801

Janssens GO, Rademakers SE, Terhaard CH et al (2012) Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. J Clin Oncol 30:1777–1783

Jenkins TC, Naylor MA, O'Neill P et al (1990) Synthesis and evaluation of alpha-[[(2-haloethyl) amino]methyl]-2- nitro- 1H-imidazole-1-ethanols as prodrugs of alpha-[(1-aziridinyl) methyl]-2- nitro-1H-imidazole-1-ethanol (RSU-1069) and its analogues which are radiosensitizers and bioreductively activated cytotoxins. J Med Chem 33:2603–2610

Johnson CA, Kilpatrick D, von Roemeling R et al (1997) Phase I trial of tirapazamine in combination with cisplatin in a single dose every 3 weeks in patients with solid tumors. J Clin Oncol 15:773–780

Jones NP, Schulze A (2012) Targeting cancer metabolism–aiming at a tumor's sweet-spot. Drug Discov Today 17:232–241

Jubb AM, Buffa FM, Harris AL (2010) Assessment of tumor hypoxia for prediction of response to therapy and cancer prognosis. J Cell Mol Med 14:18–29

Kaanders JH, Bussink J, van der Kogel AJ (2002) ARCON: a novel biology-based approach in radiotherapy. Lancet Oncol 3:728–737

Kaelin WG Jr (2005) The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer 5:689–698

Kaelin WG Jr (2008) The von Hippel-Lindau tumor suppressor protein: O2 sensing and cancer. Nat Rev Cancer 8:865–873

Kallman RF (1972) The phenomenon of reoxygenation and its implications for fractionated radiotherapy. Radiology 105:135–142

Kallman RF, Dorie MJ (1986) Tumor oxygenation and reoxygenation during radiation therapy: their importance in predicting tumor response. Int J Radiat Oncol Biol Phys 12:681–685

Karasawa K, Sunamura M, Okamoto A et al (2008) Efficacy of novel hypoxic cell sensitiser doranidazole in the treatment of locally advanced pancreatic cancer: long-term results of a placebo-controlled randomised study. Radiother Oncol 87:326–330

Kashishian A, Douangpanya H, Clark D et al (2003) DNA-dependent protein kinase inhibitors as drug candidates for the treatment of cancer. Mol Cancer Ther 2:1257–1264

Kelson AB, McNamara JP, Pandey A et al (1998) 1,2,4-Benzotriazine 1,4-dioxides. An important class of hypoxic cytotoxins with antitumor activity. Anticancer Drug Des 13:575–592

Kioi M, Vogel H, Schultz G et al (2010) Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. J Clin Invest 120:694–705

Kobori M, Shinmoto H, Tsushida T et al (1997) Phloretin-induced apoptosis in B16 melanoma 4A5 cells by inhibition of glucose transmembrane transport. Cancer Lett 119:207–212

Koch CJ (1993) Unusual oxygen concentration dependence of toxicity of SR-4233, a hypoxic cell toxin. Cancer Res 53:3992–3997

Koch CJ, Scheuermann JS, Divgi C et al (2010) Biodistribution and dosimetry of (18)F-EF5 in cancer patients with preliminary comparison of (18)F-EF5 uptake versus EF5 binding in human glioblastoma. Eur J Nucl Med Mol Imaging 37:2048–2059

Komar G, Seppanen M, Eskola O et al (2008) 18F-EF5: a new PET tracer for imaging hypoxia in head and neck cancer. J Nucl Med 49:1944–1951

Kovacs MS, Hocking DJ, Evans JW et al (1999) Cisplatin anti-tumor potentiation by tirapazamine results from a hypoxia-dependent cellular sensitization to cisplatin. Br J Cancer 80:1245–1251

Kuiper C, Molenaar IGM, Dachs GU et al (2010) Low ascorbate levels are associated with increased hypoxia-inducible factor-1 activity and an aggressive tumor phenotype in endometrial cancer. Cancer Res 70:5749–5758

Kummar S, Raffeld M, Juwara L et al (2011) Multihistology, target-driven pilot trial of oral topotecan as an inhibitor of hypoxia-inducible factor-1alpha in advanced solid tumors. Clin Cancer Res 17:5123–5131

Kurtoglu M, Maher JC, Lampidis TJ (2007) Differential toxic mechanisms of 2-deoxy-D-glucose versus 2-fluorodeoxy-D-glucose in hypoxic and normoxic tumor cells. Antioxid Redox Signal 9:1383–1390

Kyle AH, Minchinton AI (1999) Measurement of delivery and metabolism of tirapazamine to tumor tissue using the multilayered cell culture model. Cancer Chemother Pharmacol 43:213–220

Laderoute K, Wardman P, Rauth AM (1988) Molecular mechanisms for the hypoxia-dependent activation of 3-amino- 1,2,4-benzotriazine-1,4-dioxide (SR 4233). Biochem Pharmacol 37:1487–1495

Le QT, Sutphin PD, Raychaudhuri S et al (2003) Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. Clin Cancer Res 9:59–67

Le QT, Fisher R, Oliner KS et al (2012) Prognostic and predictive significance of plasma HGF and IL-8 in a phase III trial of chemoradiation with or without tirapazamine in locoregionally advanced head and neck cancer. Clin Cancer Res 18:1798–1807

Lee AE, Wilson WR (2000) Hypoxia-dependent retinal toxicity of bioreductive anticancer prodrugs in mice. Toxicol Appl Pharmacol 163:50–59

Lee DJ, Cosmatos D, Marcial VA et al (1995) Results of an RTOG phase III trial (RTOG 85-27) comparing radiotherapy plus etanidazole with radiotherapy alone for locally advanced head and neck carcinomas.[comment]. Int J Radiat Oncol Biol Phys 32:567–576

Lee N, Nehmeh S, Schoder H et al (2009) Prospective trial incorporating pre-/mid-treatment [(18) F]-misonidazole positron emission tomography for head-and-neck cancer patients undergoing concurrent chemoradiotherapy. Int J Radiat Oncol Biol Phys 75:101–108

Lim AM, Rischin D, Fisher R et al (2012) Prognostic significance of plasma osteopontin in patients with locoregionally advanced head and neck squamous cell carcinoma treated on TROG 02.02 phase III trial. Clin Cancer Res 18:301–307

Lin AJ, Cosby LA, Shanky CW et al (1972) Potential bioreductive alkylating agents. I. Benzoquinone derivatives. J Med Chem 15:1247–1252

Liu Q, Sun JD, Wang J et al (2012) TH-302, a hypoxia-activated prodrug with broad in vivo preclinical combination therapy efficacy: optimization of dosing regimens and schedules. Cancer Chemother Pharmacol 69:1487–1498

Lo SS, Fakiris AJ, Chang EL et al (2010) Stereotactic body radiation therapy: a novel treatment modality. Nat Rev Clin Oncol 7:44–54

Lohse I, Rasowski J, Cao PJ et al (2012) Targeting tumor hypoxia in patient-derived pancreatic xenografts using TH-302. In: AACR Pancreatic Cancer meeting, Lake Tahoe, 18–20 June 2012

Mabjeesh NJ, Post DE, Willard MT et al (2002) Geldanamycin induces degradation of hypoxia-inducible factor 1alpha protein via the proteosome pathway in prostate cancer cells. Cancer Res 62:2478–2482

Macheda ML, Rogers S, Best JD (2005) Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. J Cell Physiol 202:654–662

Maluf FC, Leiser AL, Aghajanian C et al (2006) Phase II study of tirapazamine plus cisplatin in patients with advanced or recurrent cervical cancer. Int J Gynecol Cancer 16:1165–1171

Mason RP, Holtzman JL (1975) The role of catalytic superoxide formation in the O2 inhibition of nitroreductase. Biochem Biophys Res Commun 67:1267–1274

Maxwell PH, Dachs GU, Gleadle JM et al (1997) Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A 94:8104–8109

Mazure NM, Chen EY, Yeh P et al (1996) Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. Cancer Res 56:3436–3440

McKeage MJ, Gu Y, Wilson WR et al (2011) A phase I trial of PR-104, a pre-prodrug of the bioreductive prodrug PR-104A, given weekly to solid tumor patients. BMC Cancer 11:432

McKeown SR, Cowen RL, Williams KJ (2007) Bioreductive drugs: from concept to clinic. Clin Oncol 19:427–442

Meng F, Evans JW, Bhupathi D et al (2012) Molecular and cellular pharmacology of the hypoxia-activated prodrug TH-302. Mol Cancer Ther 11:740–751

Milbank JB, Stevenson RJ, Ware DC et al (2009) Synthesis and evaluation of stable bidentate transition metal complexes of 1-(chloromethyl)-5-hydroxy-3-(5,6,7-trimethoxyindol-2-

ylcarbonyl)-2,3-dihydro-1*H*-pyrrolo[3,2-*f*]quinoline (*seco*-6-azaCBI-TMI) as hypoxia selective cytotoxins. J Med Chem 52:6822–6834
Minchinton AI, Tannock IF (2006) Drug penetration in solid tumors. Nat Rev Cancer 6:583–592
Minchinton AI, Lemmon MJ, Tracy M et al (1992) Second-generation 1,2,4-benzotriazine 1,4-di-N-oxide bioreductive anti- tumor agents: pharmacology and activity in vitro and in vivo. Int J Radiat Oncol Biol Phys 22:701–705
Minchinton AI, Wendt KR, Clow KA et al (1997) Multilayers of cells growing on a permeable support. An in vitro tumor model. Acta Oncol 36:13–16
Moeller BJ, Cao Y, Li CY et al (2004) Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. Cancer Cell 5:429–441
Moeller BJ, Dreher MR, Rabbani ZN et al (2005) Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell 5:99–110
Mohindra JK, Rauth AM (1976) Increased cell killing by metronidazole and nitrofurazone of hypoxic compared to aerobic mammalian cells. Cancer Res 36:930–936
Mok TS (2011) Personalized medicine in lung cancer: what we need to know. Nat Rev Clin Oncol 8:661–668
Murat A, Migliavacca E, Hussain SF et al (2009) Modulation of angiogenic and inflammatory response in glioblastoma by hypoxia. PLoS One 4:e5947
Murata R, Tsujitani M, Horsman MR (2008) Enhanced local tumor control after single or fractionated radiation treatment using the hypoxic cell radiosensitizer doranidazole. Radiother Oncol 87:331–338
Naylor MA, Stephens MA, Cole S et al (1990) Synthesis and evaluation of novel electrophilic nitrofuran carboxamides and carboxylates as radiosensitizers and bioreductively activated cytotoxins. J Med Chem 33:2508–2513
Naylor MA, Threadgill MD, Showalter HD et al (1993) Synthesis of the enantiomers of the bioreductively-activated cytotoxin RSU-1069 and its prodrug RB6145 and lack of stereoselectivity in their cytotoxicity and radiosensitization in vitro. Drug Des Discov 10:249–255
Nishimura Y, Nakagawa K, Takeda K et al (2007) Phase I/II trial of sequential chemoradiotherapy using a novel hypoxic cell radiosensitizer, doranidazole (PR-350), in patients with locally advanced non-small-cell lung cancer (WJTOG-0002). Int J Radiat Oncol Biol Phys 69:786–792
Nordsmark M, Bentzen SM, Rudat V et al (2005) Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. Radiother Oncol 77:18–24
Olive PL, Vikse CM, Banath JP (1996) Use of the comet assay to identify cells sensitive to tirapazamine in multicell spheroids and tumors in mice. Cancer Res 56:4460–4463
Olive PL, Banath JP, Sinnott LT (2004) Phosphorylated histone H2AX in spheroids, tumors, and tissues of mice exposed to etoposide and 3-amino-1,2,4-benzotriazine-1,3-dioxide. Cancer Res 64:5363–5369
Overgaard J (1994) Clinical evaluation of nitroimidazoles as modifiers of hypoxia in solid tumors. Oncol Res 6:509–518
Overgaard J (2007) Hypoxic radiosensitization: adored and ignored. J Clin Oncol 25:4066–7440
Overgaard J (2011) Hypoxic modification of radiotherapy in squamous cell carcinoma of the head and neck–a systematic review and meta-analysis. Radiother Oncol 100:22–32
Overgaard J (2012) IAEA-HypoX. Accelerated radiotherapy with or without Nimorazole in squamous cell carcinoma of the head and neck. http://clinicaltrialsgov/ct2/show/NCT01507467
Overgaard J, Horsman MR (1996) Modification of hypoxia-induced radioresistance in tumors by the use of oxygen and sensitizers. Semin Radiat Oncol 6:10–21
Overgaard J, Overgaard M, Nielsen OS et al (1982) A comparative investigation of nimorazole and misonidazole as hypoxic radiosensitizers in a C3H mammary carcinoma in vivo. Br J Cancer 46:904–911
Overgaard J, Overgaard M, Timothy AR (1983) Studies of the pharmacokinetic properties of nimorazole. Br J Cancer 48:27–34

Overgaard J, Hansen HS, Overgaard M et al (1998) A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5–85. Radiother Oncol 46:135–146

Overgaard J, Eriksen JG, Nordsmark M et al (2005) Plasma osteopontin, hypoxia, and response to the hypoxia sensitiser nimorazole in radiotherapy of head and neck cancer: results from the DAHANCA 5 randomised double-blind placebo-controlled trial. Lancet Oncol 6:757–764

Oya N, Shibamoto Y, Sasai K et al (1995) Optical isomers of a new 2-nitroimidazole nucleoside analog (PR-350 series): radiosensitization efficiency and toxicity. Int J Radiat Oncol Biol Phys 33:119–127

Palmer BD, Wilson WR, Cliffe S et al (1992) Hypoxia-selective antitumor agents. 5. Synthesis of water-soluble nitroaniline mustards with selective cytotoxicity for hypoxic mammalian cells. J Med Chem 35:3214–3222

Palmer BD, Wilson WR, Atwell GJ et al (1994) Hypoxia-selective antitumor agents. 9. Structure-activity relationships for hypoxia-selective cytotoxicity among analogues of 5-[N, N-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide. J Med Chem 37:2175–2184

Palmer BD, Wilson WR, Anderson RF et al (1996) Hypoxia-selective antitumor agents. 14. Synthesis and hypoxic cell cytotoxicity of regioisomers of the hypoxia-selective cytotoxin 5-[N, N- bis(2-chloroethyl)amino]-2,4-dinitrobenzamide. J Med Chem 39:2518–2528

Papadopoulou MV, Ji M, Rao MK et al (2003) Reductive activation of the nitroimidazole-based hypoxia-selective cytotoxin NLCQ-1 (NSC 709257). Oncol Res 14:21–29

Parker LL, Lacy SM, Farrugia LJ et al (2004) A novel design strategy for stable metal complexes of nitrogen mustards as bioreductive prodrugs. J Med Chem 47:5683–5689

Parveen I, Naughton DP, Whish WJ et al (1999) 2-nitroimidazol-5-ylmethyl as a potential bioreductively activated prodrug system: reductively triggered release of the PARP inhibitor 5-bromoisoquinolinone. Bioorg Med Chem Lett 9:2031–2036

Patel K, Lewiston D, Gu Y et al (2007) Analysis of the hypoxia-activated dinitrobenzamide mustard phosphate prodrug PR-104 and its alcohol metabolite PR-104A in plasma and tissues by liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 856:302–311

Patel K, Choy SF, Hicks KO et al (2011) A combined pharmacokinetic model for the hypoxia-targeted prodrug PR-104A in humans, dogs, rats and mice predicts species differences in clearance and toxicity. Cancer Chemother Pharmacol 67:1145–1155

Patterson LH (1993) Rationale for the use of aliphatic N-oxides of cytotoxic anthraquinones as prodrug DNA binding agents: a new class of bioreductive agent. Cancer Metastasis Rev 12:119–134

Patterson AV, Saunders MP, Chinje EC et al (1997) Overexpression of human NADPH:cytochrome c (P450) reductase confers enhanced sensitivity to both tirapazamine (SR 4233) and RSU 1069. Br J Cancer 76:1338–1347

Patterson AV, Saunders MP, Chinje EC et al (1998) Enzymology of tirapazamine metabolism: a review. Anticancer Drug Des 13:541–573

Patterson AV, Ferry DM, Edmunds SJ et al (2007) Mechanism of action and preclinical antitumor activity of the novel hypoxia-activated DNA crosslinking agent PR-104. Clin Cancer Res 13:3922–3932

Patterson AV, Jaiswal J, Syddall SP et al (2009) Cellular metabolism, murine pharmacokinetics and preclinical antitumor activity of SN29966, a novel hypoxia-activated irreversible pan-HER inhibitor. Mol Cancer Ther 8:Abstract B76

Peasland A, Wang LZ, Rowling E et al (2011) Identification and evaluation of a potent novel ATR inhibitor, NU6027, in breast and ovarian cancer cell lines. Br J Cancer 105:372–381

Phillips RM, Hendriks HR, Peters GJ (2013) EO9 (Apaziquone): from the clinic to the laboratory and back again. Br J Pharmacol 168:11–18

Pires IM, Olcina MM, Anbalagan S et al (2012) Targeting radiation-resistant hypoxic tumor cells through ATR inhibition. Br J Cancer 107:291–299

Poon E, Harris AL, Ashcroft M (2009) Targeting the hypoxia-inducible factor (HIF) pathway in cancer. Expert Rev Mol Med 11:e26

Pries AR, Cornelissen AJ, Sloot AA et al (2009) Structural adaptation and heterogeneity of normal and tumor microvascular networks. PLoS Comput Biol 5:e1000394

Pruijn FB, Sturman JR, Liyanage HDS et al (2005) Extravascular transport of drugs in tumor tissue: effect of lipophilicity on diffusion of tirapazamine analogs in multicellular layer cultures. J Med Chem 48:1079–1087

Pruijn FB, Patel K, Hay MP et al (2008) Prediction of tumor tissue diffusion coefficients of hypoxia-activated prodrugs from physicochemical parameters. Aust J Chem 61:687–693

Raleigh JA, Calkins-Adams DP, Rinker LH et al (1998) Hypoxia and vascular endothelial growth factor expression in human squamous cell carcinomas using pimonidazole as a hypoxia marker. Cancer Res 58:3765–3768

Ramanathan RK, Kirkpatrick DL, Belani CP et al (2007) A Phase I pharmacokinetic and pharmacodynamic study of PX-12, a novel inhibitor of thioredoxin-1, in patients with advanced solid tumors. Clin Cancer Res 13:2109–2114

Rapisarda A, Uranchimeg B, Scudiero DA et al (2002) Identification of small molecule inhibitors of hypoxia-inducible factor 1 transcriptional activation pathway. Cancer Res 62:4316–4324

Rapisarda A, Uranchimeg B, Sordet O et al (2004a) Topoisomerase I-mediated inhibition of hypoxia-inducible factor 1: mechanism and therapeutic implications. Cancer Res 64:1475–1482

Rapisarda A, Zalek J, Hollingshead M et al (2004b) Schedule-dependent inhibition of hypoxia-inducible factor-1alpha protein accumulation, angiogenesis, and tumor growth by topotecan in U251-HRE glioblastoma xenografts. Cancer Res 64:6845–6848

Rapisarda A, Hollingshead M, Uranchimeg B et al (2009) Increased antitumor activity of bevacizumab in combination with hypoxia inducible factor-1 inhibition. Mol Cancer Ther 8:1867–1877

Reaper PM, Griffiths MR, Long JM et al (2011) Selective killing of ATM- or p53-deficient cancer cells through inhibition of ATR. Nat Chem Biol 7:428–430

Reddy SB, Williamson SK (2009) Tirapazamine: a novel agent targeting hypoxic tumor cells. Expert Opin Investig Drugs 18:77–87

Ren H, Accili D, Duan C (2010) Hypoxia converts the myogenic action of insulin-like growth factors into mitogenic action by differentially regulating multiple signaling pathways. Proc Natl Acad Sci U S A 107:5857–5862

Rini BI (2010) New strategies in kidney cancer: therapeutic advances through understanding the molecular basis of response and resistance. Clin Cancer Res 16:1348–1354

Rischin D, Peters L, Fisher R et al (2005) Tirapazamine, cisplatin, and radiation versus fluorouracil, cisplatin, and radiation in patients with locally advanced head and neck cancer: a randomized phase II trial of the Trans-Tasman Radiation Oncology Group (TROG 98.02). J Clin Oncol 23:79–87

Rischin D, Hicks RJ, Fisher R et al (2006) Prognostic significance of [18F]-misonidazole positron emission tomography-detected tumor hypoxia in patients with advanced head and neck cancer randomly assigned to chemoradiation with or without tirapazamine: a substudy of Trans-Tasman Radiation Oncology Group Study 98.02. J Clin Oncol 24:2098–2104

Rischin D, Narayan K, Oza AM et al (2010a) Phase 1 study of tirapazamine in combination with radiation and weekly cisplatin in patients with locally advanced cervical cancer. Int J Gynecol Cancer 20:827–833

Rischin D, Peters LJ, O'Sullivan B et al (2010b) Tirapazamine, cisplatin, and radiation versus cisplatin and radiation for advanced squamous cell carcinoma of the head and neck (TROG 02.02, HeadSTART): a phase III trial of the Trans-Tasman Radiation Oncology Group. J Clin Oncol 28:2989–2995

Rockwell S, Dobrucki IT, Kim EY et al (2009) Hypoxia and radiation therapy: past history, ongoing research, and future promise. Curr Mol Med 9:442–458

Salas-Burgos A, Iserovich P, Zuniga F et al (2004) Predicting the three-dimensional structure of the human facilitative glucose transporter glut1 by a novel evolutionary homology strategy: insights on the molecular mechanism of substrate migration, and binding sites for glucose and inhibitory molecules. Biophys J 87:2990–2999

Sandler AB, Nemunaitis J, Denham C et al (2000) Phase III trial of gemcitabine plus cisplatin versus cisplatin alone in patients with locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 18:122–130

Sapra P, Zhao H, Mehlig M et al (2008) Novel delivery of SN38 markedly inhibits tumor growth in xenografts, including a camptothecin-11-refractory model. Clin Cancer Res 14:1888–1896

Sapra P, Kraft P, Pastorino F et al (2011) Potent and sustained inhibition of HIF-1alpha and downstream genes by a polyethyleneglycol-SN38 conjugate, EZN-2208, results in anti-angiogenic effects. Angiogenesis 14:245–253

Sarkaria JN, Eshleman JS (2001) ATM as a target for novel radiosensitizers. Semin Radiat Oncol 11:316–327

Saunders M, Dische S (1996) Clinical results of hypoxic cell radiosensitisation from hyperbaric oxygen to accelerated radiotherapy, carbogen and nicotinamide. Br J Cancer 27:S271–S278

Semenza GL (2003) Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3:721–732

Semenza GL (2007) Evaluation of HIF-1 inhibitors as anticancer agents. Drug Discov Today 12:853–859

Semenza GL (2010) HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev 20:51–56

Senan S, Rampling R, Graham MA et al (1997) Phase I and pharmacokinetic study of tirapazamine (SR 4233) administered every three weeks. Clin Cancer Res 3:31–38

Senra JM, Telfer BA, Cherry KE et al (2011) Inhibition of PARP-1 by olaparib (AZD2281) increases the radiosensitivity of a lung tumor xenograft. Mol Cancer Ther 10:1949–1958

Shepherd F, Koschel G, von Pawel J et al (2000) Comparison of Tirazone (tirapazamine) and cisplatin vs. etoposide and cisplatin in advanced non-small cell lung cancer (NSCLC): final results of the international phase III CATAPULT II trial. Lung Cancer 29(Suppl 1):28, abstract No. 87

Shewach DS, Lawrence TS (2007) Antimetabolite radiosensitizers. J Clin Oncol 25:4043–4050

Shinde SS, Hay MP, Patterson AV et al (2009) Spin trapping of radicals other than the *OH radical upon reduction of the anticancer agent tirapazamine by cytochrome P450 reductase. J Am Chem Soc 131:14220–14221

Shinde SS, Maroz A, Hay MP et al (2010) Characterization of radicals formed following enzymatic reduction of 3-substituted analogues of the hypoxia-selective cytotoxin 3-amino-1,2,4-benzotriazine 1,4-dioxide (tirapazamine). J Am Chem Soc 132:2591–2599

Shinohara ET, Geng L, Tan J et al (2005) DNA-dependent protein kinase is a molecular target for the development of noncytotoxic radiation-sensitizing drugs. Cancer Res 65:4987–4992

Siim BG, van Zijl PL, Brown JM (1996) Tirapazamine-induced DNA damage measured using the comet assay correlates with cytotoxicity towards hypoxic tumor cells in vitro. Br J Cancer 73:952–960

Singleton RS, Guise CP, Ferry DM et al (2009) DNA crosslinks in human tumor cells exposed to the prodrug PR-104A: relationships to hypoxia, bioreductive metabolism and cytotoxicity. Cancer Res 69:3884–3891

Song CW, Clement JJ, Levitt SH (1976) Preferential cytotoxicity of 5-thio-D-glucose against hypoxic tumor cells. J Natl Cancer Inst 57:603–605

Sun JD, Liu Q, Wang J et al (2012) Selective tumor hypoxia targeting by hypoxia-activated prodrug TH-302 inhibits tumor growth in preclinical models of cancer. Clin Cancer Res 18:758–770

Sutphin PD, Chan DA, Li JM et al (2007) Targeting the loss of the von Hippel-Lindau tumor suppressor gene in renal cell carcinoma cells. Cancer Res 67:5896–5905

Sutphin PD, Chan D, Turcotte S et al (2011) Heteroaryl benzamides, compositions and methods of use. Patent WO2011011514 A1

Tatsumi K, Kitamura S, Narai N (1986) Reductive metabolism of aromatic nitro compounds including carcinogens by rabbit liver preparations. Cancer Res 46:1089–1093

Tatum JL, Kelloff GJ, Gillies RJ et al (2006) Hypoxia: importance in tumor biology, noninvasive measurement by imaging, and value of its measurement in the management of cancer therapy. Int J Radiat Biol 82:699–757

Thomlinson RH, Gray LH (1955) The histological structure of some human lung cancers and possible implications for radiotherapy. Br J Cancer 9:539–549

Threadgill MD, Webb P, O'Neill P et al (1991) Synthesis of a series of nitrothiophenes with basic or electrophilic substituents and evaluation as radiosensitizers and as bioreductively activated cytotoxins. J Med Chem 34:2112–2120

Timothy AR, Overgaard J, Overgaard M (1984) A phase I clinical study of Nimorazole as a hypoxic radiosensitizer. Int J Radiat Oncol Biol Phys 10:1765–1768

Toustrup K, Sorensen BS, Nordsmark M et al (2011) Development of a hypoxia gene expression classifier with predictive impact for hypoxic modification of radiotherapy in head and neck cancer. Cancer Res 71:5923–5931

Toustrup K, Sorensen BS, Alsner J et al (2012) Hypoxia gene expression signatures as prognostic and predictive markers in head and neck radiotherapy. Semin Radiat Oncol 22:119–127

Turcotte S, Chan DA, Sutphin PD et al (2008) A molecule targeting VHL-deficient renal cell carcinoma that induces autophagy. Cancer Cell 14:90–102

Tuttle SW, Maity A, Oprysko PR et al (2007) Detection of reactive oxygen species via endogenous oxidative pentose phosphate cycle activity in response to oxygen concentration. J Biol Chem 282:36790–36796

Ueda O, Kitamura S, Ohashi K et al (2003) Xanthine oxidase-catalysed metabolism of 2-nitrofluorene, a carcinogenic air pollutant, in rat skin. Drug Metab Dispos 31:367–372

Urtasun RC, Band P, Chapman JD et al (1976) Radiation and high dose metronidazole in supratentorial glioblastomas. N Engl J Med 294:1364–1367

van Loon J, Janssen MHM, Ollers M et al (2010) PET imaging of hypoxia using [18F]HX4: a phase I trial. Eur J Nucl Med Mol Imaging 37:1663–1668

Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26:225–239

Vaupel P, Hockel M, Mayer A (2007) Detection and characterization of tumor hypoxia using pO2 histography. Antioxid Redox Signal 9:1221–1235

von Pawel J, von Roemeling R, Gatzemeier U et al (2000) Tirapazamine plus cisplatin versus cisplatin in advanced non-small-cell lung cancer: a report of the international CATAPULT I study group. Cisplatin and tirapazamine in subjects with advanced previously untreated non-small-cell lung tumors. J Clin Oncol 18:1351–1359

Wang H, Wang H, Powell SN et al (2004) ATR affecting cell radiosensitivity is dependent on homologous recombination repair but independent of nonhomologous end joining. Cancer Res 64:7139–7143

Wang D, Chu PC, Yang CN et al (2012a) Development of a novel class of glucose transporter inhibitors. J Med Chem 55:3827–3836

Wang J, Foehrenbacher A, Su J et al (2012b) The 2-nitroimidazole EF5 is a biomarker for oxidoreductases that activate bioreductive prodrug CEN-209 under hypoxia. Clin Cancer Res 18:1684–1695

Wardman P (2001) Electron transfer and oxidative stress as key factors in the design of drugs selectively active in hypoxia. Curr Med Chem 8:739–761

Wardman P (2007) Chemical radiosensitizers for use in radiotherapy. Clin Oncol 19:397–417

Ware DC, Palmer BD, Wilson WR et al (1993) Hypoxia-selective antitumor agents. 7. Metal complexes of aliphatic mustards as a new class of hypoxia-selective cytotoxins. Synthesis and evaluation of cobalt(III) complexes of bidentate mustards. J Med Chem 36:1839–1846

Welsh SJ, Williams RR, Birmingham A et al (2003) The thioredoxin redox inhibitors 1-methylpropyl 2-imidazolyl disulfide and pleurotin inhibit hypoxia-induced factor 1alpha and vascular endothelial growth factor formation. Mol Cancer Ther 2:235–243

Wilhelm SM, Adnane L, Newell P et al (2008) Preclinical overview of sorafenib, a multikinase inhibitor that targets both Raf and VEGF and PDGF receptor tyrosine kinase signaling. Mol Cancer Ther 7:3129–3140

Williams KJ, Telfer BA, Xenaki D et al (2005) Enhanced response to radiotherapy in tumors deficient in the function of hypoxia-inducible factor-1. Radiother Oncol 75:89–98

Williamson SK, Crowley JJ, Lara PN Jr et al (2005) Phase III trial of paclitaxel plus carboplatin with or without tirapazamine in advanced non-small-cell lung cancer: Southwest Oncology Group Trial S0003. J Clin Oncol 23:9097–9104

Wilson WR, Hay MP (2011) Targeting hypoxia in cancer therapy. Nat Rev Cancer 11:393–410
Wilson WR, van Zijl P, Denny WA (1992) Bis-bioreductive agents as hypoxia-selective cytotoxins: nitracrine N-oxide. Int J Radiat Oncol Biol Phys 22:693–696
Wilson WR, Hicks KO, Pullen SM et al (2007) Bystander effects of bioreductive drugs: potential for exploiting pathological tumor hypoxia with dinitrobenzamide mustards. Radiat Res 167:625–636
Winter SC, Buffa FM, Silva P et al (2007) Relation of a hypoxia metagene derived from head and neck cancer to prognosis of multiple cancers. Cancer Res 67:3441–3449
Wood TE, Dalili S, Simpson CD et al (2008) A novel inhibitor of glucose uptake sensitizes cells to FAS-induced cell death. Mol Cancer Ther 7:3546–3555
Wouters BG, Brown JM (1997) Cells at intermediate oxygen levels can be more important than the "hypoxic fraction" in determining tumor response to fractionated radiotherapy. Radiat Res 147:541–550
Xia Y, Choi HK, Lee K (2012) Recent advances in hypoxia-inducible factor (HIF)-1 inhibitors. Eur J Med Chem 49:24–40
Yamamoto N, Renfrew AK, Kim BJ et al (2012) Dual targeting of hypoxic and acidic tumor environments with a cobalt(III) chaperone complex. J Med Chem 55:11013–11021
Yin J, Glaser R, Gates KS (2012) On the reaction mechanism of tirapazamine reduction chemistry: unimolecular N-OH homolysis, stepwise dehydration, or triazene ring-opening. Chem Res Toxicol 25:634–645
Zeman EM, Brown JM, Lemmon MJ et al (1986) SR-4233: a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. Int J Radiat Oncol Biol Phys 12:1239–1242
Zeman EM, Hirst VK, Lemmon MJ et al (1988) Enhancement of radiation-induced tumor cell killing by the hypoxic cell toxin SR 4233. Radiother Oncol 12:209–218
Zeman EM, Baker MA, Lemmon MJ et al (1989) Structure-activity relationships for benzotriazine di-N-oxides. Int J Radiat Oncol Biol Phys 16:977–981
Zhao Y, Thomas HD, Batey MA et al (2006) Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. Cancer Res 66:5354–5362
Zhong H, Chiles K, Feldser D et al (2000) Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. Cancer Res 60:1541–1545

# Chapter 7
# Radiotherapy and the Tumor Microenvironment: Mutual Influence and Clinical Implications

**Reid F. Thompson and Amit Maity**

**Abstract** Ionizing radiation has been employed in targeted cancer treatments for more than a century because of its cytotoxic effects on cancer cells. However, the responsiveness to radiation and the behavior of tumors in vivo may differ dramatically from observed behaviors of isolated cancer cells in vitro. While not fully understood, these discrepancies are due to a complex constellation of extracellular and intercellular factors that are together termed the *tumor microenvironment*. Radiation may alter or affect the components of the adjacent tumor microenvironment in significant ways, often with consequences for cancer cells beyond the direct effects of the radiation itself. Moreover, different microenvironmental states, whether induced or at baseline, can modulate or even attenuate the effects of radiation, with consequences for therapeutic efficacy. This chapter describes this bidirectional relationship in detail, exploring the role and clinical implications of the tumor microenvironment with respect to therapeutic irradiation.

**Keywords** Radiotherapy • Radiation • Tumor microenvironment • Hypoxia • Extracellular matrix • Bystander effect • Abscopal effect

## 7.1 Introduction

Ionizing radiation has been employed in targeted cancer treatments for more than a century because of its cytotoxic effects on cancer cells. Over the years, the physical methods of delivering radiation have been refined to increase clinical effectiveness and minimize toxicity to surrounding tissues. In parallel, the biological mechanisms of radiation-induced cell killing have been studied extensively in the laboratory,

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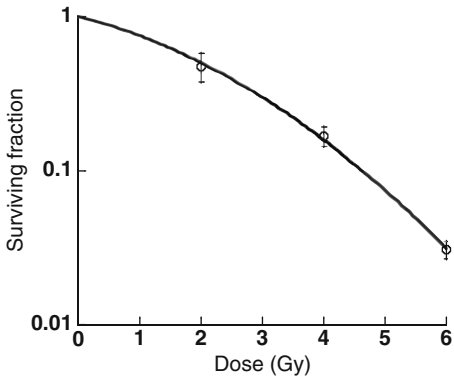


**Fig. 7.1** Typical survival curve for mammalian cells in culture exposed to x-rays. The surviving fraction of cells is plotted as a function of irradiated dose. Surviving fraction represents the fraction of single cells that ultimately give rise to colonies (corrected for plating efficiency). Dose of radiation describes single fraction x-ray exposure

with increasing understanding of the signaling pathways triggered in response to DNA damage and double-strand breaks (e.g., cellular repair machinery) (Thompson 2012; Jeggo and Lavin 2009).

One technique in particular, pioneered in the 1950s, has enabled detailed description of cellular survival as a function of radiation exposure (Puck and Marcus 1956). In these radiation survival assays, single-cell suspensions are seeded onto tissue culture dishes and irradiated with different doses, with quantification of visible clonal aggregates after a period of approximately 1–2 weeks. The surviving fraction of cells can be depicted graphically as a function of radiation dose; a standard example of such a clonogenic survival curve is shown in Fig. 7.1. Minimal cell killing is noted at very low doses of radiation, manifested by a "shoulder" on the survival curve, a phenomenon that has been ascribed to the ability of cells to repair low-level DNA damage without dying.

Although in vitro survival curves have transformed the study of radiation biology and the effects of radiation on cells, the radiation responsiveness and behavior of tumors in vivo may differ dramatically from behaviors of isolated cancer cells observed in vitro. A recent study of glioblastoma multiforme surgical specimens demonstrated significant discordance in radiation sensitivity when grown as xenografts within mouse brains compared to in vitro culture conditions (Jamal et al. 2012). Indeed, multiple cell types exhibit differential radiosensitivity under in vivo compared to in vitro conditions (Jen et al. 1991; Maruyama and Eichten 1968).

These discrepancies, although not fully understood, are likely due to a complex interplay between the tumor cells and their microenvironment. In vitro clonogenic assays do not account for interactions with surrounding cells and stroma, nor do they take into account the hypoxia that may occur in vivo because these studies are typically carried out under ambient oxygen conditions. Rather, these assays measure a cell population's "intrinsic" radiosensitivity. The tumor microenvironment functions as an "extrinsic" component influencing radiosensitivity and likely explains the discordance between in vitro and in vivo observations.

In a broad sense, the tumor microenvironment (TME) is composed of tumor cells, soluble factors, signaling molecules, the extracellular matrix, and other cells in the local vicinity, including endothelial cells, stromal cells, and immune cells (Fig. 7.2). The TME can play an important role in cancer progression by supporting

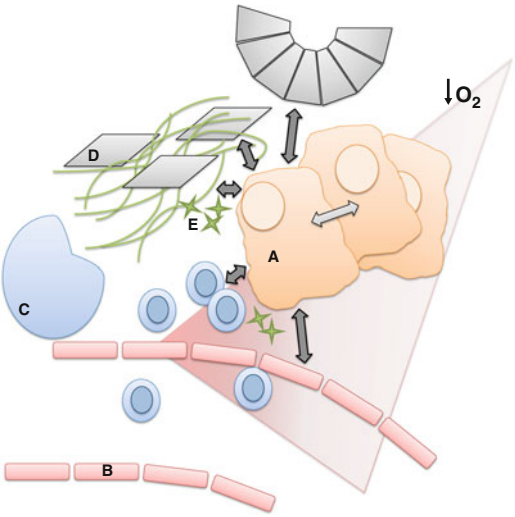


**Fig. 7.2** Schematic of components of the tumor microenvironment. Note that the relationships depicted are conceptual and not intended to portray histology, function, scale, or spatial orientation. Tumor cells are shown in *orange* (*A*), with their microenvironment interactions depicted by *shaded bidirectional arrows*. Endothelial cells are shown in *red* (*B*), whereas infiltrating and resident immune cells are shown in *blue* (*C*). Stromal cells are shaded in *gray* (*D*), whereas the extracellular matrix and diffusible signaling molecules are shown in *green* (*E*). Intratumoral oxygen gradient is depicted in *red*, with hypoxia present most distant from the microvasculature

tumor growth and invasion, insulating the tumor from host immunity, or fostering generalized resistance to tumoricidal therapy. Furthermore, the TME is neither uniform nor fixed; rather, it is considered both dynamic and heterogeneous and represents a unique niche dependent on the type, location, temporality, and immune status of the tumor and surrounding tissue as well as a number of other factors.

Therapeutic irradiation is intended to selectively target cancer cells while preserving normal tissue function. However, radiation may also alter or affect the components of the adjacent microenvironment in significant ways, often with consequences for cancer cells beyond the direct effects of the radiation itself. Moreover, different microenvironment states, whether basal or induced, can modulate or even attenuate the effects of radiation, with consequences for therapeutic efficacy. This chapter describes this bidirectional relationship, exploring the interaction between the TME and radiation, and how our improved understanding of the TME can be applied in the clinic.

## 7.2 Tumor Hypoxia and Hypoxia-Inducible Factor

As tumors increase in size, they may outgrow their native vascular supply, limiting the availability of nutrients and oxygen and restricting growth. Such focal regions of hypoxia within tumors can stimulate the secretion of vascular endothelial growth

factor (VEGF) and other factors that promote angiogenesis. However, the excessive secretion of VEGF in this nonphysiologic setting often leads to the development of dysregulated and dysfunctional vasculature, with tortuous, dilated, and saccular microvessels with poor hemodynamic flow. Moreover, supraphysiologic VEGF can increase vascular permeability, causing excessive leakiness in the tumor microvasculature (Jain 2005; Senger et al. 1983). Therefore, hypoxia-induced VEGF in tumors may paradoxically increase tumor heterogeneity and exacerbate focal areas of hypoxia (Brown and Wilson 2004).

These hypoxic regions are characterized by increased glycolysis and production of carbon dioxide, with acidification of the TME and increased resistance to therapeutic intervention (e.g., radiation and chemotherapy). The presence of hypoxia is often associated with worse outcome of radiation therapy for a variety of malignancies, including head and neck cancers (Brizel et al. 1997). In fact, cells under nearly anoxic conditions require two to three times the dose of radiation for equivalent cell killing compared to the same cells irradiated under well-oxygenated conditions (Palcic and Skarsgard 1984). This phenomenon is attributed mechanistically to the fact that the majority of DNA damage induced by x-rays is indirect, secondary to diffusible free radicals, which are formed only in the presence of molecular oxygen (Barron 1954).

In an attempt to improve therapeutic outcomes of radiation in hypoxic tumors, numerous methods have been employed in the clinic. These interventions include increasing oxygen delivery or using oxygen mimetics or hypoxic radiosensitizers. In at least one trial, concurrent hyperbaric oxygen and radiotherapy was shown to improve local control and survival (Watson et al. 1978). Similar to this, hyperoxic gas with concurrent nicotinamide (a vasoactive agent) was shown to improve regional control following radiotherapy, most prominently for hypoxic tumors (Janssens et al. 2012). A meta-analysis of 86 randomized trials with 10,108 patients treated with primary radiotherapy with curative intent showed improved locoregional control and overall survival benefit favoring the arms designed to modify tumor hypoxia (Overgaard 2007).

In addition to the effect of hypoxia on radiosensitivity, there is also evidence that hypoxia induces a variety of phenotypic changes that may select for more aggressive tumor cells (Graeber et al. 1996). Hypoxia-inducible factor (HIF)-1, a potent transcription factor activated in response to intracellular oxygen depletion, is a prime mediator of some of these changes (Wang and Semenza 1995). Hypoxia specifically upregulates the HIF-1 α subunit (the transcription factor's β subunit is constitutively expressed, irrespective of oxygen tension). Numerous downstream gene targets of HIF-1 allow for adaptation to the oxygen-poor environment, the most notable of which are VEGF, glucose transporter 1 (GLUT1), and glycolytic enzymes that allow for an increase in anaerobic metabolism. In the absence of HIF-1 induction, tumor cells demonstrate stunted growth (Maxwell et al. 1997), whereas increased aggressiveness is often correlated with increased HIF-1 expression (Zhong et al. 1999).

In some reports, radiation induces HIF-1 activity in tumor cells, with upregulation of VEGF and promotion of the survival of neighboring endothelial cells

(Moeller et al. 2004). In turn, HIF-1 activation may decrease tumor radiosensitivity (Moeller et al. 2005). As a consequence, in some studies, blockade of HIF-1 (e.g., via an inhibitor such as YC-1) following radiation exposure delays tumor growth and improves therapeutic responsiveness; note, however, that this effect may be highly dependent on the timing of radiation relative to drug exposure (Moeller et al. 2005; Harada et al. 2009). In other studies, two different inhibitors of HIF-1 also have been found to enhance the radiation sensitivity of solid tumors in vivo (Schwartz et al. 2009; Yasui et al. 2008).

The initial induction of HIF-1 in response to radiation may result from radiation-induced microvascular damage and local ischemia. Indeed, microvascular endothelial cells are directly injured by radiation therapy, with multiple potential downstream consequences, including enhanced tumor killing and exacerbation of normal tissue damage (Paris et al. 2001). In one study, genetic inhibition of endothelial apoptosis achieved by growing tumors in sphingomyelinase (asmase)-deficient or Bax-deficient mice resulted in resistance to single-dose radiation up to 20 Gy (Garcia-Barros et al. 2003).

## 7.3 Tumor Microvasculature

As discussed in the preceding section, the tumor microvasculature may play a role in the tumor response to radiotherapy. The microvasculature thus presents an attractive therapeutic target in combination with radiation. To date there have been two predominant strategies used to target tumor microvessels: vascular disruptive agents and anti-angiogenic agents (Denekamp 1993). Vascular disruptive agents are designed to preferentially destroy preexisting tumor vessels, whereas anti-angiogenic agents target the process of neovascularization by inhibiting the action of critical effector molecules (Folkman 1995).

Multiple vascular disruptive agents have been used successfully in combination with radiation therapy in preclinical models to enhance the efficiency and potency of tumor killing (Murata et al. 2001; Siemann and Rojiani 2002; Wilson et al. 1998). The effects of these vascular disruptive agents are often most pronounced in the dysregulated vasculature on the interior of solid tumors, whereas an outer rim of tumor typically retains its perfusion because of the collateral blood supply to surrounding normal tissue. Vascular disruptive agents also stimulate tumor angiogenesis and mobilize bone marrow–derived circulating endothelial progenitor cells to the viable tumor rim (Shaked et al. 2006). Thus the oxygenated tumor rim may acquire enhanced susceptibility to radiation, improving overall efficiency of tumor killing in combined modality therapy.

Numerous anti-angiogenic therapies also have been studied in the clinic, particularly those agents targeting key angiogenic factors such as VEGF. Moreover, VEGF-targeted therapies have been used effectively in combination with radiotherapy. Of note, ionizing radiation itself induces VEGF, and inhibition of this pathway has been shown to improve radiation efficacy and tumor control in preclinical models

(Gorski et al. 1999). This phenomenon is mediated by endothelial cells, and blockade of the VEGF receptor significantly decreases the number of tumor microvessels and enhances radiation sensitivity in tumor xenografts (Hess et al. 2001). The effect is dependent on the timing of treatments, and VEGF receptor inhibition following radiotherapy further enhances the degree of tumor response (Williams et al. 2004).

This phenomenon at first seems paradoxical: anti-angiogenic agents would be predicted to increase tumor hypoxia, promoting resistance to radiation therapy. However, there are reports of preclinical models in which inhibition of VEGF signaling may, under certain circumstances, decrease the vascular permeability of tumors and promote vascular "normalization," thereby enhancing blood flow and tumor oxygenation (Tong et al. 2004). In this way, anti-angiogenic therapy may enhance tumor sensitivity to therapeutic irradiation via vascular normalization (e.g., glioblastoma multiforme xenografts) (Winkler et al. 2004).

However, there is as yet few mature clinical data to support the use of combination radiation and anti-angiogenic therapy. The most commonly used anti-VEGF agent in the clinic is bevacizumab (Avastin), an antibody directed against human VEGF. Bevacizumab seems to induce vascular normalization in patients with high-grade glioma with concomitant radiographic response (Fischer et al. 2008), and the addition of therapeutic irradiation may prolong overall survival (Aguilera et al. 2013). However, these are preliminary results, and the use of radiation in combination with bevacizumab remains the subject of active clinical investigation, with multiple ongoing trials listed with the National Cancer Institute.

There are data suggesting that agents affecting other signaling pathways may indirectly modulate the tumor microvasculature, with potential clinical benefit. Inhibition of epidermal growth factor receptor can promote vascular normalization (Cerniglia et al. 2009) and has been shown to increase radiation response and overall survival in patients with locally advanced head and neck squamous cell carcinoma (Bonner et al. 2006). Phosphatidylinositol 3-kinase inhibition similarly can induce vascular normalization (Qayum et al. 2009), with additive cytoxic effects in combination with radiotherapy (Chen et al. 2008). Nelfinavir, a human immunodeficiency virus protease inhibitor that blocks Akt signaling, improves tumor oxygenation (perhaps via an anti-VEGF effect) (Pore et al. 2006) and induces radiation sensitization in vitro and in vivo (Gupta et al. 2005). Last, inhibition of the mammalian target of rapamycin pathway (downstream of Akt signaling) causes strong radiosensitization in vivo but not in vitro (Eshleman et al. 2002); this is hypothesized to be a function of enhanced vascular endothelial cellular damage (Shinohara et al. 2005).

## 7.4 Diffusible Signaling and the Extracellular Matrix

While tumors are typically thought of as an aggregation of multiple cellular populations, the acellular or intercellular components of a tumor may dictate its behavior and therapeutic response. This intercellular environment comprises both diffusible crosstalk and physical linkages among cells, influencing tissue architecture and

functional coordination between neighboring cells. Moreover, intercellular architecture is highly variable among tissue and tumor types and possesses a significant degree of plasticity and responsiveness to the surrounding milieu. Derangements in diffusible signaling or the extracellular matrix (ECM) may modulate tumor progression, metastasis, and therapeutic response.

Although radiation is canonically thought to exert its cytotoxic effects through direct induction of reactive oxygen species (ROS) and DNA double-strand breaks, radiation damage to a single cell may in fact propagate damage to neighboring cells that are not exposed to radiation (Nagasawa and Little 1992). This phenomenon (the "bystander effect") may be observed in immediately adjacent cells (Gerashchenko and Howell 2005) or even those located up to 1 mm distantly (Belyakov et al. 2005). Moreover, radiation damage may be conferred through medium transfer from an irradiated population to a separate unirradiated population of cells, implicating the presence of a diffusible damage signal (Mothersill and Seymour 1997).

However, the bystander effect is highly dependent on cell type and quality of radiation exposure, and even the nature of biological outcomes is variable. Heavy ion irradiation can induce autophagy in bystander myoblasts (Hino et al. 2010), whereas microbeams may induce DNA double-strand breaks in nearby cells (Sedelnikova et al. 2007). Unirradiated bladder cell cultures exposed to conditioned media taken from separately irradiated cultures can exhibit changes in gene expression and terminal differentiation (Vines et al. 2009). In some instances, however, radiation may actually generate a protective effect whereby bystander cells become relatively resistant to further radiation (Iyer and Lehnert 2002).

Irrespective of biological outcome, bystander cells display elevated global production of ROS (Hanot et al. 2009), and scavenging of ROS abrogates various bystander responses, implicating ROS as a common mechanism of bystander cell damage (Bishayee et al. 2001). Bystander ROS production is further dependent on cytochrome C and mitochondrial function (Yang et al. 2009). DNA repair pathways can mitigate bystander cell damage, whereas compromise of the DNA repair machinery (e.g., in malignant cells) may exacerbate bystander effects (Little et al. 2003; Mothersill et al. 2004).

The underlying mechanism and nature of the so-called damage signals released from irradiated cells in the first place, however, are not fully understood. There are reports that suggest that the diffusible signal may be short-lived (<60 s [Wang and Coderre 2005]) or may not peak for hours to days after irradiation (Belyakov et al. 2003). The signals released even from a single cell may be sufficient to induce bystander damage, although this represents a threshold effect because damage is not amplified with increasing doses of radiation (Schettino et al. 2005).

Multiple candidate molecules have been implicated as damage signals. Long-lived diffusible radicals may directly induce bystander damage (Kumagai et al. 2003), whereas waves of calcium (Shao et al. 2006) or various small toxic metabolites or cytokines (e.g., tumor necrosis factor-α [Zhou et al. 2005], transforming growth factor-β [Shao et al. 2008], and interleukin [IL]-8 [Narayanan et al. 1999]) may be produced in irradiated cells and propagate damage to neighboring cells. Furthermore, the radiation-induced bystander effect is more pronounced when cells

are in direct contact (Cummins et al. 1999) because bystander signaling can occur through direct intercellular gap junctions, which allow free exchange of molecules up to 1.5 kDa in size (Azzam et al. 2001).

The vast majority of studies have investigated diffusible bystander signaling in vitro, whether in monolayer experiments or under three-dimensional culture conditions. However, the bystander contributions to the in vivo TME are largely unknown, and complex tissue architecture, intercellular spaces, and cell-cell networks may variably limit penetration or, conversely, enhance bystander signaling. Nonetheless, emerging evidence suggests that the bystander effect may occur in vivo as well, since unirradiated tumor growth is significantly slowed by interspersed irradiated tumor cells (Xue et al. 2002). The bone marrow niche may be similarly prone to bystander-induced damage in vivo (Watson et al. 2000).

These effects have the largest potential clinical significance in areas of low-dose radiation, which typically lie outside of targeted tumors. Nonetheless, radiotherapy with a low dose rate (e.g., intraprostatic brachytherapy) may be prone to intratumoral bystander phenomena. Intratumoral crosstalk may more generally potentiate tumor control in hyperfractionated radiotherapy regimens, particularly given the increased bystander sensitivity of cells in cycle and the recruitment of stem cell cycling with fractionated radiotherapy.

Beyond diffusible intercellular crosstalk, direct cellular interactions with the ECM may further modulate radiation therapy. Moreover, radiation itself can influence cellular adhesion to and interactions with the ECM, particularly via integrin receptors, which directly constitute the cell-ECM interface (Park et al. 2003). This has been demonstrated in glioma cells, which exhibit radiation-enhanced invasive potential in vivo (Wild-Bode et al. 2001). It should be noted that this study was performed using 9L tumors grown in rat brains and that such a finding has not yet been substantiated in human patients given radiation. Ionizing radiation can also induce β1 integrin expression in several cancers, with a resultant increase in cellular resistance to further irradiation (Cordes et al. 2006).

The importance of integrins stems from the role of these proteins in promoting tumor initiation, cancer cell growth and viability, invasion, and metastasis (White et al. 2004; Fujita et al. 1995). Moreover, increased integrin expression is associated with decreased overall clinical survival in some cancers (Yao et al. 2007). Inhibition of integrin may conversely abrogate this effect, inducing selective apoptosis in malignant cells (Park et al. 2006). This phenomenon is more pronounced in cancer cells compared to normal cells and could provide an attractive target for multimodal therapy in certain cancers (Nam et al. 2010).

With respect to radiation, integrin-ECM interactions can modulate cancer cell response, and inhibition of integrin signaling enhances the tumoricidal effects of radiotherapy (Abdollahi et al. 2005). The underlying mechanism is not fully understood; however, multiple signaling cascades have been implicated, and integrin-ECM signaling is an important component of radiation response both in vitro and in vivo (Eke et al. 2012). It is interesting that radiation is also thought to induce changes in integrin signaling, which may predispose cells to metastasis in locally recurrent disease (Monnier et al. 2008). Inhibitors of integrin signaling (e.g.,

cilengitide) may attenuate this effect; hence, they are emerging as attractive clinical candidates to increase the efficacy of radiotherapy and potentially improve tumor control (Albert et al. 2006; Mikkelsen et al. 2009).

## 7.5 Stromal Cells

Tumor cells interact with and depend on neighboring stromal cells for growth cues, nutritional support, and mechanical stability. These stromal cells – which include endothelial cells, fibroblasts, adipocytes, smooth muscle cells, glial cells, and tissue stem cells – create and maintain the extracellular scaffold and compose the bulk of the TME. Moreover, the tumor stroma is highly dynamic, and the ECM may be degraded by fibroblast-secreted matrix metalloproteinases or reinforced by collagen deposition. Cancer and stromal cells may also co-evolve throughout tumorigenesis, and their interactions enable suitable metastatic niches and can promote or prevent the spread of disease.

Indeed, the role of tumor stromal cells is emerging as a key factor in determining cancer behavior. In pancreatic ductal adenocarcinoma, for example, an admixture of profibrogenic pancreatic stromal cells (stellate cells) and tumor cells promotes growth and metastasis in vivo and reduces the effectiveness of both chemo- and radiotherapy (Hwang et al. 2008). The stellate cells also mediate a dramatic desmoplastic reaction, which contributes to poor therapeutic response and integrin-dependent radioprotection in vivo (Mantoni et al. 2011).

In addition, stromal cells may promote transformation of tumor cells (e.g., epithelial-to-mesenchymal transition), enabling tumor progression (Kikuta et al. 2010). For instance, prostate cancer cells can undergo such a transition in the bone marrow microenvironment in an integrin- and cadherin-dependent manner, with acquisition of radiation resistance mediated by stromal cells (Josson et al. 2010). The mechanism of this decreased radiation sensitivity may be related to the induction of DNA damage response pathways (Chiba et al. 2012). Transforming growth factor-β signaling is also known to play a significant role (Andarawewa et al. 2007).

Much of the crosstalk between cancer cells and surrounding stromal cells is mediated by secreted factors. Production of a gastrointestinal secretory protein (trefoil factor 1) by pancreatic adenocarcinoma cells stimulates the motility of both pancreatic ductal adenocarcinoma cells and pancreatic stellate cells and also promotes overall metastatic potential (Arumugam et al. 2011). In esophageal adenocarcinoma, production of hepatocyte growth factor (HGF) by resident fibroblasts increases cancer cell migration, invasion, and metastasis, whereas HGF inhibition attenuates these phenomena (Grugan et al. 2010). In breast cancer models, stromal adipocytes can produce hormones, growth factors, and other adipokine-signaling molecules that promote neighboring tumor cell survival and radiation resistance (Bochet et al. 2011).

Just as the tumor stromal contributions may modulate radiotherapeutic efficacy, radiation can induce changes in stromal cells that have repercussions for tumor

control and behavior. Irradiation can enhance invasion of esophageal adenocarcinoma through alterations of stromal-derived HGF levels (Patel et al. 2012). In fact, radiation can up- or downregulate a number of gene expression pathways in stromal fibroblasts (Kis et al. 2006). Irradiation of fibroblasts associated with lung cancer limits the migratory and invasive potential of tumors (Hellevik et al. 2012); radiation-induced fibroblast senescence may, conversely, increase the growth of tumor cells (Papadopoulou and Kletsas 2011).

Last, radiation can modulate stromal cell function, with consequences for surrounding normal tissue in addition to the tumor itself. Radiation can stimulate stromal fibroblasts and increase fibrosis in models of mammary stroma (Qayyum and Insana 2012). Pulmonary fibrosis is also a known consequence of radiation-induced stromal changes (e.g., alveolar epithelial cell mesenchymal transition) (Almeida et al. 2013). Numerous other tissues and cell types are sensitive to radiation-induced stromal cellular damage (e.g., preadipocytes [Poglio et al. 2009] and bone marrow–derived human mesenchymal stem cells [Prendergast et al. 2011]).

## 7.6 Resident and Infiltrating Immune Cells

From the first experiments that showed immunosuppression could affect tumor engraftment, growth, and cancer-specific survival (Reiner and Southam 1966), the immune system has featured prominently in our understanding of oncology and has played an important role in cancer care. Like normal tissues, tumors can contain both resident and infiltrating immune cells, which may contribute to antitumor immune surveillance or alternatively promote local or systemic immune tolerance. Radiation can modulate these immune responses, with significant implications for cancer care.

The TME contains many different populations of immune cells. Tumor macrophages can be present in either quiescent or activated states and function as cellular scavengers. Dendritic cells may be mature or immature and function as general antigen-presenting cells, initiating immune attack or tolerance, depending on multiple factors. Regulatory T cells, helper T cells, extrafollicular B cells, natural killer cells, neutrophils, and numerous other cellular types play further roles in instigating, modulating, or implementing a complex spectrum of tumor immune responses.

Under certain conditions, the immune system can recognize tumors by the novel or mutated antigens they possess, stimulating immune infiltration and attack with improvement in overall cancer control (Galon et al. 2006). However, cancer cells may exhibit defects in antigen processing and presentation, thus evading the immune system (Sanda et al. 1995). In persistent or growing tumors, an altered equilibrium of other immune actors may promote local immune tolerance, wherein the immune system fails to recognize or react to novel antigens presented by dysregulated cancer cells. Numerous additional experiments and clinical reports have demonstrated that defects in the localized or systemic immune response predispose to all forms of cancer, even rare diseases (e.g., Kaposi sarcoma) not seen in immunocompetent individuals. A prime example of this is the propensity of patients with human immunodeficiency virus to develop malignancies (Pinzone et al. 2012).

Radiation damage to tumor cells can cause necrosis and apoptosis with the generation of cellular debris and exposure of tumor antigens to the immune system (Kotera et al. 2001). Radiation can also increase antigen presentation in intact cancer cells (Reits et al. 2006). Mature dendritic cells provide the requisite costimulatory molecules needed to initiate a T-cell-directed attack, with immune tolerance in the absence of costimulation. Dying cancer cells produce "danger signals" (e.g., uric acid), which can activate dendritic cells and thus prompt an immune response (Shi et al. 2003). Additional danger signals, including alarmin proteins, which are released from dying tumor cells following chemo- or radiotherapy, directly stimulate dendritic cells and allow for cross-presentation of tumor antigens, with improved cancer control (Apetoh et al. 2007). In the chronic setting, however, recognition of danger signals by tumor cells may drive disease progression (Sato et al. 2009).

In addition to inducing the presentation of tumor antigens, radiation also increases the expression of pro-inflammatory cytokines (e.g., tumor necrosis factor-α [Hallahan et al. 1989] and IL-1B [Ishihara et al. 1993]), with upregulation of major histocompatibility complexes and immune costimulatory molecules in both tumor cells and the surrounding stroma (Nesslinger et al. 2007). Radiation-induced damage also upregulates adhesion molecules, facilitating local T cell migration and infiltration into the TME (Matsumura et al. 2008; Lugade et al. 2005). Irradiated tumor cells express death receptors, sensitizing to antigen-specific cytotoxic T cells (Chakraborty et al. 2003).

Radiation does not, however, exhibit a uniform effect in all cases. Different fractionation schemes may favor immunostimulation or immunosuppression, with dose-dependent effects on regulatory T cell populations (Schaue et al. 2012). Furthermore, different immune cell populations are differentially susceptible to radiation exposure (Belka et al. 1999). Suppressor T cells seem to be particularly radiosensitive: very low doses of radiation are potentially able to deplete their population and increase tumor response to therapy (Hellstrom et al. 1978; Tilkin et al. 1981). In contrast, macrophages are relatively resistant to irradiation, maintaining metabolic and proliferative capacity despite higher doses (Hildebrandt et al. 1998). Regulatory T cells are also comparatively more resistant to radiation than other lymphocyte populations, resulting in a proportional increase in their numbers in irradiated tumors and potentially leading to functional changes in the immune response (Kachikwu et al. 2011; Kusunoki et al. 2010).

It is interesting that perturbations of the local tumor-immune microenvironment can have systemic implications for cancer control. This has been observed for many years in the clinic, with patients unexpectedly exhibiting systemic antitumor responses following local radiation therapy (Antoniades et al. 1977; Ehlers and Fridman 1973). This phenomenon, called the "abscopal effect," has been observed in a number of different malignancies, including hepatocellular carcinoma (Ohba et al. 1998), metastatic renal cell carcinoma (Wersall et al. 2006), and metastatic melanoma (Stamell et al. 2012), among others.

The detailed mechanism of the abscopal effect remains an area of active research; however, a functional immune system is required to mediate the systemic effects of local irradiation. Radiation induces T-cell priming in draining lymphatic tissue and increases circulating populations, with a $CD8^+$ T-cell-dependent reduction in

primary tumor and distant metastases (Lee et al. 2009; Schaue et al. 2008). In addition, radiation alters the ratio of suppressor and effector T cells, with consequences for the development of an antitumor immune response (North 1986). Thus, radiotherapy is thought to overcome immunosuppressive barriers within the TME and improve systemic antitumoral activity (Formenti and Demaria 2009).

While the abscopal effect can be dramatic at times, it remains rare and unpredictable. There are numerous efforts to increase both the frequency and durability of immune-mediated tumor clearance. One strategy involving the use of radiation with active therapeutic vaccination (i.e., recombinant vaccinia virus expressing human carcinoembryonic antigen and costimulatory molecules) was shown to enhance antitumor T-cell response and disease clearance (Chakraborty et al. 2004). A separate strategy combining radiotherapy and immunotherapy vaccination also demonstrated an antitumor immune response, with regression of metastases following treatment of the primary tumor (Hodge et al. 2012).

In addition to combined radiation and vaccination strategies, radiation has been combined with immunomodulatory antibodies and small molecules with good effect. Ipilimumab (a humanized anti-CTLA-4 antibody) is approved by the US Food and Drug Administration for the treatment of melanoma and has been shown to induce dramatic disease response in conjunction with radiation therapy (Postow et al. 2012). Anti-CTLA-4 antibodies also were shown to induce an abscopal response in conjunction with fractionated radiotherapy in preclinical carcinoma models (Dewan et al. 2009). A combination of stereotactic body radiation therapy and repeated IL-2 infusions can induce significant antitumoral immune response in metastatic melanoma and renal cell carcinoma (Seung et al. 2012).

The tumor-immune interface is extraordinarily complex and in a state of dynamic equilibrium that often favors tumor evasion of the immune response. Resident and locally infiltrating immune cells offer the opportunity for surveillance and cytotoxic response; however, they also govern immune tolerance. Radiation can alter the TME, induce antigen presentation, and ultimately alter immune balance, with consequences for disease control. The combination of radiation and immunotherapy in the treatment of cancer is rapidly evolving and offers significant promise for future care.

## 7.7 Summary and Perspective

A wealth of radiobiological understanding has been generated through the use of in vitro clonogenic survival assays. However, this radiobiological understanding is incomplete when divorced from consideration of the TME and the complexity of a tumor growing in vivo. The viewpoint that focuses on the cancer cell itself as the sole arbiter of radiation response has thus evolved with the recognition that factors extrinsic to the malignant cell or even outside of the tumor mass (e.g., stem cells within the bone marrow) can dramatically influence radioresponsiveness.

For decades we have known that hypoxia may play an important role in radiation treatments. Much effort in the radiobiology field has focused on trying to reverse the cytoprotective effects of hypoxia on radiation-induced cell killing. Over the years,

clinical trials targeting hypoxia have yielded some tantalizing results; however, when considered in aggregate, the results remain mixed. Nonetheless, for selected tumor types, the data indicating that hypoxia is associated with poor outcome following radiotherapy are strong, and a strategy targeting hypoxia may yet prove to be successful and more widely accepted in radiotherapy.

Another important element of the TME is the microvasculature, which is often dysfunctional and exacerbates the hypoxia seen in tumors. There is thus a great deal of interest in targeting the tumor vasculature, particularly by VEGF blockade. Preclinical data suggest that combining anti-VEGF agents with radiation may improve the overall efficacy of radiation; however, clinical evidence supporting this approach remains sparse.

In addition to the vasculature, the TME also contains stromal cells that modulate tumor cell responsiveness to radiation and in turn can be modulated by radiation. This may have important implications for the development of late effects, for example, radiation-induced fibrosis. The ECM also functions as part of the TME, not only providing for intercellular crosstalk but also constituting a critical foundation for overall tumor architecture. The ECM influences behaviors, including growth and metastatic potential, as well as the radiation response of tumor cells via integrins. Clinical trials investigating the combination of anti-integrin agents (e.g., Cilengitide) and radiation, in particular in the treatment of glioblastoma, are ongoing (Scaringi et al. 2012).

Resident and infiltrating immune cells constitute another key component of the TME and influence tumor behavior and treatment response far beyond the immediate site of the tumor itself. There are exciting data suggesting that large fractions of radiation used in stereotactic radiotherapy may prime the immune system, perhaps by allowing for increased antigen presentation to T cells. Radiation is therefore increasingly used in conjunction with immunomodulatory agents such as ipilimumab to induce systemic (abscopal) treatment responses. Although it remains a nascent approach, radioimmunotherapy could play a significant role in the future management of systemic disease.

Taken together, numerous components of the TME can modulate radiation response to an extraordinary degree. The clinical implications of these interactions are significant, and modulation of the TME may lead to real improvements in therapeutic ratio and overall cancer control. Numerous trials and approaches are currently underway, and the future of clinical oncology will undoubtedly integrate a deeper understanding of cancer cells and their interactions with the surrounding environment.

## References

Abdollahi A et al (2005) Inhibition of alpha(v)beta3 integrin survival signaling enhances antiangiogenic and antitumor effects of radiotherapy. Clin Cancer Res Off J Am Assoc Cancer Res 11(17):6270–6279

Aguilera DG et al (2013) Prolonged survival after treatment of diffuse intrinsic pontine glioma with radiation, temozolamide, and bevacizumab: report of 2 cases. J Pediatric Hematol Oncol 35(1):e42–e46

Albert JM et al (2006) Integrin alpha v beta 3 antagonist Cilengitide enhances efficacy of radiotherapy in endothelial cell and non-small-cell lung cancer models. Int J Radiat Oncol Biol Phys 65(5):1536–1543

Almeida C et al (2013) The role of alveolar epithelium in radiation-induced lung injury. PloS One 8(1):e53628

Andarawewa KL et al (2007) Ionizing radiation predisposes nonmalignant human mammary epithelial cells to undergo transforming growth factor beta induced epithelial to mesenchymal transition. Cancer Res 67(18):8662–8670

Antoniades J, Brady LW, Lightfoot DA (1977) Lymphangiographic demonstration of the abscopal effect in patients with malignant lymphomas. Int J Radiat Oncol Biol Phys 2(1–2):141–147

Apetoh L et al (2007) Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. Nat Med 13(9):1050–1059

Arumugam T et al (2011) Trefoil factor 1 stimulates both pancreatic cancer and stellate cells and increases metastasis. Pancreas 40(6):815–822

Azzam EI, de Toledo SM, Little JB (2001) Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. Proc Natl Acad Sci USA 98(2):473–478

Barron ES (1954) The role of free radicals and oxygen in reactions produced by ionizing radiations. Radiat Res 1(1):109–124

Belka C et al (1999) Impact of localized radiotherapy on blood immune cells counts and function in humans. Radiother Oncol J Eur Soc Ther Radiol Oncol 50(2):199–204

Belyakov OV et al (2003) A proliferation-dependent bystander effect in primary porcine and human urothelial explants in response to targeted irradiation. Brit J Cancer 88(5):767–774

Belyakov OV et al (2005) Biological effects in unirradiated human tissue induced by radiation damage up to 1 mm away. Proc Natl Acad Sci USA 102(40):14203–14208

Bishayee A et al (2001) Free radical-initiated and gap junction-mediated bystander effect due to nonuniform distribution of incorporated radioactivity in a three-dimensional tissue culture model. Radiat Res 155(2):335–344

Bochet L et al (2011) Cancer-associated adipocytes promotes breast tumor radioresistance. Biochem Biophys Res Commun 411(1):102–106

Bonner JA et al (2006) Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. New Engl J Med 354(6):567–578

Brizel DM et al (1997) Tumor hypoxia adversely affects the prognosis of carcinoma of the head and neck. Int J Radiat Oncol Biol Phys 38(2):285–289

Brown JM, Wilson WR (2004) Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 4(6):437–447

Cerniglia GJ et al (2009) Epidermal growth factor receptor inhibition modulates the microenvironment by vascular normalization to improve chemotherapy and radiotherapy efficacy. PloS one 4(8):e6539

Chakraborty M et al (2003) Irradiation of tumor cells up-regulates Fas and enhances CTL lytic activity and CTL adoptive immunotherapy. J Immunol 170(12):6338–6347

Chakraborty M et al (2004) External beam radiation of tumors alters phenotype of tumor cells to render them susceptible to vaccine-mediated T-cell killing. Cancer Res 64(12):4328–4337

Chen JS et al (2008) Characterization of structurally distinct, isoform-selective phosphoinositide 3′-kinase inhibitors in combination with radiation in the treatment of glioblastoma. Mol Cancer Ther 7(4):841–850

Chiba N et al (2012) Homeobox B9 induces epithelial-to-mesenchymal transition-associated radioresistance by accelerating DNA damage responses. Proc Natl Acad Sci USA 109(8):2760–2765

Cordes N et al (2006) beta1-integrin-mediated signaling essentially contributes to cell survival after radiation-induced genotoxic injury. Oncogene 25(9):1378–1390

Cummins RJ et al (1999) The effect of microcolony size, at time of irradiation, on colony forming ability. Int J Radiat Biol 75(2):225–232

Denekamp J (1993) Review article: angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. Brit J Radiol 66(783):181–196
Dewan MZ et al (2009) Fractionated but not single-dose radiotherapy induces an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody. Clin Cancer Res Off J Am Assoc Cancer Res 15(17):5379–5388
Ehlers G, Fridman M (1973) Abscopal effect of radiation in papillary adenocarcinoma. Brit J Radiol 46(543):220–222
Eke I, Dickreuter E, Cordes N (2012) Enhanced radiosensitivity of head and neck squamous cell carcinoma cells by beta1 integrin inhibition. Radiother Oncol J Eur Soc Ther Radiol Oncol 104(2):235–242
Eshleman JS et al (2002) Inhibition of the mammalian target of rapamycin sensitizes U87 xenografts to fractionated radiation therapy. Cancer Res 62(24):7291–7297
Fischer I et al (2008) High-grade glioma before and after treatment with radiation and Avastin: initial observations. Neuro Oncol 10(5):700–708
Folkman J (1995) Seminars in medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. New Engl J Med 333(26):1757–1763
Formenti SC, Demaria S (2009) Systemic effects of local radiotherapy. Lancet Oncol 10(7):718–726
Fujita S et al (1995) Alteration of expression in integrin beta 1-subunit correlates with invasion and metastasis in colorectal cancer. Cancer Lett 91(1):145–149
Galon J et al (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313(5795):1960–1964
Garcia-Barros M et al (2003) Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science 300(5622):1155–1159
Gerashchenko BI, Howell RW (2005) Bystander cell proliferation is modulated by the number of adjacent cells that were exposed to ionizing radiation. Cytom Part A J Int Soc Anal Cytol 66(1):62–70
Gorski DH et al (1999) Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. Cancer Res 59(14):3374–3378
Graeber TG et al (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379(6560):88–91
Grugan KD et al (2010) Fibroblast-secreted hepatocyte growth factor plays a functional role in esophageal squamous cell carcinoma invasion. Proc Natl Acad Sci USA 107(24):11026–11031
Gupta AK et al (2005) HIV protease inhibitors block Akt signaling and radiosensitize tumor cells both in vitro and in vivo. Cancer Res 65(18):8256–8265
Hallahan DE et al (1989) Increased tumor necrosis factor alpha mRNA after cellular exposure to ionizing radiation. Proc Natl Acad Sci USA 86(24):10104–10107
Hanot M et al (2009) Membrane-dependent bystander effect contributes to amplification of the response to alpha-particle irradiation in targeted and nontargeted cells. Int J Radiat Oncol Biol Phys 75(4):1247–1253
Harada H et al (2009) Treatment regimen determines whether an HIF-1 inhibitor enhances or inhibits the effect of radiation therapy. Brit J Cancer 100(5):747–757
Hellevik T et al (2012) Cancer-associated fibroblasts from human NSCLC survive ablative doses of radiation but their invasive capacity is reduced. Radiat Oncol 7(1):59
Hellstrom KE et al (1978) Regression and inhibition of sarcoma growth by interference with a radiosensitive T-cell population. J Exp Med 148(3):799–804
Hess C et al (2001) Effect of VEGF receptor inhibitor PTK787/ZK222584 [correction of ZK222548] combined with ionizing radiation on endothelial cells and tumour growth. Brit J Cancer 85(12):2010–2016
Hildebrandt G et al (1998) Mechanisms of the anti-inflammatory activity of low-dose radiation therapy. Int J Radiat Biol 74(3):367–378
Hino M et al (2010) Heavy ion irradiation induces autophagy in irradiated C2C12 myoblasts and their bystander cells. J Electron Microsc 59(6):495–501

Hodge JW, Sharp HJ, Gameiro SR (2012) Abscopal regression of antigen disparate tumors by antigen cascade after systemic tumor vaccination in combination with local tumor radiation. Cancer Biother Radiopharm 27(1):12–22

Hwang RF et al (2008) Cancer-associated stromal fibroblasts promote pancreatic tumor progression. Cancer Res 68(3):918–926

Ishihara H et al (1993) Induction of the expression of the interleukin-1 beta gene in mouse spleen by ionizing radiation. Radiation Res 133(3):321–326

Iyer R, Lehnert BE (2002) Alpha-particle-induced increases in the radioresistance of normal human bystander cells. Radiat Res 157(1):3–7

Jain RK (2005) Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science 307(5706):58–62

Jamal M et al (2012) The brain microenvironment preferentially enhances the radioresistance of CD133(+) glioblastoma stem-like cells. Neoplasia 14(2):150–158

Janssens GO et al (2012) Accelerated radiotherapy with carbogen and nicotinamide for laryngeal cancer: results of a phase III randomized trial. J Clin Oncol Off J Am Soc Clin Oncol 30(15):1777–1783

Jeggo P, Lavin MF (2009) Cellular radiosensitivity: how much better do we understand it? Int J Radiat Biol 85(12):1061–1081

Jen YM, West CM, Hendry JH (1991) The lower radiosensitivity of mouse kidney cells irradiated in vivo than in vitro: a cell contact effect phenomenon. Int J Radiat Oncol Biol Phys 20(6):1243–1248

Josson S et al (2010) Tumor-stromal interactions influence radiation sensitivity in epithelial- versus mesenchymal-like prostate cancer cells. J Oncol 2010

Kachikwu EL et al (2011) Radiation enhances regulatory T cell representation. Int J Radiat Oncol Biol Phys 81(4):1128–1135

Kikuta K et al (2010) Pancreatic stellate cells promote epithelial-mesenchymal transition in pancreatic cancer cells. Biochem Biophys Res Commun 403(3–4):380–384

Kis E et al (2006) Microarray analysis of radiation response genes in primary human fibroblasts. Int J Radiat Oncol Biol Phys 66(5):1506–1514

Kotera Y, Shimizu K, Mule JJ (2001) Comparative analysis of necrotic and apoptotic tumor cells as a source of antigen(s) in dendritic cell-based immunization. Cancer Res 61(22):8105–8109

Kumagai J et al (2003) Long-lived mutagenic radicals induced in mammalian cells by ionizing radiation are mainly localized to proteins. Radiat Res 160(1):95–102

Kusunoki Y et al (2010) T-cell immunosenescence and inflammatory response in atomic bomb survivors. Radiat Res 174(6):870–876

Lee Y et al (2009) Therapeutic effects of ablative radiation on local tumor require CD8+ T cells: changing strategies for cancer treatment. Blood 114(3):589–595

Little JB et al (2003) Involvement of the nonhomologous end joining DNA repair pathway in the bystander effect for chromosomal aberrations. Radiat Res 159(2):262–267

Lugade AA et al (2005) Local radiation therapy of B16 melanoma tumors increases the generation of tumor antigen-specific effector cells that traffic to the tumor. J Immunol 174(12): 7516–7523

Mantoni TS et al (2011) Pancreatic stellate cells radioprotect pancreatic cancer cells through beta1-integrin signaling. Cancer Res 71(10):3453–3458

Maruyama Y, Eichten JG (1968) Radiation sensitivity of spleen cells irradiated in vitro and in vivo. Am J Roentgenol Radium Ther Nucl Med 102(1):46–52

Matsumura S et al (2008) Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells. J Immunol 181(5):3099–3107

Maxwell PH et al (1997) Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA 94(15):8104–8109

Mikkelsen T et al (2009) Radiation sensitization of glioblastoma by cilengitide has unanticipated schedule-dependency. Int J Cancer 124(11):2719–2727

Moeller BJ et al (2004) Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. Cancer Cell 5(5):429–441

Moeller BJ et al (2005) Pleiotropic effects of HIF-1 blockade on tumor radiosensitivity. Cancer Cell 8(2):99–110
Monnier Y et al (2008) CYR61 and alphaVbeta5 integrin cooperate to promote invasion and metastasis of tumors growing in preirradiated stroma. Cancer Res 68(18):7323–7331
Mothersill C, Seymour C (1997) Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. Int J Radiat Biol 71(4):421–427
Mothersill C, Seymour RJ, Seymour CB (2004) Bystander effects in repair-deficient cell lines. Radiation Res 161(3):256–263
Murata R et al (2001) Interaction between combretastatin A-4 disodium phosphate and radiation in murine tumors. Radiother Oncol J Eur Soc Ther Radiol Oncol 60(2):155–161
Nagasawa H, Little JB (1992) Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res 52(22):6394–6396
Nam JM et al (2010) Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. Cancer Res 70(13):5238–5248
Narayanan PK et al (1999) Alpha particles induce the production of interleukin-8 by human cells. Radiation Res 152(1):57–63
Nesslinger NJ et al (2007) Standard treatments induce antigen-specific immune responses in prostate cancer. Clin Cancer Res Off J Am Assoc Cancer Res 13(5):1493–1502
North RJ (1986) Radiation-induced, immunologically mediated regression of an established tumor as an example of successful therapeutic immunomanipulation. Preferential elimination of suppressor T cells allows sustained production of effector T cells. J Exp Med 164(5):1652–1666
Ohba K et al (1998) Abscopal regression of hepatocellular carcinoma after radiotherapy for bone metastasis. Gut 43(4):575–577
Overgaard J (2007) Hypoxic radiosensitization: adored and ignored. J Clin Oncol Off J Am Soc Clin Oncol 25(26):4066–4074
Palcic B, Skarsgard LD (1984) Reduced oxygen enhancement ratio at low doses of ionizing radiation. Radiat Res 100(2):328–339
Papadopoulou A, Kletsas D (2011) Human lung fibroblasts prematurely senescent after exposure to ionizing radiation enhance the growth of malignant lung epithelial cells in vitro and in vivo. Int J Oncol 39(4):989–999
Paris F et al (2001) Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science 293(5528):293–297
Park CC et al (2003) Ionizing radiation induces heritable disruption of epithelial cell interactions. Proc Natl Acad Sci USA 100(19):10728–10733
Park CC et al (2006) Beta1 integrin inhibitory antibody induces apoptosis of breast cancer cells, inhibits growth, and distinguishes malignant from normal phenotype in three dimensional cultures and in vivo. Cancer Res 66(3):1526–1535
Patel ZS et al (2012) Ionizing radiation enhances esophageal epithelial cell migration and invasion through a paracrine mechanism involving stromal-derived hepatocyte growth factor. Radiat Res 177(2):200–208
Pinzone MR et al (2012) Non-AIDS-defining cancers among HIV-infected people. Eur Rev Med Pharmacol Sci 16(10):1377–1388
Poglio S et al (2009) Adipose tissue sensitivity to radiation exposure. Am J Pathol 174(1):44–53
Pore N et al (2006) Nelfinavir down-regulates hypoxia-inducible factor 1alpha and VEGF expression and increases tumor oxygenation: implications for radiotherapy. Cancer Res 66(18):9252–9259
Postow MA et al (2012) Immunologic correlates of the abscopal effect in a patient with melanoma. N Engl J Med 366(10):925–931
Prendergast AM et al (2011) Activation of DNA damage response pathways in human mesenchymal stem cells exposed to cisplatin or gamma-irradiation. Cell Cycle 10(21):3768–3777
Puck TT, Marcus PI (1956) Action of x-rays on mammalian cells. J Exp Med 103(5):653–666
Qayum N et al (2009) Tumor vascular changes mediated by inhibition of oncogenic signaling. Cancer Res 69(15):6347–6354

Qayyum MA, Insana MF (2012) Stromal responses to fractionated radiotherapy. Int J Radiat Biol 88(5):383–392
Reiner J, Southam CM (1966) Effect of immunosuppression on first-generation isotransplantation of chemically induced tumours in mice. Nature 210(5034):429–430
Reits EA et al (2006) Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. J Exp Med 203(5):1259–1271
Sanda MG et al (1995) Molecular characterization of defective antigen processing in human prostate cancer. J Natl Cancer Inst 87(4):280–285
Sato Y et al (2009) Cancer cells expressing toll-like receptors and the tumor microenvironment. Cancer Microenviron Off J Int Cancer Microenviron Soc 2(Suppl 1):205–214
Scaringi C et al (2012) Integrin inhibitor cilengitide for the treatment of glioblastoma: a brief overview of current clinical results. Anticancer Res 32(10):4213–4223
Schaue D et al (2008) T-cell responses to survivin in cancer patients undergoing radiation therapy. Clin Cancer Res Off J Am Assoc Cancer Res 14(15):4883–4890
Schaue D et al (2012) Maximizing tumor immunity with fractionated radiation. Int J Radiat Oncol Biol Phys 83(4):1306–1310
Schettino G et al (2005) Low-dose binary behavior of bystander cell killing after microbeam irradiation of a single cell with focused c(k) x rays. Radiat Res 163(3):332–336
Schwartz DL et al (2009) The selective hypoxia inducible factor-1 inhibitor PX-478 provides in vivo radiosensitization through tumor stromal effects. Mol Cancer Ther 8(4):947–958
Sedelnikova OA et al (2007) DNA double-strand breaks form in bystander cells after microbeam irradiation of three-dimensional human tissue models. Cancer Res 67(9):4295–4302
Senger DR et al (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science 219(4587):983–985
Seung SK et al (2012) Phase 1 study of stereotactic body radiotherapy and interleukin-2-tumor and immunological responses. Sci Transl Med 4(137):137ra74
Shaked Y et al (2006) Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 313(5794):1785–1787
Shao C et al (2006) Calcium fluxes modulate the radiation-induced bystander responses in targeted glioma and fibroblast cells. Radiat Res 166(3):479–487
Shao C, Folkard M, Prise KM (2008) Role of TGF-beta1 and nitric oxide in the bystander response of irradiated glioma cells. Oncogene 27(4):434–440
Shi Y, Evans JE, Rock KL (2003) Molecular identification of a danger signal that alerts the immune system to dying cells. Nature 425(6957):516–521
Shinohara ET et al (2005) Enhanced radiation damage of tumor vasculature by mTOR inhibitors. Oncogene 24(35):5414–5422
Siemann DW, Rojiani AM (2002) Enhancement of radiation therapy by the novel vascular targeting agent ZD6126. Int J Radiat Oncol Biol Phys 53(1):164–171
Stamell EF et al (2012) The abscopal effect associated with a systemic anti-melanoma immune response. Int J Radiat Oncol Biol Phys 85:293–295
Thompson LH (2012) Recognition, signaling, and repair of DNA double-strand breaks produced by ionizing radiation in mammalian cells: the molecular choreography. Mutat Res 751(2):158–246
Tilkin AF et al (1981) Reduced tumor growth after low-dose irradiation or immunization against blastic suppressor T cells. Proc Natl Acad Sci USA 78(3):1809–1812
Tong RT et al (2004) Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. Cancer Res 64(11):3731–3736
Vines AM et al (2009) Bystander effect induced changes in apoptosis related proteins and terminal differentiation in in vitro murine bladder cultures. Int J Radiat Biol 85(1):48–56
Wang R, Coderre JA (2005) A bystander effect in alpha-particle irradiations of human prostate tumor cells. Radiat Res 164(6):711–722
Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270(3):1230–1237

Watson ER et al (1978) Hyperbaric oxygen and radiotherapy: a medical research council trial in carcinoma of the cervix. Brit J Radiol 51(611):879–887
Watson GE et al (2000) Chromosomal instability in unirradiated cells induced in vivo by a bystander effect of ionizing radiation. Cancer Res 60(20):5608–5611
Wersall PJ et al (2006) Regression of non-irradiated metastases after extracranial stereotactic radiotherapy in metastatic renal cell carcinoma. Acta oncol 45(4):493–497
White DE et al (2004) Targeted disruption of beta1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. Cancer Cell 6(2):159–170
Wild-Bode C et al (2001) Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. Cancer Res 61(6):2744–2750
Williams KJ et al (2004) ZD6474, a potent inhibitor of vascular endothelial growth factor signaling, combined with radiotherapy: schedule-dependent enhancement of antitumor activity. Clin Cancer Res Off J Am Assoc Cancer Res 10(24):8587–8593
Wilson WR et al (1998) Enhancement of tumor radiation response by the antivascular agent 5,6-dimethylxanthenone-4-acetic acid. Int J Radiat Oncol Biol Phys 42(4):905–908
Winkler F et al (2004) Kinetics of vascular normalization by VEGFR2 blockade governs brain tumor response to radiation: role of oxygenation, angiopoietin-1, and matrix metalloproteinases. Cancer Cell 6(6):553–563
Xue LY et al (2002) Bystander effect produced by radiolabeled tumor cells in vivo. Proc Natl Acad Sci USA 99(21):13765–13770
Yang G et al (2009) Mitochondrial dysfunction resulting from loss of cytochrome c impairs radiation-induced bystander effect. Brit J Cancer 100(12):1912–1916
Yao ES et al (2007) Increased beta1 integrin is associated with decreased survival in invasive breast cancer. Cancer Res 67(2):659–664
Yasui H et al (2008) Inhibition of HIF-1alpha by the anticancer drug TAS106 enhances X-ray-induced apoptosis in vitro and in vivo. Brit J Cancer 99(9):1442–1452
Zhong H et al (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59(22):5830–5835
Zhou H et al (2005) Mechanism of radiation-induced bystander effect: role of the cyclooxygenase-2 signaling pathway. Proc Natl Acad Sci USA 102(41):14641–14646

# Chapter 8
# Autophagy and Cell Death to Target Cancer Cells: Exploiting Synthetic Lethality as Cancer Therapies

**Julie Reyjal, Kevin Cormier, and Sandra Turcotte**

**Abstract** Since 1940 chemotherapy has been one of the major therapies used to kill cancer cells. However, conventional standard cytotoxic agents have a low therapeutic index and often show toxicity in healthy cells. Over the past decade, progress in molecular biology and genomics has identified signaling pathways and mutations driving different types of cancer. Genetic and epigenetic alterations that characterize tumor cells have been used in the development of targeted therapy, a very active area of cancer research. Moreover, identification of synthetic lethal interactions between two altered genes in cancer cells shows much promise to target specifically tumor cells. For a long time, apoptosis was considered the principal mechanism by which cells die from chemotherapeutic agents. Autophagy, necroptosis (a programmed cell death mechanism of necrosis), and lysosomal-mediated cell death significantly improve our understanding of how malignancy can be targeted by anticancer treatments. Autophagy is a highly regulated process by which misfolded proteins and organelles reach lysosomes for their degradation. Alterations in this cellular process have been observed in several pathological conditions, including cancer. The role of autophagy in cancer raised a paradox wherein it can act as a tumor suppressor at early stage of tumor development but can also be used by cancer cells as cytoprotection to promote survival in established tumors. It is interesting that autophagy can be targeted by anticancer agents to provoke cancer cell death. This review focuses on the role of autophagy in cancer cells and its potential to therapeutically kill cancer cells.

**Keywords** Autophagy • Cancer • Cell death • Targeted therapy • Synthetic lethality • Renal cell carcinoma • von Hippel-Lindau

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## 8.1 Overview of the Autophagy Machinery

Autophagy is a self-digestive process. From the Greek *auto*, meaning "oneself," and *phagy*, meaning "eating," this process is highly conserved in organisms from yeast to mammals and acts to remove misfolded proteins, aggregates, lipids, and damaged organelles. To maintain cellular homeostasis, cytoplasmic cargoes are sequestered into vesicles that reach lysosomes, where the material is degraded (Yang and Klionsky 2010). There are different types of autophagy, ranging from nonselective macroautophagy to selective autophagy such as chaperone-mediated autophagy, microautophagy, and the type based on the origin of the sequestered cargo, including mitophagy for mitochondria. Chaperone-mediated autophagy targets specific proteins containing the KFERQ sequence across the lysosome membrane, whereas microautophagy involves the direct engulfment of cytoplasm at the lysosome surface by invagination of the lysosome membrane (Reggiori et al. 2012). In contrast, macroautophagy (referred to hereafter as autophagy) is mediated by the special organelle autophagosome that engulfs proteins, lipids, and damaged organelles into double-membraned vesicles. Then the autophagosome fuses with an endosome/lysosome, a single-membrane vesicle, where the cargo is degraded through lysosomal activity (Fig. 8.1) (Klionsky and Emr 2000). Autophagy is activated under physiological and pathological conditions, such as nutrient starvation, hypoxia, metabolic stress, and in response to drugs and radiation. This dynamic process generates cellular energy resources that allow a cell to adapt its metabolism to energy demand. Defects during any step of the autophagy process result in the accumulation of damaged proteins and/or genomic damage that can stimulate the development of many human diseases, including neurodegeneration, infectious disease, heart disease, and cancer (Levine and Kroemer 2008; Turcotte and Giaccia 2010).

### *8.1.1 Autophagosome Formation*

The unique structure of the autophagosome was first observed more than 50 years ago using electronic microscopy, and successive studies have demonstrated that autophagy is regulated through activation of autophagy-related genes (Atg) (Yang and Klionsky 2010). These genes were first identified in yeast, and many of them are found as homologs in murine and human cells (Takeshige et al. 1992). More than 15 mammalian Atg proteins have been identified and regulate the formation of autophagosomes (Table 8.1) (Mizushima et al. 2011). The initiation stage of this process engages the formation of a phagophore, followed by its elongation and closure to form an autophagosome. The origin of the phagophore is still controversial, but the endoplasmic reticulum membrane (Axe et al. 2008; Hayashi-Nishino et al. 2009; Yla-Anttila et al. 2009), mitochondrial outer membrane (Hailey et al. 2010), and plasma membrane (Ravikumar et al. 2010) have been suggested to contribute to autophagosome formation.

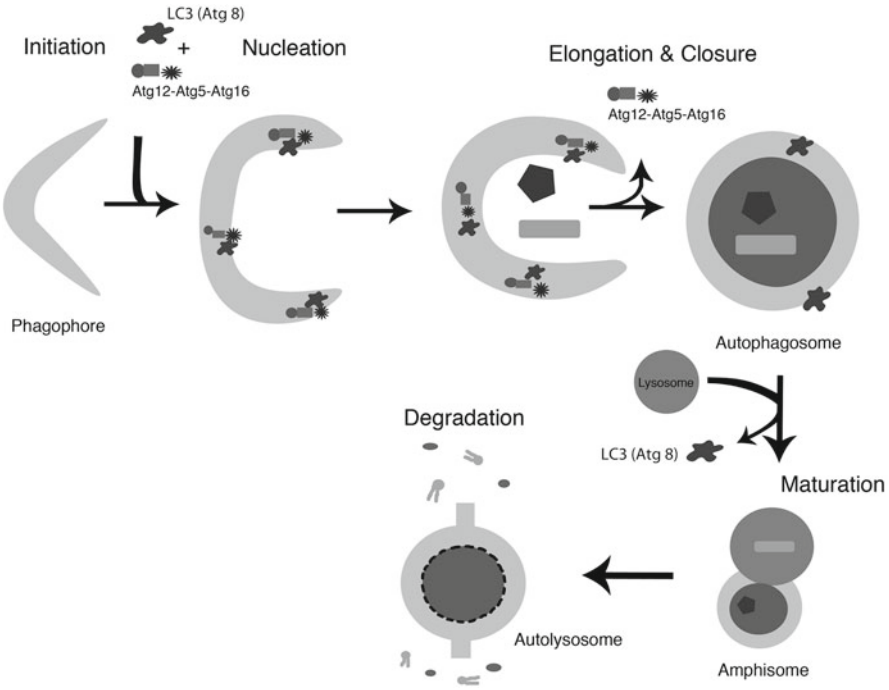


**Fig. 8.1** Principal steps regulating the autophagy process. Autophagy involves the formation of double-membrane autophagosomes that fuse with lysosomes to form autolysosomes for the degradation of intracellular proteins and organelles. Under conditions of nutrient deprivation or microenvironmental stress, initiation gives rise to a phagophore, which elongates while being regulated by a series of autophagy-related genes. The phagophore closes into an autophagosome. This autophagosome then fuses with a lysosome to become an amphisome, which will mature and give rise to an autolysosome, where the encapsulated material is degraded via lysosomal activity

The activity of the autophagic machinery is regulated by different complexes: the ULK1/2 kinase complex, the vacuolar sorting protein (Vps) 34/Beclin-1 complex, the shuttling of the Atg9 protein (the only transmembrane Atg) between organelles including endosomes, and the two ubiquitin-conjugation systems, the Atg5-Atg12-Atg16 and Atg8/LC3 complexes (Fig. 8.2) (Orsi et al. 2012; Lamb et al. 2013; Rubinsztein et al. 2012). *ULK1* and *ULK2*, two Atg1 homologs, are associated with Atg13 and FIP200 in a large complex that integrates stress signals from the mammalian target of rapamycin (mTOR) complex 1 (mTORC1) (Jung et al. 2009; Mizushima 2010). Many signals, including growth factors, amino acids, glucose, and energy status, regulate mTORC1. Upon inhibition of mTORC1 induced by starvation or chemotherapeutic agents targeting mTOR, *ULK1* and *ULK2* are phosphorylated and activated, initiating the autophagy cascade. Other complexes essential to autophagosome formation is Beclin-1, the Atg6 homolog, and Vps34, a class III phosphoinositide 3-kinase (PI3K), which recruit autophagy proteins such

**Table 8.1** Autophagy-related genes involved in autophagosome formation

| Yeast name | Human orthologs | Functions |
|---|---|---|
| Atg1 | ULK1/2 | Serine protein kinase |
| | | Component of complex ULK1-FIP200-Atg13 |
| Atg2 | Atg2a | Autophagosome closure |
| | Atg2b | Component of complex Atg9-Atg2-Atg18 |
| Atg3 | Atg3 | E2-like enzyme required for LC3 lipidation |
| Atg4 | Atg4A, 4B, 4C, 4D | Cysteine protease involved in LC3 lipidation |
| Atg5 | Atg5 | Atg5-Atg12 ubiquitin conjugation complex |
| Atg6 | Beclin-1 | Component of the PI3K-Vps34-Beclin complex |
| Atg7 | Atg7 | E1-like enzyme activates LC3 and Atg12 |
| Atg8 | LC3A, LC3B, LC3C | Autophagosome marker, ubiquitin-like protein conjugated to phosphatidylethanolamine |
| | GABARAP, GABARAPL1, GABARAPL2 | |
| Atg9 | Atg9a | Transmembrane protein |
| | Atg9b | Component of Atg9-Atg2-Atg18 complex |
| Atg10 | Atg10 | E2-like enzyme conjugates Atg12 to Atg5 |
| Atg12 | Atg12 | Ubiquitin-like protein conjugated to Atg5 |
| Atg13 | Atg13 | Response to mTOR signaling |
| | | Component of complex ULK1-Atg13-FIP200 |
| Atg14 | Atg14 | Component of the PI3K-Vps34-Beclin complex |
| Atg16 | Atg16L1 | Component of Atg5-Atg12-Atg16 complex |
| Atg17 | FIP200 | Component of ULK1-Atg13-FIP200 complex |
| Atg18 | WIP1/2 | Component of Atg9-Atg2-Atg18 complex |

*mTOR* mammalian target of rapamycin; *PI3K* phosphoinositide 3-kinase; *Vps* vacuolar sorting protein

as *UVRAG* (ultraviolet irradiation resistance-associated gene), *Ambra-1*, *Bif-1*, and *Barkor* (Kroemer et al. 2010). Furthermore, Beclin-1 binds to anti-apoptotic proteins of the BCL-2 family, such as BCL-$X_{L,}$ through a BCL-2 homology 3 domains and inhibits autophagy (Pattingre et al. 2005; Erlich et al. 2007). In response to starvation, phosphorylation on Bcl-2 by Jun kinase 1 dissociates the binding between Bcl-2 and Beclin-1 and allow Beclin-1 to induce autophagy (Wei et al. 2008; Pattingre et al. 2009). BCL-2 homology 3 mimics can also disrupt Bcl-2 and Beclin-1 binding. Finally, there are two ubiquitin conjugation systems that have been associated with autophagosome formation: Atg12-Atg5-Atg16 and Atg8/LC3. Atg5 and Atg12 were the first Atgs identified in mammals by Mizushima et al. (1998), who reported that the Atg5-Atg12-Atg16 conjugation system was conserved. The other ubiquitin conjugation system is MAP1LC3 (also called LC3), the mammalian Atg8 homolog (Kabeya et al. 2000). In unstressed cells, LC3 is present in cytoplasm in an unprocessed form, LC3I, which is converted into a phosphatidylethanolamine-conjugated form, LC3II, associated with completed autophagosomes. LC3II remains associated with the double-membraned vesicle until fusion with lysosomes. The identification of LC3 is an important finding that is routinely used to monitor autophagy in eukaryote cells. Moreover, LC3 binds the p62/sequestome1 (SQSMT1) protein via its LC3-interactin region domain and prevents its accumulation (Pankiv et al. 2007). p62 Is an adaptor protein involved in protein trafficking to

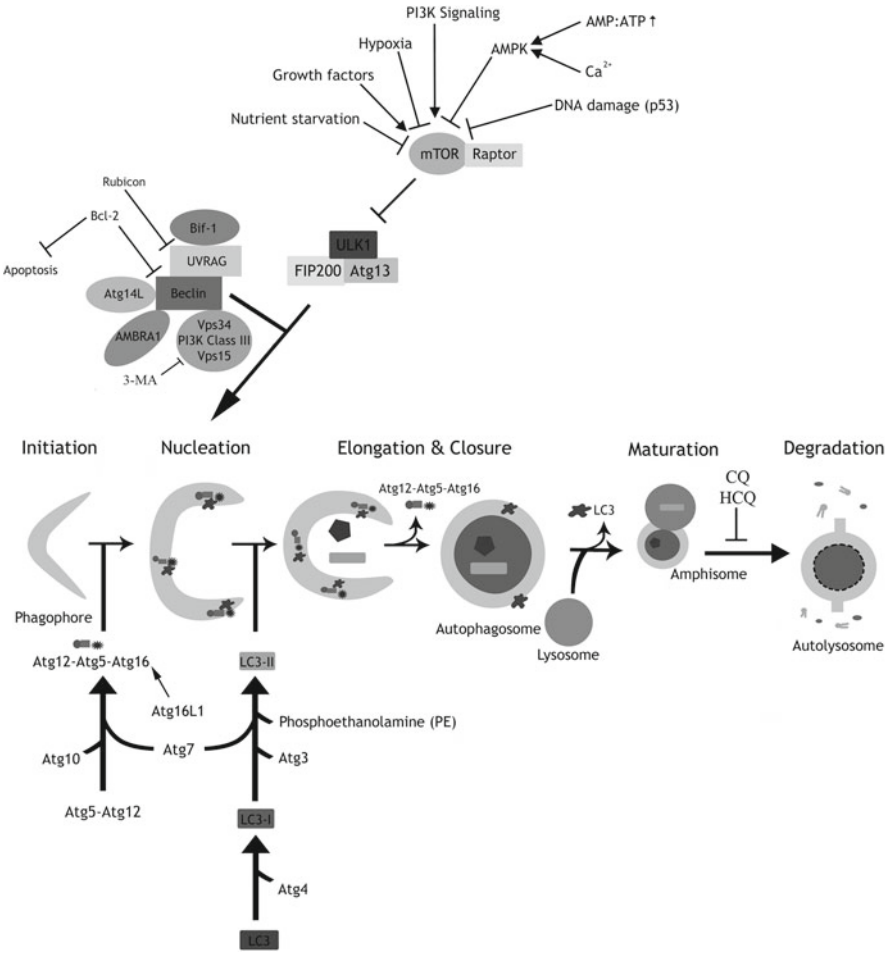


**Fig. 8.2** Overview of the complexes involved in autophagosome formation. At least four important functional groups of autophagy-related gene proteins are required for autophagy: ULK1 protein-kinase complex and vacuolar sorting protein 34–Beclin 1 class III phosphoinositide 3-kinase (PI3K) complex regulate autophagy initiation; the Atg9-Atg2-Atg18 complex regulates the expansion of the phagophore assembly site; and the Atg5-Atg12-Atg16 and LC3 conjugation systems regulate the elongation of autophagosome membranes. Phosphatidylethanolamine (*PE*)-conjugated LC3 (called *LC3-II*) remains on the isolation membranes and autophagosome membranes, whereas the Atg12-Atg5-Atg16 complex transiently associates with the isolation membranes and dissociates from the autophagosome membranes. Pharmacological inhibitors of the autophagy process are 3-methyladenine, which inhibits PI3K, and autophagosome formation, while chloroquine (*CQ*) and hydroxychloroquine (*HCQ*) block autophagosome maturation by increasing the pH of the lysosomes

the proteasome and facilitates autophagic degradation of ubiquitinated protein aggregates. It is known to activate the nuclear factor erythroid 2-related factor 2 (NRF2) (Inami et al. 2011). This transcription factor turns on the antioxidant gene transcription that allows cells to protect themselves from oxidative stress.

### 8.1.2 Maturation of the Autophagosome Through the Endocytic Pathway

Autophagosomes are subsequently transformed to an amphisome after fusion with an endosome/lysosome. During this step, endocytosis and autophagy share machinery for the maturation of the autophagosome. A functional endocytic pathway from the early endosomes to the late endosomes and including multivesicular bodies is essential to maintaining an efficient autophagic flux. Several proteins, including members of the Rab GTPase family, Vps, and endosomal sorting complexes required for transport, have been identified as regulating each step of this process and are described in recent reviews (Lamb et al. 2013). Rab7 is an important element that controls endosomal maturation and lysosome traffic, and its activity is regulated in part by its GTPase-activating proteins and by the PI3K complex formed by Rubicon-UVRAG-Rab7 (Liang et al. 2008; Sun et al. 2010). Rab7 activity is inhibited by its binding with Rubicon and UVRAG (Liang et al. 2008). However, when the level of Rab7 increases until a threshold point, binding with Rubicon is lost and UVRAG can activate the HOPS (homotypic fusion and Vps) complex, which further increases Rab7 activity, promoting fusion with lysosomes (Zlatic et al. 2011; Peralta et al. 2010).

### 8.1.3 The End of the Road Through the Lysosome

Lysosomes have emerged as an important platform of mTORC1 signaling and regulation. It has been shown that lysosomal genes are regulated by the transcription factor EB (TFEB), which also controls the major steps of the autophagy pathway (autophagosome formation, autophagosome fusion with lysosomes, and degradation of cargo) linking autophagy to lysosomal biogenesis (Sardiello et al. 2009; Settembre et al. 2011). Under stress or aberrant lysosomal storage conditions, TFEB translocates from the cytoplasm to the nucleus and induces lysosomal biogenesis (Settembre et al. 2012). Other groups demonstrated that the lysosomal reformation that occurs during autophagy is regulated by mTORC1 and that TFEB phosphorylation and nuclear translocation are coordinately regulated by mTORC1 (Yu et al. 2010; Pena-Llopis et al. 2011). At the peak of autophagy, lysosomes are consumed by their fusion with autophagosomes, but after a prolonged period of autophagy, mTORC1 is reactivated (inhibits autophagy) and induces lysosomal biogenesis through TFEB activation (Yu et al. 2010).

The mTORC1 pathway that regulates cell growth in response to numerous cues, including amino acids, has been found on the lysosomal surface, its site of activation (Pena-Llopis et al. 2011; Korolchuk et al. 2011). Although the mechanism that elucidates every step of this process is not completely understood, elegant studies indicate that Rag GTPases (a heterodimeric complex of RagA/B and RagC/D GTPases), also located on the lysosomes, and vacuolar-type H+-ATPase (V-ATPase)

form a signaling system that is necessary for amino acid sensing by mTORC1 (Bar-Peled et al. 2012; Zoncu et al. 2011; Settembre et al. 2012; Sancak et al. 2010). Under nutrient-rich conditions, mTOR is located on peripheral lysosomes, where it becomes activated and promotes cell growth and inhibits autophagy, whereas mTOR and lysosomes are clustered in the perinuclear area during starvation, leading to induction of autophagy. This location facilitates the fusion of autophagosomes with lysosomes and autophagosome synthesis (by inhibiting mTOR activity) (Korolchuk and Rubinsztein 2011). The lysosome distribution depends, in part, on their being transported along microtubules, a process mediated by Arl8 (a small GTPase) and KIF2 (a kinesis family member) (Korolchuk et al. 2011). pHi has been shown to affect lysosome positioning, where acidification redistributes lysosomes from their predominantly perinuclear location toward the cell periphery and correlates with increased mTOR activity and inhibition of autophagy (Korolchuk et al. 2011; Heuser 1989).

## 8.2 Role of Autophagy in Cancer

Cells with defects in autophagy accumulate misfolded proteins, ubiquitinated aggregates, lipid droplets, and damaged organelles (mostly mitochondria, peroxisomes, and endoplasmic reticulum) that could lead to accumulation of reactive oxygen species (ROS), metabolic stress, and toxicity. Disruption of autophagy has been associated with cancer. The consequences of autophagy defects in cancer are complex, and new advances indicate that it could be linked to the tumor stages (White 2012; Mah and Ryan 2012; Janku et al. 2011). Autophagy can suppress tumors by preventing accumulation of toxic waste and tumor initiation, but it can also help cancer cells survive under metabolic stress and promote tumors once the tumor is established. Understanding the role of autophagy in cancer is critical because inhibition or activation of autophagy can be therapeutically applicable to killing cancer cells.

### *8.2.1 Autophagy in Tumor Suppression and Tumor Initiation*

Genetic deletion of Beclin-1 is among the first evidence that autophagy can prevent tumor formation: mice with allelic loss of Beclin-1 are partially defective for autophagy and have increased spontaneous malignancies (Qu et al. 2003; Yue et al. 2003). Similarly, humans with Beclin-1 deletion have a higher frequency of leukemia, lymphomas, and tumors of the liver, lung, breast, ovarian, and prostate (Liang et al. 1999; Aita et al. 1999). Further studies of knockout mice demonstrated that basal autophagy is essential for viability because deletion of both Beclin-1 alleles induces embryonic lethality. In addition, the activation of Beclin-1 inhibits cell proliferation in vitro and tumor growth. Moreover, mice deficient in Atg4C develop fibrosarcomas (Marino et al. 2007), whereas a loss of Atg5 and Atg7 improve the risk of benign liver tumors (Takamura et al. 2011).

It has been shown that autophagy activation can prevent necrotic cell death in apoptosis-deficient cells, a process that may cause local inflammation and promote tumor growth (White et al. 2010). One explanation for the role of autophagy in tumor suppression has been linked to its ability to removed toxic waste during the initiation stage of tumorigenesis. Cells with deregulation in autophagy cause impaired mitochondria and accumulation of ROS, which promote genotoxic stress through DNA damage (Mathew et al. 2007; Degenhardt et al. 2006). This could lead to the loss of mitochondrial potential membrane, activation of phosphatase and tensin homolog–induced putative linase-1 (*PINK1*) and induction of *PARK2*, an E3 ubiquitin ligase involved in mitophagy (Arena et al. 2013). *PARK2* is a tumor suppressor gene, and mutations of it have been observed in glioblastomas and colon and lung cancers (Veeriah et al. 2010; Poulogiannis et al. 2010).

Another possibility by which autophagy may prevent cancer is through p62 (Mathew et al. 2009). In unstressed cells, NRF2 activity is inhibited by its binding to kelch-like ECH-associated protein 1 (*KEAP1*), which inactivates the antioxidant defense genes and stimulates proteasomal degradation (Copple et al. 2010; Lau et al. 2010). In autophagy-defective cells or in the presence of oxidative stress, *KEAP1* is modified and its binding with NRF2 is lost (Lau et al. 2010). Then, *p62* can bind and sequester *KEAP1*, promoting NRF2 activation, antioxidant defense, and survival. Therefore, autophagy is necessary to prevent p62 accumulation and NRF2 activation that could promote tumorigenesis.

### *8.2.2 Autophagy in Tumor Progression*

Autophagy is induced as an alternative source of energy and metabolites to maintain cell survival during nutrient starvation or metabolic or other stress such as hypoxia, ischemia, and proteasome inhibition. Almost all of these conditions are observed in established tumors. Under stress conditions, autophagy protects dormant cells from damage (White 2012). When the conditions are more favorable or return to normal, these cells can recover and grow. Then, autophagy can provide a survival advantage to tumor cells, allowing them to adapt to metabolic stress found in the tumor microenvironment; a variety of mechanisms have been proposed to support this. It has been shown that the Bcl-2/adenovirus E1B interacting protein (*BNIP3*), a downstream target of hypoxia-inducible factor (HIF)-1α, can induce autophagy by disrupting the Beclin-1–Bcl-2 complex to release Beclin-1 in response to a hypoxic microenvironment (Bellot et al. 2009). Amino acid and glucose deprivation found in the tumor microenvironment have been correlated with a higher level of autophagosomes and deletion of essential Atgs, which induces tumor cell death associated with the hypoxic regions. Recent studies indicate that human cancer tissues with a low level of Beclin-1 have been associated with worse prognosis in esophageal (Chen et al. 2009), colon (Li et al. 2009), and pancreatic cancer (Kim et al. 2011). In addition, tumors from Beclin-1-deficient mice are more aggressive under hypoxic conditions, a mechanism that could be regulated through the HIF-2α

(Lee et al. 2011). Other studies reported that autophagy is triggered to protect cancer cells from nutrient deprivation by activation of AMP-activated protein kinase (AMPK), a sensor of energy status. AMPK activation limits translation initiation and protein synthesis through the inhibition of elongation factor 2 (EF2) and the inhibition of mTOR, leading to the induction of autophagy (Horbinski et al. 2010).

By studying the role of autophagy in cancer, several groups have noticed that cancer cells have a high level of basal autophagy, even in unstressed conditions. White and colleagues showed that activated cells expressing Ras are dependent on autophagy to survive starvation, and biallelic deletion of Atg5 or Atg7 decrease tumor growth of RAS-transformed epithelial cells in the kidneys of nude mice (Guo et al. 2011). This study indicated that autophagy is required to maintain functional mitochondrial and oxidative metabolism necessary to Ras-expressing tumor growth. Autophagy can also promote metastasis and cell survival in response to microenvironmental stresses (Kenific et al. 2010). High expression of *LC3* and *Beclin-1* are correlated with poor survival and a shorter disease-free period in pancreatic and nasopharyngeal carcinomas, respectively (Fujii et al. 2008; Wan et al. 2010). It is interesting to note that γ-aminobutyric acid type A receptor-associated protein (*GABARAP*), a member of the *LC3* family, is a new prognostic marker for colorectal carcinoma because its overexpression is associated with reduced survival (Miao et al. 2010).

## 8.3 Autophagy and Cell Death as Targets for Anticancer Therapy

There are a number of molecules targeting various proteins of the apoptosis pathway. Some groups of these molecules – such as ABT-263 (www.clinicaltrials.gov identifier NCT00743028), AT-101 (NCT00275431), and GX15-070MS or Obatoclax (NCT00600964) – affect the activation or balance of the Bcl-2 protein family, tipping the scale toward apoptosis, while others block the inhibitor apoptosis proteins, including AT-406 (NCT01078649), ENZ-3042 (NCT01186328), HGS-1029 (NCT00708006), and LCL-161 (NCT01098838), thus inducing the apoptosis pathway. On the other hand, elucidation of the molecular mechanisms involved in autophagy indicates crosstalk between the apoptotic and autophagic pathways (Amelio et al. 2011; Ouyang et al. 2012). For example, inhibition of apoptosis can induce autophagy, whereas inhibition of autophagy can stimulate apoptosis (Maiuri et al. 2007). In addition, both pathways can be activated through similar proteins, among them, the complex formed by Beclin-1 and Bcl-2 (Kang et al. 2011). Depending on the anticancer agents and the cell type, drugs can have a lethal effect in response to autophagy induction through the influence of the anti-apoptotic effect of Bcl-2 or the phosphorylation of Jun kinase (Wei et al. 2008). Among other proteins that could be involved in the crosstalk between apoptosis and autophagy are the activation of *p53*, which transcriptionally increases the signaling of AMPK; death-associated protein kinase (DAPK1); tuberous sclerosis protein 2 (TSC2); and ULK1/2 (Feng 2010). Autophagy may also protect against tumorigenesis by

**Table 8.2** Clinical trials of monotherapeutic agents that induce autophagy

| Agents | Target | Condition | Clinical trial |
|---|---|---|---|
| Autophagosome formation | | | |
| Imatinimb | Bcr-Abl | Leukemia | NCT00079313 |
| Temsirolimus | mTOR | Renal cell carcinoma | NCT00494091 |
| Everolimus | mTOR | Renal cell carcinoma | NCT00422344 |
| Amiodarone | mTOR | Atrial fibrillation | NCT00845780 |
| Sunitinib | VEGFR | Renal cell carcinoma | NCT01441661 |
| AZD8055 | mTOR | Solid tumors | NCT00973076 |
| Sorafenib | VEGFR | Renal cell carcinoma | NCT00478114 |
| Arsenic trioxide | BNIP3 | Liver | NCT00582400 |
| Perifosine | Akt | Prostate cancer | NCT00058214 |
| Metformin | AMPK | Ovarian cancer | NCT01208740 |
| Autophagosome maturation | | | |
| STF-62247 | Unknown | Renal cell carcinoma | |
| CQ | Lysosomotropic agent | Small-cell lung cancer<br>Ductal carcinoma | NCT01575782<br>NCT01023477 |
| HCQ | Lysosomotropic agent | Renal cell carcinoma | NCT01144169 |

Data are taken from www.ClinicalTrials.gov. *Akt* protein kinase B; *AMPK* AMP-activated protein kinase; *BNIP3* BCL2/adenovirus E1B 19-kDa interacting protein 3; *CQ* chloroquine; *HCQ* hydroxychloroquine, *mTOR* mammalian target of rapamycin; *VEGFR* vascular endothelial growth factor receptor

limiting necrosis and chronic inflammation in response to metabolic stress, which is associated with the release of the proinflammatory HMGB1 (Degenhardt et al. 2006). A hypoxic tumor microenvironment, nutrient or amino acid levels, as well as the signaling pathway can influence the final outcome between cell death and survival when autophagy is induced. Whether cells can die from autophagy (autophagic cell death) or as a consequence of autophagy induction needs to be addressed.

### *8.3.1 Autophagy to Induce Cell Death*

Various chemotherapeutic agents have been shown to induce autophagy and participate in the induction of cell death. Therefore, inhibition of autophagy using small interfering RNA targeting Atg5, Atg7, or Beclin-1 reduces death, suggesting that autophagy can eliminate tumor cells (Amaravadi et al. 2011; Janku et al. 2011). Table 8.2 summarize agents that have been reported to have anticancer effects as monotherapies. One of the most targeted approaches to killing cancer cells in response to autophagy is through the mTOR pathway. This process regulates cell proliferation and protein translation, and its inhibition induces autophagy as well as cell cycle arrest and apoptosis. The strong induction of autophagy in vivo in response to everolimus, a chemotherapeutic agent targeting mTOR, reduces the growth of advanced pancreatic tumors (Yao et al. 2010) and leukemia (Crazzolara et al. 2009).

**Table 8.3** Clinical trials of combined agents modulating autophagy

| Molecule name | Condition | Clinical stage | Clinical trials identifier |
|---|---|---|---|
| HCQ-docetaxel | Prostate cancer | Phase II | NCT00786682 |
| HCQ-gemcitabine | Pancreatic cancer | Phase I | NCT01506973 |
| HCQ-MK2206 | Advanced solid tumors and prostate and kidney cancers | Phase I | NCT01480154 |
| HCQ-everolimus | Renal cell carcinoma | Phase I | NCT01510119 |
| HCQ-rapamycin | Relapsed or refractory myeloma | Phase I | NCT01689987 |
| HCQ-erlotinib | Lung cancer | Phase II | NCT01026844 |
| HCQ-sirolimus or vorinostat | Advanced solid cancers | Phase I | NCT01266057 NCT01023737 |
| HCQ-temozolomide | Advanced solid tumors | Phase I | |
| HCQ-sunitinib | Advanced solid tumors | Phase I | NCT00813423 |
| HCQ-bortezomib | Multiple myeloma | Phase I/II | NCT00568880 |
| Rapamycin-sunitinib | Advanced non-small-cell lung cancer | Phase I | NCT00555256 |
| Everolimus-BEZ235 | Advanced solid tumors, metastatic breast cancer, and metastatic renal cell carcinoma | Phase I | NCT01482156 |
| Rapamycin-trastuzumab | Metastatic breast cancer | Phase II | NCT00411788 |

Data are taken from (www.ClinicalTrials.gov)

Furthermore, temsirolimus and everolimus have been approved for the treatment of renal cell carcinoma (RCC). In addition, radiation as well as many chemotherapeutic agents inducing DNA damage and *p53* activation have demonstrated a synergic effect in combination with everolimus to kill cancer cells (O'Reilly et al. 2011). Other drugs inhibiting Bcl-2 and activating Beclin-1 in apoptosis-defective cells show a potential effect on cell killing by the formation of autophagosomes. Obatoclax is a Bcl-2 inhibitor that induces cell death. However, when apoptosis is functional, Obatoclax could promote both autophagy and apoptosis to kill acute lymphoblastic leukemia and non-small-lung cancer (Heidari et al. 2010; McCoy et al. 2010).

### *8.3.2 Inhibition of Autophagy to Improve Anticancer Treatments*

As an alternative, autophagy could be associated with chemoresistance by protecting the survival of cancer cells. Thus, inhibition of the autophagic flux synergized the killing effect of chemotherapeutic agents in many tumor types. The mechanism by which autophagy inhibition increases cell death could be associated with a switch toward other types of cell death, such as apoptosis, necrosis, or necroptosis. Chloroquine (CQ) and its analog hydroxychloroquine (HCQ) are antimalarial agents that increase the pH of the lysosome and then inhibit the fusion between autophagosome and lysosome (Amaravadi et al. 2011) (Table 8.3). For example, administration of the Akt inhibitor MK2206 in combination with HCQ is in clinical

trials of pancreatic, kidney, and many advanced tumors. HCQ with everolimus is in a phase I clinical trial of RCC. The combination of HCQ, radiation, and temozolomide are in clinical trials of patients with glioblastomas (www.ClinicalTrials.gov identifier NCT00486603). In chronic myelogenous leukemia, cell death is observed by the combined treatment with CQ and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (Carew et al. 2007). Finally, HCQ has been shown to potentiate the anticancer effect of 5-fluorouracil in colon cancer (Sasaki et al. 2010). Two other autophagy inhibitors have recently been identified in preclinical trials. The first inhibitor is lucanthone, or Myricil D, an existing drug that is used for the treatment of schistosomal parasites (Clarkson and Erasmus 1984). While earlier investigations have shown that lucanthone inhibits topoisomerase 2 activity, a more recent study defined a novel mechanism of action for lucanthone that includes the disruption of lysosomal function, inhibition of autophagy, and induction of apoptosis (Carew et al. 2011). In breast carcinoma cell lines, lucanthone is tenfold more potent that CQ and shows a better safety profile than CQ or HCQ. The second autophagy inhibitor is Lys05. This new drug accumulates more easily within the lysosome, increasing pH more effectively compared to HCQ (McAfee et al. 2012). Similar to lucanthone, Lys05 displayed significantly higher anticancer activity than CQ or HCQ in preclinical models, without inducing significant observable toxicity. These two new autophagy inhibitors need to be further investigated as potential therapeutic anticancer agents.

## 8.4 Synthetic Lethality and Autophagy in Anticancer Drug Discovery

### *8.4.1 Synthetic Lethality in the Context of Cancer*

Advances in cell and molecular biology have improved our knowledge of the mechanism by which cells escape death to become cancerous. The expansion of "omics" technology, from genomic through metabolomic, have identified specific mutations of genes or altered RNA and protein signaling that are responsible for different types of cancer. As discussed earlier, targeted therapy is an active area of research that has expanded the type and modality of treatments (alone or in combination). It is unfortunate that few of them show clinical efficacy, but the ones that received approval from the US Food and Drug Administration have improved survival of inflexible cancers, including RCC (Motzer et al. 2006, 2007, 2008; Gu et al. 2005; Hudes et al. 2007; Escudier et al. 2007a, b), pancreatic cancers (Moore et al. 2007), and non-small-cell lung cancers (Ansari et al. 2009; Shepherd et al. 2005). One promising approach to develop targeted therapy against tumor cells and spare normal tissue is based on synthetic lethality, which targets specific mutations in cancer genes that are not altered in normal cells (Chan and Giaccia 2011). Synthetic lethality is the genetic interaction of two genes, both of which are involved in essential processes (Hartman

et al. 2001). When either gene is mutated alone, the cell remains viable. However, the combination of these two mutations induces cell death (Hartman et al. 2001; Kaelin 2005; Hartwell et al. 1997). Chemical or RNA interference screens have made it possible to search for synthetic lethal interactions in mammalian cells (Farmer et al. 2005; Jiang et al. 2009). Thus, deregulation of an oncogene or inactivation of a tumor suppressor gene can be specifically targeted through synthetic lethality to kill tumor cells. This approach could be advantageous and facilitate the development of treatment with a single agent because only cancer cells with the specific mutation will die. The normal cells will not be affected by the therapy, and side effects from chemotherapy will be reduced. Synthetic lethality could also be used in combination with drugs and/or radiation or in patients with relapsed cancer, providing the opportunity to use lower doses of cytotoxic drugs, improve the therapeutic index of cytotoxic drugs, and reduce off-target effects. Driving mutation in cancer cells can change at different stages of tumor development – from the primary tumor to metastases – and therefore synthetic lethality could be useful to target the epithelial-to-mesenchymal transition as well as metastatic disease for which there are few options of effective treatment.

The first example of synthetic lethal interaction in cancer cells came from the mutation affecting the gene *BRCA1/2* and the enzyme poly (ADP ribose) polymerase (PARP). The tumor suppressor protein BRCA is an important player in the reparation of double-strand DNA breaks, and mutations affecting these genes have been reported in breast and ovarian cancers (Hall et al. 1992; Casey et al. 1993; Parikh and Advani 1996). In addition, PARP is an important protein that repairs single-stand DNA breaks (Petermann et al. 2005). By using pharmacological inhibitors or small interfering/small hairpin RNA targeting PARP in *BRCA*-mutated cells, studies indicate that these cells were not able to repair double-strand DNA breaks and recombination lesions and that they die by apoptosis (Bryant et al. 2005; Farmer et al. 2005). The identification of the lethal interaction between *BRCA* mutations and PARP inhibitors has been investigated in cancer cells, and several PARP inhibitors are currently in clinical trials (phase I/II/III) for the treatment of breast and ovarian cancer with the inactivated *BRCA1/2* gene (Tutt et al. 2010; Fong et al. 2009; Hutchinson 2010). These studies demonstrated proof of the concept that synthetic lethality can be useful in (and are possible for) targeting cancer cells. Some researchers and pharmaceutical companies are working to develop this killing approach in association with other oncogenes that are frequently disrupted in cancer, such as the oncogenes *Ras* and *Myc* (Chan and Giaccia 2011). New drugs (triphenyltetrazolium and a sulphinylcytidine derivative) (Torrance et al. 2001), the inhibitor apoptosis protein survivin (Sarthy et al. 2007), and cyclin-dependent kinase 4 (Puyol et al. 2010) have been identified by independent screening and demonstrate some potential as *KRAS* inhibitors. Otherwise, other large screens performed in *Ras*-mutated cells and pathways governing the mitotic machinery or the proteasomes showed synthetic lethal interaction with *Ras* (Scholl et al. 2009; Luo et al. 2009). Among other examples of synthetic lethal interaction, inhibition of aurora kinase B or death receptor 5 agonists induced killing in cells overexpressing *Myc* (Wang et al. 2004; Yang et al. 2010).

### *8.4.2 Synthetic Lethality and Autophagy in RCC*

RCC, the most common form of kidney cancer, is particularly challenging because it is resistant to standard cytotoxic therapies. The overall 5-year survival rate ranges from 85 % in patients with local tumors treated by partial or total nephrectomy to 10 % in patients with advanced or metastatic RCC (Motzer et al. 1996). There is no curative treatment for RCC, and these patients are diagnosed at an advanced stage because no symptoms are associated with kidney tumors until they are quite large. Current targeted therapies used to treat RCC (e.g., bevacizumab, sunitinib) have focused on anti-angiogenic agents targeting vascular endothelial growth factor and its receptor and agents that inhibit mTOR (e.g., temsirolimus, everolimus). Although these agents demonstrate efficiency in RCC, the clinical response to these therapies is generally short-lived, suggesting that tumor growth might be supported by alternative sources of nutrients, such as autophagy (Patel et al. 2006).

Biallelic inactivation of the von Hippel-Lindau (*VHL*) tumor suppressor gene arises in up to 85 % of RCC cases. Mutation and/or hypermethylation, which inactivate the *VHL* gene, are also responsible for the hereditary VHL cancer syndrome that affects 1 in 36,000 individuals (Maher 2004). These patients inherit a faulty allele of *VHL* and are predisposed to the development of renal cysts, RCC, retinal and central nervous system hemangioblastomas, and pheochromocytomas (Maher 2004; Kaelin 2008). Tumor development is caused by somatic inactivation of the remaining wild-type allele (Young et al. 2009; Nickerson et al. 2008; Patard et al. 2009). Because VHL is a common and early event in the development of RCC, targeting its inactivation represents a promising target for the development of new therapies. High-throughput screening using a small interfering RNA library or small molecules have been performed in *VHL*-deficient RCC in two independent studies. The first approach used a library of small hairpin RNA against about 100 different kinases and distinguished CDK6, hepatocyte growth factor receptor (also known as MET), and mitogen-activated protein kinase 1 (MAP2K1), which have the ability to reduce growth of *VHL*-inactivated cells (Bommi-Reddy et al. 2008). A recent study reported that microRNA-1826 reduced the expression of β-catenin and *MAP2K1* in RCC and inhibits the proliferation of *VHL*-deficient cells by inducing $G_1$ arrest and apoptosis (Hirata et al. 2012).

The second approach used a library of 64,000 small molecules to find drugs that specifically kill RCC lacking *VHL* without affecting the viability of the cells with the functional *VHL* gene (Turcotte et al. 2008). This study identified two classes of compounds: ST-31 inhibited the survival of *VHL*-deficient cells through GLUT1 and HIF-1α (Chan et al. 2011), whereas STF-62247 killed *VHL*-mutated cells by inducing autophagy (Turcotte et al. 2008). Moreover, they showed that reducing levels of Atg5, Atg7, and Atg9 rescued the survival of *VHL*-deficient cells in response to STF-62247, indicating that autophagy induction is required for cell death. Turcotte et al. recently investigated the autophagy machinery and found that the in vitro and in vivo sensitivity of *VHL*-deficient RCC in response to STF-62247 is associated with a default in the autophagic process involving lysosomal

degradation, which ultimately leads to cell death. In accordance with this, cells lacking *VHL* expression accumulate autophagic vacuoles that are not degraded by lysosomes, thus interfering with the clearance of damaged organelles and misfolded or aggregated proteins in response to STF-62247. Furthermore, lysosomes in these cells undergo labialization or lysosome permeabilization, which also contributes to cell death. Production of ROS that are not detoxified by the cells, lysosomotropic agents, microtubule-stabilizing agents, protein kinase C, phospholipase $A_2$, and lipids are the mechanisms speculated to induce lysosome permeabilization (Kreuzaler and Watson 2012).

## 8.5 Conclusion and Future Directions

The field of cancer research has made significant progress in recent years. New techniques have identified genetic alterations associated with different types of cancer. In parallel, advances in drug screening using small interfering RNA libraries and/or small molecules have expanded drug design and the development of targeted therapies. Using these approaches, new anticancer agents or novel uses of existing drugs are in clinical trials or have been approved for treatment. Exciting drugs exploiting synthetic lethality have gained attention as a new type of anticancer therapy. Searching for synthetic lethal interaction between two genes or drug-gene interactions represent a promising approach to kill tumor cells and leave normal cells healthy. Cancer cells evade programmed cell death to initiate tumor formation, and research has identified nonapoptotic mechanisms for how cells survive or die in response to a drug. The role of autophagy in cancer is complex: it can help prevent tumor initiation, overcome resistance to anticancer therapy, promote cytoprotection in established tumors, and may help to eradicate malignant cells. Inhibitors of the autophagic flux, including CQ and HCQ, used alone or in combination with chemotherapeutic agents and/or radiation, are currently in clinical trials of several types of cancer. In addition, drugs that induce autophagy and provoke cell death show encouraging results. Overall, other screens using synthetic lethality and our knowledge of cell death mechanisms could open a new field of oncology, helping to design monotherapy agents or a combination of cytotoxic chemotherapy and radiation.

## References

Aita VM, Liang XH, Murty VV, Pincus DL, Yu W, Cayanis E, Kalachikov S, Gilliam TC, Levine B (1999) Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 59(1):59–65. doi:10.1006/geno.1999.5851

Amaravadi RK, Lippincott-Schwartz J, Yin XM, Weiss WA, Takebe N, Timmer W, DiPaola RS, Lotze MT, White E (2011) Principles and current strategies for targeting autophagy for cancer treatment. Clin Cancer Res 17(4):654–666. doi:10.1158/1078-0432.CCR-10-2634

Amelio I, Melino G, Knight RA (2011) Cell death pathology: cross-talk with autophagy and its clinical implications. Biochem Biophys Res Commun 414(2):277–281. doi:10.1016/j.bbrc.2011.09.080

Ansari J, Palmer DH, Rea DW, Hussain SA (2009) Role of tyrosine kinase inhibitors in lung cancer. Anticancer Agents Med Chem 9(5):569–575

Arena G, Gelmetti V, Torosantucci L, Vignone D, Lamorte G, De Rosa P, Cilia E, Jonas EA, Valente EM (2013) PINK1 protects against cell death induced by mitochondrial depolarization, by phosphorylating Bcl-xL and impairing its pro-apoptotic cleavage. Cell Death Differ. doi:10.1038/cdd.2013.19

Axe EL, Walker SA, Manifava M, Chandra P, Roderick HL, Habermann A, Griffiths G, Ktistakis NT (2008) Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum. J Cell Biol 182(4):685–701. doi:10.1083/jcb.200803137

Bar-Peled L, Schweitzer LD, Zoncu R, Sabatini DM (2012) Ragulator is a GEF for the rag GTPases that signal amino acid levels to mTORC1. Cell 150(6):1196–1208. doi:10.1016/j.cell.2012.07.032

Bellot G, Garcia-Medina R, Gounon P, Chiche J, Roux D, Pouyssegur J, Mazure NM (2009) Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. Mol Cell Biol 29(10):2570–2581. doi:10.1128/MCB.00166-09

Bommi-Reddy A, Almeciga I, Sawyer J, Geisen C, Li W, Harlow E, Kaelin WG Jr, Grueneberg DA (2008) Kinase requirements in human cells: III. Altered kinase requirements in VHL–/– cancer cells detected in a pilot synthetic lethal screen. Proc Natl Acad Sci U S A 105(43):16484–16489. doi:10.1073/pnas.0806574105

Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, Kyle S, Meuth M, Curtin NJ, Helleday T (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. Nature 434(7035):913–917. doi:10.1038/nature03443

Carew JS, Nawrocki ST, Kahue CN, Zhang H, Yang C, Chung L, Houghton JA, Huang P, Giles FJ, Cleveland JL (2007) Targeting autophagy augments the anticancer activity of the histone deacetylase inhibitor SAHA to overcome Bcr-Abl-mediated drug resistance. Blood 110(1):313–322. doi:10.1182/blood-2006-10-050260

Carew JS, Espitia CM, Esquivel JA 2nd, Mahalingam D, Kelly KR, Reddy G, Giles FJ, Nawrocki ST (2011) Lucanthone is a novel inhibitor of autophagy that induces cathepsin D-mediated apoptosis. J Biol Chem 286(8):6602–6613. doi:10.1074/jbc.M110.151324

Casey G, Plummer S, Hoeltge G, Scanlon D, Fasching C, Stanbridge EJ (1993) Functional evidence for a breast cancer growth suppressor gene on chromosome 17. Hum Mol Genet 2(11):1921–1927

Chan DA, Giaccia AJ (2011) Harnessing synthetic lethal interactions in anticancer drug discovery. Nat Rev Drug Discov 10(5):351–364. doi:10.1038/nrd3374

Chan DA, Sutphin PD, Nguyen P, Turcotte S, Lai EW, Banh A, Reynolds GE, Chi JT, Wu J, SolowCordero DE, Bonnet M, Flanagan JU, Bouley DM, Graves EE, Denny WA, Hay MP, Giaccia AJ (2011) Targeting GLUT1 and the Warburg effect in renal cell carcinoma by chemical synthetic lethality. Sci Transl Med 3(94):94ra70. doi:10.1126/scitranslmed.3002394

Chen Y, Lu Y, Lu C, Zhang L (2009) Beclin-1 expression is a predictor of clinical outcome in patients with esophageal squamous cell carcinoma and correlated to hypoxia-inducible factor (HIF)-1alpha expression. Pathol Oncol Res 15(3):487–493. doi:10.1007/s12253-008-9143-8

Clarkson J, Erasmus DA (1984) Schistosoma mansoni: an in vivo study of drug-induced autophagy in the gastrodermis. J Helminthol 58(1):59–68

Copple IM, Lister A, Obeng AD, Kitteringham NR, Jenkins RE, Layfield R, Foster BJ, Goldring CE, Park BK (2010) Physical and functional interaction of sequestosome 1 with Keap1 regulates the Keap1-Nrf2 cell defense pathway. J Biol Chem 285(22):16782–16788. doi:10.1074/jbc.M109.096545

Crazzolara R, Bradstock KF, Bendall LJ (2009) RAD001 (Everolimus) induces autophagy in acute lymphoblastic leukemia. Autophagy 5(5):727–728

Degenhardt K, Mathew R, Beaudoin B, Bray K, Anderson D, Chen G, Mukherjee C, Shi Y, Gelinas C, Fan Y, Nelson DA, Jin S, White E (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. Cancer Cell 10(1):51–64. doi:10.1016/j.ccr.2006.06.001

Erlich S, Mizrachy L, Segev O, Lindenboim L, Zmira O, Adi-Harel S, Hirsch JA, Stein R, Pinkas-Kramarski R (2007) Differential interactions between Beclin 1 and Bcl-2 family members. Autophagy 3(6):561–568

Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, Negrier S, Chevreau C, Solska E, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, Freeman S, Schwartz B, Shan M, Simantov R, Bukowski RM (2007a) Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med 356(2):125–134. doi:10.1056/NEJMoa060655

Escudier B, Pluzanska A, Koralewski P, Ravaud A, Bracarda S, Szczylik C, Chevreau C, Filipek M, Melichar B, Bajetta E, Gorbunova V, Bay JO, Bodrogi I, Jagiello-Gruszfeld A, Moore N (2007b) Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. Lancet 370(9605):2103–2111. doi:10.1016/S0140-6736(07)61904-7

Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, Ashworth A (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. Nature 434(7035):917–921. doi:10.1038/nature03445

Feng Z (2010) p53 regulation of the IGF-1/AKT/mTOR pathways and the endosomal compartment. Cold Spring Harb Perspect Biol 2(2):a001057. doi:10.1101/cshperspect.a001057

Fong PC, Boss DS, Yap TA, Tutt A, Wu P, Mergui-Roelvink M, Mortimer P, Swaisland H, Lau A, O'Connor MJ, Ashworth A, Carmichael J, Kaye SB, Schellens JH, de Bono JS (2009) Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. N Engl J Med 361(2):123–134. doi:10.1056/NEJMoa0900212

Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, Kinoshita T, Ueno T, Esumi H, Ochiai A (2008) Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Sci 99(9):1813–1819. doi:10.1111/j.1349-7006.2008.00893.x

Gu J, Ruppen ME, Cai P (2005) lipase-catalyzed regioselective esterification of rapamycin: synthesis of temsirolimus (CCI-779). Org Lett 7(18):3945–3948. doi:10.1021/ol0514395

Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, Kamphorst JJ, Chen G, Lemons JM, Karantza V, Coller HA, Dipaola RS, Gelinas C, Rabinowitz JD, White E (2011) Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes Dev 25(5):460–470. doi:10.1101/gad.2016311

Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, Lippincott-Schwartz J (2010) Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell 141(4):656–667. doi:10.1016/j.cell.2010.04.009

Hall JM, Friedman L, Guenther C, Lee MK, Weber JL, Black DM, King MC (1992) Closing in on a breast cancer gene on chromosome 17q. Am J Hum Genet 50(6):1235–1242

Hartman JL, Garvik B, Hartwell L (2001) Principles for the buffering of genetic variation. Science 291(5506):1001–1004

Hartwell LH, Szankasi P, Roberts CJ, Murray AW, Friend SH (1997) Integrating genetic approaches into the discovery of anticancer drugs. Science 278(5340):1064–1068

Hayashi-Nishino M, Fujita N, Noda T, Yamaguchi A, Yoshimori T, Yamamoto A (2009) A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat Cell Biol 11(12):1433–1437. doi:10.1038/ncb1991

Heidari N, Hicks MA, Harada H (2010) GX15-070 (obatoclax) overcomes glucocorticoid resistance in acute lymphoblastic leukemia through induction of apoptosis and autophagy. Cell Death Dis 1:e76. doi:10.1038/cddis.2010.53

Heuser J (1989) Changes in lysosome shape and distribution correlated with changes in cytoplasmic pH. J Cell Biol 108(3):855–864

Hirata H, Hinoda Y, Ueno K, Nakajima K, Ishii N, Dahiya R (2012) MicroRNA-1826 directly targets beta-catenin (CTNNB1) and MEK1 (MAP2K1) in VHL-inactivated renal cancer. Carcinogenesis 33(3):501–508. doi:10.1093/carcin/bgr302

Horbinski C, Mojesky C, Kyprianou N (2010) Live free or die: tales of homeless (cells) in cancer. Am J Pathol 177(3):1044–1052. doi:10.2353/ajpath.2010.091270

Hudes G, Carducci M, Tomczak P, Dutcher J, Figlin R, Kapoor A, Staroslawska E, Sosman J, McDermott D, Bodrogi I, Kovacevic Z, Lesovoy V, Schmidt-Wolf IG, Barbarash O, Gokmen E, O'Toole T, Lustgarten S, Moore L, Motzer RJ (2007) Temsirolimus, interferon alfa, or both for advanced renal-cell carcinoma. N Engl J Med 356(22):2271–2281. doi:10.1056/NEJMoa066838

Hutchinson L (2010) Targeted therapies: PARP inhibitor olaparib is safe and effective in patients with BRCA1 and BRCA2 mutations. Nat Rev Clin Oncol 7(10):549. doi:10.1038/nrclinonc.2010.143

Inami Y, Waguri S, Sakamoto A, Kouno T, Nakada K, Hino O, Watanabe S, Ando J, Iwadate M, Yamamoto M, Lee MS, Tanaka K, Komatsu M (2011) Persistent activation of Nrf2 through p62 in hepatocellular carcinoma cells. J Cell Biol 193(2):275–284. doi:10.1083/jcb.201102031

Janku F, McConkey DJ, Hong DS, Kurzrock R (2011) Autophagy as a target for anticancer therapy. Nat Rev Clin Oncol 8(9):528–539. doi:10.1038/nrclinonc.2011.71

Jiang H, Reinhardt HC, Bartkova J, Tommiska J, Blomqvist C, Nevanlinna H, Bartek J, Yaffe MB, Hemann MT (2009) The combined status of ATM and p53 link tumor development with therapeutic response. Genes Dev 23(16):1895–1909. doi:10.1101/gad.1815309

Jung CH, Jun CB, Ro SH, Kim YM, Otto NM, Cao J, Kundu M, Kim DH (2009) ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell 20(7):1992–2003. doi:10.1091/mbc.E08-12-1249

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19(21):5720–5728. doi:10.1093/emboj/19.21.5720

Kaelin WG Jr (2005) The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer 5(9):689–698

Kaelin WG Jr (2008) The von Hippel-Lindau tumour suppressor protein: $O_2$ sensing and cancer. Nat Rev Cancer 8(11):865–873

Kang R, Zeh HJ, Lotze MT, Tang D (2011) The Beclin 1 network regulates autophagy and apoptosis. Cell Death Differ 18(4):571–580. doi:10.1038/cdd.2010.191

Kenific CM, Thorburn A, Debnath J (2010) Autophagy and metastasis: another double-edged sword. Curr Opin Cell Biol 22(2):241–245. doi:10.1016/j.ceb.2009.10.008

Kim HS, Lee SH, Do SI, Lim SJ, Park YK, Kim YW (2011) Clinicopathologic correlation of beclin-1 expression in pancreatic ductal adenocarcinoma. Pathol Res Pract 207(4):247–252. doi:10.1016/j.prp.2011.02.007

Klionsky DJ, Emr SD (2000) Autophagy as a regulated pathway of cellular degradation. Science 290(5497):1717–1721

Korolchuk VI, Rubinsztein DC (2011) Regulation of autophagy by lysosomal positioning. Autophagy 7(8):927–928

Korolchuk VI, Saiki S, Lichtenberg M, Siddiqi FH, Roberts EA, Imarisio S, Jahreiss L, Sarkar S, Futter M, Menzies FM, O'Kane CJ, Deretic V, Rubinsztein DC (2011) Lysosomal positioning coordinates cellular nutrient responses. Nat Cell Biol 13(4):453–460. doi:10.1038/ncb2204

Kreuzaler P, Watson CJ (2012) Killing a cancer: what are the alternatives? Nat Rev Cancer 12(6):411–424. doi:10.1038/nrc3264

Kroemer G, Marino G, Levine B (2010) Autophagy and the integrated stress response. Mol Cell 40(2):280–293. doi:10.1016/j.molcel.2010.09.023

Lamb CA, Dooley HC, Tooze SA (2013) Endocytosis and autophagy: shared machinery for degradation. Bioessays 35(1):34–45. doi:10.1002/bies.201200130

Lau A, Wang XJ, Zhao F, Villeneuve NF, Wu T, Jiang T, Sun Z, White E, Zhang DD (2010) A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. Mol Cell Biol 30(13):3275–3285. doi:10.1128/MCB.00248-10

Lee SJ, Kim HP, Jin Y, Choi AM, Ryter SW (2011) Beclin 1 deficiency is associated with increased hypoxia-induced angiogenesis. Autophagy 7(8):829–839

Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132(1):27–42

Li BX, Li CY, Peng RQ, Wu XJ, Wang HY, Wan DS, Zhu XF, Zhang XS (2009) The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers. Autophagy 5(3):303–306

Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402(6762):672–676. doi:10.1038/45257

Liang C, Lee JS, Inn KS, Gack MU, Li Q, Roberts EA, Vergne I, Deretic V, Feng P, Akazawa C, Jung JU (2008) Beclin1-binding UVRAG targets the class C Vps complex to coordinate autophagosome maturation and endocytic trafficking. Nat Cell Biol 10(7):776–787. doi:10.1038/ncb1740

Luo J, Emanuele MJ, Li D, Creighton CJ, Schlabach MR, Westbrook TF, Wong KK, Elledge SJ (2009) A genome-wide RNAi screen identifies multiple synthetic lethal interactions with the Ras oncogene. Cell 137(5):835–848. doi:10.1016/j.cell.2009.05.006

Mah LY, Ryan KM (2012) Autophagy and cancer. Cold Spring Harb Perspect Biol 4(1):a008821. doi:10.1101/cshperspect.a008821

Maher ER (2004) Von Hippel-Lindau disease. Curr Mol Med 4(8):833–842

Maiuri MC, Zalckvar E, Kimchi A, Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8(9):741–752. doi:10.1038/nrm2239

Marino G, Salvador-Montoliu N, Fueyo A, Knecht E, Mizushima N, Lopez-Otin C (2007) Tissue-specific autophagy alterations and increased tumorigenesis in mice deficient in Atg4C/autophagin-3. J Biol Chem 282(25):18573–18583. doi:10.1074/jbc.M701194200

Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E (2007) Autophagy suppresses tumor progression by limiting chromosomal instability. Genes Dev 21(11):1367–1381. doi:10.1101/gad.1545107

Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C, Dipaola RS, Karantza-Wadsworth V, White E (2009) Autophagy suppresses tumorigenesis through elimination of p62. Cell 137(6):1062–1075. doi:10.1016/j.cell.2009.03.048

McAfee Q, Zhang Z, Samanta A, Levi SM, Ma XH, Piao S, Lynch JP, Uehara T, Sepulveda AR, Davis LE, Winkler JD, Amaravadi RK (2012) Autophagy inhibitor Lys05 has single-agent antitumor activity and reproduces the phenotype of a genetic autophagy deficiency. Proc Natl Acad Sci U S A 109(21):8253–8258. doi:10.1073/pnas.1118193109

McCoy F, Hurwitz J, McTavish N, Paul I, Barnes C, O'Hagan B, Odrzywol K, Murray J, Longley D, McKerr G, Fennell DA (2010) Obatoclax induces Atg7-dependent autophagy independent of beclin-1 and BAX/BAK. Cell Death Dis 1:e108. doi:10.1038/cddis.2010.86

Miao Y, Zhang Y, Chen Y, Chen L, Wang F (2010) GABARAP is overexpressed in colorectal carcinoma and correlates with shortened patient survival. Hepatogastroenterology 57(98):257–261

Mizushima N (2010) The role of the Atg1/ULK1 complex in autophagy regulation. Curr Opin Cell Biol 22(2):132–139. doi:10.1016/j.ceb.2009.12.004

Mizushima N, Sugita H, Yoshimori T, Ohsumi Y (1998) A new protein conjugation system in human. The counterpart of the yeast Apg12p conjugation system essential for autophagy. J Biol Chem 273(51):33889–33892

Mizushima N, Yoshimori T, Ohsumi Y (2011) The role of Atg proteins in autophagosome formation. Annu Rev Cell Dev Biol 27:107–132. doi:10.1146/annurev-cellbio-092910-154005

Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W (2007) Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada clinical trials group. J Clin Oncol 25(15):1960–1966. doi:10.1200/JCO.2006.07.9525

Motzer RJ, Bander NH, Nanus DM (1996) Renal-cell carcinoma. N Engl J Med 335(12):865–875. doi:10.1056/NEJM199609193351207

Motzer RJ, Michaelson MD, Redman BG, Hudes GR, Wilding G, Figlin RA, Ginsberg MS, Kim ST, Baum CM, DePrimo SE, Li JZ, Bello CL, Theuer CP, George DJ, Rini BI (2006) Activity of SU11248, a multitargeted inhibitor of vascular endothelial growth factor receptor and platelet-derived growth factor receptor, in patients with metastatic renal cell carcinoma. J Clin Oncol 24(1):16–24. doi:10.1200/JCO.2005.02.2574

Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, Oudard S, Negrier S, Szczylik C, Kim ST, Chen I, Bycott PW, Baum CM, Figlin RA (2007) Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med 356(2):115–124. doi:10.1056/NEJMoa065044

Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grunwald V, Thompson JA, Figlin RA, Hollaender N, Urbanowitz G, Berg WJ, Kay A, Lebwohl D, Ravaud A (2008) Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. Lancet 372(9637):449–456. doi:10.1016/S0140-6736(08)61039-9

Nickerson ML, Jaeger E, Shi Y, Durocher JA, Mahurkar S, Zaridze D, Matveev V, Janout V, Kollarova H, Bencko V, Navratilova M, Szeszenia-Dabrowska N, Mates D, Mukeria A, Holcatova I, Schmidt LS, Toro JR, Karami S, Hung R, Gerard GF, Linehan WM, Merino M, Zbar B, Boffetta P, Brennan P, Rothman N, Chow WH, Waldman FM, Moore LE (2008) Improved identification of von Hippel-Lindau gene alterations in clear cell renal tumors. Clin Cancer Res 14(15):4726–4734

O'Reilly T, McSheehy PM, Wartmann M, Lassota P, Brandt R, Lane HA (2011) Evaluation of the mTOR inhibitor, everolimus, in combination with cytotoxic antitumor agents using human tumor models in vitro and in vivo. Anticancer Drugs 22(1):58–78. doi:10.1097/CAD.0b013e3283400a20

Orsi A, Razi M, Dooley HC, Robinson D, Weston AE, Collinson LM, Tooze SA (2012) Dynamic and transient interactions of Atg9 with autophagosomes, but not membrane integration, are required for autophagy. Mol Biol Cell 23(10):1860–1873. doi:10.1091/mbc.E11-09-0746

Ouyang L, Shi Z, Zhao S, Wang FT, Zhou TT, Liu B, Bao JK (2012) Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. Cell Prolif 45(6):487–498. doi:10.1111/j.1365-2184.2012.00845.x

Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T (2007) p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 282(33):24131–24145. doi:10.1074/jbc.M702824200

Parikh B, Advani S (1996) Pattern of second primary neoplasms following breast cancer. J Surg Oncol 63(3):179–182. doi:10.1002/(SICI)1096-9098(199611)63:3<179::AID-JSO8>3.0.CO;2-A

Patard JJ, Rioux-Leclercq N, Masson D, Zerrouki S, Jouan F, Collet N, Dubourg C, Lobel B, Denis M, Fergelot P (2009) Absence of VHL gene alteration and high VEGF expression are associated with tumour aggressiveness and poor survival of renal-cell carcinoma. Br J Cancer 101(8):1417–1424

Patel PH, Chadalavada RS, Chaganti RS, Motzer RJ (2006) Targeting von Hippel-Lindau pathway in renal cell carcinoma. Clin Cancer Res 12(24):7215–7220

Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell 122(6):927–939. doi:10.1016/j.cell.2005.07.002

Pattingre S, Bauvy C, Carpentier S, Levade T, Levine B, Codogno P (2009) Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. J Biol Chem 284(5):2719–2728. doi:10.1074/jbc.M805920200

Pena-Llopis S, Vega-Rubin-de-Celis S, Schwartz JC, Wolff NC, Tran TA, Zou L, Xie XJ, Corey DR, Brugarolas J (2011) Regulation of TFEB and V-ATPases by mTORC1. EMBO J 30(16):3242–3258. doi:10.1038/emboj.2011.257

Peralta ER, Martin BC, Edinger AL (2010) Differential effects of TBC1D15 and mammalian Vps39 on Rab7 activation state, lysosomal morphology, and growth factor dependence. J Biol Chem 285(22):16814–16821. doi:10.1074/jbc.M110.111633

Petermann E, Keil C, Oei SL (2005) Importance of poly(ADP-ribose) polymerases in the regulation of DNA-dependent processes. Cell Mol Life Sci 62(7–8):731–738. doi:10.1007/s00018-004-4504-2

Poulogiannis G, McIntyre RE, Dimitriadi M, Apps JR, Wilson CH, Ichimura K, Luo F, Cantley LC, Wyllie AH, Adams DJ, Arends MJ (2010) PARK2 deletions occur frequently in sporadic colorectal cancer and accelerate adenoma development in Apc mutant mice. Proc Natl Acad Sci U S A 107(34):15145–15150. doi:10.1073/pnas.1009941107

Puyol M, Martin A, Dubus P, Mulero F, Pizcueta P, Khan G, Guerra C, Santamaria D, Barbacid M (2010) A synthetic lethal interaction between K-Ras oncogenes and Cdk4 unveils a therapeutic strategy for non-small cell lung carcinoma. Cancer Cell 18(1):63–73. doi:10.1016/j.ccr.2010.05.025

Qu X, Yu J, Bhagat G, Furuya N, Hibshoosh H, Troxel A, Rosen J, Eskelinen EL, Mizushima N, Ohsumi Y, Cattoretti G, Levine B (2003) Promotion of tumorigenesis by heterozygous disruption of the beclin 1 autophagy gene. J Clin Invest 112(12):1809–1820. doi:10.1172/JCI20039

Ravikumar B, Moreau K, Jahreiss L, Puri C, Rubinsztein DC (2010) Plasma membrane contributes to the formation of pre-autophagosomal structures. Nat Cell Biol 12(8):747–757. doi:10.1038/ncb2078

Reggiori F, Komatsu M, Finley K, Simonsen A (2012) Selective types of autophagy. Int J Cell Biol 2012:156272. doi:10.1155/2012/156272

Rubinsztein DC, Codogno P, Levine B (2012) Autophagy modulation as a potential therapeutic target for diverse diseases. Nat Rev Drug Discov 11(9):709–730. doi:10.1038/nrd3802

Sancak Y, Bar-Peled L, Zoncu R, Markhard AL, Nada S, Sabatini DM (2010) Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell 141(2):290–303. doi:10.1016/j.cell.2010.02.024

Sardiello M, Palmieri M, di Ronza A, Medina DL, Valenza M, Gennarino VA, Di Malta C, Donaudy F, Embrione V, Polishchuk RS, Banfi S, Parenti G, Cattaneo E, Ballabio A (2009) A gene network regulating lysosomal biogenesis and function. Science 325(5939):473–477. doi:10.1126/science.1174447

Sarthy AV, Morgan-Lappe SE, Zakula D, Vernetti L, Schurdak M, Packer JC, Anderson MG, Shirasawa S, Sasazuki T, Fesik SW (2007) Survivin depletion preferentially reduces the survival of activated K-Ras-transformed cells. Mol Cancer Ther 6(1):269–276. doi:10.1158/1535-7163.MCT-06-0560

Sasaki K, Tsuno NH, Sunami E, Tsurita G, Kawai K, Okaji Y, Nishikawa T, Shuno Y, Hongo K, Hiyoshi M, Kaneko M, Kitayama J, Takahashi K, Nagawa H (2010) Chloroquine potentiates the anti-cancer effect of 5-fluorouracil on colon cancer cells. BMC Cancer 10:370. doi:10.1186/1471-2407-10-370

Scholl C, Frohling S, Dunn IF, Schinzel AC, Barbie DA, Kim SY, Silver SJ, Tamayo P, Wadlow RC, Ramaswamy S, Dohner K, Bullinger L, Sandy P, Boehm JS, Root DE, Jacks T, Hahn WC, Gilliland DG (2009) Synthetic lethal interaction between oncogenic KRAS dependency and STK33 suppression in human cancer cells. Cell 137(5):821–834. doi:10.1016/j.cell.2009.03.017

Settembre C, Di Malta C, Polito VA, Garcia Arencibia M, Vetrini F, Erdin S, Erdin SU, Huynh T, Medina D, Colella P, Sardiello M, Rubinsztein DC, Ballabio A (2011) TFEB links autophagy to lysosomal biogenesis. Science 332(6036):1429–1433. doi:10.1126/science.1204592

Settembre C, Zoncu R, Medina DL, Vetrini F, Erdin S, Huynh T, Ferron M, Karsenty G, Vellard MC, Facchinetti V, Sabatini DM, Ballabio A (2012) A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J 31(5):1095–1108. doi:10.1038/emboj.2012.32

Shepherd FA, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P, Seymour L (2005) Erlotinib in previously treated non-small-cell lung cancer. N Engl J Med 353(2):123–132. doi:10.1056/NEJMoa050753

Sun Q, Westphal W, Wong KN, Tan I, Zhong Q (2010) Rubicon controls endosome maturation as a Rab7 effector. Proc Natl Acad Sci U S A 107(45):19338–19343. doi:10.1073/pnas.1010554107

Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, Eishi Y, Hino O, Tanaka K, Mizushima N (2011) Autophagy-deficient mice develop multiple liver tumors. Genes Dev 25(8):795–800. doi:10.1101/gad.2016211

Takeshige K, Baba M, Tsuboi S, Noda T, Ohsumi Y (1992) Autophagy in yeast demonstrated with proteinase-deficient mutants and conditions for its induction. J Cell Biol 119(2):301–311

Torrance CJ, Agrawal V, Vogelstein B, Kinzler KW (2001) Use of isogenic human cancer cells for high-throughput screening and drug discovery. Nat Biotechnol 19(10):940–945. doi:10.1038/nbt1001-940

Turcotte S, Giaccia AJ (2010) Targeting cancer cells through autophagy for anticancer therapy. Curr Opin Cell Biol 22(2):246–251. doi:10.1016/j.ceb.2009.12.007

Turcotte S, Chan DA, Sutphin PD, Hay MP, Denny WA, Giaccia AJ (2008) A molecule targeting VHL-deficient renal cell carcinoma that induces autophagy. Cancer Cell 14(1):90–102. doi:10.1016/j.ccr.2008.06.004

Tutt A, Robson M, Garber JE, Domchek SM, Audeh MW, Weitzel JN, Friedlander M, Arun B, Loman N, Schmutzler RK, Wardley A, Mitchell G, Earl H, Wickens M, Carmichael J (2010) Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with BRCA1 or BRCA2 mutations and advanced breast cancer: a proof-of-concept trial. Lancet 376(9737):235–244. doi:10.1016/S0140-6736(10)60892-6

Veeriah S, Taylor BS, Meng S, Fang F, Yilmaz E, Vivanco I, Janakiraman M, Schultz N, Hanrahan AJ, Pao W, Ladanyi M, Sander C, Heguy A, Holland EC, Paty PB, Mischel PS, Liau L, Cloughesy TF, Mellinghoff IK, Solit DB, Chan TA (2010) Somatic mutations of the Parkinson's disease-associated gene PARK2 in glioblastoma and other human malignancies. Nat Genet 42(1):77–82. doi:10.1038/ng.491

Wan XB, Fan XJ, Chen MY, Xiang J, Huang PY, Guo L, Wu XY, Xu J, Long ZJ, Zhao Y, Zhou WH, Mai HQ, Liu Q, Hong MH (2010) Elevated Beclin 1 expression is correlated with HIF-1alpha in predicting poor prognosis of nasopharyngeal carcinoma. Autophagy 6(3):395–404

Wang Y, Engels IH, Knee DA, Nasoff M, Deveraux QL, Quon KC (2004) Synthetic lethal targeting of MYC by activation of the DR5 death receptor pathway. Cancer Cell 5(5):501–512

Wei Y, Pattingre S, Sinha S, Bassik M, Levine B (2008) JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. Mol Cell 30(6):678–688. doi:10.1016/j.molcel.2008.06.001

White E (2012) Deconvoluting the context-dependent role for autophagy in cancer. Nat Rev Cancer 12(6):401–410. doi:10.1038/nrc3262

White E, Karp C, Strohecker AM, Guo Y, Mathew R (2010) Role of autophagy in suppression of inflammation and cancer. Curr Opin Cell Biol 22(2):212–217. doi:10.1016/j.ceb.2009.12.008

Yang Z, Klionsky DJ (2010) Eaten alive: a history of macroautophagy. Nat Cell Biol 12(9):814–822. doi:10.1038/ncb0910-814

Yang D, Liu H, Goga A, Kim S, Yuneva M, Bishop JM (2010) Therapeutic potential of a synthetic lethal interaction between the MYC proto-oncogene and inhibition of aurora-B kinase. Proc Natl Acad Sci U S A 107(31):13836–13841. doi:10.1073/pnas.1008366107

Yao JC, Lombard-Bohas C, Baudin E, Kvols LK, Rougier P, Ruszniewski P, Hoosen S, St Peter J, Haas T, Lebwohl D, Van Cutsem E, Kulke MH, Hobday TJ, O'Dorisio TM, Shah MH, Cadiot G, Luppi G, Posey JA, Wiedenmann B (2010) Daily oral everolimus activity in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy: a phase II trial. J Clin Oncol 28(1):69–76. doi:10.1200/JCO.2009.24.2669

Yla-Anttila P, Vihinen H, Jokitalo E, Eskelinen EL (2009) 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy 5(8):1180–1185

Young AC, Craven RA, Cohen D, Taylor C, Booth C, Harnden P, Cairns DA, Astuti D, Gregory W, Maher ER, Knowles MA, Joyce A, Selby PJ, Banks RE (2009) Analysis of VHL gene alterations and their relationship to clinical parameters in sporadic conventional renal cell carcinoma. Clin Cancer Res Off J Am Assoc Cancer Res 15(24):7582–7592. doi:10.1158/1078-0432.CCR-09-2131

Yu L, McPhee CK, Zheng L, Mardones GA, Rong Y, Peng J, Mi N, Zhao Y, Liu Z, Wan F, Hailey DW, Oorschot V, Klumperman J, Baehrecke EH, Lenardo MJ (2010) Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature 465(7300):942–946. doi:10.1038/nature09076

Yue Z, Jin S, Yang C, Levine AJ, Heintz N (2003) Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proc Natl Acad Sci U S A 100(25):15077–15082. doi:10.1073/pnas.2436255100

Zlatic SA, Tornieri K, L'Hernault SW, Faundez V (2011) Metazoan cell biology of the HOPS tethering complex. Cell Logist 1(3):111–117. doi:10.4161/cl.1.3.17279

Zoncu R, Bar-Peled L, Efeyan A, Wang S, Sancak Y, Sabatini DM (2011) mTORC1 senses lysosomal amino acids through an inside-out mechanism that requires the vacuolar H(+)-ATPase. Science 334(6056):678–683. doi:10.1126/science.1207056

# Chapter 9
# Intratumoral Hypoxia as the Genesis of Genetic Instability and Clinical Prognosis in Prostate Cancer

**Daria Taiakina, Alan Dal Pra, and Robert G. Bristow**

**Abstract** Intratumoral hypoxia is prevalent in many solid tumors and is a marker of poor clinical prognosis in prostate cancer. The presence of hypoxia is associated with increased chromosomal instability, gene amplification, downregulation of DNA damage repair pathways, and altered sensitivity to agents that damage DNA. These genomic changes could also lead to oncogene activation or tumor suppressor gene inactivation during prostate cancer progression. We review here the concept of repair-deficient hypoxic tumor cells that can adapt to low oxygen levels and acquire an aggressive "unstable mutator" phenotype. We speculate that hypoxia-induced genomic instability may also be a consequence of aberrant mitotic function in hypoxic cells, which leads to increased chromosomal instability and aneuploidy. Because both hypoxia and aneuploidy are prognostic factors in prostate cancer, a greater understanding of these biological states in prostate cancer may lead to novel prognostic and predictive tests and drive new therapeutic strategies in the context of personalized cancer medicine.

**Keywords** Hypoxia • Genomic instability • Prostate cancer • Radiotherapy • Aneuploidy • DNA repair • Prognosis • Predictive assays • Mutator phenotype

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## 9.1 Clinical Impact of Hypoxia in Prostate Cancer Treatment

The tumor microenvironment consists of subregions of abnormal cell metabolism with dynamic and differential gradients of oxygen consumption. Chronic (diffusion-limited) tumor hypoxia develops in solid tumors because of the irregular distribution of tumor vessels and limited diffusion of oxygen through the tumor interstitium at distances of more than 150 μm. Acute or cycling hypoxia occurs because of fluctuating anoxia and subsequent reoxygenation as a consequence of vascular instability and transient variability in microregional tumor perfusion (Chan et al. 2007). The biological and clinical effects of tumor hypoxia include increased rates of genomic instability, increased capacity for systemic metastases, and resistance to chemotherapy and radiotherapy (Bristow and Hill 2008). Hypoxic cells historically have been documented as being resistant to ionizing radiation (IR), since oxygen renders radiation up to two to three times more efficient at causing lethal DNA damage (e.g., the oxygen enhancement ratio is 2–3) (Chan et al. 2007). Increased resistance to chemotherapy occurs because of a decrease in the perfusion of agents across diminished oxygen gradients, reduced cell death of hypoxic cells in the $G_0$-$G_1$ state by proliferation-dependent drugs, and altered multidrug resistance and DNA repair (Chan et al. 2007). However, as we describe below, recent data suggest that hypoxia can modify the DNA damage response and, in some cases, hypoxic cells are rendered as DNA repair-deficient cells with reduced oxygen enhancement ratio values and differential sensitivity to certain types of chemotherapy and radiotherapy (Sprong et al. 2006; Chan et al. 2008; Bristow and Hill 2008). An increased capacity for metastases in hypoxic tumor cells is associated with multiple mechanisms, including increased hypoxia-activated genes involved in metastasis and angiogenesis (e.g., *VEGF*, *LOX*) and selection of genetically unstable metastatic clones during tumor progression (Bristow and Hill 2008).

When taken together, these aggressive biological properties of hypoxia cells lead to a clinical scenario in which the presence of intratumoral hypoxia is an adverse prognostic factor in cancer (Bristow and Hill 2008). This is particularly true for prostate cancer (the most common noncutaneous malignancy in men): several clinical studies have shown an association between hypoxia and poor clinical outcome following radiotherapy or radical prostatectomy (see Table 9.1).

As can be seen in Table 9.1, different methodologies have been used to assess intraglandular tumor hypoxia, including direct electrode measurements of oxygen partial pressure ($pO_2$), use of the hypoxia biomarker, pimonidazole, and immunohistochemistry for hypoxia-activated protein expression. Using a needle-electrode technique, Turaka et al. (2012) studied 57 patients with more than 8 years of follow-up and showed that a decreased prostate-to-muscle oxygen ratio was an important predictor of early biochemical recurrence following brachytherapy. These authors suggested that hypoxia was driving early occurrence because it was associated with an increased likelihood of occult metastases at the time of treatment. Using a similar methodology, Milosevic et al. (2012) directly measured intraprostatic oxygen levels in the largest study to date of 247 prostate cancer patients with localized intermediate-risk disease (Milosevic et al. 2012). This large study showed that hypoxia is associated with early biochemical relapse and local recurrence in the prostate gland (see Fig. 9.1a).

**Table 9.1** Selected clinical studies of hypoxia in prostate cancer

| Study | Patients (n) | Risk group | Method | Comments |
|---|---|---|---|---|
| Turaka et al. (2012) | 57 | cT1-3 | $pO_2$ probe | Lower prostate/muscle $pO_2$ ratio predicted early biochemical failure after brachytherapy |
| Milosevic et al. (2012) | 247 | cT1-2 | $pO_2$ probe | Largest study showing that hypoxia predicted early biochemical relapse after radiotherapy and local recurrence |
| Vergis et al. (2008) | 201 (Radiotherapy)<br>289 (Surgery) | cT1-3 | IHC: VEGF, HIF-1α, OPN | Increased expression of VEGF, HIF-1α, and, in patients treated with surgery, OPN, identified patients at high risk of biochemical failure |
| Carnell et al. (2006) | 43 | cT1-3 | IHC: PIMO | Demonstrated a positive correlation of PIMO +3 binding with Gleason score |
| Boddy et al. (2005) | 149 | cT1-3 | IHC: VEGF, HIF-1α | There was a significant correlation between expression of HIF-1 α and HIF-2 α and with androgen receptor and VEGF expression. VEGF also was significantly related to the androgen receptor, whereas PHD2 was inversely related to HIF-2α expression. No significant association was shown between HIF-1α or HIF-2v and time to recurrence of PSA |
| Green et al. (2007) | 50 | cT3 | IHC | High VEGF expression was associated with lower disease-specific survival |
| Thoms et al. (2012) | 199 (T1-3)<br>37 (M1) | cT1-T3 and M1 | ELISA: OPN | Within localized prostate cancers plasma OPN was not predictive of more aggressive disease. For patients with metastatic CRPC, OPN was |
| Weber et al. (2012) | 103 | cT1-3 | IHC | High nuclear expression of HIF-1α and low expression of EGFR was associated with a good prognosis in diagnostic biopsies of patients with prostate cancer who were treated with radiotherapy ± androgen deprivation therapy |

*cT1* Clinical (preradiotherapy) T-category, *HIF* hypoxia-inducible factor, *IHC* immunohistochemistry, *PIMO* pimonidazole, *$pO_2$* measured with $pO_2$ electrode, *pT* pathologic (after surgery) T-category, *VEGF* vascular endothelial growth factor, *OPN* osteopontin, *PHD* prolyl hydroxylase enzymes, *PSA* prostate-specific antigen, *CRPC* castrate resistant prostate cancer

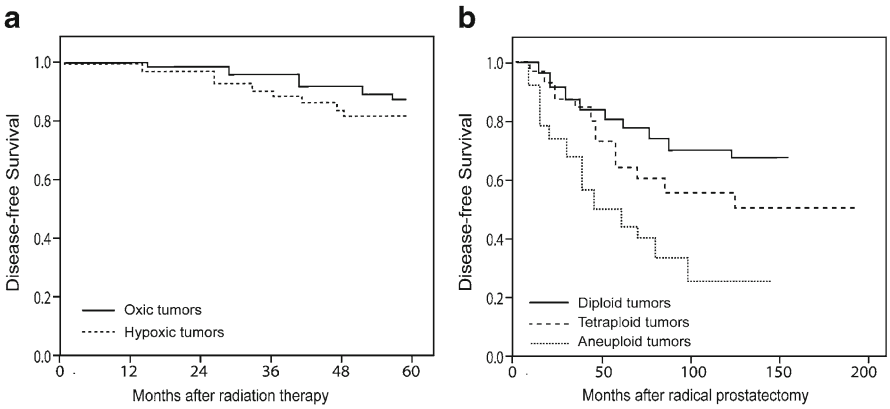


**Fig. 9.1** Hypoxia and aneuploidy are markers of poor clinical prognosis. (**a**) Patients with more hypoxic prostate tumors had higher rates of biochemical relapse after radiation therapy (Adapted from Milosevic et al. 2012). (**b**) Patients with tetraploid or aneuploid tumors had increased rates of disease relapse after radical prostatectomy relative to patients with diploid tumors (Adapted from Pretorius et al. 2009)

Prostate tumor hypoxia can also be assessed in situ using immunohistochemistry of biopsies or postsurgical specimens. Tumor cells adapt to a hypoxic microenvironment via upregulation of the transcription factor hypoxia-inducible factor (HIF)-1α (Semenza 2012). HIF-1α stabilization leads to increased transcription of several genes that are responsible for tumor cell survival in the low-oxygen environment, including vascular endothelial growth factor (*VEGF*), Glucose transporter 1 (*GLUT1*), and osteopontin (*OPN*) (Wilson and Hay 2011). Using immunohistochemistry, Vergis et al. (2008) showed that increased expression of hypoxia-induced proteins (e.g., HIF-1α, *VEGF*, and *OPN* in surgical patients and HIF-1α and *VEGF* in radiotherapy patients) predicted treatment failure, independent of clinical factors of tumor stage, Gleason score, serum prostate-specific antigen, and radiotherapy dose. In this study, the observation that the prognostic value of low $pO_2$ and increased expression of hypoxia-associated markers in situ are independent of radiation dose suggest that eradication of the aggressive hypoxic subfraction may require escalation in both local and systemic therapies. For example, this could lead to the use of combined modality therapies using precision surgery or radiotherapy plus androgen deprivation therapy (Milosevic et al. 2007) and/or selective and hypoxia-targeted systemic agents (Chan et al. 2010; Ahn and Brown 2007; Meng et al. 2012; Chan and Bristow 2010).

## 9.2 Chromosomal Instability and Prostate Cancer Prognosis

To understand a possible link between hypoxia and genetic instability in prostate cancer, one first needs to define "genome instability." Although often used interchangeably, chromosomal instability (CIN) and tumor cell aneuploidy are not the same. CIN is the dynamic process of constant loss or gain of chromosomes (or parts

of chromosomes), whereas aneuploidy defines a more static concept of chromosomal alteration (Geigl et al. 2008). Both CIN and aneuploidy are associated with cancer progression and poor prognosis (McGranahan et al. 2012). For example, Pretorius et al. (2009) showed that patients with tetraploid or aneuploid prostate tumors had decreased disease-free survival following radical prostatectomy when compared to patients with diploid tumors (see Fig. 9.1b). Table 9.2 summarizes the many clinical studies that have linked aneuploidy to poor prognosis in prostate cancer.

As we will see, hypoxia leads to downregulation of mechanisms to repair DNA damage and genomic instability (Bristow and Hill 2008; Vergis et al. 2008). Such repair-deficient hypoxic tumor cells could adapt to low oxygen levels and acquire an aggressive "mutator" phenotype, leading to clonal selection for resistant phenotypes (Bristow and Hill 2008; Luoto et al. 2013). As such, the intersection between intratumoral hypoxia, genomic instability, and aneuploidy as hallmarks of aggressive prostate cancer deserves further discussion because it may have important clinical implications. The next section describes potential mechanisms by which hypoxic cells can acquire defects in DNA repair and increased susceptibility for CIN.

## 9.3 Mechanisms for Hypoxia-Mediated Genomic Instability

One model of hypoxia-mediated tumor progression incorporates the concept of hypoxia driving the accumulation of mutations and chromosomal aberrations during cellular adaption. These hypoxic cells continue to proliferate under low oxygen conditions, with an increased likelihood of generating an unstable genome if genetic alterations accumulate during DNA replication and mitosis. For example, CIN can occur as a result of defects during the repair of DNA double-strand breaks (DSBs) and mitotic aberrations (McGranahan et al. 2012). DNA mutations can also occur secondary to microsatellite instability (MIN) (Michor et al. 2005). Microsatellites are repeat sequences one to six base pairs in length, and MIN presents as changes in the number of microsatellite repeats. MIN occurs because of defects in nucleotide mismatch repair (MMR) during the process of DNA replication, which causes elevated rates of nucleotide-level mutation (Kinzler and Vogelstein 1996). Discussed next are the data that link defects in DSBs and MMR pathways to the hypoxic cellular state.

### *9.3.1 The Effect of Hypoxia on DNA DSB Repair: The Homologous Recombination and Nonhomologous End-Joining Subpathways*

DNA double-strand breaks can be lethal to cells when not repaired or repaired incorrectly. DSBs occur as a result of exogenous DNA damage in the form of radiotherapy or chemotherapy (e.g., bleomycin) or endogenous DNA damage during DNA replication, which can produce unrepaired DNA breaks induced by replication stress

**Table 9.2** Selected clinical studies of aneuploidy in prostate cancer

| Study | Patients (n) | Risk group | Method | Comments |
|---|---|---|---|---|
| Pretorius et al. (2009) | 186 | | Image cytometry of Feulgen-stained tissue | On multivariate analysis, DNA ploidy was shown to be an independently predictor of disease recurrence. In cases with a Gleason score of 7 ($n$=68), DNA ploidy was a significant predictor of disease recurrence |
| Wirth et al. (1991) | 80 | Stage C | DNA flow cytometry | DNA ploidy was a strong prognostic indicator independent of tumor grade and tumor stage. Patients with diploid tumors did significantly better than those with an aneuploid or tetraploid tumor pattern |
| Di Silverio et al. (1996) | 85 | Stage C-D1 | DNA flow cytometry | DNA aneuploidy conferred a relative risk 2.3 times higher than diploidy for local and distant recurrences |
| Ross et al. (1994) | 89 | Early clinical stage (A2–B2) | Image analysis of Feulgen-stained tissue sections | DNA content analysis of ploidy status in needle biopsy specimens directly correlated with radical prostatectomy specimens and is associated independently with the presence of metastasis, disease recurrence, and extracapsular extension |
| Amling et al. (1999) | 108 | pT2-4N0-1 | DNA flow cytometry | DNA ploidy predicted cancer-specific and progression-free survival |
| Song et al. (1992) | 65 | cT1-3N0 | DNA flow cytometry and image analysis of Feulgen-stained tissue sections | DNA content was the most important independent variable for cancer-specific survival |
| Pollack et al. (1994b) | 76 | cT1-3N0 | DNA flow cytometry | In a cohort treated with RT, DNA ploidy (near-diploid vs. diploid and nondiploid tumors) was an independent prognostic factor for recurrence |
| Pollack et al. (1994a) | 76 | cT1-3N0 | DNA flow cytometry | In the same RT cohort (above), a significant correlation of DNA ploidy with PSA-DT was observed. Nondiploid tumors were associated with shorter PSA-DT (higher actuarial rates of disease relapse at 3 years) |
| Pollack (2003) | 149 | T2-3N0-N1 | Image analysis of Feulgen-stained tissue sections | On the basis of the RTOG 8610 (RT alone vs. RT plus short-course ADT), nondiploidy was associated with shorter overall survival, which seemed to be related to reduced response to salvage hormone therapy for those previously exposed to short-term ADT |

*cT1* clinical (preradiotherapy) T-category, *pT* pathologic (after surgery) T-category, *RT* radiotherapy, *ADT* androgen deprivation therapy

(Kuzminov 2001; Mills et al. 2003). Unrepaired/misrepaired DSBs can lead to the loss or gain of partial or whole chromosomes and chromosome translocations (Helleday et al. 2007; Jeggo and Löbrich 2007). There are two subpathways of DSB repair: the fast but error-prone nonhomologous end-joining (NHEJ) pathway is active throughout the cell cycle; the second pathway is homologous recombination (HR), which is a more stringent and time-consuming pathway but essentially error-free. HR requires an intact homologous chromosome as a template, and therefore it is active only during the S and $G_2$ phases of the cell cycle (Helleday et al. 2007; Rothkamm et al. 2003). Hypoxia causes downregulation of the expression of a number of HR and NHEJ genes, including *RAD51*, *BRCA1*, *BRCA2*, and *PRKDC*, and their protein products and has been linked to functional HR defects (Meng et al. 2005; Chan et al. 2008). In our laboratory we found that despite lower levels of initial DSB formation following ionizing radiation of hypoxic cells, DNA damage repair under continuous hypoxia led to increased residual and unrepaired DSBs and associated chromosomal aberrations at first mitosis following ionizing radiation (Kumareswaran et al. 2012). Hypoxia is also known to induce common fragile sites, which are chromosomal regions prone to breakage and deletions (Coquelle et al. 1998; Arlt et al. 2006; Schwartz et al. 2005). We speculate that this compromise in DSB repair in hypoxic cells leads to the resulting increase in chromosome aberrations and drives genomic instability and CIN. CIN would then lead to aneuploidy as well as an increase in loss of heterozygosity (LOH) (Michor et al. 2005) in cells that have mutated or lost alleles, which leads to a loss of function for genetic loci. Indeed, LOH can elevate the rate of tumor suppressor gene inactivation and contribute to cancer progression (Michor et al. 2005), and it would be of interest to compare rates of LOH in normoxic and hypoxic cells to support this hypothesis.

### *9.3.2 Other DNA Repair Pathways Modified by Hypoxia: MMR and Nucleotide Excision Repair*

Hypoxia also causes defects in other DNA damage repair pathways including MMR, nucleotide excision repair (NER), and the Fanconi anemia (FA) pathway. MMR is the mechanism of repair for the mismatch and misalignment of bases that occurs during DNA replication (Hsieh and Yamane 2008). The MMR pathway is suppressed by hypoxia because of downregulation of MMR proteins, including MLH1 and MSH2 (Shahrzad et al. 2005; Mihaylova et al. 2003; Nakamura et al. 2008). Defects in MMR have been shown to cause MIN because of accumulation of unrepaired replication errors. Tumors with MIN generally have less large-scale genomic alteration and gene mutation profiles that are distinct from those observed in CIN tumors (Geiersbach and Samowitz 2011).

Bulky DNA adducts or crosslinks caused by chemotherapeutic drugs, such as cisplatin, can be repaired by the NER pathway (Nouspikel 2009). It is important to note that HIF-1α binds to the hypoxia-responsive elements within the gene promoters of two NER proteins, XPC and ERCC2, and exhibits negative transcriptional regulation on these genes under hypoxic conditions (Rezvani et al. 2010). HIF-1α also

downregulates the NER protein RAD23B under hypoxia via activation of the microRNA miR373 (Crosby et al. 2009). FA is a hereditary cancer predisposition disorder caused by mutations in any of 14 *FANC* genes that participate in DNA interstrand crosslink repair (Kitao and Takata 2011). Less is known about the function of the FA pathway under hypoxic conditions, but work has linked cells deficient in *FANCD2* to differential DNA repair and radiosensitivity under hypoxia versus normoxia (Sprong et al. 2006; Kuhnert et al. 2009). The global reduction in numerous DNA repair pathways in hypoxic cells can therefore contribute to an accumulation of different mutations and translocations, which, if they are not lethal and provide a relative growth advantage, may drive aggressive tumor phenotypes after clonal selection.

### *9.3.3 Using Hypoxia-Mediated DNA Repair Defects as an Achilles' Heel for Cancer Treatment: The Concept of Contextual Synthetic Lethality*

Treatment-resistant and aggressive tumor phenotypes associated with DNA repair-deficient hypoxic cells may be uniquely targeted using the knowledge of the DNA repair defect. For example, our laboratory and others have suggested that DNA repair-deficient hypoxic tumor cells can be targeted using the concept of contextual synthetic lethality (Chan and Bristow 2010; Chan et al. 2010). Two genes are synthetically lethal if a mutation of either gene alone is compatible with viability but mutation of both genes leads to cell death (Kaelin 2005). This is termed *genetic synthetic lethality* because it was originally based on yeast genetics (Kaelin 2005). However, this observation could also apply to hypoxic cells deficient in HR or MMR (Chan and Bristow 2010; Chan et al. 2010). For example, hypoxic cells deficient in HR can be sensitized by inhibitors of poly (ADP-ribose) polymerase (PARP) proteins, which function in single-strand breaks and base-excision repair (Hegan et al. 2010; Chalmers et al. 2010; Chan et al. 2010). This is similar to the use of PARP inhibitors in ovarian cancers, which are defective in HR because of a loss of function of the *BRCA1* or *BRCA2* gene (Fong et al. 2009). Contextual synthetic lethality can also potentially be used to target downregulation of MMR in hypoxic cells. Genetic disruption of DNA polymerases POLB and POLG has been shown to be synthetically lethal in cells with deficiency in MSH2 and MLH1, the MMR proteins known to be downregulated by hypoxia (Martin et al. 2010). In addition, repair-deficient hypoxic tumor cells can have increased sensitivity to specific drugs that are selectively toxic to repair-deficient cells (e.g., MMC or cisplatin with HR-defective cells) (Chan and Bristow 2010; Chan et al. 2008). Therefore, defining the presence and fraction of repair-defective hypoxic cells in human tumors using biomarkers of hypoxia and DNA repair defects could lead to personalized treatment and achieve a high therapeutic ratio, given that these abnormalities are likely to be tumor-specific (Bristow and Hill 2008; Chan and Bristow 2010).

## 9.4 Hypoxia as a Model for Mitotic Control

### *9.4.1 Causes and Consequences of CIN*

CIN is highly prevalent in cancer and leads to aneuploidy (Geigl et al. 2008). Numerical CIN is classified as deletion or addition of whole chromosomes, whereas structural CIN is associated with intrachromosome breaks (Geigl et al. 2008). Structural CIN can occur in tumor cells when there is a genetic inactivation of one or more cell-cycle checkpoints that normally act to prevent genome instability (e.g., abrogation of the G1 checkpoint in cells with a *TP53* mutation) (Gisselsson 2003; Smith and Fornance 1995). Disruption of mitotic function, such as defects in sister chromatid cohesion, bypass of the spindle assembly checkpoint, and/or centrosome aberrations, can lead to both numeric and structural CIN (Thompson et al. 2010). Structural chromosome rearrangements can also result in gene fusion products as well as gene amplification (McGranahan et al. 2012). A link between intratumoral hypoxia and abnormal centrosome biology or CIN could therefore lead to LOH during prostate tumor progression (Baker et al. 2009; McGranahan et al. 2012) and a functional loss of tumor suppressors.

### *9.4.2 The Effect of Hypoxia on Mitotic Function: A Possible Mechanism of Hypoxia-Induced Genomic Instability*

In animal cells, centrosomes are organelles that serve as the main microtubule organizing center. During mitosis, centrosomes play a crucial role in mitotic spindle assembly, proper segregation of sister chromatids, and cytokinesis (Mihaylova et al. 2003). Disrupted centrosome function has been recognized as one of the leading causes of CIN and aneuploidy in many cancer types (Nigg 2002; Krämer et al. 2002; Chan 2011). Centrosome aberrations are common in many human cancers (Krämer et al. 2002; Nigg 2002; Chan 2011), including prostate cancer. Pihan et al. (2001) demonstrated that centrosome abnormalities are evident in the majority of prostate carcinomas and are enhanced in poorly differentiated tumors; as such, higher levels of centrosome may contribute to prostate cancer aggression.

Centrosome amplification describes the various processes through which a cell acquires supernumerary centrosomes. Centrosome amplification can occur as a premitotic stress response after DNA damage during prolonged arrest of the $G_2$ phase (Dodson et al. 2004). During the $G_2$ phase, DNA damage response and repair proteins such as RAD51, BRCA1, BRCA2, XRCC2, MDC1, and BRIT1 colocalize to centrosomes (Rai et al. 2006, 2008; Cappelli et al. 2011; Hsu and White 1998). Depletion or mutation of these genes enhances centrosome amplification (see Table 9.3) (Dodson et al. 2004; Rai et al. 2008; Saladino et al. 2009; Ko et al. 2006; Shimada et al. 2010). The proteins ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related (ATR) are phosphatidylinositol 3-kinase-related kinases (PIKKs) that are involved in DSB sensing and subsequent activation of cell-cycle arrest as

**Table 9.3** DNA repair proteins that may have a role in centrosome function and are downregulated by hypoxia

| Gene/ protein | Genetic model | Protein function | Result | References |
|---|---|---|---|---|
| Rad51 | Knockdown | HR | Increase in centrosome amplification<br>Colocalizes with centrosomes | Cappelli et al. (2011), Dodson et al. (2004) |
| BRCA1<br>BRCA2 | Null mutant/ knockout | HR | Increased centrosome amplification in the absence of any treatment<br>Enhanced IR-induced centrosome amplification<br>Colocalizes with centrosomes | Cappelli et al. (2011), Hsu and White (1998), Saladino et al. (2009) |
| DNA-PK, Ku70 | Null mutant/ knockout | DDR | IR-induced centrosome amplification is reduced | Saladino et al. (2009) |
| DNA-PK, Ku70 | Knockout | NHEJ | Slightly increased IR-induced centrosome amplification | Shimada et al. (2010) |
| NBS1 | Knockout | DSB sensing | Slightly increased IR-induced centrosome amplification | Shimada et al. (2010) |
| XRCC2 | Deficient (irs1 hamster cells) | HR | Centrosome disruption (without IR-induced DNA damage)<br>Co-immunoprecipitates with BRCA1 and γ-tubulin<br>Colocalizes with centrosomes | Cappelli et al. (2011) |

*HR* homologous recombination, *NHEJ* nonhomologous end-joining, *IR* ionizing radiation, *DDR* DNA damage response, *DSB* double-strand break

well as the recruitment of DNA repair proteins to the sites of DNA damage. These two kinases have complementary roles in limiting centrosome amplification in response to DNA damage (Bourke et al. 2007; Dodson et al. 2004).

The link between the DNA damage repair pathways and centrosome amplification is of interest in the context of tumor hypoxia. Since the disruption of DNA damage repair proteins can lead to centrosome amplification, we speculate that hypoxia may also lead to centrosome aberrations. Centrosome aberrations and subsequent mitotic abnormalities may be an additional mechanism of hypoxia-induced genomic instability (see Fig. 9.2). However, there is currently almost no literature on the effects of hypoxia on centrosome function. The only direct link between hypoxia and centrosome function that has been found so far is an increase in centrosome amplification in cells overexpressing miR-210, a microRNA upregulated by the HIF-1α pathway and hypoxic conditions (Nakada et al. 2011). If hypoxia modulates mitotic function, then some mitotic aberrations may be specific to the hypoxic tumor state. Drugs targeting mitosis and centrosomes are under investigation as potential cancer therapeutics (Mazzorana et al. 2011). Centrosome-associated kinases such as polo-like kinase 1 (*PLK1*), aurora kinase A (*AURKA*), and cyclin-dependent kinase 1 (*CDK1*) which regulate centrosome function, are all targets of compounds that are in advanced stages of clinical trials for cancer therapy

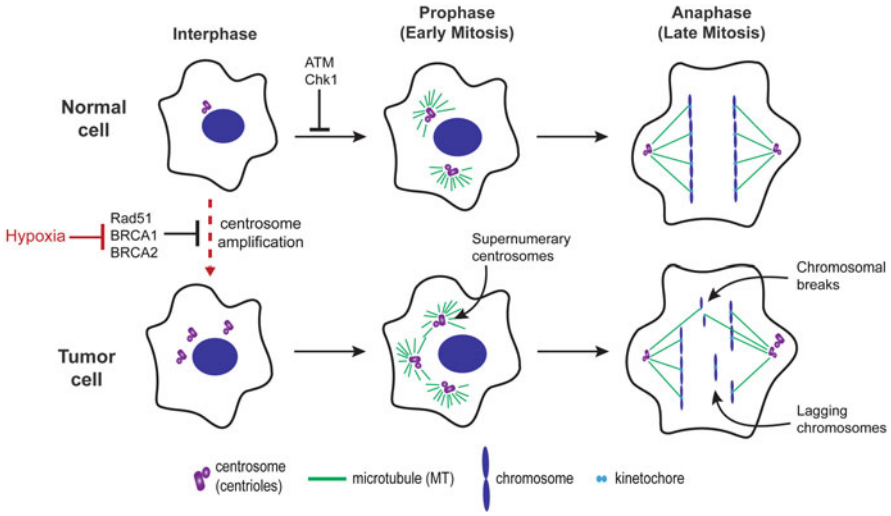


**Fig. 9.2** Mitotic and centrosome abnormalities in cancer and their potential relationship with intratumoral hypoxia. Hypoxia downregulates DNA damage repair proteins (e.g., RAD51, BRCA1, BRCA2) involved in both homologous recombination (HR) and centrosome biology. Defective HR and centrosome disruption leads to increased mutation burden, centrosome amplification, and supernumerary centrosomes. As a cell progresses through mitosis, the presence of supernumerary centrosomes and centrosome aberrations can lead to improper microtubule-kinetochore attachments and result in lagging chromosomes and DNA-chromosomal breaks. As chromosome aberrations accumulate, chromosomal instability and aneuploidy increase during tumor progression

(Mazzorana et al. 2011). Our laboratory is currently exploring the role of centrosome biology in normoxic and hypoxic prostate cancers as a potential biomarker of the efficacy of these compounds.

## 9.5 Conclusions and Outstanding Questions

In this chapter we summarized the role of hypoxia in genomic instability and clinical prognosis using the example of prostate cancer. Hypoxia can lead to increased metastasis and poor clinical prognosis as a result of a "mutator" tumor phenotype aligned with cellular adaption and clonal selection. A potential model of the interaction between hypoxia, CIN, and mitotic function that leads to cancer aggression is shown in Fig. 9.3.

By identifying the pathways affected specifically in hypoxic tumor cells, clinicians may be able to selectively target these cells and improve cancer treatment outcomes. However, there are many outstanding questions regarding hypoxia and genetic instability that require further investigation and are the subject of current investigations within our laboratory:

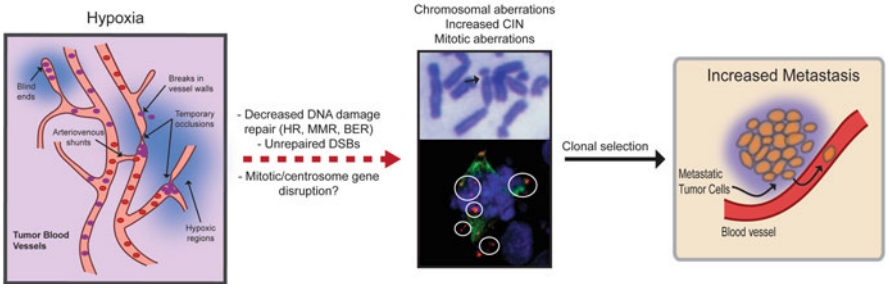


**Fig. 9.3** A mechanistic model for the effect of intratumoral hypoxia on genomic instability and cancer metastasis. One model of hypoxia-induced genomic instability suggests that the decrease in protein expression and function associated with DNA damage responses and repair leads to increased chromosomal aberrations and instability and drives abnormal mitosis. The clonal selection and expansion of these unstable mutant cells leads to aggressive tumor phenotypes and an increased capacity for metastasis (Adapted from Brown and Wilson 2004)

- Is aberrant mitotic function enhanced or specific to hypoxic tumor cells and do these abnormalities lead to increased tumor CIN?
- How can we address the challenge of direct assessment of $pO_2$ changes within the tumor and correlate hypoxic conditions to mitotic function?
- Is there a driving mechanism by which hypoxia leads to aneuploidy that can be specifically targeted with agents that target mutated mitosis or centrosome genes to prevent clonal selection/adaption and metastases?

Providing answers to these questions will provide a greater understanding as to why hypoxic subregions within the tumor microenvironment lead to adverse prognosis and provide new avenues for individualized therapeutic approaches.

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## References

Ahn G, Brown M (2007) Targeting tumors with hypoxia-activated cytotoxins. Front Biosci 12:3483–3501

Amling CL, Lerner SE, Martin SK, Slezak JM, Blute ML, Zincke H (1999) Deoxyribonucleic acid ploidy and serum prostate specific antigen predict outcome following salvage prostatectomy for radiation refractory prostate cancer. J Urol 161:857–863

Arlt MF, Durkin SG, Ragland RL, Glover TW (2006) Common fragile sites as targets for chromosome rearrangements. DNA Repair 5:1126–1135

Baker DJ, Jin F, Jeganathan KB, Van Deursen JM (2009) Whole chromosome instability caused by Bub1 insufficiency drives tumorigenesis through tumor suppressor gene loss of heterozygosity. Cancer Cell 16:475–486

Boddy JL, Fox SB, Han C, Campo L, Turley H, Kanga S, Malone PR, Harris AL (2005) The androgen receptor is significantly associated with vascular endothelial growth factor and hypoxia sensing via hypoxia-inducible factors HIF-1a, HIF-2a, and the prolyl hydroxylases in human prostate cancer. Clin Cancer Res 11:7658–7663. doi:10.1158/1078-0432.CCR-05-0460

Bourke E, Dodson H, Merdes A, Cuffe L, Zachos G, Walker M, Gillespie D, Morrison CG (2007) DNA damage induces Chk1-dependent centrosome amplification. EMBO Rep 8:603–609

Bristow RG, Hill RP (2008) Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. Nat Rev Cancer 8:180–192

Brown JM, Wilson WR (2004) Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 4(6):437–447

Cappelli E, Townsend S, Griffin C, Thacker J (2011) Homologous recombination proteins are associated with centrosomes and are required for mitotic stability. Exp Cell Res 317:1203–1213. doi:10.1016/j.yexcr.2011.01.021

Carnell DM, Smith RE, Daley FM, Saunders MI, Bentzen SM, Hoskin PJ (2006) An immunohistochemical assessment of hypoxia in prostate carcinoma using pimonidazole: implications for radioresistance. Int J Radiat Oncol Biol Phys 65:91–99. doi:10.1016/j.ijrobp.2005.11.044

Chalmers AJ, Lakshman M, Chan N, Bristow RG (2010) Poly(ADP-ribose) polymerase inhibition as a model for synthetic lethality in developing radiation oncology targets. Semin Radiat Oncol 20:274–281

Chan JY (2011) A clinical overview of centrosome amplification in human cancers. Int J Biol Sci 7:1122–1144

Chan N, Bristow RG (2010) "Contextual" synthetic lethality and/or loss of heterozygosity: tumor hypoxia and modification of DNA repair. Clin Cancer Res 16:4553–4560

Chan N, Milosevic M, Bristow RG (2007) Tumor hypoxia, DNA repair and prostate cancer progression: new targets and new therapies. Future Oncol (London, England) 3:329–341

Chan N, Koritzinsky M, Zhao H, Bindra R, Glazer PM, Powell S, Belmaaza A, Wouters B, Bristow RG (2008) Chronic hypoxia decreases synthesis of homologous recombination proteins to offset chemoresistance and radioresistance. Cancer Res 68:605–614

Chan N, Pires IM, Bencokova Z et al (2010) Contextual synthetic lethality of cancer cell kill based on the tumor microenvironment. Cancer Res 70:8045–8054

Coquelle A, Toledo F, Stern S, Bieth A, Dabatisse M (1998) A new role for hypoxia in tumor progression: induction of fragile site triggering genomic rearrangements and formation of complex DMs and HSRs. Mol Cell 2:259–265

Crosby ME, Kulshreshtha R, Ivan M, Glazer PM (2009) MicroRNA regulation of DNA repair gene expression in hypoxic stress. Cancer Res 69:1221–1229

Di Silverio F, D'Eramo G, Buscarini M, Sciarra A, Casale P, Di Nicola S, Loreto A, Seccareccia F, De Vita R (1996) DNA ploidy, Gleason score, pathological stage and serum PSA levels as predictors of disease-free survival in C-D1 prostatic cancer patients submitted to radical retropubic prostatectomy. Eur Urol 30:316–321

Dodson H, Bourke E, Jeffers LJ, Vagnarelli P, Sonoda E, Takeda S, Earnshaw WC, Merdes A, Morrison C (2004) Centrosome amplification induced by DNA damage occurs during a prolonged G2 phase and involves ATM. EMBO J 23:3864–3873. doi:10.1038/sj.emboj.7600393

Fong PC, Boss DS, Yap TA et al (2009) Inhibition of poly(ADP-ribose) polymerase in tumors from BRCA mutation carriers. New Engl J Med 361:123–134

Geiersbach KB, Samowitz WS (2011) Microsatellite instability and colorectal cancer. Arch Pathol Lab Med 135:1269–1277

Geigl JB, Obenauf AC, Schwarzbraun T, Speicher MR (2008) Defining "chromosomal instability". Trends Genet 24:64–69

Gisselsson D (2003) Chromosome instability in cancer: how, when, and why? Adv Cancer Res 87:1–29

Green MML, Hiley CT, Shanks JH, Bottomley IC, West CML, Cowan RA, Stratford IJ (2007) Expression of vascular endothelial growth factor (VEGF) in locally invasive prostate cancer is prognostic for radiotherapy outcome. Int J Radiat Oncol Biol Phys 67:84–90. doi:10.1016/j.ijrobp.2006.08.077

Hegan DC, Lu Y, Stachelek GC, Crosby ME, Bindra RS, Glazer PM (2010) Inhibition of poly(ADP-ribose) polymerase down-regulates BRCA1 and RAD51 in a pathway mediated by E2F4 and p130. Proc Natl Acad Sci U S A 107:2201–2206

Helleday T, Lo J, Van Gent DC, Engelward BP (2007) DNA double-strand break repair: from mechanistic understanding to cancer treatment. DNA Repair 6:923–935

Hsieh P, Yamane K (2008) DNA mismatch repair: molecular mechanism, cancer, and ageing. Mech Ageing Dev 129:391–407

Hsu LC, White RL (1998) BRCA1 is associated with the centrosome during mitosis. Proc Natl Acad Sci U S A 95:12983–12988

Jeggo P, Löbrich M (2007) DNA double-strand breaks: their cellular and clinical impact? Oncogene 26:7717–7719

Kaelin WG (2005) The concept of synthetic lethality in the context of anticancer therapy. Nat Rev Cancer 5:689–698

Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. Cell 87:159–170

Kitao H, Takata M (2011) Fanconi anemia: a disorder defective in the DNA damage response. Int J Hematol 93:417–424

Ko MJ, Murata K, Hwang D-S, Parvin JD (2006) Inhibition of BRCA1 in breast cell lines causes the centrosome duplication cycle to be disconnected from the cell cycle. Oncogene 25:298–303

Krämer A, Neben K, Ho A (2002) Centrosome replication, genomic instability and cancer. Leukemia 16:767–775

Kuhnert VM, Kachnic L, Li L, Purschke M, Gheorghiu L, Lee R, Held KD, Willers H (2009) FANCD2-deficient human fibroblasts are hypersensitive to ionising radiation at oxygen concentrations of 0% and 3% but not under normoxic conditions. Int J Radiat Biol 85:523–531

Kumareswaran R, Ludkovski O, Meng A, Sykes J, Pintilie M, Bristow RG (2012) Chronic hypoxia compromises repair of DNA double-strand breaks to drive genetic instability. J Cell Sci 125:189–199

Kuzminov A (2001) Single-strand interruptions in replicating chromosomes cause double-strand breaks. Proc Natl Acad Sci U S A 98:8241–8246

Luoto K, Kumareswaran R, Bristow RG (2013) Tumor Hypoxia as a Driving Force in Genetic Instability In Press. Genome Integrity

Martin S, McCabe N, Mullarkey M, Cummins R, Burgess DJ, Nakabeppu Y, Oka S, Kay E, Lord CJ, Ashworth A (2010) DNA polymerases as potential therapeutic targets for cancers deficient in the DNA mismatch repair proteins MSH2 or MLH1. Cancer Cell 17:235–248

Mazzorana M, Montoya G, Mortuza GB (2011) The centrosome: a target for cancer therapy. Curr Cancer Drug Targets 11:600–612

McGranahan N, Burrell R, Endesfelder D, Novelli MR, Swanton C (2012) Cancer chromosomal instability: therapeutic and diagnostic challenges. EMBO Rep 13:528–538

Meng AX, Jalali F, Cuddihy A, Chan N, Bindra RS, Glazer PM, Bristow RG (2005) Hypoxia down-regulates DNA double strand break repair gene expression in prostate cancer cells. Radiother Oncol J Eur Soc Ther Radiol Oncol 76:168–176

Meng F, Evans JW, Bhupathi D et al (2012) Molecular and cellular pharmacology of the hypoxia-activated prodrug TH-302. Mol Cancer Ther 11:740–751

Michor F, Iwasa Y, Vogelstein B, Lengauer C, Nowak M (2005) Can chromosomal instability initiate tumorigenesis? Semin Cancer Biol 15:43–49

Mihaylova VT, Bindra RS, Yuan J, Campisi D, Narayana L, Jensen R, Giordano F, Randall JS, Rockwell S, Glazer PM (2003) Decreased expression of the DNA mismatch repair gene Mlh1 under hypoxic stress in mammalian cells. Mol Cell Biol 23:3265–3273

Mills KD, Ferguson DO, Alt FW (2003) The role of DNA breaks in genomic instability and tumorigenesis. Immunol Rev 194:77–95

Milosevic M, Chung P, Parker C et al (2007) Androgen withdrawal in patients reduces prostate cancer hypoxia: implications for disease progression and radiation response. Cancer Res 67:6022–6025

Milosevic M, Warde P, Ménard C et al (2012) Tumor hypoxia predicts biochemical failure following radiotherapy for clinically localized prostate cancer. Clin Cancer Res 18:2108–2114. doi:10.1158/1078-0432.CCR-11-2711

Nakada C, Tsukamoto Y, Matsuura K, Nguyen TL, Hijiya N, Uchida T, Sato F, Mimata H, Seto M, Moriyama M (2011) Overexpression of miR-210, a downstream target of HIF1α, causes centrosome amplification in renal carcinoma cells. J Pathol 224:280–288

Nakamura H, Tanimoto K, Hiyama K, Yunokawa M, Kawamoto T, Kato Y, Yoshiga K, Poellinger L, Hiyama E, Nishiyama M (2008) Human mismatch repair gene, MLH1, is transcriptionally repressed by the hypoxia-inducible transcription factors, DEC1 and DEC2. Oncogene 27:4200–4209

Nigg EA (2002) Centrsome aberrations: cause or consequence of cancer progression? Nat Rev Cancer 2:1–11

Nouspikel T (2009) Nucleotide excision repair: variations on versatility. Cell Mol Life Sci 66:994–1009

Pihan GA, Purohit A, Wallace J, Malhotra R, Liotta L, and Doxsey SJ (2001) Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. Cancer Res 61:2212–2219

Pollack A (2003) Prostate cancer DNA ploidy and response to salvage hormone therapy after radiotherapy with or without short-term total androgen blockade: an analysis of RTOG 8610. J Clin Oncol 21:1238–1248. doi:10.1200/JCO.2003.02.025

Pollack A, Zagars GK, El-Naggar AK, Gauwitz MD, Terry NHA (1994a) Near-diploidy: a new prognostic factor for clinically localized prostate cancer treated with external beam radiation therapy. Cancer 73:1895–1903. doi:10.1002/1097-0142(19940401)73:7<1895::AID-CNCR2820730721>3.0.CO;2-0

Pollack A, Zagars GK, El-Naggar K, Terry NH (1994b) Relationship of tumor DNA-ploidy to serum prostate-specific antigen doubling time after radiotherapy for prostate cancer. Urology 44:711–718

Pretorius ME, Waehre H, Abeler VM, Davidson B, Vlatkovic L, Lothe R, Giercksky K-E, Danielsen HE (2009) Large scale genomic instability as an additive prognostic marker in early prostate cancer. Cell Oncol 31:251–259. doi:10.3233/CLO-2009-0463

Rai R, Dai H, Multani AS et al (2006) BRIT1 regulates early DNA damage response, chromosomal integrity, and cancer. Cancer Cell 10:145–157

Rai R, Phadnis A, Haralkar S, Badwe R, Dai H, Li K, Lin S-Y (2008) Differential regulation of centrosome integrity by DNA damage response proteins. Cell Cycle 7:2225–2233

Rezvani HR, Mahfouf W, Ali N, Chemin C, Ged C, Kim AL, De Verneuil H, Taïeb A, Bickers DR, Mazurier F (2010) Hypoxia-inducible factor-1alpha regulates the expression of nucleotide excision repair proteins in keratinocytes. Nucl Acids Res 38:797–809

Ross JS, Figge H, Bui HX, Del Rosario D, Jennings T, Rifkin MD, Fisher H (1994) Prediction of pathologic stage and postprostatectomy disease recurrence by DNA ploidy analysis of initial needle biopsy specimens of prostate cancer. Cancer 74:2811–2818

Rothkamm K, Krüger I, Thompson LH, Kru I, Lo M (2003) Pathways of DNA double-strand break repair during the mammalian cell cycle pathways of DNA double-strand break repair during the mammalian cell cycle. Mol Cell Biol. doi:10.1128/MCB.23.16.5706

Saladino C, Bourke E, Conroy PC, Morrison CG (2009) Centriole separation in DNA damage-induced centrosome amplification. Environ Mol Mutagen 50:725–732. doi:10.1002/em

Schwartz M, Zlotorynski E, Goldberg M, Ozeri E, Rahat A, Sage C, Chen BPC, Chen DJ, Agami R, Kerem B (2005) Homologous recombination and nonhomologous end-joining repair pathways regulate fragile site stability. Genes Dev 19:2715–2726

Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. Cell 148:399–408

Shahrzad S, Quayle L, Stone C, Plumb C, Shirasawa S, Rak JW, Coomber BL (2005) Ischemia-induced K-ras mutations in human colorectal cancer cells: role of microenvironmental regulation of MSH2 expression. Cancer Res 65:8134–8141

Shimada M, Kobayashi J, Hirayama R, Komatsu K (2010) Differential role of repair proteins, BRCA1/NBS1 and Ku70/DNA-PKcs, in radiation-induced centrosome overduplication. Cancer Sci 101:2531–2537. doi:10.1111/j.1349-7006.2010.01702.x

Smith M, Fornance A (1995) Genomic instability and the role of p53 mutations in cancer cells. Curr Opin Oncol 7:69–75

Song J, Cheng WS, Cupps RE, Earle JD (1992) Nuclear deoxyribonucleic acid content measured by static cytometry: important prognostic association for patients with clinically localized prostate carcinoma treated by external beam radiotherapy. J Urol 147:794–797

Sprong D, Janssen HL, Vens C, Begg AC (2006) Resistance of hypoxic cells to ionizing radiation is influenced by homologous recombination status. Int J Radiat Oncol Biol Phys 64:562–572

Thompson SL, Bakhoum SF, Compton D (2010) Mechanisms of chromosomal instability. Curr Biol 20:R285–R295

Thoms JW, Dal Pra A, Anborgh PH et al (2012) Plasma osteopontin as a biomarker of prostate cancer aggression: relationship to risk category and treatment response. Brit J Cancer 107:840–846. doi:10.1038/bjc.2012.345

Turaka A, Buyyounouski MK, Hanlon AL, Horwitz EM, Greenberg RE, Movsas B (2012) Hypoxic prostate/muscle PO2 ratio predicts for outcome in patients with localized prostate cancer: long-term results. Int J Radiat Oncol Biol Phys 82:e433–e439. doi:10.1016/j.ijrobp.2011.05.037

Vergis R, Corbishley CM, Norman AR et al (2008) Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. Lancet Oncol 9:342–351. doi:10.1016/S1470-2045(08)70076-7

Weber DC, Tille J-C, Combescure C, Egger J-F, Laouiti M, Hammad K, Granger P, Rubbia-Brandt L, Miralbell R (2012) The prognostic value of expression of HIF1α, EGFR and VEGF-A, in localized prostate cancer for intermediate- and high-risk patients treated with radiation therapy with or without androgen deprivation therapy. Radiat Oncol (London, England) 7:66. doi:10.1186/1748-717X-7-66

Wilson W, Hay M (2011) Targeting hypoxia in cancer therapy. Nat Rev Cancer 11:393–410

Wirth M, Muller H, Manseck A, Muller J, Frohmuler H (1991) Value of nuclear DNA ploidy patterns in patients with prostate cancer after radical prostatectomy. Eur Urol 20:248–252

# Chapter 10
# miR-210: Fine-Tuning the Hypoxic Response

**Mircea Ivan and Xin Huang**

**Abstract** Hypoxia is a central component of the tumor microenvironment and represents a major source of therapeutic failure in cancer therapy. Recent work has provided a wealth of evidence that noncoding RNAs and, in particular, microRNAs, are significant members of the adaptive response to low oxygen in tumors. All published studies agree that miR-210 specifically is a robust target of hypoxia-inducible factors, and the induction of miR-210 is a consistent characteristic of the hypoxic response in normal and transformed cells. Overexpression of miR-210 is detected in most solid tumors and has been linked to adverse prognosis in patients with soft-tissue sarcoma, breast, head and neck, and pancreatic cancer. A wide variety of miR-210 targets have been identified, pointing to roles in the cell cycle, mitochondrial oxidative metabolism, angiogenesis, DNA damage response, and cell survival. Additional microRNAs seem to be modulated by low oxygen in a more tissue-specific fashion, adding another layer of complexity to the vast array of protein-coding genes regulated by hypoxia.

**Keywords** Hypoxia • microRNA • Cancer • Biomarker • miR-210 • Mitochondria • Apoptosis • Metabolism

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## 10.1 Introduction

Tissue hypoxia is a dynamic feature of virtually all solid tumors (Semenza 2010a). The adaptive response to low oxygen encompasses complex biochemical and cellular processes, such as energy metabolism, cell survival and proliferation, angiogenesis, adhesion, and motility (Ruan et al. 2009). These, in turn, shape the natural history of cancer and constitute a major source of therapeutic failure in oncology (Brown and Giaccia 1998). During the past two decades, clinical research and animal models have provided strong evidence that tumors with extensive low oxygen tension are more likely exhibit poor prognosis (Vaupel and Mayer 2007). Therefore, a deep understanding of cellular adaptation to oxygen deprivation is key for developing more efficient therapeutic strategies (Wilson and Hay 2011).

Cells react to hypoxia in part via a transcriptional program orchestrated by an oxygen-monitoring machinery that is centered around the hypoxia-inducible factors (HIFs) (Wang and Semenza 1993; Wang et al. 1995; Wang and Semenza 1995). HIFs are heterodimers consisting of an oxygen-sensitive α-subunit (HIFα) and a constitutively expressed β-subunit (HIF1β, also called aryl hydrocarbon receptor nuclear translocator). Among the three homologous HIFα genes, HIF-1α, HIF-2α, and HIF-3α, the functions of HIF-1α and HIF-2α are best characterized. Under normoxic conditions, HIFα is hydroxylated by prolyl-4-hydroxylases, targeting it for proteasomal destruction mediated by the von Hippel-Lindau (VHL) protein-containing E3 ubiquitin ligase (Ivan et al. 2001; Jaakkola et al. 2001). When oxygen becomes limiting, decreased prolyl-4-hydroxylase activity leads to HIFα stabilization and heterodimerization with the β-subunit, followed by translocation to the nucleus and activation of hundreds of target genes (Semenza 2012). The protein-coding components of the HIF-mediated response are discussed in detail elsewhere in this book.

## 10.2 Noncoding RNA: Wide Roles in Physiology and Pathology

While protein coding sequences account for only approximately 1.1 % of the entire human genome (Venter et al. 2001), during the past decade it has become apparent that the vast majority of the noncoding sequences are, in fact, actively transcribed (Djebali et al. 2012). Given the long history of evolution that has shaped the human genome, it is unlikely that these transcripts are results of "background transcriptional noise." During the past decade, largely because of rapid advancements in microarray and next-generation sequencing technologies, the "dark matter" of the human genome has been found to encode tens of thousands of noncoding RNAs (ncRNAs). This is a highly heterogeneous superfamily, including diverse entities such as ribosomal RNAs, small nucleolar RNAs, small nuclear RNAs, transfer RNA, small interfering RNAs, piwi-associated RNAs, microRNA (miRNA), unusually small RNA, and long ncRNAs. Despite failing to be translated into proteins, it already has been demonstrated that a small percentage of ncRNAs exhibit important

biological functions, and many more are suspected to do so (Hüttenhofer et al. 2005). By far the best-characterized ncRNAs, both in general as well as in the particular context of hypoxia, are miRNAs, which are the main focus of this chapter.

### *10.2.1 MicroRNAs*

miRNAs are single-stranded, small ncRNA molecules (~22 nucleotides in length) that regulate gene expression by inhibiting messenger RNA (mRNA) translation or by facilitating cleavage of the target mRNA (Valencia-Sanchez et al. 2006). Our understanding of miRNA biology was relatively slow to emerge. The first miRNA, lin-4, was discovered in *Caenorhabditis elegans* in 1993 (Lee et al. 1993; Wightman et al. 1993), followed by the second miRNA, let-7, 7 years later (Reinhart et al. 2000). The finding that let-7 is well conserved in a wide range of animal species (Pasquinelli et al. 2000) spurred an accelerated expansion of miRNA discovery that is still ongoing. To date more than 2,200 miRNAs have been identified in the human genome (miRBase Release 19, http://www.mirbase.org), and at least one-third of all protein-encoding genes are now predicted to be regulated by miRNAs (Lewis et al. 2005). miRNAs are widely recognized as important regulators in developmental, physiological, and pathological settings, including cell growth, differentiation, metabolism, viral infection, and tumorigenesis (Bushati and Cohen 2007). In fact, one would be hard pressed to name a biomedical field that has not been affected in one way or another by miRNA research.

Genes encoding miRNAs are initially transcribed by RNA polymerase II as part of much longer primary transcripts (pri-miRNAs) (Lee et al. 2002) that typically contain the cap structure and the poly(A) tails (Lee et al. 2004). This feature predicts the presence of a wealth of pri-miRNAs alongside mRNA in most whole transcriptome databases. In the second step, pri-miRNAs are processed by the nuclear RNase III Drosha, leading to ~70 nucleotide hairpin-shaped intermediates, called precursor miRNAs (pre-miRNAs). Pre-miRNAs are subsequently exported out of the nucleus and cleaved by the cytoplasmic RNase III Dicer into a short miRNA duplex. One strand of this short-lived duplex is degraded, while the other strand is retained as mature miRNA and incorporated into the RNA-induced silencing complex (RISC), an RNA-protein complex with proteins from the Argonaute family (Schwarz et al. 2003).

The mature miRNA guides the RISC to recognize its target mRNA based on sequence complementarity, most important between the "seed region" of mature miRNAs, nucleotides 2–8, and the 3′ untranslated regions (UTRs) of their target genes, which generally leads to translation inhibition and/or mRNA degradation (Djuranovic et al. 2011, 2012). Because a perfect sequence complementarity is usually only required between the seed region of a miRNA and the 3′ UTR of its target mRNA, a single miRNA can theoretically regulate multiple mRNAs (often hundreds) (Fig. 10.1). Conversely, the 3′ UTR of a given mRNA may contain several miRNA recognition sequences. This relative lack of specificity poses significant challenges for the miRNA research field, in particular in identifying biologically meaningful miRNA targets.

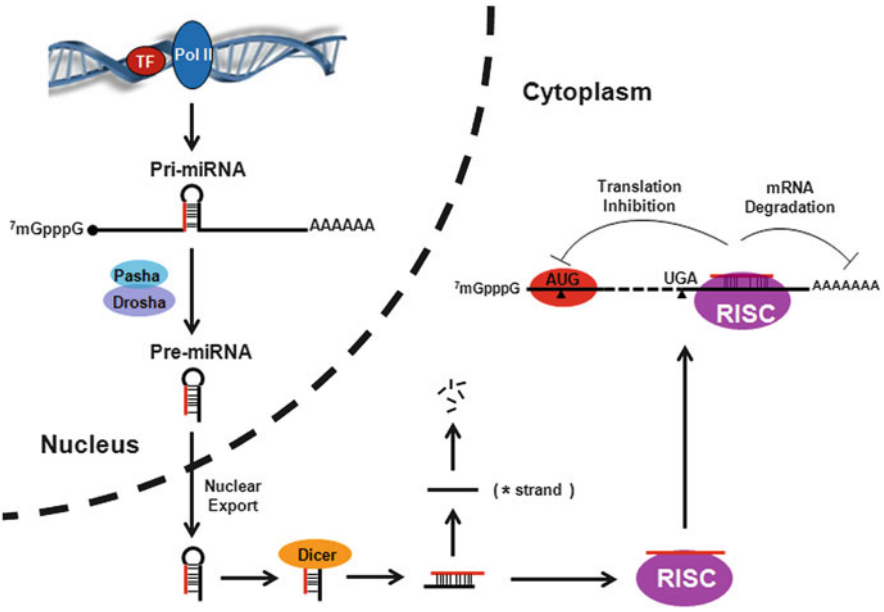


**Fig. 10.1** Schematic view of microRNA (miRNA) biogenesis and action. RNA polymerase II (*Pol II*) transcribes genes that encode miRNAs into primary miRNAs, which usually have a 5′ cap structure and a 3′ poly(A) tail as protein-coding messenger RNAs (mRNAs). The pri-miRNA is first processed in the nucleus by the microprocessor by two partners, Pasha and Drosha, into precursor miRNAs (pre-miRNAs) that are approximately 70 nucleotides in length and have a stem-loop structure. The pre-miRNA is then exported into cytoplasm and further processed by a type III RNA endonuclease Dicer to generate a mature miRNA duplex (~22 nucleotides in length). The sense strand of the miRNA duplex is then loaded into the RNA-induced silencing complex (RISC), whereas the complementary "star" strand (*) of the miRNA duplex is degraded. The RISC regulates gene expression through the inhibition of RNA translation or degradation of target mRNA by base pairing the "seed region" of an miRNA to the 3′ untranslated region of its target mRNA. This figure is modified from Huang et al. (2010)

While highly meaningful for normal cell function, miRNAs have been investigated in depth in most pathological settings, with cancer arguably leading the way. Deregulated miRNA expression has been demonstrated in virtually all neoplasms investigated. It is interesting to note that different cancer types tend to exhibit specific miRNA signatures (Lu et al. 2005; Calin and Croce 2006), including cancers of the colon (Michael et al. 2003), breast (Iorio et al. 2005), brain (Ciafre et al. 2005), liver (Murakami et al. 2006), and lung (Yanaihara et al. 2006). Although the elucidation of the mechanisms behind the specific shifts of profiles in tumors remains a work in progress, recent data on miRNA responses to microenvironmental stresses and oncogenic alterations have provided critical clues.

During the past 6 years multiple reports have demonstrated that miRNAs are involved in the hypoxic response and contribute to the repression of specific genes

under low oxygen conditions (Donker et al. 2007; Kulshreshtha et al. 2007a; Camps et al. 2008; Fasanaro et al. 2008; Giannakakis et al. 2008; Huang et al. 2009; Gee et al. 2010). The next section summarizes the current knowledge about the involvement of miRNAs in cellular hypoxic response, discusses challenges for elucidation of their biological functions, and speculates on potential opportunities for cancer diagnosis, prognosis, and treatment.

## 10.3 Hypoxia-Regulated miRNAs: The New Paradigm of Cellular Response to a Hypoxic Microenvironment

Several groups from diverse fields embarked on genome-wide miRNA profiling, with the goal to identify hypoxia-regulated miRNAs in a variety of cellular contexts. Expression of several dozen mature miRNAs, including miR-210, -21, -23, -24, -26, -103/107, and -181, was found to be induced under hypoxic conditions (Kulshreshtha et al. 2007b; Camps et al. 2008; Fasanaro et al. 2008; Crosby et al. 2009; Huang et al. 2009; Sarkar et al. 2010; Chen et al. 2012). Although these miRNA were reported in at least two publications, relatively limited overall overlap was noticed among these studies. With the caveat that different technologies were employed by different groups, this variability suggests a cell-specific component of miRNA induction and maturation. This in turn may contribute to the well-recognized variations in the magnitude of coding gene responses in hypoxia. Moreover, a large number of mature miRNAs were found to be downregulated in hypoxia (Kulshreshtha et al. 2007a), and their role may be to de-repress expression of specific genes in low oxygen conditions.

In sharp contrast, hypoxic-induced miR-210 stands out as the only miRNA agreed on by all the studies to date (Huang et al. 2010). This is drastically different from the case of classic protein-coding genes in which a plethora of mRNAs with diverse functions are induced by hypoxia, with relatively good overlap between different cell types (Denko et al. 2003). According to most groups that investigated the hypoxic regulation of miR-210, it seems to be a rather specific HIF-1 target (Camps et al. 2008; Crosby et al. 2009; Huang et al. 2009; Kim et al. 2009). However, this specificity is not absolute because HIF-2-dependent regulation of miR-210 also has been reported (Zhang et al. 2009). As is the case for the classic genes, HIF-1 directly binds to a hypoxia-responsive element (HRE) on the proximal miR-210 promoter (Huang et al. 2009). When the miR-210 core promoter is compared across species, this HRE site is highly conserved, indicating the importance of hypoxia/HIFs in regulating miR-210 expression during evolution. Consistent with this observation, the induction of mouse miR-210 under hypoxia is dependent on HIF-1α (Crosby et al. 2009). This highly conserved HRE was recently confirmed to be the functional HRE that is responsible for the robust induction of mouse miR-210 expression under hypoxic conditions (Cicchillitti et al. 2012).

### 10.3.1 miR-210: A Mirror of HIF Activity with Clinical Implications

miR-210 is upregulated in most solid tumors investigated to date, and its levels generally correlate with a negative clinical outcome (Kulshreshtha et al. 2007b; Porkka et al. 2007; Zhang et al. 2007; Fasanaro et al. 2008; Foekens et al. 2008; Lawrie et al. 2008; Gee et al. 2010; Rothe et al. 2011; Hong et al. 2012). Moreover, miR-210 levels are correlated with a gene expression signature of hypoxia (Camps et al. 2008; Huang et al. 2009), suggesting that its overexpression in tumors is the direct consequence of decreased oxygen tension in the microenvironment. In addition to tissue expression, in our laboratory, we observed a significant increase of circulating miR-210 in patients with pancreatic cancer compared to healthy controls (Ho et al. 2010). This is consistent with our knowledge of miR-210's responsiveness to hypoxia, since pancreatic adenocarcinomas are usually highly hypoxic (Koong et al. 2000).

Whether miR-210 independently increases tumor aggressiveness and/or decreases the response to therapy is still a matter of debate, although there is preliminary evidence to suggest such an effect (Camps et al. 2008). An emerging paradigm is that miR-210 expression is an accurate readout of HIF-1 activity in vivo, as it is in vitro (Fasanaro et al. 2008; Huang et al. 2009; Devlin et al. 2011).

One particular disease that is connected closely with the HIF pathway is clear-cell renal cell carcinomas (ccRCCs), which is commonly associated with the inactivation of the *VHL* tumor suppressor gene (Presti et al. 1991; Brugarolas 2007). Mutations and loss of heterozygosity of the *VHL* gene have been found in 57 % and 98 % of sporadic renal cell carcinoma cases, respectively (Gnarra et al. 1994). The VHL tumor suppressor gene product functions as the adaptor subunit of the E3 ubiquitin ligase complex that targets hydroxylated HIF-1α and HIF-2α for ubiquitination and subsequent degradation by the 26S proteasome (Ivan et al. 2001; Jaakkola et al. 2001). Given its close relationship with HIF, it is not surprising that miR-210 is particularly overexpressed in ccRCCs (Juan et al. 2010; White et al. 2011; Redova et al. 2012). In addition, elevated levels of circulating miR-210 have been found in patients with ccRCC compared to healthy controls (Zhao et al. 2013). Although the origin of circulating miRNAs remains a much-debated subject, the existence of high-level miR-210 in circulation in these patients suggests that miR-210 may serve as a novel biomarker for noninvasive detection of highly hypoxic cancers.

While our own work has focused on the emerging roles of miR-210 in tumors, the impact of this miRNA most likely extends well beyond cancer biology, most notably in cardiac cerebrovascular diseases (Semenza 2010b), cardiac hypertrophy and failure (van Rooij et al. 2006; Thum et al. 2007; Greco et al. 2012), transient focal brain ischemia (Jeyaseelan et al. 2008), limb ischemia (Jeyaseelan et al. 2008; Pulkkinen et al. 2008), ischemic wounds (Biswas et al. 2010), acute myocardial infarction (Bostjancic et al. 2009), atherosclerosis obliterans (Li et al. 2011), and preeclampsia (Pineles et al. 2007; Zhu et al. 2009; Enquobahrie et al. 2011).

### *10.3.2 miR-210 Targets: A Growing and Diverse List*

Identification of biologically relevant targets is an essential step toward understanding the functions of miR-210. A frequently employed approach begins with computational prediction using a growing number of programs available online. These are based on algorithms that search for complementarity between 3′ UTR sequences of annotated coding genes and the seed region sequence of the miRNA (Bartel 2009). Among the most popular programs employed to this end are miRanda (Betel et al. 2008), TargetScan (Lewis et al. 2003), Pictar (Krek et al. 2005), PITA (Kertesz et al. 2007), MicroCosm (Griffiths-Jones et al. 2008), and Dianalab (Maragkakis et al. 2009). Drawing the line after several years of experience with these resources, none stands out as the most accurate predictor of real targets. To further complicate matters, the lists of candidates generated by these programs usually exhibit limited overlap. This seems to be generally true for most miRNAs investigated and is exemplified by a proteomic study comparing the accuracy of different computational predictions of miR-223 targets (Baek et al. 2008). Since the miRNA seed region only consists of 6 or 7? nucleotides, false-positive prediction is a major limitation of this approach. Any given miRNA, including miR-210, may be predicted to regulate hundreds, if not thousands, of coding genes. When the search is extended to the 5′ UTR and the coding region, the number of targets is expected to be even higher. Finally, Fasanaro et al. (2012) have provided recent experimental evidence for a "seedless" target of miR-210 on the basis of complementarity between sequences of the *ROD1* (regulator of differentiation 1) gene and 10 consecutive bases in the central portion of miR-210. It is interesting that two widely employed algorithms, PicTar and TargetScan, predict relatively few targets for human miR-210, and, conversely, most of the experimentally validated targets are not predicted by any of these programs with a high score. It is also becoming increasingly apparent that "seed" binding is not always sufficient, as other features of the surrounding sequences can affect binding efficacy (Lewis et al. 2005). In conclusion, while computational predictions still represent powerful tools in the search for targets, they suffer from clear limitations, and exclusive reliance on them can lead to long lists of limited use.

In the particular case of miR-210, a number of genes appearing on these lists have been confirmed, but the success was largely due to the addition of a strong experimental arm. Such confirmed target genes are involved in cell proliferation, mitochondrial metabolism, DNA repair, chromatin remodeling, and cell migration (Camps et al. 2008; Fasanaro et al. 2008; Giannakakis et al. 2008; Pulkkinen et al. 2008; Chan et al. 2009; Crosby et al. 2009; Mizuno et al. 2009; Chen et al. 2010; Qin et al. 2010).

A widely used approach for identification of miRNA targets is a combination of expression profiling following miRNA manipulation using mimics and antagomirs, followed by expression profiling and comparisons with the results of computational predictions. This is a feasible strategy because directing mRNA degradation is a major mechanism that miRNAs use to downregulate the corresponding target genes

(Lim et al. 2005; Guo et al. 2010). This effect is usually robust enough to be detected by microarray analysis or whole transcriptome RNA sequencing (RNA-Seq). For example, both Zhang et al. (2009) and Puissegur et al. (2011) began their search by identifying transcripts that were down-modulated upon forced miR-210 expression in colorectal and lung adenocarcinoma cancer cell lines, respectively. The down-regulated genes, which also contained a predicted miR-210 binding site, were analyzed further to confirm that *MNT* (Zhang et al. 2009), *NDUFA4*, and *SDHD* (Puissegur et al. 2011) are bona fide miR-210 targets.

However, because miRNA frequently regulates its target gene by inhibiting protein translation rather than altering mRNA abundance (Selbach et al. 2008), complete reliance on mRNA profiling is likely to miss authentic miRNA targets. An ideal sensitive and robust proteomic approach should identify miRNA targets directly when combined with computational prediction. However, lack of sufficient sensitivity, heavy bias for abundant proteins, and high costs still preclude the broader application of this promising technology.

A recent approach has emerged that, at least in part, allows us to overcome the above-mentioned limitations. miRNAs is known to recruit the mRNAs of their target genes to the RISC complex, of which Argonaute family proteins, especially Argonaute 2, are essential components (Kawamata and Tomari 2010). Argonaute protein immunoprecipitation (miRNP-IP) methods have been developed that capture the mRNAs recruited to the complex. This pull-down can be followed by microarray or RNA-Seq and help identify targets that are regulated by both translational blockade or message degradation (Karginov et al. 2007). miRNP-IP can "freeze miRNAs in action" and thus greatly reduce the number of nonspecific targets and the secondary effect that are commonly seen in other approaches. Our groups have successfully pursued this approach in cells overexpressing miR-210 as part of more integrative strategies to identify targets (Fasanaro et al. 2009; Huang et al. 2009). In one of the studies we used a combination of computational prediction, proteomic, transcriptomic, and miRNP-IP approaches in human umbilical vein endothelial cells overexpressing miR-210 (Fasanaro et al. 2009). Proteomic profiling identified 11 downregulated proteins, whereas transcriptome profiling identified 51 transcripts that are induced upon miR-210 knockdown and downregulated by miR-210 overexpression. Despite the fact that 42 of the 62 genes were enriched with miR-210 seed-complementary sequences in their 3′ UTRs, surprisingly few were predicted by the miRNA target identification algorithms listed above. To distinguish between direct and indirect targets, analysis of the miRNP-IP content was analyzed by quantitative reverse transcriptase polymerase chain reaction, revealing that 16 potential targets were enriched in the RISC. An ncRNA (*XIST*) involved in X chromosome inactivation was identified in the miRNP-IP experiment, indicating a previously unsuspected layer of interaction between ncRNAs. This finding lends support to the recently proposed competing endogenous RNA theory (Salmena et al. 2011).

Using a similar miRNP-IP strategy, we also compared mRNAs enriched in the RISC after exposing immortalized human breast MCF10A cells to low oxygen for 24 hours? versus normoxic controls (Huang et al. 2009). More than 200 mRNAs

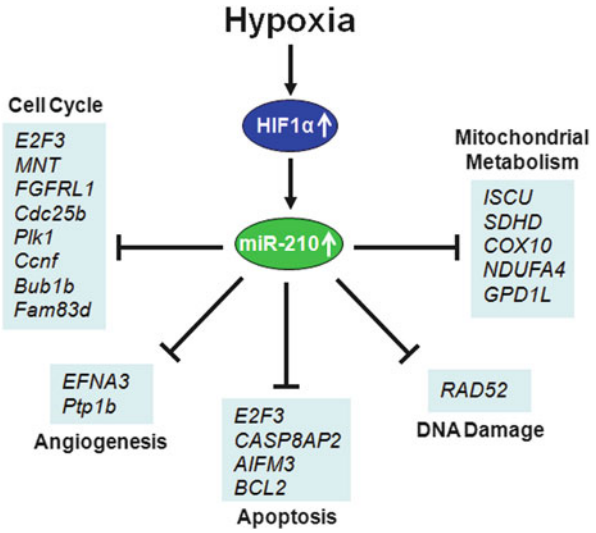


**Fig. 10.2** Experimentally identified micro RNA target genes implicated in cancer biology. Genes that have been experimentally identified as miR-210 targets can be classified into five major functional categories: mitochondrial metabolism, cell cycle control, angiogenesis, apoptosis, and DNA damage repair, indicating a complex functional network regulated by miR-210 in the hypoxic microenvironment in which tumor cells reside

were enriched, and several algorithms predicted that 50 of these were direct miR-210 targets. Three of five randomly selected genes from the list of 50 genes were confirmed as bona fide miR-210 targets by functional assays.

It is notable that there are few targets in common between these two studies, leading to the hypothesis that miR-210 regulates different sets of target genes in these two cell types. This is not necessarily surprising: comprehensive proteomic studies indicated that miRNAs act as rheostats by performing fine-scale adjustments to the output of hundreds of proteins (Baek et al. 2008; Selbach et al. 2008). Thus, only minor changes at the protein and/or mRNA levels are expected for the majority of miRNA targets, which translate into low fold changes (e.g.,1.2–1.5) in target expression when measured by microarray or RNA-Seq. This inherent variability of our experimental systems to measure such small changes may account at least in part for the discrepancy between our studies.

The current paradigm of miRNA function states that they suppress target gene expression via inhibiting protein translation, degrading mRNA, or both (Bartel 2004). Several reports have provided the first evidence for exceptions to this rule, identifying genes that are upregulated by miRNA transduction (Vasudevan et al. 2007; Place et al. 2008). Although no direct evidence for this has been provided for miR-210, upregulated gene expression was observed in our study (Fasanaro et al. 2009). At this point there is no evidence that this represents more than secondary waves of regulation.

We anticipate that improved understanding of miRNA function will be followed by the increased accuracy of predicting miRNA targets. Moreover, with the lowering cost of next-generation sequencing, the combination of computational tools and advanced experimental approaches will provide a more complete identification of physiologically relevant miR-210 targets. Figure 10.2 summarizes experimentally

validated miR-210 targets and their potential regulatory functions. Some of the better-validated miR-210 targets are reviewed below, and their function in hypoxic response and cancer biology are discussed.

### *10.3.3 miR-210 Regulates Mitochondrial Metabolism and Oxidative Stress*

Under normoxic conditions, mitochondria are the "energy factories" of a cell; they generate the majority of adenosine triphosphate through the oxidative phosphorylation pathway using oxygen as an electron acceptor. However, when the oxygen supply is limited, cells switch to glycolysis to produce adenosine triphosphate (the Pasteur effect). During this process, HIF-1 not only upregulates expression of most glycolytic enzymes but also downregulates mitochondrial respiration and biogenesis (Zhang et al. 2007; Denko 2008). Results from several groups have demonstrated that hypoxic induction of miR-210 significantly contributes to this metabolic shift by downregulating the activity of mitochondrial electron transport chains (ETCs). A well-accepted branch of this mechanism is targeting by miR-210 of the iron-sulfur cluster scaffold proteins (*ISCU*) (Chan et al. 2009; Fasanaro et al. 2009; Chen et al. 2010; Favaro et al. 2010). One of the most consistent miR-210 targets, *ISCU* is part of an ancient mechanism that catalyzes the assembly of iron-sulfur clusters that are critical for enzymes, such as aconitase, that function in the tricarboxylic acid cycle, as well as for the function of mitochondrial ETC complexes I, II, and III (Tong and Rouault 2006). The role of *ISCU* as a metabolic regulator has strong backing from clinical data: its mutations are associated with hereditary lactic acidosis characterized by myopathy and exercise intolerance (Mochel et al. 2008).

Further strengthening the case for *ISCU* as a biologically relevant target, its levels are inversely correlated with miR-210 in multiple tumor data sets (Favaro et al. 2010). In contrast to a poor prognosis predicted by higher levels of miR-210, the expression of *ISCU* is predictive of a favorable prognosis, at least in breast cancer, indicating that *ISCU* is regulated by miR-210 in vivo.

While *ISCU* itself is not a primarily mitochondrial protein, several integral components of the mitochondrial ETC have been found to be miR-210 targets: NADH dehydrogenase (ubiquinone) 1α subcomplex 4 (*NDUFA4*) (Giannakakis et al. 2008), succinate dehydrogenase complex, subunit D (*SDHD*) (Puissegur et al. 2011), and cytochrome C oxidase assembly homolog 10 (*COX10*) (Chen et al. 2010). *NDUFA4* was initially considered to be part of ETC complex I until recent work demonstrated that it actually resides in complex IV (Balsa et al. 2012). SDHD is a subunit of complex II and a well-documented tumor suppressor gene product (Baysal et al. 2000; Gottlieb and Tomlinson 2005). Targeting of this gene by miR-210 provides additional support to a protumorigenic role for miR-210. *COX10*, which encodes a heme A:farnesyltransferase, is another recently discovered target of miR-210. Although *COX10* is not a structural subunit of COX (mitochondrial ETC complex IV), it is required for the expression of a functional

COX complex (Antonicka et al. 2003). To summarize, by targeting multiple ETC components and regulators, miR-210 may act as a key HIF-1 effector in attenuating oxygen consumption in hypoxia. One unanswered question is whether miR-210 plays biological roles in anoxia, where decreasing oxygen consumption is not a factor.

Another intriguing target predicted by several programs and experimentally confirmed is glycerol-3-phosphate dehydrogenase 1-like (*GPD1L*) (Fasanaro et al. 2009). *GPD1L* is highly homologous to glycerol-3-phosphate dehydrogenases that transfer electrons from cytoplasmic NADH to the mitochondrial ETC (Bunoust et al. 2005). Thus, *GPD1L* may also be involved in the Pasteur effect by regulating $NAD^+$-to-NADH ratios (Liu et al. 2010). Recent insights from Kelly et al. (2011) identified a feedback mechanism based on *GPD1L* repression, which in turn inactivates HIF prolyl hydroxylase activity, leading to stabilization of HIF-1α. Therefore, miR-210 may be present in or at both downstream and upstream of HIF-1α signaling. Puissegur et al. (2011) also showed that high levels of miR-210 participate to stabilize HIF-1α during hypoxia.

The generation of mitochondrial reactive oxygen species (ROS) is a consequence of electron leakage during electron transport (Murphy 2009). Increased ROS production has been reported in hypoxia, potentially as a result of ETC dysfunction (Guzy and Schumacker 2006); however, whether cell reoxygenation during the ROS assay contributes to this increase is still being debated. We reported that miR-210 increases oxidative stress at least in part by *ISCU* suppression in normoxic MCF7 cells (Chan et al. 2009; Favaro et al. 2010). However, in hypoxia, and in other cell types, the effects of miR-210 are still a subject of controversy. We observed hypoxic induction of ROS in cancer cell lines, and an miR-210 antagonist reversed this effect to almost normoxic levels (Favaro et al. 2010). Conversely, Chan et al. (2009), working on endothelial cells, did not detect any change in ROS production after exposing the cells to hypoxia and noted a burst of ROS when miR-210 was blocked. This discrepancy is sure to promote additional investigation and may reflect underlying differences between normal versus cancer cells. In addition, miR-210 may exhibit differential effects on various ROS species, a hypothesis that needs to be addressed in future studies.

Aside from its robust response to oxygen deprivation, the HIF pathway plays an increasingly clear role in the Warburg effect (Kim and Dang 2006). HIF is also induced in tumors as part of oncogenic signaling networks, even in the absence of hypoxia (Zundel et al. 2000), so it is tempting to speculate that elevated miR-210 may contribute to the Warburg effect in these tumors by helping to stabilize HIF-1α to promote aerobic glycolysis. Expression of miR-210 is frequently elevated in cancers, including glioblastomas (Malzkorn et al. 2009), melanomas (Satzger et al. 2009; Zhang et al. 2009), ccRCCs (Juan et al. 2010; White et al. 2011), lung (Miko et al. 2009; Raponi et al. 2009), pancreatic cancers (Greither et al. 2009), and breast cancers (Camps et al. 2008; Foekens et al. 2008). However, whether elevated miR-210 expression within the tumor can occur outside of the hypoxic areas remains unclear at this stage, and future laser-capture, microdissection-based analyses may shed critical light on this dilemma.

### *10.3.4 miR-210 as a Regulator of Angiogenesis*

Angiogenesis is a complex, multistep process that normally occurs during embryonic development and rarely in adults. Exceptions include normal repair processes such as wound healing and pathological settings such as in tumor growth and ischemic disorders (Semenza 2003). Tumor growth is highly dependent on the formation of neovessels to establish nutrient and oxygen supplies for cell viability and proliferation. Imbalance between oxygen consumption by tumor cells with high metabolic activities (Gatenby and Gillies 2004) and oxygen delivery by dysfunctional vasculature (Brown and Giaccia 1998) leads to hypoxia and stimulates compensatory angiogenesis (Liu et al. 1995).

Multiple miRNAs seem to be part of the various steps of the angiogenic response, either as positive or negative regulators (Wang and Olson 2009; Wu et al. 2009). On the basis of its involvement in the hypoxic response, it is hardly surprising that miR-210 has been investigated as a candidate angiogenic regulator. Consistent with this hypothesis, miR-210 expression was found to correlate closely with vascular endothelial growth factor (*VEGF*) expression, hypoxia, and angiogenesis in patients with breast cancer (Foekens et al. 2008). Transduction of miR-210 in human umbilical vein endothelial cells using miRNA mimics functionally stimulates the formation of capillary-like structures as well as *VEGF*-induced cell migration (Fasanaro et al. 2008; Lou et al. 2012). Conversely, inhibiting miR-210 using the corresponding antagomir blocks both tubulogenesis and *VEGF*-mediated endothelial chemotaxis. The receptor tyrosine kinase ligand Ephrin-A3 (*EFNA3*) was identified as a candidate mediator for these effects (Fasanaro et al. 2008), consistent with the knowledge that ephrin ligands and their receptors are important in the development of the cardiovascular system and in vascular remodeling (Kuijper et al. 2007). Overall, *EFNA3* seems to be one of the most consistently reported miR-210 target genes (Fasanaro et al. 2008; Fasanaro et al. 2009; Greither et al. 2009; Huang et al. 2009). To complicate matters, regulation of *EFNA3* is more complex when ischemic responses are examined (Pulkkinen et al. 2008). Contrary to the expectation of miR210-mediated repression, *EFNA3* was present at higher levels in mouse hippocampus after ischemia. It is interesting that *EFNA3* transcription is also induced by hypoxia (Fasanaro et al. 2008), suggesting that miR-210 fine-tunes primarily *EFNA3* protein translation. Thus, abundance of the *EFNA3* protein may reflect the balance between hypoxic induction of mRNA and repression of miR-210, which conceivably varies in different pathological contexts.

The tyrosine phosphatase *PTP1B* was identified as another miR-210 target that promotes angiogenesis and inhibits apoptosis after myocardial infarction in a mouse model (Hu et al. 2010). *PTP1B* is documented to negatively regulate activation of the VEGF receptor 2 and stabilize cell-cell adhesions through reducing tyrosine phosphorylation of vascular endothelial cadherin (Nakamura et al. 2008). Thus, by inhibiting *EFNA3 and PTP1B*, both negative regulators of angiogenesis, miR-210 could promote angiogenesis.

An interesting recent study also suggests positive feedback between *VEGF* and miR-210. $CD34^+$ cells in umbilical cord blood expanded in VEGF-containing medium

upregulated miR-210 expression, and when these cells were transplanted into the ischemic hind limbs of mice tissue perfusion/capillary density were significantly improved, whereas an miR-210 inhibitor abolished the effect (Alaiti et al. 2012).

While the above-mentioned studies were not conducted in the context of cancer, they may nevertheless shed new light on possible roles of miR-210 in tumor angiogenesis. It will be of great interest to investigate whether feedback involving hypoxia, miR-210, and *VEGF* also occurs in tumors in vivo. This may have implications for developing strategies to increase the efficacy of anti-VEGF therapy, for example, by adding miR-210 inhibitors.

### *10.3.5 miR-210 Regulation of DNA Damage Response*

Genome integrity is challenged by a variety of stresses, including radiation, mutagens, ROS, ultraviolet light, and chemo- or radiotherapeutic agents. Cellular responses to DNA damage involve a complex network of processes that detect and repair genomic lesions. miRNAs have been demonstrated to participate in these processes (Simone et al. 2009; Landau and Slack 2011; Wan et al. 2011). Zhang et al. (2011) provided direct evidence that more than 20 % of examined miRNAs are significantly induced upon DNA damage. While it is not robustly induced by irradiation, miR-210 nevertheless seems to be relevant for this complex process because it targets *RAD52* (Crosby et al. 2009; Fasanaro et al. 2009), a key component in the homologous recombination–mediated repair of double-strand breaks (Benson et al. 1998; Shinohara and Ogawa 1998). Suppression of *RAD52* by miR-210 may provide an additional mechanism to help explain compromised homologous recombination repair activity in hypoxic cells (Bindra et al. 2007). Consistent with this hypothesis, forced expression of miR-210 was found to lead to double-strand DNA breaks in cultured cells (Faraonio et al. 2012).

### *10.3.6 miR-210 Regulation of Apoptosis*

Cellular stresses, including hypoxia, are well known triggers of programmed cells death, a process also called apoptosis. Evasion from apoptotic responses is critical for tumor progression: transformed cells need to overcome the adverse conditions present in their microenvironment (Hanahan and Weinberg 2011). Thus, it is not surprising that miR-210 has been investigated for possible effects on apoptotic responses. In general, the available evidence suggests an anti-apoptotic role of miR-210 in a variety of cell types. On the one hand, overexpression of miR-210 can protect cells from apoptosis (Kulshreshtha et al. 2007a; Kim et al. 2009; Hu et al. 2010; Mutharasan et al. 2011; Nie et al. 2011); on the other hand, downregulation of miR-210 during hypoxia promotes apoptosis (Cheng et al. 2005; Kulshreshtha et al. 2007b; Fasanaro et al. 2008; Gou et al. 2012; Liu et al. 2012; Yang et al. 2012).

While many gaps remain in our understanding of this process, several relevant targets have been identified to help explain such effect: *E2F3* (Gou et al. 2012), *Ptp1b* (Hu et al. 2010), caspase-8-associated protein-2 (*CASP8AP2*) (Kim et al. 2009), and apoptosis-inducing factor, mitochondrion-associated 3 (*AIFM3*) (Yang et al. 2012). However, the caveat is that, apart from *CASP8AP2* (Kim et al. 2012), none of the other genes has been verified to mediate miR-210's anti-apoptotic function by an independent study. In a recent report, although *AIFM3* was found to be an miR-210 target, its overexpression failed to overcome the cytoprotective effects of the miRNA, suggesting that cooperation with other targets may be necessary (Mutharasan et al. 2011). Despite the evidence of an anti-apoptotic role cited above, a recent report suggested that miR-210 may also exhibit a pro-apoptotic function, at least in hypoxic neuroblastoma cells, by targeting the anti-apoptotic gene *BCL2* (Chio et al. 2013).

In summary, evidence for miR-210-mediated regulation of apoptosis in hypoxia is emerging for various cell types. However, it is still premature to state that this miRNA represents a major protector against hypoxia-induced cell death.

### *10.3.7 miR-210 Effects on the Cell Cycle*

In many cell types, extended exposure to hypoxia leads to downregulation of a large number of cell cycle genes, including cyclins and other positive regulators of cell cycle transition (Hammer et al. 2007), while other cells tend to proliferate better under low oxygen conditions (Krick et al. 2005). One of the better-characterized miR-210 targets that belong to cell cycle control is *E2F3*, a promoter of $G_1$/S transition (Lees et al. 1993; Leone et al. 1998). *E2F3* was first reported as an miR-210 target in ovarian cancer (Giannakakis et al. 2008). Later, several independent studies confirmed that *E2F3* was an miR-210 target in various cell types, including ccRCC (Nakada et al. 2011), keratinocytes and in a murine model of ischemic wounds (Biswas et al. 2010), and HEK293 cells (Fasanaro et al. 2009). Despite these findings, the relative contribution of *E2F3* to tumor cell cycle alterations in a hypoxic microenvironment remains largely unknown. In addition to *E2F3*, fibroblast growth factor receptor-like 1 (*FGFRL1*) also was identified as an miR-210 target involved in cell cycle control (Tsuchiya et al. 2011), consistent with our earlier observation that *FGFRL1* is robustly repressed by miR-210 (Huang et al. 2009). De-repression of *FGFRL1* by using anti-miR-210 accelerates cell cycle progression, whereas overexpression of miR-210 leads to cell cycle arrest in the $G_1/G_0$ and $G_2$/M phases (Tsuchiya et al. 2011). The effects of miR-210 on the cells cycle may, in fact, be significantly broader, including a group of mitosis-related genes, such as *Plk1*, *Cdc25B*, cyclin F (*CCNF*), *Bub1B*, and *Fam83D* (He et al. 2013). Whether all these represent direct targets or more indirect responders downstream of the genes discussed above remains unclear.

As is the case with hypoxic cell death, under some circumstances miR-210 may promote cell cycle progression, for example, by downregulating *MNT* (Zhang et al. 2009), a member of the *Myc*/*MAX*/*MAD* network with a basic-Helix-Loop-Helix-zipper domain. *MNT* functions as an antagonist of *c-Myc* and represses *Myc* target genes by binding the E box DNA sequence (CANNTG) after forming heterodimers

with *MAX* (Hurlin et al. 1997; Meroni et al. 1997). As HIF-1 regulates cell proliferation and metabolism in part by interacting with *c-Myc* (Gordan et al. 2007), miR-210 may fine-tune the latter under hypoxic conditions to regulate cell cycle progression.

### *10.3.8 miR-210 as a Candidate Cancer Biomarker*

Past studies have demonstrated that miRNAs are frequently dysregulated in human cancers (Ventura and Jacks 2009). Tumor-specific miRNA expression signatures can distinguish between normal and malignant tissues as well as classify cancer subtypes (Garzon et al. 2009). When used to classify poorly differentiated tumors, miRNA expression profiling outperformed mRNA expression profiling (Lu et al. 2005), pointing toward considerable potential as a biomarkers. Expression of miR-210 has been associated with poor clinical outcome in soft-tissue sarcoma, breast, head and neck, and pancreatic tumors (Camps et al. 2008; Foekens et al. 2008; Greither et al. 2009; Gee et al. 2010; Greither et al. 2011; Rothe et al. 2011; Hong et al. 2012; Toyama et al. 2012). However, whether high miR-210 only serves as an indicator of tumor hypoxia or actively promotes a more aggressive disease remains unclear (Huang et al. 2010).

The status of miR-210 expression in tumors may have therapeutic implications. Preliminary in vitro evidence was provided by Chen et al. (2010), who reported that overexpressing miR-210 rendered cells significantly more susceptible to killing by 3-bromo-pyruvate, an inhibitor of the glycolytic pathway. Molecules of this class, such as 2-deoxyglucose and dichloroacetate, have been considered promising therapeutic agents; however, they have yet to fulfill their promise in clinical settings. Therefore, miR-210 may help identify subsets of patients who can benefit from such agents in the future.

Recent publications report that miRNAs are exceptionally stable and can be readily detected in the systemic circulation and other body fluids of healthy subjects and patients with malignant diseases (Chen et al. 2008; Gilad et al. 2008; Lawrie et al. 2008; Mitchell et al. 2008; Taylor and Gercel-Taylor 2008; Weber et al. 2010). It has been suggested that the high stability of miRNAs may be partially attributed to the exosomal miRNA packaging (Valadi et al. 2007). The release of exosomes into the extracellular environment provides an opportunity to use exosome components in body fluids as a proxy to monitor molecular events occurring in tumor cells (Iguchi et al. 2010). Pilot studies assessing the use of circulating miRNAs as cancer biomarkers have attracted broad interest in the field and to date at least 79 miRNAs have been reported as plasma or serum biomarker candidates for solid and hematologic tumors (Allegra et al. 2012). miR-210 was found to be increased in the serum of patients with diffuse large B-cell lymphoma (Lawrie et al. 2008), ccRCC (Zhao et al. 2013), and pancreatic cancer (Wang et al. 2009; Ho et al. 2010). It is interesting that hypoxia has been demonstrated to promote the release of exosomes from cultured breast cancer cells (King et al. 2012); therefore, one can speculate that the elevated levels of circulating miR-210 may directly reflect the hypoxic state of tumor cells.

Circulating miR-210 levels were also correlated with sensitivity to trastuzumab (a human epidermal growth factor receptor 2 monoclonal antibody), tumor presence, and lymph node metastases in patients with breast cancer (Jung et al. 2012). This provides proof of the concept that plasma miR-210 may also be used to monitor response to anticancer therapies (Cortez et al. 2011).

### *10.3.9 miR-210: A Viable Cancer Therapeutic Target?*

Recent development of anti-miRNA agents such as locked nucleic acids or peptide nucleic acids represent significant steps for the therapeutic targeting of miRNAs in vivo (Stenvang et al. 2008; Lanford et al. 2010; Fabbri et al. 2011a, b; Gambari et al. 2011; Iorio and Croce 2012). It is conceivable that inactivation of miRNAs involved in hypoxic adaptation, in combination with other anticancer agents, may be a viable strategy to target a tumor compartment that poses significant therapeutic challenges. At present, efficient delivery of miRNA-related reagents to solid tumors, and in particular to poorly perfused areas, remains a significant hurdle. However, rapid advances in nanoparticle-based nucleic acid delivery (Tabernero et al. 2013) are providing realistic expectations that such limitations eventually will be overcome.

## 10.4 Concluding Remarks

On the basis of the experimental evidence summarized in this chapter, miR-210 plays complex roles in the cellular responses to hypoxia and in cancer biology. Given the diversity of genes that seem to respond as bona fide miR-210 targets – some with opposing effects on specific cellular functions – a simple and universal model of miR-210 function will be challenging to develop using exclusively in vitro approaches. Answering some of the key questions regarding miR-210 functions will require data from more sophisticated systems such as genetic inactivation of the corresponding locus in animal models.

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## References

Alaiti MA, Ishikawa M et al (2012) Up-regulation of miR-210 by vascular endothelial growth factor in ex vivo expanded CD34+ cells enhances cell-mediated angiogenesis. J Cell Mol Med 16(10):2413–2421

Allegra A, Alonci A et al (2012) Circulating microRNAs: new biomarkers in diagnosis, prognosis and treatment of cancer (review). Int J Oncol 41(6):1897–1912

Antonicka H, Leary SC et al (2003) Mutations in COX10 result in a defect in mitochondrial heme a biosynthesis and account for multiple, early-onset clinical phenotypes associated with isolated COX deficiency. Hum Mol Genet 12(20):2693–2702
Baek D, Villen J et al (2008) The impact of microRNAs on protein output. Nature 455(7209):64–71
Balsa E, Marco R et al (2012) NDUFA4 is a subunit of complex IV of the mammalian electron transport chain. Cell Metab 16(3):378–386
Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2):281–297
Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136(2):215–233
Baysal BE, Ferrell RE et al (2000) Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. Science 287(5454):848–851
Benson FE, Baumann P et al (1998) Synergistic actions of Rad51 and Rad52 in recombination and DNA repair. Nature 391(6665):401–404
Betel D, Wilson M et al (2008) The microRNA.org resource: targets and expression. Nucleic Acids Res 36(suppl 1):D149–D153
Bindra R, Crosby M et al (2007) Regulation of DNA repair in hypoxic cancer cells. Cancer Metastasis Rev 26(2):249–260
Biswas S, Roy S et al (2010) Hypoxia inducible microRNA 210 attenuates keratinocyte proliferation and impairs closure in a murine model of ischemic wounds. Proc Natl Acad Sci 107(15):6976–6981
Bostjancic E, Zidar N et al (2009) MicroRNA microarray expression profiling in human myocardial infarction. Dis Markers 27(6):255–268
Brown JM, Giaccia AJ (1998) The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 58(7):1408–1416
Brugarolas J (2007) Renal-cell carcinoma – molecular pathways and therapies. N Engl J Med 356(2):185–187
Bunoust O, Devin A et al (2005) Competition of electrons to enter the respiratory chain: a new regulatory mechanism of oxidative metabolism in Saccharomyces cerevisiae. J Biol Chem 280(5):3407–3413
Bushati N, Cohen SM (2007) MicroRNA functions. Annu Rev Cell Dev Biol 23(1):175–205
Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. Nat Rev Cancer 6(11):857–866
Camps C, Buffa FM et al (2008) hsa-miR-210 is induced by hypoxia and is an independent prognostic factor in breast cancer. Clin Cancer Res 14(5):1340–1348
Chan SY, Zhang YY et al (2009) MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. Cell Metab 10(4):273–284
Chen X, Ba Y et al (2008) Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18(10):997–1006
Chen Z, Li Y et al (2010) Hypoxia-regulated microRNA-210 modulates mitochondrial function and decreases ISCU and COX10 expression. Oncogene 29(30):4362–4368
Chen H-Y, Lin Y-M et al (2012) miR-103/107 promote metastasis of colorectal cancer by targeting the metastasis suppressors DAPK and KLF4. Cancer Res 72(14):3631–3641
Cheng AM, Byrom MW et al (2005) Antisense inhibition of human miRNAs and indications for an involvement of miRNA in cell growth and apoptosis. Nucleic Acids Res 33(4):1290–1297
Chio C-C, Lin J-W et al (2013) MicroRNA-210 targets antiapoptotic Bcl-2 expression and mediates hypoxia-induced apoptosis of neuroblastoma cells. Arch Toxicol 87(3):459–468
Ciafre SA, Galardi S et al (2005) Extensive modulation of a set of microRNAs in primary glioblastoma. Biochem Biophys Res Commun 334(4):1351–1358
Cicchillitti L, Di Stefano V et al (2012) Hypoxia-inducible factor 1-a induces miR-210 in normoxic differentiating myoblasts. J Biol Chem 287(53):44761–44771
Cortez MA, Bueso-Ramos C et al (2011) MicroRNAs in body fluids–the mix of hormones and biomarkers. Nat Rev Clin Oncol 8(8):467–477

Crosby ME, Kulshreshtha R et al (2009) MicroRNA regulation of DNA repair gene expression in hypoxic stress. Cancer Res 69(3):1221–1229
Denko NC (2008) Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 8(9):705–713
Denko NC, Fontana LA et al (2003) Investigating hypoxic tumor physiology through gene expression patterns. Oncogene 22(37):5907–5914
Devlin C, Greco S et al (2011) miR-210: more than a silent player in hypoxia. IUBMB Life 63(2):94–100
Djebali S, Davis CA et al (2012) Landscape of transcription in human cells. Nature 489(7414):101–108
Djuranovic S, Nahvi A et al (2011) A parsimonious model for gene regulation by miRNAs. Science 331(6017):550–553
Djuranovic S, Nahvi A et al (2012) MiRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science 336(6078):237–240
Donker RB, Mouillet JF et al (2007) The expression of Argonaute2 and related microRNA biogenesis proteins in normal and hypoxic trophoblasts. Mol Hum Reprod 13(4):273–279
Enquobahrie DA, Abetew DF et al (2011) Placental microRNA expression in pregnancies complicated by preeclampsia. Am J Obstet Gynecol 204(2):178 e112–121
Fabbri E, Brognara E et al (2011a) MiRNA therapeutics: delivery and biological activity of peptide nucleic acids targeting miRNAs. Epigenomics 3(6):733–745
Fabbri E, Manicardi A et al (2011b) Modulation of the biological activity of microRNA-210 with peptide nucleic acids (PNAs). ChemMedChem 6(12):2192–2202
Faraonio R, Salerno P et al (2012) A set of miRNAs participates in the cellular senescence program in human diploid fibroblasts. Cell Death Differ 19(4):713–721
Fasanaro P, D'Alessandra Y et al (2008) MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand ephrin-A3. J Biol Chem 283(23):15878–15883
Fasanaro P, Greco S et al (2009) An integrated approach for experimental target identification of hypoxia-induced miR-210. J Biol Chem 284(50):35134–35143
Fasanaro P, Romani S et al (2012) ROD1 is a seedless target gene of hypoxia-induced miR-210. PLoS One 7(9):e44651
Favaro E, Ramachandran A et al (2010) MicroRNA-210 regulates mitochondrial free radical response to hypoxia and Krebs cycle in cancer cells by targeting iron sulfur cluster protein ISCU. PLoS One 5(4):e10345
Foekens JA, Sieuwerts AM et al (2008) Four miRNAs associated with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer. Proc Natl Acad Sci 105(35):13021–13026
Gambari R, Fabbri E et al (2011) Targeting microRNAs involved in human diseases: a novel approach for modification of gene expression and drug development. Biochem Pharmacol 82(10):1416–1429
Garzon R, Calin GA et al (2009) MicroRNAs in cancer. Annu Rev Med 60(1):167–179
Gatenby RA, Gillies RJ (2004) Why do cancers have high aerobic glycolysis? Nat Rev Cancer 4(11):891–899
Gee HE, Camps C et al (2010) hsa-miR-210 is a marker of tumor hypoxia and a prognostic factor in head and neck cancer. Cancer 116(9):2148–2158
Giannakakis A, Sandaltzopoulos R et al (2008) miR-210 links hypoxia with cell cycle regulation and is deleted in human epithelial ovarian cancer. Cancer Biol Ther 7(2):255–264
Gilad S, Meiri E et al (2008) Serum microRNAs are promising novel biomarkers. PLoS One 3(9):e3148
Gnarra JR, Tory K et al (1994) Mutations of the VHL tumour suppressor gene in renal carcinoma. Nat Genet 7(1):85–90
Gordan JD, Thompson CB et al (2007) HIF and c-Myc: sibling rivals for control of cancer cell metabolism and proliferation. Cancer Cell 12(2):108–113

Gottlieb E, Tomlinson IP (2005) Mitochondrial tumour suppressors: a genetic and biochemical update. Nat Rev Cancer 5(11):857–866
Gou D, Ramchandran R et al (2012) miR-210 has an antiapoptotic effect in pulmonary artery smooth muscle cells during hypoxia. Am J Physiol Lung Cell Mol Physiol 303(8):L682–L691
Greco S, Fasanaro P et al (2012) MicroRNA dysregulation in diabetic ischemic heart failure patients. Diabetes 61(6):1633–1641
Greither T, Grochola LF et al (2009) Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int J Cancer 126(1):73–80
Greither T, Würl P et al (2011) Expression of microRNA 210 associates with poor survival and age of tumor onset of soft-tissue sarcoma patients. Int J Cancer 130(5):1230–1235
Griffiths-Jones S, Saini HK et al (2008) MiRBase: tools for microRNA genomics. Nucleic Acids Res 36(suppl 1):D154–D158
Guo H, Ingolia NT et al (2010) Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 466(7308):835–840
Guzy RD, Schumacker PT (2006) Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol 91(5):807–819
Hammer S, To KK et al (2007) Hypoxic suppression of the cell cycle gene CDC25A in tumor cells. Cell Cycle 6(15):1919–1926
Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674
He J, Wu J et al (2013) MiR-210 disturbs mitotic progression through regulating a group of mitosis-related genes. Nucleic Acids Res 41(1):498–508
Ho AS, Huang X et al (2010) Circulating miR-210 as a novel hypoxia marker in pancreatic cancer. Transl Oncol 3(2):109–113
Hong L, Yang J et al (2012) High expression of miR-210 predicts poor survival in patients with breast cancer: a meta-analysis. Gene 507(2):135–138
Hu S, Huang M et al (2010) MicroRNA-210 as a novel therapy for treatment of ischemic heart disease. Circulation 122(Suppl 1):S124–S131
Huang X, Ding L et al (2009) Hypoxia-inducible mir-210 regulates normoxic gene expression involved in tumor initiation. Mol Cell 35(6):856–867
Huang X, Le Q-T et al (2010) MiR-210 – micromanager of the hypoxia pathway. Trends Mol Med 16(5):230–237
Hurlin PJ, Queva C et al (1997) Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. Genes Dev 11(1):44–58
Hüttenhofer A, Schattner P et al (2005) Non-coding RNAs: hope or hype? Trends Genet 21(5):289–297
Iguchi H, Kosaka N et al (2010) Versatile applications of microRNA in anti-cancer drug discovery: from therapeutics to biomarkers. Curr Drug Discov Technol 7(2):95–105
Iorio MV, Croce CM (2012) MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. EMBO Mol Med 4(3):143–159
Iorio MV, Ferracin M et al (2005) MicroRNA gene expression deregulation in human breast cancer. Cancer Res 65(16):7065–7070
Ivan M, Kondo K et al (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292(5516):464–468
Jaakkola P, Mole DR et al (2001) Targeting of HIF-alpha to the von hippel-lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292(5516):468–472
Jeyaseelan K, Lim KY et al (2008) MicroRNA expression in the blood and brain of rats subjected to transient focal ischemia by middle cerebral artery occlusion. Stroke 39(3):959–966
Juan D, Alexe G et al (2010) Identification of a MicroRNA panel for clear-cell kidney cancer. Urology 75(4):835–841
Jung EJ, Santarpia L et al (2012) Plasma microRNA 210 levels correlate with sensitivity to Trastuzumab and tumor presence in breast cancer patients. Cancer 118(10):2603–2614
Karginov FV, Conaco C et al (2007) A biochemical approach to identifying microRNA targets. Proc Natl Acad Sci 104(49):19291–19296

Kawamata T, Tomari Y (2010) Making RISC. Trends Biochem Sci 35(7):368–376
Kelly TJ, Souza AL et al (2011) A hypoxia-induced positive feedback loop promotes hypoxia-inducible factor 1alpha stability through miR-210 suppression of glycerol-3-phosphate dehydrogenase 1-like. Mol Cell Biol 31(13):2696–2706
Kertesz M, Iovino N et al (2007) The role of site accessibility in microRNA target recognition. Nat Genet 39(10):1278–1284
Kim JW, Dang CV (2006) Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 66(18):8927–8930
Kim HW, Haider HK et al (2009) Ischemic preconditioning augments survival of stem cells via miR-210 expression by targeting caspase-8-associated protein 2. J Biol Chem 284(48):33161–33168
Kim HW, Mallick F et al (2012) Concomitant activation of miR-107/PDCD10 and hypoxamir-210/Casp8ap2 and their role in cytoprotection during ischemic preconditioning of stem cells. Antioxid Redox Signal 17(8):1053–1065
King HW, Michael MZ et al (2012) Hypoxic enhancement of exosome release by breast cancer cells. BMC Cancer 12(1):421
Koong AC, Mehta VK et al (2000) Pancreatic tumors show high levels of hypoxia. Int J Radiat Oncol Biol Phys 48(4):919–922
Krek A, Grun D et al (2005) Combinatorial microRNA target predictions. Nat Genet 37(5):495–500
Krick S, Hanze J et al (2005) Hypoxia-driven proliferation of human pulmonary artery fibroblasts: cross-talk between HIF-1alpha and an autocrine Angiotensin system. FASEB J 19(7):857–859
Kuijper S, Turner CJ et al (2007) Regulation of angiogenesis by Eph–ephrin interactions. Trends Cardiovasc Med 17(5):145–151
Kulshreshtha R, Ferracin M et al (2007a) Regulation of microRNA expression: the hypoxic component. Cell Cycle 6(12):1426–1431
Kulshreshtha R, Ferracin M et al (2007b) A MicroRNA signature of hypoxia. Mol Cell Biol 27(5):1859–1867
Landau DA, Slack FJ (2011) MicroRNAs in mutagenesis, genomic instability, and DNA repair. Semin Oncol 38(6):743–751
Lanford RE, Hildebrandt-Eriksen ES et al (2010) Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science 327(5962):198–201
Lawrie CH, Gal S et al (2008) Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 141(5):672–675
Lee RC, Feinbaum RL et al (1993) The C. Elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75(5):843–854
Lee Y, Jeon K et al (2002) MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 21(17):4663–4670
Lee Y, Kim M et al (2004) MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23(20):4051–4060
Lees JA, Saito M et al (1993) The retinoblastoma protein binds to a family of E2F transcription factors. Mol Cell Biol 13(12):7813–7825
Leone G, DeGregori J et al (1998) E2F3 activity is regulated during the cell cycle and is required for the induction of S phase. Genes Dev 12(14):2120–2130
Lewis BP, Shih IH et al (2003) Prediction of mammalian MicroRNA targets. Cell 115(7):787–798
Lewis BP, Burge CB et al (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are MicroRNA targets. Cell 120(1):15–20
Li T, Cao H et al (2011) Identification of miR-130a, miR-27b and miR-210 as serum biomarkers for atherosclerosis obliterans. Clin Chim Acta 412(1–2):66–70
Lim LP, Lau NC et al (2005) Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433(7027):769–773
Liu Y, Cox SR et al (1995) Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells: identification of a 5′ enhancer. Circ Res 77(3):638–643

Liu M, Liu H et al (2010) Reactive oxygen species originating from mitochondria regulate the cardiac sodium channel. Circ Res 107(8):967–974
Liu Y, Han Y et al (2012) Synthetic miRNA-mowers targeting miR-183-96-182 cluster or miR-210 inhibit growth and migration and induce apoptosis in bladder cancer cells. PLoS One 7(12):e52280
Lou YL, Guo F et al (2012) miR-210 activates notch signaling pathway in angiogenesis induced by cerebral ischemia. Mol Cell Biochem 370(1–2):45–51
Lu J, Getz G et al (2005) MicroRNA expression profiles classify human cancers. Nature 435(7043):834–838
Malzkorn B, Wolter M et al (2009) Identification and functional characterization of microRNAs involved in the malignant progression of gliomas. Brain Pathol 20(3):539–550
Maragkakis M, Reczko M et al (2009) DIANA-microT web server: elucidating microRNA functions through target prediction. Nucleic Acids Res 37(suppl 2):W273–W276
Meroni G, Reymond A et al (1997) Rox, a novel bHLHZip protein expressed in quiescent cells that heterodimerizes with Max, binds a non-canonical E box and acts as a transcriptional repressor. EMBO J 16(10):2892–2906
Michael MZ, O'Connor SM, et al (2003) Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res 1(12):882–891
Miko E, Czimmerer Z et al (2009) Differentially expressed microRNAs in small cell lung cancer. Exp Lung Res 35(8):646–664
Mitchell PS, Parkin RK et al (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci 105(30):10513–10518
Mizuno Y, Tokuzawa Y et al (2009) miR-210 promotes osteoblastic differentiation through inhibition of AcvR1b. FEBS Lett 583(13):2263–2268
Mochel F, Knight MA et al (2008) Splice mutation in the iron-sulfur cluster scaffold protein ISCU causes myopathy with exercise intolerance. Am J Hum Genet 82(3):652–660
Murakami Y, Yasuda T et al (2006) Comprehensive analysis of microRNA expression patterns in hepatocellular carcinoma and non-tumorous tissues. Oncogene 25(17):2537–2545
Murphy MP (2009) How mitochondria produce reactive oxygen species. Biochem J 417(1):1–13
Mutharasan RK, Nagpal V et al (2011) MicroRNA-210 is upregulated in hypoxic cardiomyocytes through Akt- and p53-dependent pathways and exerts cytoprotective effects. Am J Physiol Heart Circ Physiol 301(4):H1519–H1530
Nakada C, Tsukamoto Y et al (2011) Overexpression of miR-210, a downstream target of HIF1α, causes centrosome amplification in renal carcinoma cells. J Pathol 224(2):280–288
Nakamura Y, Patrushev N et al (2008) Role of protein tyrosine phosphatase 1B in vascular endothelial growth factor signaling and cell–cell adhesions in endothelial cells. Circ Res 102(10):1182–1191
Nie Y, Han B-M et al (2011) Identification of MicroRNAs involved in hypoxia- and serum deprivation-induced apoptosis in mesenchymal stem cells. Int J Biol Sci 7:762–768
Pasquinelli AE, Reinhart BJ et al (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408(6808):86–89
Pineles BL, Romero R et al (2007) Distinct subsets of microRNAs are expressed differentially in the human placentas of patients with preeclampsia. Am J Obstet Gynecol 196(3):261 e261–266
Place RF, Li L-C et al (2008) MicroRNA-373 induces expression of genes with complementary promoter sequences. Proc Natl Acad Sci 105(5):1608–1613
Porkka KP, Pfeiffer MJ et al (2007) MicroRNA expression profiling in prostate cancer. Cancer Res 67(13):6130–6135
Presti JC Jr, Rao PH et al (1991) Histopathological, cytogenetic, and molecular characterization of renal cortical tumors. Cancer Res 51(5):1544–1552
Puissegur MP, Mazure NM et al (2011) miR-210 is overexpressed in late stages of lung cancer and mediates mitochondrial alterations associated with modulation of HIF-1 activity. Cell Death Differ 18(3):465–478
Pulkkinen K, Malm T et al (2008) Hypoxia induces microRNA miR-210 in vitro and in vivo ephrin-A3 and neuronal pentraxin 1 are potentially regulated by miR-210. FEBS Lett 582(16):2397–2401

Qin L, Chen Y et al (2010) A deep investigation into the adipogenesis mechanism: profile of microRNAs regulating adipogenesis by modulating the canonical Wnt/beta-catenin signaling pathway. BMC Genomics 11:320
Raponi M, Dossey L et al (2009) MicroRNA classifiers for predicting prognosis of squamous cell lung cancer. Cancer Res 69(14):5776–5783
Redova M, Poprach A et al (2012) MiR-210 expression in tumor tissue and in vitro effects of its silencing in renal cell carcinoma. Tumor Biol 34(1):481–491
Reinhart BJ, Slack FJ et al (2000) The 21-nucleotide let-7 RNA regulates developmental timing in caenorhabditis elegans. Nature 403(6772):901–906
Rothe F, Ignatiadis M et al (2011) Global MicroRNA expression profiling identifies MiR-210 associated with tumor proliferation, invasion and poor clinical outcome in breast cancer. PLoS One 6(6):e20980
Ruan K, Song G et al (2009) Role of hypoxia in the hallmarks of human cancer. J Cell Biochem 107(6):1053–1062
Salmena L, Poliseno L et al (2011) A ceRNA hypothesis: the Rosetta stone of a hidden RNA language? Cell 146(3):353–358
Sarkar J, Gou D et al (2010) MicroRNA-21 plays a role in hypoxia-mediated pulmonary artery smooth muscle cell proliferation and migration. Am J Physiol Lung Cell Mol Physiol 299(6):L861–L871
Satzger I, Mattern A et al (2009) MicroRNA-15b represents an independent prognostic parameter and is correlated with tumor cell proliferation and apoptosis in malignant melanoma. Int J Cancer 126(11):2553–2562
Schwarz DS, Hutvágner G et al (2003) Asymmetry in the assembly of the RNAi enzyme complex. Cell 115(2):199–208
Selbach M, Schwanhausser B et al (2008) Widespread changes in protein synthesis induced by microRNAs. Nature 455(7209):58–63
Semenza GL (2003) Angiogenesis ischemic and neoplastic disorders. Annu Rev Med 54(1):17–28
Semenza GL (2010a) Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. Oncogene 29(5):625–634
Semenza GL (2010b) Vascular responses to hypoxia and ischemia. Arterioscler Thromb Vasc Biol 30(4):648–652
Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. Cell 148(3):399–408
Shinohara A, Ogawa T (1998) Stimulation by Rad52 of yeast Rad51-mediated recombination. Nature 391(6665):404–407
Simone NL, Soule BP et al (2009) Ionizing radiation-induced oxidative stress alters miRNA expression. PLoS One 4(7):e6377
Stenvang J, Silahtaroglu AN et al (2008) The utility of LNA in microRNA-based cancer diagnostics and therapeutics. Semin Cancer Biol 18(2):89–102
Tabernero J, Shapiro GI et al (2013) First-in-man trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Disco 3(4):406–417
Taylor DD, Gercel-Taylor C (2008) MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 110(1):13–21
Thum T, Galuppo P et al (2007) MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. Circulation 116(3):258–267
Tong WH, Rouault TA (2006) Functions of mitochondrial ISCU and cytosolic ISCU in mammalian iron-sulfur cluster biogenesis and iron homeostasis. Cell Metab 3(3):199–210
Toyama T, Kondo N et al (2012) High expression of microRNA-210 is an independent factor indicating a poor prognosis in Japanese triple-negative breast cancer patients. Jpn J Clin Oncol 42(4):256–263
Tsuchiya S, Fujiwara T et al (2011) MicroRNA-210 regulates cancer cell proliferation through targeting fibroblast growth factor receptor-like 1 (FGFRL1). J Biol Chem 286(1):420–428
Valadi H, Ekstrom K et al (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9(6):654–659

Valencia-Sanchez MA, Liu J et al (2006) Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 20(5):515–524
van Rooij E, Sutherland LB et al (2006) A signature pattern of stress-responsive microRNAs that can evoke cardiac hypertrophy and heart failure. Proc Natl Acad Sci U S A 103(48):18255–18260
Vasudevan S, Tong Y et al (2007) Switching from repression to activation: MicroRNAs can up-regulate translation. Science 318(5858):1931–1934
Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26(2):225–239
Venter JC, Adams MD et al (2001) The sequence of the human genome. Science 291(5507):1304–1351
Ventura A, Jacks T (2009) MicroRNAs and cancer: short RNAs go a long way. Cell 136(4):586–591
Wan G, Mathur R et al (2011) MiRNA response to DNA damage. Trends Biochem Sci 36(9):478–484
Wang S, Olson EN (2009) AngiomiRs–key regulators of angiogenesis. Current Opin Genet Dev 19(3):205–211
Wang GL, Semenza GL (1993) General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A 90(9):4304–4308
Wang GL, Semenza GL (1995) Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270(3):1230–1237
Wang GL, Jiang BH et al (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A 92(12):5510–5514
Wang J, Chen J et al (2009) MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. Cancer Prev Res 2(9):807–813
Weber JA, Baxter DH et al (2010) The MicroRNA spectrum in 12 body fluids. Clin Chem 56(11):1733–1741
White NMA, Bao TT et al (2011) MiRNA profiling for clear cell renal cell carcinoma: biomarker discovery and identification of potential controls and consequences of miRNA dysregulation. J Urol 186(3):1077–1083
Wightman B, Ha I et al (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. Elegans. Cell 75(5):855–862
Wilson WR, Hay MP (2011) Targeting hypoxia in cancer therapy. Nat Rev Cancer 11(6):393–410
Wu F, Yang Z et al (2009) Role of specific microRNAs for endothelial function and angiogenesis. Biochem Biophys Res Commun 386(4):549–553
Yanaihara N, Caplen N et al (2006) Unique microRNA molecular profiles in lung cancer diagnosis and prognosis. Cancer Cell 9(3):189–198
Yang W, Sun T et al (2012) Downregulation of miR-210 expression inhibits proliferation, induces apoptosis and enhances radiosensitivity in hypoxic human hepatoma cells in vitro. Exp Cell Res 318(8):944–954
Zhang H, Gao P et al (2007) HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer Cell 11(5):407–420
Zhang Z, Sun H et al (2009) MicroRNA miR-210 modulates cellular response to hypoxia through the MYC antagonist MNT. Cell Cycle 8(17):2756–2768
Zhang X, Wan G et al (2011) The ATM kinase induces microRNA biogenesis in the DNA damage response. Mol Cell 41(4):371–383
Zhao A, Li G et al (2013) Serum miR-210 as a novel biomarker for molecular diagnosis of clear cell renal cell carcinoma. Exp Mol Pathol 94(1):115–120
Zhu XM, Han T et al (2009) Differential expression profile of microRNAs in human placentas from preeclamptic pregnancies vs normal pregnancies. Am J Obstet Gynecol 200(6):661 e661–667
Zundel W, Schindler C et al (2000) Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev 14(4):391–396

# Chapter 11
# The Role of Complement in Tumor Growth

**Ruben Pio, Leticia Corrales, and John D. Lambris**

**Abstract** Complement is a central part of the immune system that has developed as a first defense against non-self cells. Neoplastic transformation is accompanied by an increased capacity of the malignant cells to activate complement. In fact, clinical data demonstrate complement activation in cancer patients. On the basis of the use of protective mechanisms by malignant cells, complement activation has traditionally been considered part of the body's immunosurveillance against cancer. Inhibitory mechanisms of complement activation allow cancer cells to escape from complement-mediated elimination and hamper the clinical efficacy of monoclonal antibody–based cancer immunotherapies. To overcome this limitation, many strategies have been developed with the goal of improving complement-mediated effector mechanisms. However, significant work in recent years has identified new and surprising roles for complement activation within the tumor microenvironment. Recent reports suggest that complement elements can promote tumor growth in the context of chronic inflammation. This chapter reviews the data describing the role of complement activation in cancer immunity, which offers insights that may aid the development of more effective therapeutic approaches to control cancer.

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A link between cancer and inflammation was first proposed by Rudolf Virchow in the nineteenth century (Grivennikov et al. 2010). Virchow observed that chronic inflammation established an environment that promoted the initiation and growth of malignancy (Balkwill and Mantovani 2001). Since then, a number of epidemiological studies have provided evidence that chronic inflammation predisposes individuals to various types of cancer (Mantovani et al. 2008). Inflammation affects every step of tumorigenesis and fosters multiple hallmarks of cancer (Hanahan and Weinberg 2011), inducing proliferation and survival, promoting angiogenesis and metastasis, evading adaptive immunity, and reducing the response to therapeutic agents. The main features of cancer-related inflammation include infiltration of white blood cells, predominantly tumor-associated macrophages (TAMs), and the presence of pro-inflammatory cytokines and chemokines (Colotta et al. 2009). Many studies have found activation of the complement system in tumors and an elevation of complement activity in the sera of patients with neoplastic diseases. It is interesting that some of the most powerful proinflammatory molecules (e.g., anaphylatoxin C5a) are generated by the activation of the complement system. This chapter reviews the evidence for complement activation within the tumor microenvironment and discusses the implications of its biological actions for cancer progression and anticancer therapy.

## 11.1 The Complement System and Its Regulation

The complement system has classically been recognized as a central part of the innate immune response, which serves as a first defense against microbes and unwanted host molecules. Physiological functions of complement include defending against pyogenic bacterial infection and disposing of immune complexes and products of inflammatory injury (Walport 2001). However, more recent findings have revealed that complement orchestrates many more immunological and inflammatory processes that contribute substantially to homeostasis (Ricklin et al. 2010). Complement participates in such diverse processes as control of adaptive immunity, enhancement of humoral immunity, removal of apoptotic cells, regulation of the coagulation system, maturation of synapses, angiogenesis, mobilization of hematopoietic stem-progenitor cells, regeneration of tissue, and lipid metabolism. All these activities are mediated by more than 50 circulating or cell surface–bound proteins. These proteins can be zymogens (which become active enzymes upon activation of complement), effectors, receptors, or control proteins that help maintain well-balanced activation and inhibition of the system. There are three well-established

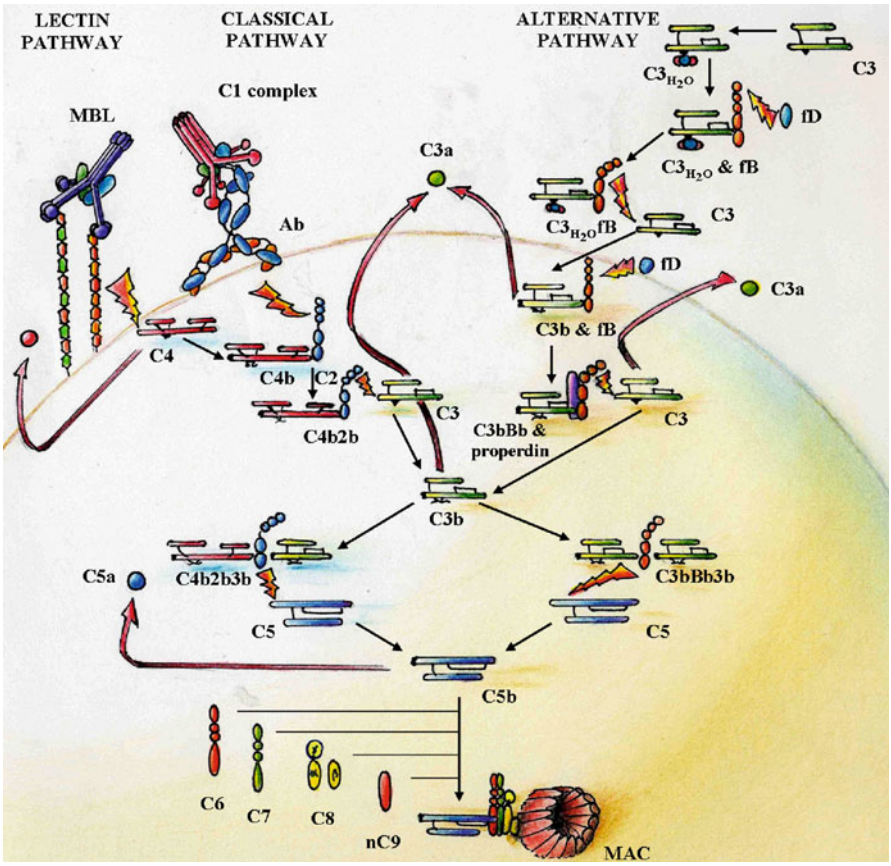


**Fig. 11.1** Cascade of events during the activation of the complement system

mechanisms of complement activation: the classical, lectin, and alternative pathways (Fig. 11.1). The three complement pathways share the common step of activating the central component C3 but differ according to their activation mechanisms of target recognition.

### *11.1.1 The Classical Pathway*

The first component of the classical pathway is a complex formed by the hexameric C1q together with C1r and C1s, two serine protease proenzymes (Kojouharova et al. 2010). Activation of this pathway is initiated by the recognition by C1q of the antibody constant regions of μ chains (immunoglobulin [Ig] M) and some γ chains (IgG) bound to target antigens. The classical pathway can also be activated in the

absence of antibodies because C1q can recognize endogenous ligands such as dying cells, extracellular matrix proteins, pentraxins, amyloid deposits, prions, and DNA (Nauta et al. 2002; Sjoberg et al. 2009; McGrath et al. 2006; Ying et al. 1993; Mitchell et al. 2007; Jiang et al. 1992). Binding of C1q activates C1s and C1r. C1s is a serine protease that cleaves C4 into two fragments: C4b, which binds to the cell surface through a thioester bond, and C4a, a soluble small fragment of unknown function that diffuses away. Next, complement C2 binds to C4b and becomes a target for C1s as well. The cleavage of C2 generates two fragments: C2a and C2b, which remains bound to C4b, forming the classical pathway C3 convertase (C4bC2b). In the complex C2b acts as a serine protease that cleaves C3 to C3b and C3a. C3b binds covalently to the cell membrane through its thioester bond and joins to the C3 convertase to form the classical pathway C5 convertase, C4bC2bC3b (Pangburn and Rawal 2002).

### *11.1.2 The Lectin Pathway*

The lectin pathway is analogous to the classical pathway. This pathway is activated by proteins homologous to C1q: mannose-binding lectin (MBL) and H-, L- or M-ficolins (Thiel 2007). These proteins recognize repetitive carbohydrate patterns on pathogens, such as mannose and N-acetyl-glucosamine. After their binding, these proteins form a C1q-like complex with MBL-associated serine protease-2 (MASP-2), cleaving the complement components C4 and C2 to form the C3 convertase C4bC2b, which is common to the classical pathway activation route (Matsushita et al. 2000).

### *11.1.3 The Alternative Pathway*

The alternative pathway is mechanistically distinct from the classical and lectin pathways. It provides an initial line of innate immune defense, being initiated by spontaneous low-level hydrolysis of C3 (Pangburn et al. 1981). The spontaneous hydrolysis of C3, also known as the "tickover" of C3, forms C3($H_2O$). C3($H_2O$) can bind to factor B, which is cleaved by factor D to form the initial alternative pathway C3 convertase, C3($H_2O$)Bb (Bexborn et al. 2008). This complex begins to convert C3 into C3b and C3a. In most cases this C3b is rapidly inactivated; however, some C3b can bind to complement-activating surfaces and associate with factor B, which, again, in complex with C3b, can be cleaved by factor D, forming the predominant alternative pathway C3 convertase (C3bBb). The stability of this convertase is enhanced by the binding of properdin (Hourcade 2008). The fragment Bb on the C3 convertase cleaves more C3 and initiates an amplification loop, generating more C3b that can create new alternative C3 convertases and the C5 convertase (C3bBbC3b).

### 11.1.4 Nonenzymatic Assembly of the Terminal Pathway Components

C5 cleavage by the C5 convertases (C4bC2bC3b or C3bBbC3b) initiates the second phase of complement activation. C5 is cleaved into C5a, the most potent anaphylatoxin in the arsenal, and C5b, which begins the nonenzymatic assembly of the terminal pathway components C5b-9 (membrane attack complex [MAC]), which may lead to lysis in a process known as complement-dependent cytotoxicity (CDC). It is interesting to note that the MAC can also induce other responses in the cell: it can activate immune cells to release inflammatory molecules, increase the resistance of cells to further lytic attack, drive cells to proliferate, and make cells more resistant to apoptosis (Cole and Morgan 2003).

### 11.1.5 Alternative Routes of Complement Activation

Apart from the three major pathways, there are several bypass routes that have been shown to trigger complement activation at various stages. The lectin pathway can be activated directly by the binding of MBL to natural IgM antibodies bound to ischemic antigens in endothelial cells after ischemia/reperfusion injury (McMullen et al. 2006; Zhang et al. 2006). In the absence of C2/C4, but in the presence of alternative pathway components, some antigen-antibody complexes or certain oligosaccharides can lead to C3 activation (Selander et al. 2006). C3 can be cleaved and activated by extrinsic proteases, such as thrombin or kallikrein, pointing to a crosstalk between the complement system and the coagulation cascade (Markiewski et al. 2007). C5 can also be cleaved by thrombin, bypassing C3 (Huber-Lang et al. 2006). Finally, C5 can be cleaved by silica and asbestos fibers through mechanisms involving the generation of free radicals (Governa et al. 2002).

### 11.1.6 Complement Regulators

Regulation of complement activation is of critical importance for the homeostasis of the organism. Control proteins regulate complement at three main levels: they can inhibit the protease activity of the proteins involved in the activation cascade, they can facilitate the decay and destruction of convertases, and they can control MAC formation (Fig. 11.2). Many regulators share a varying number of a repeating motifs called short consensus repeats (SCRs), complement control protein repeats, or sushi domains. SCRs are globular domains containing approximately 60 amino acids and have a framework of conserved residues that includes four invariant cysteines, an almost invariant tryptophan, and highly conserved prolines, glycines, and hydrophobic residues (Kirkitadze and Barlow 2001). These domains are thought to play a role in binding to C3 and C4 and their breakdown products.

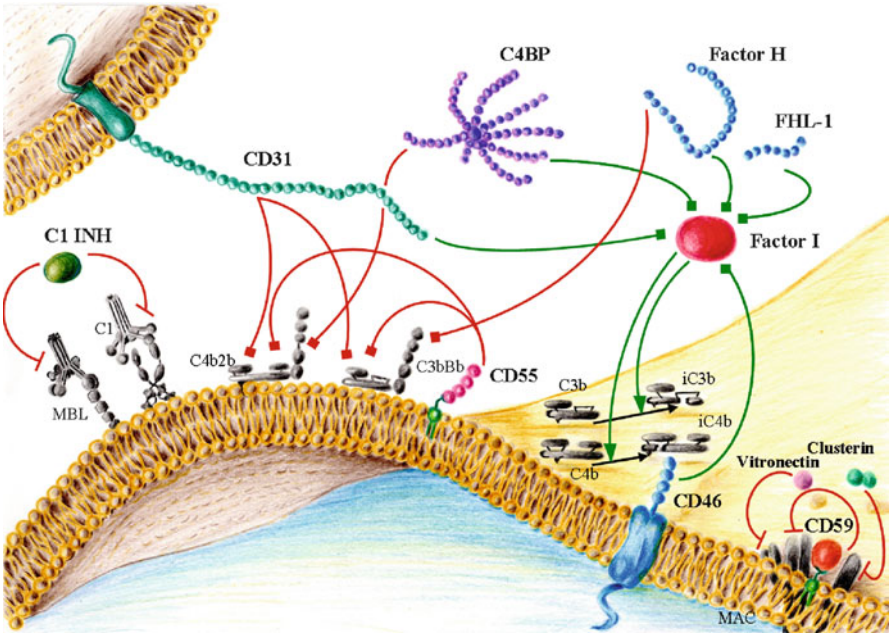


**Fig. 11.2** Main complement inhibitors: soluble proteins and membrane-bound complement regulatory proteins. *Red lines* represent inhibitory activity (when ending in a *bar*) or accelerated decay activity (when ending in a *square*). *Green lines* represent cofactor activity (when ending in a *square*) or protease activity (when ending in an *arrowhead*)

Complement regulators have traditionally been grouped into two categories: soluble regulators and membrane-bound regulators. At least six complement regulators can be found in soluble form in plasma: C1 inhibitor, factor I, C4b-binding protein (C4BP), factor H, vitronectin (S protein), and clusterin (SP40,40). C1 inhibitor is a member of the serine family of protease inhibitors that inactivates C1r, C1s, and MASP-2 (Davis et al. 2008). Factor I cleaves and inactivates C4b and C3b (Sim et al. 1993). C4BP is a heterogeneous oligomeric protein that controls the classical complement pathway. After binding to C4b, C4BP inhibits complement by three different mechanisms. It prevents the assembly of the C3 convertase, accelerates the decay of the classical C3 and C5 convertases, and functions as a cofactor in the factor I–mediated inactivation of C4b (Blom et al. 2004). Factor H, with its alternatively spliced variant factor H-like protein 1 (FHL-1), is mostly known as an inhibitor of the alternative pathway. Through its binding to C3b, factor H competes with factor B in the formation of the C3 and C5 convertases, displaces the Bb subunit from the convertases, and acts as a cofactor for factor I in the cleavage of C3b (Jozsi and Zipfel 2008).

Several recent studies have described the association of genetic variations in complement factor H with various diseases. Mutations or polymorphisms that alter the binding of factor H to C3b and polyanions are associated with atypical

hemolytic uremic syndrome, whereas mutations that disrupt the plasma activity of factor H, leading to unrestricted activation of the alternative pathway, are associated with membranoproliferative glomerulonephritis type II (de Cordoba and de Jorge 2008). A polymorphism at the factor H locus that causes a Tyr402His amino acid substitution in SCR7 confers a significantly increased risk for age-related macular degeneration (Shaw et al. 2012). Five complement factor H–related proteins encoded by genes closely linked to the factor H locus have been identified. These proteins are involved in complement regulation, but their exact functions are not well-defined (Jozsi and Zipfel 2008). Vitronectin and clusterin inhibit the insertion of the MAC into the membrane (Podack and Muller-Eberhard 1979; Jenne and Tschopp 1989). Clusterin can also modulate cell differentiation and regulate the production of major pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 (Falgarone and Chiocchia 2009).

Complement activation is also controlled by membrane-bound complement regulatory proteins (mCRPs) such as complement receptor (CR) type 1 (CR1; CD35), membrane cofactor protein (CD46), decay-accelerating factor (CD55), and CD59 (protectin). CR1 is expressed by erythrocytes, neutrophils, eosinophils, monocytes, follicular dendritic cells, glomerular podocytes, B lymphocytes, and some T lymphocytes (Fischer et al. 1986); it functions as a cofactor for the factor I–mediated cleavage of C3b and C4b and accelerates the decay of the classical and alternative convertases (Fearon 1979). CD46 is expressed in most cells (except erythrocytes) and acts as a cofactor of factor I in C3b/C4b cleavage (Liszewski et al. 1991). CD46 also has been implicated in the regulation of T cells (Marie et al. 2002; Kemper et al. 2003). CD55 is attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor and is present in all blood elements and most other cell types (Medof et al. 1987). CD55 accelerates the decay of the classical and alternative C3 and C5 convertases (Lublin and Atkinson 1989). CD55 binds to CD97, which is expressed on macrophages, granulocytes, dendritic cells, and activated T and B cells, and simultaneously regulates innate and adaptive immune responses (Abbott et al. 2007). CD59, a GPI-anchored protein, is expressed by all circulating cells, vascular endothelium, epithelium, and most other cell organs (Morgan 1999). CD59 binds C8 during the formation of MAC and inhibits the insertion of C9 into the lipid bilayer (Meri et al. 1990). Alternative roles for CD59, related to its GPI anchor signaling properties, also have been demonstrated in T cells, natural killer cells, and B cells (Kimberley et al. 2007).

### *11.1.7 Opsonization by C3b and Its Related Fragments*

Activation of all three complement pathways results in the conversion of C3 into C3b. The nascent C3b molecule can trigger complement amplification, but it can also be inactivated by proteolysis. Initial inactivation of C3b is mediated by factor I and its cofactors. Factor I catalyzes the proteolysis of two peptide bonds in the α′ polypeptide chain of C3b. The resulting products are the membrane-bound iC3b

and a small peptide, C3f, which is released from the molecule. A third cleavage, catalyzed by factor I, generates the inert C3c and C3dg; C3c is released, and C3dg is retained on the cell membrane (Law and Dodds 1997). Although further complement amplification is abolished, recognition of C3b and its fragments by complement receptors on cells promotes phagocytosis. Complement receptors are grouped into three families: the SCR family members CR1 and CR2, the β2 integrin family members CR3 and CR4, and the Ig superfamily member CRIg. CR1 (CD35) is expressed in the majority of peripheral blood cells and recognizes C3b, C4b, iC3b, C3dg, C1q, and mannose-binding protein (Klickstein et al. 1997; Ghiran et al. 2000). The main function of CR1 is to capture immune complexes on erythrocytes for transport and clearance by the liver (Taylor et al. 1997). CR1 also promotes the secretion of pro-inflammatory molecules, such as IL-1α, IL-1β, and prostaglandins; it also plays a role in the presentation of antigens to B cells and inhibits both the classical and alternative pathways via its decay-accelerating activity and cofactor activity in C3b/C4b cleavage (Krych-Goldberg and Atkinson 2001). CR2 (CD21) is an evolutionary homolog of CR1 but binds only to iC3b, C3d, and C3dg. On B cells, CR2 forms a coreceptor with CD19 and CD81. Binding of this coreceptor to the B-cell antigen receptor lowers the threshold for B-cell activation (Fearon and Carter 1995). CR3 (CD11b/CD18) and CR4 (CD11c/CD18) are expressed by leukocytes and stimulate phagocytosis when bound to iC3b. In addition, they contribute to leukocyte trafficking, adhesion, migration, and costimulation (van Lookeren et al. 2007). CRIg has recently been identified as a complement receptor. It is expressed on a restricted subset of tissue-resident macrophages and may play an important role in phagocytosis (Helmy et al. 2006; He et al. 2008).

### *11.1.8 Biological Effects Mediated by Anaphylatoxins*

During complement activation, soluble active fragments are released from C3, and C5. These bioactive peptides, C3a and C5a, were called anaphylatoxins because they were found to be potent multifunctional pro-inflammatory molecules, acting as chemotaxins and leukocyte activators (Kohl 2001). Anaphylatoxin receptors belong to the superfamily of G-protein-coupled receptors. They share high sequence homology but differ in ligand specificity, signal transduction capacity, and function. The anaphylatoxin receptors are C3aR for C3a and C5aR and C5L2 for C5a. For C5a binding, the first recognized receptor was C5aR-1 (Boulay et al. 1991), also known as CD88. The orphan receptor GPR77 was later identified as a second C5a receptor and was called C5a-like receptor 2 (C5L2) (Ohno et al. 2000). C5aR is a classic G protein-coupled receptor, whereas C5L2 is an enigmatic receptor deficient in G-protein coupling. This fact, together with the fact that the pathway for C5L2 after C5a binding is unknown, has prompted the suggestion that C5L2 is a default receptor that attenuates C5a biological responses by competing with C5aR-1. Nevertheless, this role for C5L2 has been challenged by results that point to its function as a positive modulator for both C5a- and C3a-anaphylatoxin-induced responses (Chen et al. 2007).

C3aR and C5aR are expressed in both myeloid and nonmyeloid cell types. The activities of anaphylatoxins are related to the cell types that express their receptors. They can increase vascular permeability; promote smooth muscle contraction; induce leukocyte recruitment; increase chemotaxis, migration, and phagocytosis in white blood cells; and promote the production and release of other pro-inflammatory mediators (e.g., histamine) (Haas and van Strijp 2007). Anaphylatoxins have been implicated in brain development (Benard et al. 2004) and tissue regeneration and fibrosis (Strey et al. 2003). As described below, in recent years C5a activity also has been connected to cancer progression.

## 11.2 Cancer Immunity

A growing body of evidence supports the proposed capacity of the immune system to recognize malignant cells and regulate tumor growth. Cancer cells acquire several sequential genetic and epigenetic abnormalities that dictate malignant growth and produce changes in cell morphology, generating tumor-associated antigens that distinguish malignant cells from their normal counterparts. Those changes can induce the recognition of malignant cells by immune defense mechanisms mediated by T and B cells, protecting the host against the development of cancers (Pardoll 2003). Tumor cells can also become susceptible to natural killer cells as a result of the decreased expression of self-class I major histocompatability complex (Karre et al. 1986), the expression of stress-induced proteins (Bauer et al. 1999), and the presence of mitosis-associated alterations of the cell membrane (Nolte-'t Hoen et al. 2007). Today, immune surveillance in cancer is supported by both epidemiological data and cancer models. Still, immune surveillance represents only one dimension of the complex relationship between the immune system and cancer (Dunn et al. 2004a).

Immune surveillance creates a selective pressure in the tumor microenvironment that can ultimately edit tumor immunogenicity. This idea has prompted the development of the cancer immunoediting hypothesis to explain the dynamic relationship established between cancer and immunity (Dunn et al. 2004b). Cancer immunoediting is a multistep process comprising different phases: recognition, elimination, equilibrium, and escape (Fig. 11.3). Through the process of transformation, normal cells express distinct tumor-specific markers and generate pro-inflammatory "danger" signals that are recognized by the immune system and initiate the process of cancer immunoediting. Once these signals are recognized, cells and molecules of innate and adaptive immunity, which compose the cancer immune surveillance network, can eradicate the nascent tumor cells, protecting the host from tumor formation. This stage is characterized by a lack of clinical evidence of disease; therefore, it is difficult to determine how often tumors are naturally eradicated. In addition, the tumor antigens and the immune mechanisms that underlie this process remain poorly understood (Matsushita et al. 2012). In any case, it is clinically evident that the immune system is unable to get rid of all emerging malignant cells.

When the elimination process is unsuccessful, tumor cells are capable of colonizing sites in the tissue microenvironment and enter the equilibrium phase, in

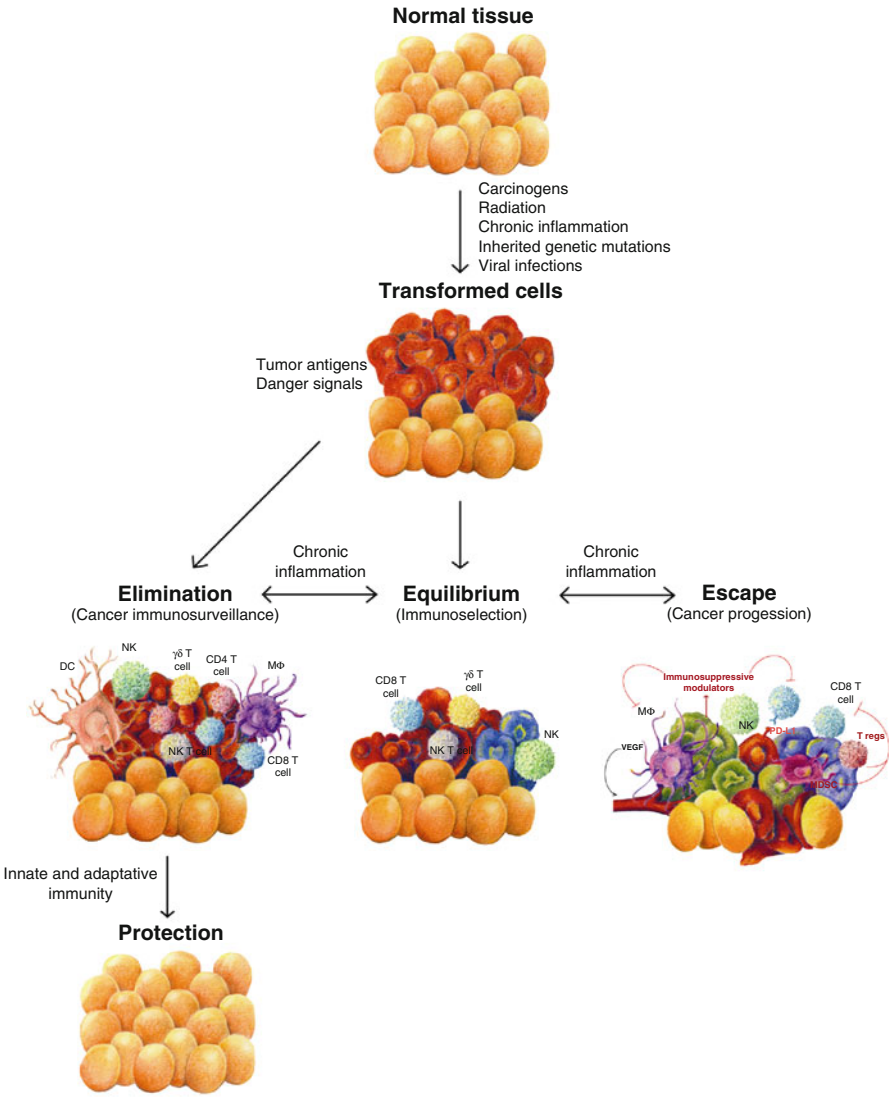


**Fig. 11.3** Steps of cancer immunity

which they may either be maintained chronically or be immunologically induced to change and produce new populations of tumor variants that are less immunogenic or possess mechanisms to control immune activation. In this phase, tumor cells can grow, although the immune system is still capable of controlling tumor progression. The escape phase refers to the final outgrowth of tumors that eventually evolve into a state in which they can effectively evade, suppress, and overcome control by the immune system. Hanahan and Weinberg (2011) have included "evading immune destruction" as an emerging hallmark of cancer, in addition to the previously established capabilities acquired during the multistep development of human tumors.

Many immunomodulatory mechanisms operate in tumors. These include the selection of tumor cells that no longer provoke a T cell–mediated immune response as a result of the loss of expression or presentation of tumor antigens (DuPage et al. 2012). At this point, tumors can progress and become clinically detectable. Moreover, at this stage, tumor cells can take advantage of the inflammatory microenvironment associated with the immune response and use it to promote carcinogenesis (Grivennikov et al. 2010). In fact, most solid malignancies trigger an intrinsic inflammatory response that builds up a protumorigenic microenvironment (Mantovani et al. 2008). The heterogeneous role of the immune response in the pathogenesis of cancer is exemplified by the divergent functions of immune cells found within the tumor microenvironment. Immune infiltrates can be located in the center of the tumor, in the invasive margin, or in the adjacent tissue. This component of the tumor microenvironment encompasses a wide variety of immune cells that is extremely diverse from patient to patient; however, most of the cells are macrophages and T cells.

Considerable evidence has been accumulated to support a dual role for TAMs in the regulation of tumor cell proliferation, invasion, and angiogenesis and immune control (Kataki et al. 2002; Lewis and Pollard 2006). High TAM content is generally correlated with poor prognosis (Quatromoni and Eruslanov 2012), but, depending on their stage of differentiation and activation, tissue macrophages have the ability to promote or inhibit neoplasia (Montuenga and Pio 2007). T cells can also exert both tumor-suppressive and tumor-promoting effects (Fridman et al. 2012). Tumor-infiltrating T cells can attenuate the metastatic potential of tumor cells and are correlated with better survival in many different tumor types (Galon et al. 2006; Laghi et al. 2009). However, many T-cell subsets found in solid tumors are involved in tumor promotion, progression, or metastasis (Roberts et al. 2007; Aspord et al. 2007).

In particular, regulatory T cells are considered the most powerful suppressors of antitumor immunity (Zou 2006). Regulatory T cells promote immunosuppression via direct effects on activated T cells or via the secretion of immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)-β (Thornton and Shevach 1998; Hawrylowicz and O'Garra 2005). An increased number of these cells in the tumor microenvironment confers growth and metastatic advantages and predicts a marked reduction in patient survival (Curiel et al. 2004; Shimizu et al. 2010). Therefore, the immune microenvironment surrounding the tumor comprises a highly heterogeneous population of immune cells with pro- and antitumor activities.

Whether the immune system limits or promotes tumor growth depends on a delicate balance between opposing forces. As is shown in the next sections, this duality in tumor immunity is also seen in the interrelationship between cancer and complement activation.

## 11.3 Complement in Immune Surveillance Against Tumors

Once a threatening body is recognized by the complement system, the activating steps initiate an inflammatory reaction, the opsonization of the target cell, and, in some cases, its killing. This conventional role of complement may have an effect on

the control of tumor growth. The numerous genetic and epigenetic alterations associated with carcinogenesis dramatically change the morphology and composition of the cell membrane. Altered glycosylation is considered a hallmark of cancer cells (Hakomori 2002; Hollingsworth and Swanson 2004; Miyagi et al. 2012), and progression of epithelial cells from a normal to malignant phenotype is associated with an aberrant increase in the metabolism of membrane phospholipids (Costello and Franklin 2005; Glunde and Serkova 2006; Griffin and Kauppinen 2007).

Although there is no irrefutable evidence for the existence of an effective immune surveillance mediated by complement, these changes in the composition of cell membranes may target tumor cells for complement recognition. In fact, several observations support the capacity of complement to recognize malignant cells. In a recent report, lung cancer cell lines were shown to deposit C5 and generate C5a more efficiently than bronchial epithelial cells (Corrales et al. 2012). Moreover, a significant increase in C5a was found in the plasma samples of patients with non-small-cell lung cancer, suggesting that the local generation of C5a within tumors may be followed by its systemic diffusion (Corrales et al. 2012). In primary lung tumors, C3b (but not MAC deposition) can be detected by immunohistochemistry (Niehans et al. 1996). C3c and C4 are elevated in patients with lung cancer (Gminski et al. 1992), and complement levels correlate with tumor size (Nishioka et al. 1976). Several studies of other cancer types also have suggested that the complement system is activated in response to the expression of tumor-associated antigens, with the subsequent deposition of complement components on tumor tissue (Guidi et al. 1988; Zurlo et al. 1989; Niculescu et al. 1992; Baatrup et al. 1994; Yamakawa et al. 1994; Lucas et al. 1996; Gasque et al. 1996; Bu et al. 2007). Elevated levels of C3a and soluble C5b-9 are present in the intraperitoneal ascitic fluid of patients with ovarian cancer (Bjorge et al. 2005). The lectin pathway of complement activation has been found to be significantly increased in patients with colorectal cancer when compared to healthy subjects (Ytting et al. 2004), and the MASP-2 concentration in serum has been reported to be an independent prognostic marker for poor survival (Ytting et al. 2005). Higher complement hemolytic activity and C3 levels have been observed in serum samples from children with neuroblastoma (Carli et al. 1979) and elevated complement levels have similarly been reported in patients with carcinomas of the digestive tract (Maness and Orengo 1977) or with brain tumors (Matsutani et al. 1984). In vivo alterations in the activation of the classical pathway have been described in patients with chronic lymphatic leukemia (Fust et al. 1987; Schlesinger et al. 1996), with a strong positive correlation between survival and the initial activity of the classical pathway of complement (Varga et al. 1995).

All these observations support the capacity of complement to recognize malignant cells. However, little is known about the tumor-associated antigens that are involved in the recognition of cancer cells by complement and the exact mechanisms that drive this activation. In the TC-1 syngeneic mouse model of cervical cancer, the classical pathway was found to be the main contributor to complement activation (Markiewski et al. 2008). Evidence for the classical pathway of complement activation also has been found in patients with papillary thyroid carcinoma (Lucas et al. 1996), follicular lymphoma, and mucosa-associated lymphoid tissue

lymphoma (Bu et al. 2007). In contrast, the alternative complement pathway has been found to be activated in lymphoblastoid cell lines (Budzko et al. 1976; Theofilopoulos and Perrin 1976; McConnell et al. 1978) and patients with multiple myeloma (Kraut and Sagone 1981). In childhood acute lymphoblastic leukemia, amplification of the alternative pathway after activation of the classical pathway has been suggested (Kalwinsky et al. 1976). On the other hand, the capacity of lung cancer cell lines to produce C5a in the absence of an exogenous source of complement components (i.e., serum), suggests that, apart from the traditional pathways of complement activation, cancer cells may have the capacity to activate complement by an extrinsic activation mechanism (Corrales et al. 2012). The production of anaphylatoxins by cancer cells may be mediated by soluble and membrane-bound proteases, such as serine proteases of the coagulation and fibrinolysis systems or cell-bound proteases (Huber-Lang et al. 2002, 2006; Amara et al. 2008). A better analysis of the pathways by which cancer cells activate complement would greatly improve our understanding of the interplay between complement and cancer and may be of value in identifying new diagnostic biomarkers and molecular targets for anticancer therapies. Changes in plasma complement components as part of the host's response to chemotherapy may also be useful as early predictive markers of a response to treatment (Michlmayr et al. 2011).

## 11.4 Mechanisms for Adaptation and Control of Complement Activation: Implications for Cancer Immunotherapy

There is sufficient basis to propose that neoplastic transformation is accompanied by an increased capacity to activate complement. However, cancer cells exhibit a number of strategies to resist complement attack. Many of these resistance mechanisms also are used by normal cells to avoid accidental activation or bystander effects from a local activation of complement. However, cancer cells develop additional mechanisms to inhibit complement activation (Fig. 11.4). Cancer-associated resistance mechanisms can be divided into two categories: extracellular and intracellular (Jurianz et al. 1999). One of the best characterized extracellular mechanisms is the expression of mCRPs. This research area has been extensively reviewed (Gorter and Meri 1999; Fishelson et al. 2003; Yan et al. 2008; Gancz and Fishelson 2009; Kolev et al. 2011).

With the exception of CR1, most cancers – whatever their tissue origin – express at least two, if not three, mCRPs. In several cancer types, increased levels of CD59 have been found to be associated with resistance to CDC (Brasoveanu et al. 1996; Jarvis et al. 1997; Chen et al. 2000; Coral et al. 2000), increased metastatic potential (Loberg et al. 2005), or poor prognosis (Xu et al. 2005; Watson et al. 2006). For example, prostatic tumors and medullary thyroid carcinomas overexpress the regulator CD55 and its receptor CD97 (Loberg et al. 2005; Mustafa et al. 2004). A deficiency of CD55 in mice significantly enhances T-cell responses (Liu et al. 2005).

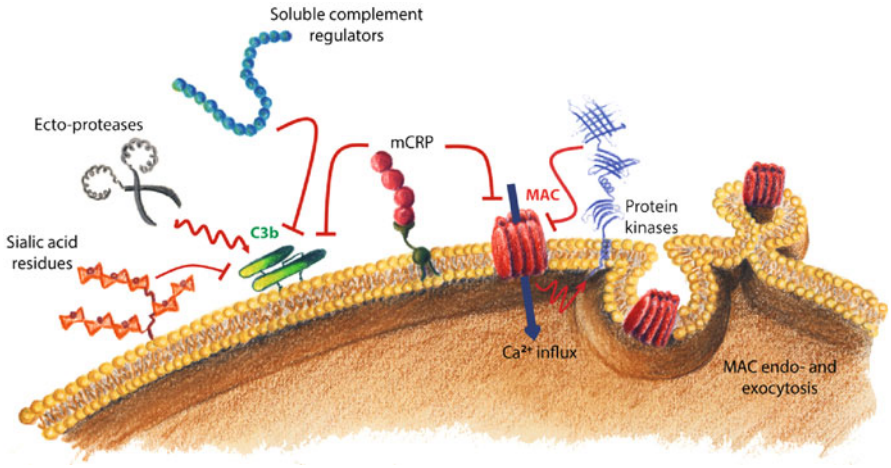


**Fig. 11.4** Mechanisms used by cancer cells to resist complement activation. *Red lines* represent inhibitory activity and *green lines* represent activation

In colorectal carcinoma, the expression of CD55 is associated with poor prognosis (Durrant et al. 2003). In contrast, the loss of CD55 has been related to poor prognosis in breast cancer (Madjd et al. 2004). CD46 is perhaps the mCRP with the lowest level of variation between tumors and normal tissue. Nevertheless, CD46 levels are correlated with tumor grade and recurrence in breast tumors (Rushmere et al. 2004; Madjd et al. 2005). Cell lines from various cancer types release soluble forms of the mCRPs (Bjorge et al. 2005; Brasoveanu et al. 1997; Hindmarsh and Marks 1998; Nasu et al. 1998; Jurianz et al. 2001; Li et al. 2001; Morgan et al. 2002; Donin et al. 2003), and many of these forms also have been detected in patients with cancer (Niehans et al. 1996; Li et al. 2001; Morgan et al. 2002; Seya et al. 1995; Sadallah et al. 1999; Gelderman et al. 2002a; Kawada et al. 2003; Hakulinen et al. 2004; Kohno et al. 2005). These forms of mCRPs are able to bind to tumor cells and should be considered contributors to the resistance of tumor cells to complement activation.

Soluble complement regulators, including factor H and FHL-1, are also important in the resistance of tumor cells to complement activation and CDC (Bjorge et al. 2005; Reiter and Fishelson 1989; Ollert et al. 1995; Junnikkala et al. 2000; Ajona et al. 2004). A clinically approved immunoassay for the detection of bladder cancer in urine is based on the quantification of factor H (Kinders et al. 1998; Cheng et al. 2005). H2 glioblastoma cells are able to bind factor H and FHL-1, promoting the inactivation of C3b (Junnikkala et al. 2000). In the melanoma cell line SK-MEL-93-2, factor H seems to be the dominant factor regulating the activation of complement (Ollert et al. 1995). An anti–factor H antibody enhances the complement-mediated killing of cells obtained from a Burkitt's lymphoma (Corey et al. 1997). Some cancer cells are protected from complement attack by sequestration of factor H to the cell surface through members of the SIBLING family (Fedarko et al. 2000; Jain et al. 2002). Factor H and FHL-1 are highly expressed by ovarian

carcinomas, and both proteins are abundantly present in ascites from these tumors (Junnikkala et al. 2002). In vitro studies have shown that lung cancer cell lines are more resistant to CDC than are human nasal epithelium primary cell cultures (Varsano et al. 1996; Varsano et al. 1998). This resistance may be mediated by the expression and secretion of factor H and FHL-1 to the extracellular milieu (Ajona et al. 2004). Downregulation of factor H reduces the growth of lung cancer cells in vivo (Ajona et al. 2007), and its expression in lung adenocarcinomas may be associated with worse prognosis (Cui et al. 2011).

Non-small-cell lung cancer cell lines also express factor I and C4BP, which efficiently support the cleavage of C3b and C4b in vitro (Okroj et al. 2008). Lung cancer cell lines downregulate the expression of factor H, factor I, CD46, and CD55 under hypoxic conditions and during hypoxia/reoxygenation, implying that, under these conditions, cancer cells reduce their reliance on mechanisms to control complement activation while keeping free from CDC (Okroj et al. 2009). In patients with various nonmetastatic solid tumors, C4BP plasma levels were found to be significantly higher than in control subjects (Battistelli et al. 2005). C4BP is able to bind to SK-OV-3, SW626, and Caov-3 ovarian adenocarcinoma cell lines, and this binding may lead to an increased control of classical pathway activation (Holmberg et al. 2001). Other soluble complement regulatory proteins such as C1 inhibitor (Gasque et al. 1996; Bjorge et al. 2005; Jurianz et al. 2001; Morris et al. 1982; Buo et al. 1993) and clusterin (Trougakos and Gonos 2002) may also be involved in the protection of cancer cells from complement activation.

In addition to the expression of mCRP and soluble regulators, there are several alternative mechanisms that can be used by cancer cells to control complement activation. Tumor cells can release proteases that cleave complement components (Ollert et al. 1990) or express them in their cell membrane (Paas et al. 1999; Bohana-Kashtan et al. 2005). Tumor cells are able to eliminate the MAC by endocytosis or vesiculation (Morgan 1992; Moskovich and Fishelson 2007). Sublytic doses of the MAC can, surprisingly, provide intracellular protection against complement attack. Insertion of the MAC into the cell membrane causes a variety of biological effects, including entrance into the cell cycle, resistance to apoptosis, expression of adhesion molecules, or augmentation of complement resistance (Morgan 1989; Liu et al. 2012). The mechanisms responsible for this protection are poorly understood but involve an increase in intracellular concentrations of calcium and the activation of protein kinases (Carney et al. 1990; Soane et al. 2001; Kraus et al. 2001). The signaling activation triggered by sublytic doses of MAC is discussed in greater detail in the next section.

The effectiveness of complement regulators in protecting tumor tissues from complement injury has led to the idea that inhibiting the function of these regulatory proteins will enhance monoclonal antibody–based immunotherapy. The number of monoclonal antibodies approved for cancer treatment has rapidly increased since rituximab, an anti-CD20 monoclonal antibody used for treatment of malignant lymphoma, was first used for the treatment of lymphomas (Schrama et al. 2006) (Table 11.1). Monoclonal antibodies normally use a combination of mechanisms to direct cytotoxic effects to a tumor cell (Weiner et al. 2010). They target

**Table 11.1** Therapeutic monoclonal antibodies (unconjugated) approved for use in cancer treatment

| Name (trade name) | Isotype | Target | Cancer indication |
|---|---|---|---|
| Rituximab (Rituxan) | Chimeric IgG1 | CD20 | Non-Hodgkin's and follicular lymphoma |
| Trastuzumab (Herceptin) | Humanized IgG1 | HER2/neu | Breast |
| Cetuximab (Erbitux) | Chimeric IgG1 | EGFR | Colorectal and head and neck |
| Bevacizumab (Avastin) | Humanized IgG1 | VEGF | Colorectal, lung, kidney, and brain |
| Alemtuzumab (Campath) | Humanized IgG1 | CD52 | Chronic lymphocytic leukemia |
| Panitumumab (Vectibix) | Human IgG2 | EGFR | Colorectal |
| Ofatumumab (Arzerra) | Human IgG1 | CD20 | Chronic lymphocytic leukemia |
| Ipilimumab (Yervoy) | Human IgG1 | CTLA-4 | Melanoma |

*Ig* immunoglobulin; *EGFR* epidermal growth factor receptor; VEGF vascular endothelial growth factor; *CTLA-4* cytotoxic T-lymphocyte-associated protein 4

tumor-specific and tumor-associated antigens and block important cancer activities. In addition, many of them are able to activate the immune system and mediate Fc domain–based reactions, such as antibody-dependent cellular cytotoxicity and complement fixation (Kolev et al. 2011). Successful complement activation by these therapeutic antibodies can have multiple effects on the immune response against tumors (i.e., the formation of the MAC, opsonization, and release of proinflammatory anaphylatoxins). However, the above-described protective mechanisms against complement activation hamper the clinical efficacy of cancer therapies based on the use of monoclonal antibodies that can activate complement. For example, rituximab exerts its effects against malignant lymphomas through a variety of mechanisms, including CDC (Di Gaetano et al. 2003; Cragg and Glennie 2004; Beum et al. 2008). The efficacy of rituximab seems to be limited by the expression of complement regulatory proteins in B-cell lymphoma cell lines (Golay et al. 2000; Cardarelli et al. 2002) Therefore, it is logical to assume that the anticancer efficacy of monoclonal antibodies would be enhanced by overcoming the protection exerted by complement regulators.

Several strategies to overcome this protection have been tested experimentally in vitro and in animal models (Fishelson et al. 2003; Gancz and Fishelson 2009; Kolev et al. 2011) (Table 11.2). These strategies include blockade of the activity of the regulators, downregulation of their expression, or their removal from the cell surface (Brasoveanu et al. 1996; Ajona et al. 2007; Di Gaetano et al. 2001; Andoh et al. 2002; Blok et al. 2003; Nagajothi et al. 2004; Terui et al. 2006; Shi et al. 2009; Gao et al. 2009; Hsu et al. 2010; Geis et al. 2010; Bellone et al. 2012). However, targeting inhibitory molecules to complement regulators in vivo is technically challenging and may have unwelcome consequences for normal cells. To limit the inhibitory effect on the tumor microenvironment, some researchers have proposed strategies such as the use of a biotin-avidin system (Macor et al. 2006) or bispecific monoclonal antibodies that target a tumor antigen and simultaneously block a major complement regulatory protein (Gelderman et al. 2002b, 2004a, 2005). All these strategies are limited by the fact that each tumor may be equipped with specific mechanisms of cell protection, and a concerted action against different protective mechanisms may be needed.

**Table 11.2** Strategies for the improvement of complement-mediated immunotherapy

| Goal | Strategy |
|---|---|
| *To overcome the protected capacity of complement regulators* | |
| Blockade of the regulatory activity | Neutralizing mono- or bispecific antibodies |
| Downregulation of the regulator expression | RNA interference |
| | Antisense oligonucleotides |
| | Pharmacological agents |
| | Cytokines |
| Removal of the regulatory capacity from the cell membrane | Phosphatidylinositol-specific phospholipase C |
| | Desialyzation |
| Inhibition of MAC removal | Downregulation of mortalin |
| | Inhibition of heat shock proteins |
| *To improve complement-mediated effector mechanisms of monoclonal antibodies* | |
| Antibody modification | Engineering of the Fc region antibody |
| Bispecific antibodies | Targeting a cancer-associated antigen and a complement regulator |
| Conjugation with complement-activation molecules | Conjugation with CVF |
| | Conjugation with C3b |
| Cocktail of antibodies | Targeting distinct epitopes of the same antigen |
| Fusion proteins including Fc domains | CR2-Fc fusion |
| Immunomodulators | β-glucan |

*CVF* cobra venom factor; *CR2* complement receptor 2

An alternative approach would be to improve the complement-mediated effector mechanisms of monoclonal antibodies through genetic engineering or conjugation. Several strategies have been devised for turning a non-complement-fixing antibody into a complement-fixing antibody to be employed in immunotherapy, including the selection of the Ig subclasses (IgG1 and IgG3) that are most efficient in activating complement and the production of IgG1-containing recombinant variants of Fc that exhibit increased capacity to induce CDC or antibody-dependent cytotoxicity (Macor and Tedesco 2007). Heteroconjugates comprising antitumor antibodies and molecules such as cobra venom factor, C3b, or iC3b have been used (Reiter and Fishelson 1989; Gelderman et al. 2002b; Juhl et al. 1990, 1995, 1997; Yefenof et al. 1990). Alteration of the glycosylation pattern has been shown to enhance the lytic potential of monoclonal antibodies without affecting their affinity or specificity (Schuster et al. 2005). The Fc region can be engineered to enhance the CDC activity of therapeutic antibodies (Moore et al. 2010), and bispecific antibodies have been engineered to recruit complement effector functions (Holliger et al. 1997). Mixtures of several antibodies have been proposed (Macor et al. 2006; Spiridon et al. 2002; Kennedy et al. 2003). β-Glucan has been used to induce CR3-dependent cellular cytotoxicity (Gelderman et al. 2004b). In addition, the extracellular domain of the CR2 fused to an IgG Fc domain has been successfully used in syngeneic mouse

tumor models (Elvington et al. 2012). The molecular architecture of the antigens selected for immunotherapy and the antibody concentration also seem to be essential for the proper induction of CDC (Ragupathi et al. 2005; Livingston et al. 2005; Beurskens et al. 2012).

## 11.5 Complement Activation Can Promote Carcinogenesis

In the cancer setting, researchers have traditionally focused on the role of complement in the tagging and elimination of tumor cells. However, recent work has challenged this conventional view. The fact that mice deficient in C3 or C5aR show decreased tumor growth when compared to wild-type mice suggests that complement proteins may perversely promote malignancy (Corrales et al. 2012; Markiewski et al. 2008; Nunez-Cruz et al. 2012). In line with this hypothesis, several studies have demonstrated a role for activated components of the complement system in the various stages of carcinogenesis. Complement can assist the escape of tumor cells from immunosurveillance, promote angiogenesis, activate mitogenic signaling pathways, sustain cellular proliferation and insensitivity to apoptosis, and participate in tumor cell invasion and migration (Rutkowski et al. 2010a) (Fig. 11.5).

### *11.5.1 Complement and Immunosuppression*

Activation of specific T cells against tumor-associated antigens has been demonstrated in cancer patients and mouse models (Boon et al. 1997; Peterson et al. 2003). However, multiple evasion mechanisms in tumor and stromal cells reduce immune function, causing a miscarriage of tumor rejection by effector immune cells

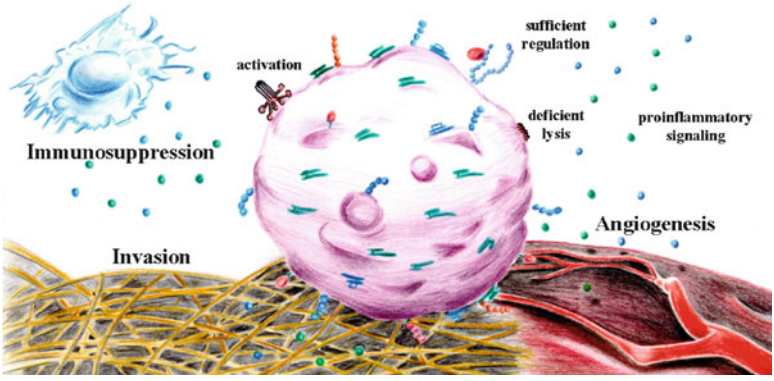


**Fig. 11.5** Potential tumor-promoting roles of complement proteins in the tumor microenvironment

(Umansky and Sevko 2013). Tumor-derived immunosuppressive mechanisms can be summarized as the downregulation (or loss) of major histocompatibility complex class I molecules, tumor-associated antigens, or danger signals and as the secretion of immunosuppressive factors such as vascular endothelial growth factor (VEGF), TGF-β, IL-10, reactive oxygen species, and prostaglandins (Kim et al. 2006). Immunosuppression is orchestrated by cells of lymphoid and myeloid origin that are recruited and activated in the tumor microenvironment. These immunosuppressive cells include regulatory T cells, TAMs, regulatory/tolerogenic dendritic cells, and myeloid-derived suppressor cells (MDSCs) (Zou 2005). Recent studies have linked complement activation to the induction of a suppressive immune response. Differentiation of regulatory T cells is correlated with the C5a concentration within the tumor (Gunn et al. 2012). It also has been proposed that the generation of inducible regulatory T cells can be mediated by co-engagement of CD3 and the complement regulator CD46 in the presence of IL-2 (Kemper et al. 2003). Nevertheless, the effect of this complement receptor (highly expressed on lymphocytes) on the generation of inducible regulatory T cells within tumors has not yet been demonstrated. On the other hand, several studies have emphasized the pivotal role of MDSCs in tumor immunosuppression (Gabrilovich and Nagaraj 2009; Ostrand-Rosenberg and Sinha 2009). Like their mature counterparts (monocytes and neutrophils), MDSCs also respond to C5a anaphylatoxin. C5a released as a result of complement activation on tumor cells is connected to the recruitment and activation of MDSCs into tumors (Markiewski et al. 2008). Among the various mechanisms used by MDSCs to inhibit T-cell function, the production and release of reactive oxygen and nitrogen species seem to be critical for their suppressive capabilities. C5a may play a key role as a chemoattractant for a subpopulation of MDSCs that is morphologically related to neutrophils (polymorphonuclear MDSCs) and as an activator of the production of reactive oxygen and nitrogen species in the monocyte-like subpopulation (Markiewski et al. 2008). The role of C5a in the immunosuppressive function of MDSCs was confirmed ex vivo when isolated MDSCs from C5aR-deficient mice were unable to inhibit T-cell proliferation (Markiewski et al. 2008). Moreover, in a lung cancer mouse model, the blockade of C5a signaling downregulated the expression of key immunosuppressive molecules within the tumors. These molecules included ARG1, IL-10, IL-6, CTLA4, LAG3, and PDL1 (Corrales et al. 2012). All these studies suggest that C5a can suppress the T-cell-mediated antitumor response by promoting an immunosuppressive microenvironment and recruiting regulatory T cells and MDSCs into the tumor.

### *11.5.2 Complement and Angiogenesis*

Angiogenesis, the creation of new vessels from preexisting ones, is a key mechanism of carcinogenesis that is directly related to the aggressiveness of the tumor (Carmeliet 2003). Complement-activated factors have been related, either directly or indirectly, to neovascularization in several diseases. Because of the heterogeneity

of the studies and diseases examined, there is some controversy about the pro- or anti-angiogenic role of the complement system in neovascularization.

An anti-angiogenic effect of C3 and C5 was observed in a model of retinopathy of prematurity, in which C5a stimulated macrophages toward an angiogenesis-inhibitory phenotype and induced the secretion of the anti-angiogenic soluble VEGF receptor-1 (Langer et al. 2010). This anti-angiogenic factor also was upregulated in monocytes by complement activation products in an antibody-independent model of spontaneous miscarriage and intrauterine growth restriction (Girardi et al. 2006). The soluble receptor was able to sequester circulating VEGF and placental growth factor, altering the balance of angiogenic factors in pregnancy (Girardi et al. 2006).

In contrast, a role for complement in the activation of angiogenesis has been demonstrated in age-related macular degeneration, a disease caused by choroidal neovascularization. Both C3a and C5a are present in the lipoproteinaceous deposits, also called drusen, that appear between the choroid and the retinal pigmented epithelium in patients with age-related macular degeneration and in animals with laser-induced choroidal neovascularization (Nozaki et al. 2006). Both anaphylatoxins seem to be involved in the induction of VEGF expression in retinal pigmented epithelium cells, thereby promoting the generation of new vessels. In addition, MAC and C3 are deposited in the eyes of animals with laser-induced choroidal neovascularization, concomitant with an increase in the expression of the angiogenic factors VEGF, TGF-β2, and basic fibroblast growth factor (Bora et al. 2005). In a mouse model of epithelial ovarian cancer, a genetic C3 deficiency impaired tumor vascularization by altering the function of endothelial cells (Nunez-Cruz et al. 2012). However, in end point tumor specimens in the murine TC-1 cervical cancer model, C5aR blockade did not impair tumor angiogenesis (Markiewski et al. 2008). These results suggest that complement activation may be important in the promotion of angiogenesis only during the early steps of tumor formation. In vitro studies using endothelial cells support this conclusion. C5a stimulates chemotaxis and the formation of tube-like structures in gelled Matrigel in both human umbilical endothelial cells (Corrales et al. 2012; Schraufstatter et al. 2002) and human microvascular endothelial cells (Nunez-Cruz et al. 2012). Like the response to TNF-α and lipopolysaccharide, the endothelial cell response to C5a involves the activation of genes that participate in endothelial adhesion, migration, and angiogenesis (Albrecht et al. 2004).

### *11.5.3 Complement and Tumor Cell Signaling*

Various complement factors have been linked to the activation of signaling pathways in tumor cells. Deposition of C5b-9 has been demonstrated in various human malignancies (Vlaicu et al. 2013). However, as mentioned earlier, tumor cells acquire resistance to complement attack, leading to the deposition of sublytic doses of the MAC on the cell membrane. Whereas a lytic dose of MAC is detrimental to cells because it induces an influx of $Ca^{2+}$, mitochondrial damage, and adenosine triphosphate depletion (Kim et al. 1987), sublytic doses of the MAC play a role in

cell activation, proliferation, differentiation and the inhibition of apoptosis (Tegla et al. 2011). These effects may be a result of the regulation of cell cycle genes activated by the phosphoinositide 3-kinase/Akt and extracellular signal-regulated kinase (ERK) 1 pathways (Vlaicu et al. 2013). Sublytic MACs activate several prooncogenic pathways such as the mitogen-activated protein kinase family of proteins, ERKs, p38 mitogen-activated protein kinases, and Jun N-terminal kinases (Kraus et al. 2001); the phosphatidylinositol 3-kinase pathway (Niculescu et al. 1999); Ras (Niculescu et al. 1997); p70 S6 kinase; and the Janus kinase/signal transducers and activators of transcription pathway (Niculescu et al. 1999). C5b-9 also inhibits apoptosis by inducing the phosphorylation of Bad and blocking the activation of FLIP, caspase-8, and Bid (Tegla et al. 2011). Several genes are regulated by sublytic doses of complement in oligodendrocytes (Badea et al. 1998). Among these genes, designated response genes to complement (RGCs), is RGC-32, which was shown to bind and increase the kinase activity of CDC2/cyclin B1 and thus regulate the cell cycle (Badea et al. 2002). It is likely that RGC-32 is involved in cell proliferation in vivo because it is overexpressed in malignant tumors of the human colon, kidney, stomach, and ovary (Fosbrink et al. 2005). Finally, some complement-activated factors have been linked to the production of growth factors and cytokines that support neoplastic transformation. Signaling via C3aR and C5aR has been shown to be necessary for the survival of liver cells after partial hepatectomy through the induction of the cytokines IL-6 and TNF-α, both of which are necessary for liver regeneration and (in the case of IL-6) hepatoprotection (Markiewski et al. 2009). Moreover, in several animal models of central nervous system pathology, C5a has been shown to mediate neuroprotection and exert an antiapoptotic effect (Mukherjee and Pasinetti 2001; Mukherjee et al. 2008). Overall, it is becoming more evident that complement factors can trigger oncogenic pathways, establishing the basis for the use of complement inhibitors in the treatment of cancer.

### *11.5.4 Complement and Tumor Cell Invasion and Migration*

Metastasis is estimated to be the responsible for ~90 % of cancer deaths. This multistep process involves genetic and molecular changes in tumor and stromal cells, leading to local invasion, intravasation into the tumor vasculature, transit within the blood, extravasation into secondary sites, and, finally, the formation of metastases (Hanahan and Weinberg 2011).

Complement proteins can participate in some of the processes that orchestrate invasion and metastasis. One of the key events in this process is the generation of mesenchymal derivatives from epithelial phenotypes, also called the epithelial-to-mesenchymal transition (EMT). Activation of an EMT program during tumorigenesis often requires signaling between cancer and stromal cells such as fibroblasts, myofibroblasts, granulocytes, macrophages, mesenchymal stem cells, and lymphocytes. These cells create a “reactive” stroma that seems to result in the release of EMT-inducing signals (Chaffer and Weinberg 2011). Complement activation by

tumor cells releases anaphylatoxins that can recruit stromal cells to the tumor. C3a and C5a have been shown to promote the chemotaxis of human bone marrow–derived mesenchymal stem cells and robustly activate ERK1/2 and Akt (Schraufstatter et al. 2009). In the development of tubulointerstitial injury, C3a release seems to induce the EMT, at least partially in response to a decrease in the expression of E-cadherin (Tang et al. 2009). It is well documented that the expression of E-cadherin antagonizes invasion and metastasis, whereas a decrease in its expression has the opposite effect (Hanahan and Weinberg 2011).

Complement proteins can also promote the degradation of the extracellular matrix (Rutkowski et al. 2010b). Although C1s can directly degrade collagens and gelatin in human cartilage, complement activation can also induce the release and activation of proteases such as matrix metalloproteinase (MMP)-2 and -9 (Bandyopadhyay and Rohrer 2012). In particular, C5a signaling through C5aR promotes the release of MMP-9 by macrophages (Gonzalez et al. 2011). Proteases can, conversely, inactivate complement proteins, protecting tumor cells from complement attack. Overexpression of MMP-1 in a murine melanoma cell line protected these cells from the damaging effects of complement and promoted the formation of lung metastasis in vivo (Rozanov et al. 2006). In melanoma cells, the overexpression of procathepsin-L, another protease with anticomplement capacity, similarly increased the tumorigenicity of the cells and switched their phenotype from nonmetastatic to highly metastatic (Frade et al. 1998). Therefore, the context-dependent interaction between matrix proteases and complement activation illustrates once again the duality in the relationship between cancer and the complement system.

## 11.6 Concluding Remarks

An anticancer function for complement is well illustrated by its contribution to the clinical efficacy of monoclonal antibodies for the treatment of neoplasias. However, the biological functions of the complement system are much more diverse than a simple elimination of target cells. In fact, complement recognition of cancer cells may be an element of immunosurveillance, with complement taking part in the elimination of tumors and at the same time serving as a force for immunoselection. This idea is entirely consistent with our growing recognition of the homeostatic function of the complement system. Furthermore, in the context of chronic inflammation, complement elements can promote tumor growth. Recent reports of the role of complement activation in the pathogenesis of cancer stress this duality. The expression of immune modulators in the tumor microenvironment dictates the balance between antitumor and tumor-promoting complement activities. It is clinically evident that when complement fails to protect an organism from growing tumors, this balance is tilted toward protumor inflammation. However, to date, we still have only fragmentary knowledge concerning the interplay between complement activation and tumor cells. We need to both identify those tumor-associated antigens that are able to stimulate complement and better understand the intricate mechanisms of activation and resistance. These studies will permit the development of new

therapeutic strategies for cancer that are aimed at modulating this interaction and enhancing immunologically based cancer therapies. Additional in vivo models are needed to validate the strategy of using complement inhibitors to treat cancer.

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## References

Abbott RJ, Spendlove I, Roversi P et al (2007) Structural and functional characterization of a novel T cell receptor co-regulatory protein complex, CD97-CD55. J Biol Chem 282(30): 22023–22032

Ajona D, Castano Z, Garayoa M et al (2004) Expression of complement factor H by lung cancer cells: effects on the activation of the alternative pathway of complement. Cancer Res 64(17):6310–6318

Ajona D, Hsu YF, Corrales L et al (2007) Down-regulation of human complement factor H sensitizes non-small cell lung cancer cells to complement attack and reduces in vivo tumor growth. J Immunol 178(9):5991–5998

Albrecht EA, Chinnaiyan AM, Varambally S et al (2004) C5a-Induced gene expression in human umbilical vein endothelial cells. Am J Pathol 164(3):849–859

Amara U, Rittirsch D, Flierl M et al (2008) Interaction between the coagulation and complement system. Adv Exp Med Biol 632:71–79

Andoh A, Shimada M, Araki Y et al (2002) Sodium butyrate enhances complement-mediated cell injury via down-regulation of decay-accelerating factor expression in colonic cancer cells. Cancer Immunol Immunother 50(12):663–672

Aspord C, Pedroza-Gonzalez A, Gallegos M et al (2007) Breast cancer instructs dendritic cells to prime interleukin 13-secreting CD4+ T cells that facilitate tumor development. J Exp Med 204(5):1037–1047

Baatrup G, Qvist N, Junker A et al (1994) Activity and activation of the complement system in patients being operated on for cancer of the colon. Eur J Surg 160(9):503–510

Badea TC, Niculescu FI, Soane L et al (1998) Molecular cloning and characterization of RGC-32, a novel gene induced by complement activation in oligodendrocytes. J Biol Chem 273(41):26977–26981

Badea T, Niculescu F, Soane L et al (2002) RGC-32 increases p34CDC2 kinase activity and entry of aortic smooth muscle cells into S-phase. J Biol Chem 277(1):502–508

Balkwill F, Mantovani A (2001) Inflammation and cancer: back to Virchow? Lancet 357(9255):539–545

Bandyopadhyay M, Rohrer B (2012) Matrix metalloproteinase activity creates pro-angiogenic environment in primary human retinal pigment epithelial cells exposed to complement. Invest Ophthalmol Vis Sci 53(4):1953–1961

Battistelli S, Vittoria A, Cappelli R et al (2005) Protein S in cancer patients with non-metastatic solid tumours. Eur J Surg Oncol 31(7):798–802

Bauer S, Groh V, Wu J et al (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285(5428):727–729

Bellone S, Roque D, Cocco E et al (2012) Down regulation of membrane complement inhibitors CD55 and CD59 by siRNA sensitises uterine serous carcinoma overexpressing Her2/neu to complement and antibody-dependent cell cytotoxicity in vitro: implications for Trastuzumab-based immunotherapy. Br J Cancer 106(9):1543–1550

Benard M, Gonzalez BJ, Schouft MT et al (2004) Characterization of C3a and C5a receptors in rat cerebellar granule neurons during maturation. Neuroprotective effect of C5a against apoptotic cell death. J Biol Chem 279(42):43487–43496

Beum PV, Lindorfer MA, Beurskens F et al (2008) Complement activation on B lymphocytes opsonized with rituximab or ofatumumab produces substantial changes in membrane structure preceding cell lysis. J Immunol 181(1):822–832

Beurskens FJ, Lindorfer MA, Farooqui M et al (2012) Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. J Immunol 188(7):3532–3541

Bexborn F, Andersson PO, Chen H et al (2008) The tick-over theory revisited: formation and regulation of the soluble alternative complement C3 convertase (C3(H2O)Bb). Mol Immunol 45(8):2370–2379

Bjorge L, Hakulinen J, Vintermyr OK et al (2005) Ascitic complement system in ovarian cancer. Br J Cancer 92(5):895–905

Blok VT, Gelderman KA, Tijsma OH et al (2003) Cytokines affect resistance of human renal tumour cells to complement-mediated injury. Scand J Immunol 57(6):591–599

Blom AM, Villoutreix BO, Dahlback B (2004) Complement inhibitor C4b-binding protein-friend or foe in the innate immune system? Mol Immunol 40(18):1333–1346

Bohana-Kashtan O, Pinna LA, Fishelson Z (2005) Extracellular phosphorylation of C9 by protein kinase CK2 regulates complement-mediated lysis. Eur J Immunol 35(6):1939–1948

Boon T, Coulie PG, Van den Eynde B (1997) Tumor antigens recognized by T cells. Immunol Today 18(6):267–268

Bora PS, Sohn JH, Cruz JM et al (2005) Role of complement and complement membrane attack complex in laser-induced choroidal neovascularization. J Immunol 174(1):491–497

Boulay F, Mery L, Tardif M et al (1991) Expression cloning of a receptor for C5a anaphylatoxin on differentiated HL-60 cells. Biochemistry 30(12):2993–2999

Brasoveanu LI, Altomonte M, Fonsatti E et al (1996) Levels of cell membrane CD59 regulate the extent of complement-mediated lysis of human melanoma cells. Lab Invest 74(1):33–42

Brasoveanu LI, Fonsatti E, Visintin A et al (1997) Melanoma cells constitutively release an anchor-positive soluble form of protectin (sCD59) that retains functional activities in homologous complement-mediated cytotoxicity. J Clin Invest 100(5):1248–1255

Bu X, Zheng Z, Wang C et al (2007) Significance of C4d deposition in the follicular lymphoma and MALT lymphoma and their relationship with follicular dendritic cells. Pathol Res Pract 203(3):163–167

Budzko DB, Lachmann PJ, McConnell I (1976) Activation of the alternative complement pathway by lymphoblastoid cell lines derived from patients with Burkitt's lymphoma and infectious mononucleosis. Cell Immunol 22(1):98–109

Buo L, Karlsrud TS, Dyrhaug G et al (1993) Differential diagnosis of human ascites: inhibitors of the contact system and total proteins. Scand J Gastroenterol 28(9):777–782

Cardarelli PM, Quinn M, Buckman D et al (2002) Binding to CD20 by anti-B1 antibody or F(ab') (2) is sufficient for induction of apoptosis in B-cell lines. Cancer Immunol Immunother 51(1):15–24

Carli M, Bucolo C, Pannunzio MT et al (1979) Fluctuation of serum complement levels in children with neuroblastoma. Cancer 43(6):2399–2404

Carmeliet P (2003) Angiogenesis in health and disease. Nat Med 9(6):653–660

Carney DF, Lang TJ, Shin ML (1990) Multiple signal messengers generated by terminal complement complexes and their role in terminal complement complex elimination. J Immunol 145(2):623–629

Chaffer CL, Weinberg RA (2011) A perspective on cancer cell metastasis. Science 331(6024):1559–1564

Chen S, Caragine T, Cheung NK et al (2000) CD59 expressed on a tumor cell surface modulates decay-accelerating factor expression and enhances tumor growth in a rat model of human neuroblastoma. Cancer Res 60(11):3013–3018

Chen NJ, Mirtsos C, Suh D et al (2007) C5L2 Is critical for the biological activities of the anaphylatoxins C5a and C3a. Nature 446(7132):203–207
Cheng ZZ, Corey MJ, Parepalo M et al (2005) Complement factor H as a marker for detection of bladder cancer. Clin Chem 51(5):856–863
Cole DS, Morgan BP (2003) Beyond lysis: how complement influences cell fate. Clin Sci (Lond) 104(5):455–466
Colotta F, Allavena P, Sica A et al (2009) Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30(7):1073–1081
Coral S, Fonsatti E, Sigalotti L et al (2000) Overexpression of protectin (CD59) down-modulates the susceptibility of human melanoma cells to homologous complement. J Cell Physiol 185(3):317–323
Corey MJ, Kinders RJ, Brown LG et al (1997) A very sensitive coupled luminescent assay for cytotoxicity and complement-mediated lysis. J Immunol Methods 207(1):43–51
Corrales L, Ajona D, Rafail S et al (2012) Anaphylatoxin c5a creates a favorable microenvironment for lung cancer progression. J Immunol 189(9):4674–4683
Costello LC, Franklin RB (2005) 'Why do tumour cells glycolyse?': from glycolysis through citrate to lipogenesis. Mol Cell Biochem 280(1–2):1–8
Cragg MS, Glennie MJ (2004) Antibody specificity controls in vivo effector mechanisms of anti-CD20 reagents. Blood 103(7):2738–2743
Cui T, Chen Y, Knosel T et al (2011) Human complement factor H is a novel diagnostic marker for lung adenocarcinoma. Int J Oncol 39(1):161–168
Curiel TJ, Coukos G, Zou L et al (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10(9):942–949
Davis AE 3rd, Mejia P, Lu F (2008) Biological activities of C1 inhibitor. Mol Immunol 45(16):4057–4063
de Cordoba SR, de Jorge EG (2008) Translational mini-review series on complement factor H: genetics and disease associations of human complement factor H. Clin Exp Immunol 151(1):1–13
Di Gaetano N, Xiao Y, Erba E et al (2001) Synergism between fludarabine and rituximab revealed in a follicular lymphoma cell line resistant to the cytotoxic activity of either drug alone. Br J Haematol 114(4):800–809
Di Gaetano N, Cittera E, Nota R et al (2003) Complement activation determines the therapeutic activity of rituximab in vivo. J Immunol 171(3):1581–1587
Donin N, Jurianz K, Ziporen L et al (2003) Complement resistance of human carcinoma cells depends on membrane regulatory proteins, protein kinases and sialic acid. Clin Exp Immunol 131(2):254–263
Dunn GP, Old LJ, Schreiber RD (2004a) The three Es of cancer immunoediting. Annu Rev Immunol 22:329–360
Dunn GP, Old LJ, Schreiber RD (2004b) The immunobiology of cancer immunosurveillance and immunoediting. Immunity 21(2):137–148
DuPage M, Mazumdar C, Schmidt LM et al (2012) Expression of tumour-specific antigens underlies cancer immunoediting. Nature 482(7385):405–409
Durrant LG, Chapman MA, Buckley DJ et al (2003) Enhanced expression of the complement regulatory protein CD55 predicts a poor prognosis in colorectal cancer patients. Cancer Immunol Immunother 52(10):638–642
Elvington M, Huang Y, Morgan BP et al (2012) A targeted complement-dependent strategy to improve the outcome of mAb therapy, and characterization in a murine model of metastatic cancer. Blood 119(25):6043–6051
Falgarone G, Chiocchia G (2009) Chapter 8: clusterin: a multifacet protein at the crossroad of inflammation and autoimmunity. Adv Cancer Res 104:139–170
Fearon DT (1979) Regulation of the amplification C3 convertase of human complement by an inhibitory protein isolated from human erythrocyte membrane. Proc Natl Acad Sci U S A 76(11):5867–5871

Fearon DT, Carter RH (1995) The CD19/CR2/TAPA-1 complex of B lymphocytes: linking natural to acquired immunity. Annu Rev Immunol 13:127–149
Fedarko NS, Fohr B, Robey PG et al (2000) Factor H binding to bone sialoprotein and osteopontin enables tumor cell evasion of complement-mediated attack. J Biol Chem 275(22):16666–16672
Fischer E, Appay MD, Cook J et al (1986) Characterization of the human glomerular C3 receptor as the C3b/C4b complement type one (CR1) receptor. J Immunol 136(4):1373–1377
Fishelson Z, Donin N, Zell S et al (2003) Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Mol Immunol 40(2–4):109–123
Fosbrink M, Cudrici C, Niculescu F et al (2005) Overexpression of RGC-32 in colon cancer and other tumors. Exp Mol Pathol 78(2):116–122
Frade R, Rodrigues-Lima F, Huang S et al (1998) Procathepsin-L, a proteinase that cleaves human C3 (the third component of complement), confers high tumorigenic and metastatic properties to human melanoma cells. Cancer Res 58(13):2733–2736
Fridman WH, Pages F, Sautes-Fridman C et al (2012) The immune contexture in human tumours: impact on clinical outcome. Nat Rev Cancer 12(4):298–306
Fust G, Miszlay Z, Czink E et al (1987) C1 and C4 abnormalities in chronic lymphocytic leukaemia and their significance. Immunol Lett 14(3):255–259
Gabrilovich DI, Nagaraj S (2009) Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 9(3):162–174
Galon J, Costes A, Sanchez-Cabo F et al (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313(5795):1960–1964
Gancz D, Fishelson Z (2009) Cancer resistance to complement-dependent cytotoxicity (CDC): problem-oriented research and development. Mol Immunol 46(14):2794–2800
Gao LJ, Guo SY, Cai YQ et al (2009) Cooperation of decay-accelerating factor and membrane cofactor protein in regulating survival of human cervical cancer cells. BMC Cancer 9:384
Gasque P, Thomas A, Fontaine M et al (1996) Complement activation on human neuroblastoma cell lines in vitro: route of activation and expression of functional complement regulatory proteins. J Neuroimmunol 66(1–2):29–40
Geis N, Zell S, Rutz R et al (2010) Inhibition of membrane complement inhibitor expression (CD46, CD55, CD59) by siRNA sensitizes tumor cells to complement attack in vitro. Curr Cancer Drug Targets 10(8):922–931
Gelderman KA, Blok VT, Fleuren GJ et al (2002a) The inhibitory effect of CD46, CD55, and CD59 on complement activation after immunotherapeutic treatment of cervical carcinoma cells with monoclonal antibodies or bispecific monoclonal antibodies. Lab Invest 82(4):483–493
Gelderman KA, Kuppen PJ, Bruin W et al (2002b) Enhancement of the complement activating capacity of 17-1A mAb to overcome the effect of membrane-bound complement regulatory proteins on colorectal carcinoma. Eur J Immunol 32(1):128–135
Gelderman KA, Kuppen PJ, Okada N et al (2004a) Tumor-specific inhibition of membrane-bound complement regulatory protein crry with bispecific monoclonal antibodies prevents tumor outgrowth in a rat colorectal cancer lung metastases model. Cancer Res 64(12):4366–4372
Gelderman KA, Tomlinson S, Ross GD et al (2004b) Complement function in mAb-mediated cancer immunotherapy. Trends Immunol 25(3):158–164
Gelderman KA, Lam S, Gorter A (2005) Inhibiting complement regulators in cancer immunotherapy with bispecific mAbs. Expert Opin Biol Ther 5(12):1593–1601
Ghiran I, Barbashov SF, Klickstein LB et al (2000) Complement receptor 1/CD35 is a receptor for mannan-binding lectin. J Exp Med 192(12):1797–1808
Girardi G, Yarilin D, Thurman JM et al (2006) Complement activation induces dysregulation of angiogenic factors and causes fetal rejection and growth restriction. J Exp Med 203(9):2165–2175
Glunde K, Serkova NJ (2006) Therapeutic targets and biomarkers identified in cancer choline phospholipid metabolism. Pharmacogenomics 7(7):1109–1123
Gminski J, Mykala-Ciesla J, Machalski M et al (1992) Immunoglobulins and complement components levels in patients with lung cancer. Rom J Intern Med 30(1):39–44
Golay J, Zaffaroni L, Vaccari T et al (2000) Biologic response of B lymphoma cells to anti-CD20 monoclonal antibody rituximab in vitro: CD55 and CD59 regulate complement-mediated cell lysis. Blood 95(12):3900–3908

Gonzalez JM, Franzke CW, Yang F et al (2011) Complement activation triggers metalloproteinases release inducing cervical remodeling and preterm birth in mice. Am J Pathol 179(2):838–849
Gorter A, Meri S (1999) Immune evasion of tumor cells using membrane-bound complement regulatory proteins. Immunol Today 20(12):576–582
Governa M, Fenoglio I, Amati M et al (2002) Cleavage of the fifth component of human complement and release of a split product with C5a-like activity by crystalline silica through free radical generation and kallikrein activation. Toxicol Appl Pharmacol 179(3):129–136
Griffin JL, Kauppinen RA (2007) Tumour metabolomics in animal models of human cancer. J Proteome Res 6(2):498–505
Grivennikov SI, Greten FR, Karin M (2010) Immunity, inflammation, and cancer. Cell 140(6):883–899
Guidi L, Baroni R, Bartoloni C et al (1988) Immune complexes in solid tumours precipitable by 3.5% Polyethylene glycol: analysis of some nonspecific components. Diagn Clin Immunol 5(6):284–288
Gunn L, Ding C, Liu M et al (2012) Opposing roles for complement component C5a in tumor progression and the tumor microenvironment. J Immunol 189(6):2985–2994
Haas PJ, van Strijp J (2007) Anaphylatoxins: their role in bacterial infection and inflammation. Immunol Res 37(3):161–175
Hakomori S (2002) Glycosylation defining cancer malignancy: new wine in an old bottle. Proc Natl Acad Sci U S A 99(16):10231–10233
Hakulinen J, Junnikkala S, Sorsa T et al (2004) Complement inhibitor membrane cofactor protein (MCP; CD46) is constitutively shed from cancer cell membranes in vesicles and converted by a metalloproteinase to a functionally active soluble form. Eur J Immunol 34(9):2620–2629
Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5):646–674
Hawrylowicz CM, O'Garra A (2005) Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. Nat Rev Immunol 5(4):271–283
He JQ, Wiesmann C, van Lookeren CM (2008) A role of macrophage complement receptor CRIg in immune clearance and inflammation. Mol Immunol 45(16):4041–4047
Helmy KY, Katschke KJ Jr, Gorgani NN et al (2006) CRIg: a macrophage complement receptor required for phagocytosis of circulating pathogens. Cell 124(5):915–927
Hindmarsh EJ, Marks RM (1998) Decay-accelerating factor is a component of subendothelial extracellular matrix in vitro, and is augmented by activation of endothelial protein kinase C. Eur J Immunol 28(3):1052–1062
Holliger P, Wing M, Pound JD et al (1997) Retargeting serum immunoglobulin with bispecific diabodies. Nat Biotechnol 15(7):632–636
Hollingsworth MA, Swanson BJ (2004) Mucins in cancer: protection and control of the cell surface. Nat Rev Cancer 4(1):45–60
Holmberg MT, Blom AM, Meri S (2001) Regulation of complement classical pathway by association of C4b-binding protein to the surfaces of SK-OV-3 and caov-3 ovarian adenocarcinoma cells. J Immunol 167(2):935–939
Hourcade DE (2008) Properdin and complement activation: a fresh perspective. Curr Drug Targets 9(2):158–164
Hsu YF, Ajona D, Corrales L et al (2010) Complement activation mediates cetuximab inhibition of non-small cell lung cancer tumor growth in vivo. Mol Cancer 9:139
Huber-Lang M, Younkin EM, Sarma JV et al (2002) Generation of C5a by phagocytic cells. Am J Pathol 161(5):1849–1859
Huber-Lang M, Sarma JV, Zetoune FS et al (2006) Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med 12(6):682–687
Jain A, Karadag A, Fohr B et al (2002) Three SIBLINGs (small integrin-binding ligand, N-linked glycoproteins) enhance factor H's cofactor activity enabling MCP-like cellular evasion of complement-mediated attack. J Biol Chem 277(16):13700–13708
Jarvis GA, Li J, Hakulinen J et al (1997) Expression and function of the complement membrane attack complex inhibitor protectin (CD59) in human prostate cancer. Int J Cancer 71(6):1049–1055

Jenne DE, Tschopp J (1989) Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. Proc Natl Acad Sci U S A 86(18):7123–7127

Jiang H, Cooper B, Robey FA et al (1992) DNA binds and activates complement via residues 14–26 of the human C1q a chain. J Biol Chem 267(35):25597–25601

Jozsi M, Zipfel PF (2008) Factor H family proteins and human diseases. Trends Immunol 29(8):380–387

Juhl H, Petrella EC, Cheung NK et al (1990) Complement killing of human Neuroblastoma cells: a cytotoxic monoclonal antibody and its F(ab')2-cobra venom factor conjugate are equally cytotoxic. Mol Immunol 27(10):957–964

Juhl H, Sievers M, Baltzer K et al (1995) A monoclonal antibody-cobra venom factor conjugate increases the tumor-specific uptake of a 99mTc-labeled anti-carcinoembryonic antigen antibody by a two-step approach. Cancer Res 55(23 Suppl):5749s–5755s

Juhl H, Petrella EC, Cheung NK et al (1997) Additive cytotoxicity of different monoclonal antibody-cobra venom factor conjugates for human Neuroblastoma cells. Immunobiology 197(5):444–459

Junnikkala S, Jokiranta TS, Friese MA et al (2000) Exceptional resistance of human H2 glioblastoma cells to complement-mediated killing by expression and utilization of factor H and factor H-like protein 1. J Immunol 164(11):6075–6081

Junnikkala S, Hakulinen J, Jarva H et al (2002) Secretion of soluble complement inhibitors factor H and factor H-like protein (FHL-1) by ovarian tumour cells. Br J Cancer 87(10):1119–1127

Jurianz K, Ziegler S, Garcia-Schuler H et al (1999) Complement resistance of tumor cells: basal and induced mechanisms. Mol Immunol 36(13–14):929–939

Jurianz K, Ziegler S, Donin N et al (2001) K562 Erythroleukemic cells are equipped with multiple mechanisms of resistance to lysis by complement. Int J Cancer 93(6):848–854

Kalwinsky DK, Urmson JR, Stitzel AE et al (1976) Activation of the alternative pathway of complement in childhood acute lymphoblastic leukemia. J Lab Clin Med 88(5):745–756

Karre K, Ljunggren HG, Piontek G et al (1986) Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature 319(6055):675–678

Kataki A, Scheid P, Piet M et al (2002) Tumor infiltrating lymphocytes and macrophages have a potential dual role in lung cancer by supporting both host-defense and tumor progression. J Lab Clin Med 140(5):320–328

Kawada M, Mizuno M, Nasu J et al (2003) Release of decay-accelerating factor into stools of patients with colorectal cancer by means of cleavage at the site of glycosylphosphatidylinositol anchor. J Lab Clin Med 142(5):306–312

Kemper C, Chan AC, Green JM et al (2003) Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. Nature 421(6921):388–392

Kennedy AD, Solga MD, Schuman TA et al (2003) An anti-C3b(i) mAb enhances complement activation, C3b(i) deposition, and killing of CD20+ cells by rituximab. Blood 101(3):1071–1079

Kim SH, Carney DF, Hammer CH et al (1987) Nucleated cell killing by complement: effects of C5b-9 channel size and extracellular Ca2+ on the lytic process. J Immunol 138(5):1530–1536

Kim R, Emi M, Tanabe K et al (2006) Tumor-driven evolution of immunosuppressive networks during malignant progression. Cancer Res 66(11):5527–5536

Kimberley FC, Sivasankar B, Paul Morgan B (2007) Alternative roles for CD59. Mol Immunol 44(1–3):73–81

Kinders R, Jones T, Root R et al (1998) Complement factor H or a related protein is a marker for transitional cell cancer of the bladder. Clin Cancer Res 4(10):2511–2520

Kirkitadze MD, Barlow PN (2001) Structure and flexibility of the multiple domain proteins that regulate complement activation. Immunol Rev 180:146–161

Klickstein LB, Barbashov SF, Liu T et al (1997) Complement receptor type 1 (CR1, CD35) is a receptor for C1q. Immunity 7(3):345–355

Kohl J (2001) Anaphylatoxins and infectious and non-infectious inflammatory diseases. Mol Immunol 38(2–3):175–187

Kohno H, Mizuno M, Nasu J et al (2005) Stool decay-accelerating factor as a marker for monitoring the disease activity during leukocyte apheresis therapy in patients with refractory ulcerative colitis. J Gastroenterol Hepatol 20(1):73–78
Kojouharova M, Reid K, Gadjeva M (2010) New insights into the molecular mechanisms of classical complement activation. Mol Immunol 47(13):2154–2160
Kolev M, Towner L, Donev R (2011) Complement in cancer and cancer immunotherapy. Arch Immunol Ther Exp (Warsz) 59(6):407–419
Kraus S, Seger R, Fishelson Z (2001) Involvement of the ERK mitogen-activated protein kinase in cell resistance to complement-mediated lysis. Clin Exp Immunol 123(3):366–374
Kraut EH, Sagone AL Jr (1981) Alternative pathway of complement in multiple myeloma. Am J Hematol 11(4):335–345
Krych-Goldberg M, Atkinson JP (2001) Structure-function relationships of complement receptor type 1. Immunol Rev 180:112–122
Laghi L, Bianchi P, Miranda E et al (2009) CD3+ Cells at the invasive margin of deeply invading (pT3-T4) colorectal cancer and risk of post-surgical metastasis: a longitudinal study. Lancet Oncol 10(9):877–884
Langer HF, Chung KJ, Orlova VV et al (2010) Complement-mediated inhibition of neovascularization reveals a point of convergence between innate immunity and angiogenesis. Blood 116(22):4395–4403
Law SK, Dodds AW (1997) The internal thioester and the covalent binding properties of the complement proteins C3 and C4. Protein Sci 6(2):263–274
Lewis CE, Pollard JW (2006) Distinct role of macrophages in different tumor microenvironments. Cancer Res 66(2):605–612
Li L, Spendlove I, Morgan J et al (2001) CD55 is over-expressed in the tumour environment. Br J Cancer 84(1):80–86
Liszewski MK, Post TW, Atkinson JP (1991) Membrane cofactor protein (MCP or CD46): newest member of the regulators of complement activation gene cluster. Annu Rev Immunol 9:431–455
Liu J, Miwa T, Hilliard B et al (2005) The complement inhibitory protein DAF (CD55) suppresses T cell immunity in vivo. J Exp Med 201(4):567–577
Liu L, Li W, Li Z et al (2012) Sublytic complement protects prostate cancer cells from tumour necrosis factor-alpha-induced cell death. Clin Exp Immunol 169(2):100–108
Livingston PO, Hood C, Krug LM et al (2005) Selection of GM2, fucosyl GM1, globo H and polysialic acid as targets on small cell lung cancers for antibody mediated immunotherapy. Cancer Immunol Immunother 54(10):1018–1025
Loberg RD, Wojno KJ, Day LL et al (2005) Analysis of membrane-bound complement regulatory proteins in prostate cancer. Urology 66(6):1321–1326
Lublin DM, Atkinson JP (1989) Decay-accelerating factor: biochemistry, molecular biology, and function. Annu Rev Immunol 7:35–58
Lucas SD, Karlsson-Parra A, Nilsson B et al (1996) Tumor-specific deposition of immunoglobulin G and complement in papillary thyroid carcinoma. Hum Pathol 27(12):1329–1335
Macor P, Tedesco F (2007) Complement as effector system in cancer immunotherapy. Immunol Lett 111(1):6–13
Macor P, Mezzanzanica D, Cossetti C et al (2006) Complement activated by chimeric anti-folate receptor antibodies is an efficient effector system to control ovarian carcinoma. Cancer Res 66(7):3876–3883
Madjd Z, Durrant LG, Bradley R et al (2004) Loss of CD55 is associated with aggressive breast tumors. Clin Cancer Res 10(8):2797–2803
Madjd Z, Durrant LG, Pinder SE et al (2005) Do poor-prognosis breast tumours express membrane cofactor proteins (CD46)? Cancer Immunol Immunother 54(2):149–156
Maness PF, Orengo A (1977) Serum complement levels in patients with digestive tract carcinomas and other neoplastic diseases. Oncology 34(2):87–89
Mantovani A, Allavena P, Sica A et al (2008) Cancer-related inflammation. Nature 454(7203):436–444

Marie JC, Astier AL, Rivailler P et al (2002) Linking innate and acquired immunity: divergent role of CD46 cytoplasmic domains in T cell induced inflammation. Nat Immunol 3(7):659–666
Markiewski MM, Nilsson B, Ekdahl KN et al (2007) Complement and coagulation: strangers or partners in crime? Trends Immunol 28(4):184–192
Markiewski MM, DeAngelis RA, Benencia F et al (2008) Modulation of the antitumor immune response by complement. Nat Immunol 9(11):1225–1235
Markiewski MM, DeAngelis RA, Strey CW et al (2009) The regulation of liver cell survival by complement. J Immunol 182(9):5412–5418
Matsushita M, Thiel S, Jensenius JC et al (2000) Proteolytic activities of two types of mannose-binding lectin-associated serine protease. J Immunol 165(5):2637–2642
Matsushita H, Vesely MD, Koboldt DC et al (2012) Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. Nature 482(7385):400–404
Matsutani M, Suzuki T, Hori T et al (1984) Cellular immunity and complement levels in hosts with brain tumours. Neurosurg Rev 7(1):29–35
McConnell I, Klein G, Lint TF et al (1978) Activation of the alternative complement pathway by human B cell lymphoma lines is associated with Epstein-Barr virus transformation of the cells. Eur J Immunol 8(7):453–458
McGrath FD, Brouwer MC, Arlaud GJ et al (2006) Evidence that complement protein C1q interacts with C-reactive protein through its globular head region. J Immunol 176(5):2950–2957
McMullen ME, Hart ML, Walsh MC et al (2006) Mannose-binding lectin binds IgM to activate the lectin complement pathway in vitro and in vivo. Immunobiology 211(10):759–766
Medof ME, Walter EI, Rutgers JL et al (1987) Identification of the complement decay-accelerating factor (DAF) on epithelium and glandular cells and in body fluids. J Exp Med 165(3):848–864
Meri S, Morgan BP, Davies A et al (1990) Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. Immunology 71(1):1–9
Michlmayr A, Bachleitner-Hofmann T, Baumann S et al (2011) Modulation of plasma complement by the initial dose of Epirubicin/docetaxel therapy in breast cancer and its predictive value. Br J Cancer 103(8):1201–1208
Mitchell DA, Kirby L, Paulin SM et al (2007) Prion protein activates and fixes complement directly via the classical pathway: implications for the mechanism of scrapie agent propagation in lymphoid tissue. Mol Immunol 44(11):2997–3004
Miyagi T, Takahashi K, Moriya S et al (2012) Altered expression of sialidases in human cancer. Adv Exp Med Biol 749:257–267
Montuenga LM, Pio R (2007) Tumour-associated macrophages in nonsmall cell lung cancer: the role of interleukin-10. Eur Respir J 30(4):608–610
Moore GL, Chen H, Karki S et al (2010) Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions. MAbs 2(2):181–189
Morgan BP (1989) Complement membrane attack on nucleated cells: resistance, recovery and non-lethal effects. Biochem J 264(1):1–14
Morgan BP (1992) Effects of the membrane attack complex of complement on nucleated cells. Curr Top Microbiol Immunol 178:115–140
Morgan BP (1999) Regulation of the complement membrane attack pathway. Crit Rev Immunol 19(3):173–198
Morgan J, Spendlove I, Durrant LG (2002) The role of CD55 in protecting the tumour environment from complement attack. Tissue Antigens 60(3):213–223
Morris KM, Aden DP, Knowles BB et al (1982) Complement biosynthesis by the human hepatoma-derived cell line HepG2. J Clin Invest 70(4):906–913
Moskovich O, Fishelson Z (2007) Live cell imaging of outward and inward vesiculation induced by the complement c5b-9 complex. J Biol Chem 282(41):29977–29986
Mukherjee P, Pasinetti GM (2001) Complement anaphylatoxin C5a neuroprotects through mitogen-activated protein kinase-dependent inhibition of caspase 3. J Neurochem 77(1):43–49

Mukherjee P, Thomas S, Pasinetti GM (2008) Complement anaphylatoxin C5a neuroprotects through regulation of glutamate receptor subunit 2 in vitro and in vivo. J Neuroinflammation 5:5
Mustafa T, Klonisch T, Hombach-Klonisch S et al (2004) Expression of CD97 and CD55 in human medullary thyroid carcinomas. Int J Oncol 24(2):285–294
Nagajothi N, Matsui WH, Mukhina GL et al (2004) Enhanced cytotoxicity of rituximab following genetic and biochemical disruption of glycosylphosphatidylinositol anchored proteins. Leuk Lymphoma 45(4):795–799
Nasu J, Mizuno M, Uesu T et al (1998) Cytokine-stimulated release of decay-accelerating factor (DAF;CD55) from HT-29 human intestinal epithelial cells. Clin Exp Immunol 113(3):379–385
Nauta AJ, Trouw LA, Daha MR et al (2002) Direct binding of C1q to apoptotic cells and cell blebs induces complement activation. Eur J Immunol 32(6):1726–1736
Niculescu F, Rus HG, Retegan M et al (1992) Persistent complement activation on tumor cells in breast cancer. Am J Pathol 140(5):1039–1043
Niculescu F, Rus H, van Biesen T et al (1997) Activation of Ras and mitogen-activated protein kinase pathway by terminal complement complexes is G protein dependent. J Immunol 158(9):4405–4412
Niculescu F, Badea T, Rus H (1999) Sublytic C5b-9 induces proliferation of human aortic smooth muscle cells: role of mitogen activated protein kinase and phosphatidylinositol 3-kinase. Atherosclerosis 142(1):47–56
Niehans GA, Cherwitz DL, Staley NA et al (1996) Human carcinomas variably express the complement inhibitory proteins CD46 (membrane cofactor protein), CD55 (decay-accelerating factor), and CD59 (protectin). Am J Pathol 149(1):129–142
Nishioka K, Kawamura K, Hirayama T et al (1976) The complement system in tumor immunity: significance of elevated levels of complement in tumor bearing hosts. Ann N Y Acad Sci 276:303–315
Nolte-'t Hoen EN, Almeida CR, Cohen NR et al (2007) Increased surveillance of cells in mitosis by human NK cells suggests a novel strategy for limiting tumor growth and viral replication. Blood 109(2):670–673
Nozaki M, Raisler BJ, Sakurai E et al (2006) Drusen complement components C3a and C5a promote choroidal neovascularization. Proc Natl Acad Sci U S A 103(7):2328–2333
Nunez-Cruz S, Gimotty PA, Guerra MW et al (2012) Genetic and pharmacologic inhibition of complement impairs endothelial cell function and ablates ovarian cancer neovascularization. Neoplasia 14(11):994–1004
Ohno M, Hirata T, Enomoto M et al (2000) A putative chemoattractant receptor, C5L2, is expressed in granulocyte and immature dendritic cells, but not in mature dendritic cells. Mol Immunol 37(8):407–412
Okroj M, Hsu YF, Ajona D et al (2008) Non-small cell lung cancer cells produce a functional set of complement factor I and its soluble cofactors. Mol Immunol 45(1):169–179
Okroj M, Corrales L, Stokowska A et al (2009) Hypoxia increases susceptibility of non-small cell lung cancer cells to complement attack. Cancer Immunol Immunother 58(11):1771–1780
Ollert MW, Frade R, Fiandino A et al (1990) C3-Cleaving membrane proteinase. A new complement regulatory protein of human melanoma cells. J Immunol 144(10):3862–3867
Ollert MW, David K, Bredehorst R et al (1995) Classical complement pathway activation on nucleated cells. Role of factor H in the control of deposited C3b. J Immunol 155(10):4955–4962
Ostrand-Rosenberg S, Sinha P (2009) Myeloid-derived suppressor cells: linking inflammation and cancer. J Immunol 182(8):4499–4506
Paas Y, Bohana-Kashtan O, Fishelson Z (1999) Phosphorylation of the complement component, C9, by an ecto-protein kinase of human leukemic cells. Immunopharmacology 42(1–3):175–185
Pangburn MK, Rawal N (2002) Structure and function of complement C5 convertase enzymes. Biochem Soc Trans 30(Pt 6):1006–1010
Pangburn MK, Schreiber RD, Muller-Eberhard HJ (1981) Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. J Exp Med 154(3):856–867

Pardoll D (2003) Does the immune system see tumors as foreign or self? Annu Rev Immunol 21:807–839
Peterson AC, Harlin H, Gajewski TF (2003) Immunization with melan-a peptide-pulsed peripheral blood mononuclear cells plus recombinant human interleukin-12 induces clinical activity and T-cell responses in advanced melanoma. J Clin Oncol 21(12):2342–2348
Podack ER, Muller-Eberhard HJ (1979) Isolation of human S-protein, an inhibitor of the membrane attack complex of complement. J Biol Chem 254(19):9808–9814
Quatromoni JG, Eruslanov E (2012) Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. Am J Transl Res 4(4):376–389
Ragupathi G, Liu NX, Musselli C et al (2005) Antibodies against tumor cell glycolipids and proteins, but not mucins, mediate complement-dependent cytotoxicity. J Immunol 174(9):5706–5712
Reiter Y, Fishelson Z (1989) Targeting of complement to tumor cells by heteroconjugates composed of antibodies and of the complement component C3b. J Immunol 142(8):2771–2777
Ricklin D, Hajishengallis G, Yang K et al (2010) Complement: a key system for immune surveillance and homeostasis. Nat Immunol 11(9):785–797
Roberts SJ, Ng BY, Filler RB et al (2007) Characterizing tumor-promoting T cells in chemically induced cutaneous carcinogenesis. Proc Natl Acad Sci U S A 104(16):6770–6775
Rozanov DV, Savinov AY, Golubkov VS et al (2006) Interference with the complement system by tumor cell membrane type-1 matrix metalloproteinase plays a significant role in promoting metastasis in mice. Cancer Res 66(12):6258–6263
Rushmere NK, Knowlden JM, Gee JM et al (2004) Analysis of the level of mRNA expression of the membrane regulators of complement, CD59, CD55 and CD46, in breast cancer. Int J Cancer 108(6):930–936
Rutkowski MJ, Sughrue ME, Kane AJ et al (2010a) Cancer and the complement cascade. Mol Cancer Res 8(11):1453–1465
Rutkowski MJ, Sughrue ME, Kane AJ et al (2010b) The complement cascade as a mediator of tissue growth and regeneration. Inflamm Res 59(11):897–905
Sadallah S, Lach E, Schwarz S et al (1999) Soluble complement receptor 1 is increased in patients with leukemia and after administration of granulocyte colony-stimulating factor. J Leukoc Biol 65(1):94–101
Schlesinger M, Broman I, Lugassy G (1996) The complement system is defective in chronic lymphatic leukemia patients and in their healthy relatives. Leukemia 10(9):1509–1513
Schrama D, Reisfeld RA, Becker JC (2006) Antibody targeted drugs as cancer therapeutics. Nat Rev Drug Discov 5(2):147–159
Schraufstatter IU, Trieu K, Sikora L et al (2002) Complement c3a and c5a induce different signal transduction cascades in endothelial cells. J Immunol 169(4):2102–2110
Schraufstatter IU, Discipio RG, Zhao M et al (2009) C3a and C5a are chemotactic factors for human mesenchymal stem cells, which cause prolonged ERK1/2 phosphorylation. J Immunol 182(6):3827–3836
Schuster M, Umana P, Ferrara C et al (2005) Improved effector functions of a therapeutic monoclonal Lewis Y-specific antibody by glycoform engineering. Cancer Res 65(17):7934–7941
Selander B, Martensson U, Weintraub A et al (2006) Mannan-binding lectin activates C3 and the alternative complement pathway without involvement of C2. J Clin Invest 116(5):1425–1434
Seya T, Hara T, Iwata K et al (1995) Purification and functional properties of soluble forms of membrane cofactor protein (CD46) of complement: identification of forms increased in cancer patients' sera. Int Immunol 7(5):727–736
Shaw PX, Zhang L, Zhang M et al (2012) Complement factor H genotypes impact risk of age-related macular degeneration by interaction with oxidized phospholipids. Proc Natl Acad Sci U S A 109(34):13757–13762
Shi XX, Zhang B, Zang JL et al (2009) CD59 silencing via retrovirus-mediated RNA interference enhanced complement-mediated cell damage in ovary cancer. Cell Mol Immunol 6(1):61–66
Shimizu K, Nakata M, Hirami Y et al (2010) Tumor-infiltrating Foxp3+ regulatory T cells are correlated with cyclooxygenase-2 expression and are associated with recurrence in resected non-small cell lung cancer. J Thorac Oncol 5(5):585–590

Sim RB, Day AJ, Moffatt BE et al (1993) Complement factor I and cofactors in control of complement system convertase enzymes. Methods Enzymol 223:13–35
Sjoberg AP, Manderson GA, Morgelin M et al (2009) Short leucine-rich glycoproteins of the extracellular matrix display diverse patterns of complement interaction and activation. Mol Immunol 46(5):830–839
Soane L, Cho HJ, Niculescu F et al (2001) C5b-9 Terminal complement complex protects oligodendrocytes from death by regulating Bad through phosphatidylinositol 3-kinase/Akt pathway. J Immunol 167(4):2305–2311
Spiridon CI, Ghetie MA, Uhr J et al (2002) Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antigrowth activity of a human breast cancer cell line in vitro and in vivo. Clin Cancer Res 8(6):1720–1730
Strey CW, Markiewski M, Mastellos D et al (2003) The proinflammatory mediators C3a and C5a are essential for liver regeneration. J Exp Med 198(6):913–923
Tang Z, Lu B, Hatch E et al (2009) C3a Mediates epithelial-to-mesenchymal transition in proteinuric nephropathy. J Am Soc Nephrol 20(3):593–603
Taylor RP, Ferguson PJ, Martin EN et al (1997) Immune complexes bound to the primate erythrocyte complement receptor (CR1) via anti-CR1 mAbs are cleared simultaneously with loss of CR1 in a concerted reaction in a rhesus monkey model. Clin Immunol Immunopathol 82(1):49–59
Tegla CA, Cudrici C, Patel S et al (2011) Membrane attack by complement: the assembly and biology of terminal complement complexes. Immunol Res 51(1):45–60
Terui Y, Sakurai T, Mishima Y et al (2006) Blockade of bulky lymphoma-associated CD55 expression by RNA interference overcomes resistance to complement-dependent cytotoxicity with rituximab. Cancer Sci 97(1):72–79
Theofilopoulos AN, Perrin LH (1976) Binding of components of the properdin system to cultured human lymphoblastoid cells and B lymphocytes. J Exp Med 143(2):271–289
Thiel S (2007) Complement activating soluble pattern recognition molecules with collagen-like regions, mannan-binding lectin, ficolins and associated proteins. Mol Immunol 44(16):3875–3888
Thornton AM, Shevach EM (1998) CD4+CD25+ Immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. J Exp Med 188(2):287–296
Trougakos IP, Gonos ES (2002) Clusterin/apolipoprotein J in human aging and cancer. Int J Biochem Cell Biol 34(11):1430–1448
Umansky V, Sevko A (2013) Tumor microenvironment and myeloid-derived suppressor cells. Cancer Microenviron 6(2):169–177
van Lookeren CM, Wiesmann C, Brown EJ (2007) Macrophage complement receptors and pathogen clearance. Cell Microbiol 9(9):2095–2102
Varga L, Czink E, Miszlai Z et al (1995) Low activity of the classical complement pathway predicts short survival of patients with chronic lymphocytic leukaemia. Clin Exp Immunol 99(1):112–116
Varsano S, Frolkis I, Rashkovsky L et al (1996) Protection of human nasal respiratory epithelium from complement-mediated lysis by cell-membrane regulators of complement activation. Am J Respir Cell Mol Biol 15(6):731–737
Varsano S, Rashkovsky L, Shapiro H et al (1998) Human lung cancer cell lines express cell membrane complement Inhibitory proteins and are extremely resistant to complement-mediated lysis; a comparison with normal human respiratory epithelium in vitro, and an insight into mechanism(s) of resistance. Clin Exp Immunol 113(2):173–182
Vlaicu SI, Tegla CA, Cudrici CD et al (2013) Role of C5b-9 complement complex and response gene to complement-32 (RGC-32) in cancer. Immunol Res 56(1):109–121
Walport MJ (2001) Complement. First of two parts. N Engl J Med 344(14):1058–1066
Watson NF, Durrant LG, Madjd Z et al (2006) Expression of the membrane complement regulatory protein CD59 (protectin) is associated with reduced survival in colorectal cancer patients. Cancer Immunol Immunother 55(8):973–980
Weiner LM, Surana R, Wang S (2010) Monoclonal antibodies: versatile platforms for cancer immunotherapy. Nat Rev Immunol 10(5):317–327

Xu C, Jung M, Burkhardt M et al (2005) Increased CD59 protein expression predicts a PSA relapse in patients after radical prostatectomy. Prostate 62(3):224–232

Yamakawa M, Yamada K, Tsuge T et al (1994) Protection of thyroid cancer cells by complement-regulatory factors. Cancer 73(11):2808–2817

Yan J, Allendorf DJ, Li B et al (2008) The role of membrane complement regulatory proteins in cancer immunotherapy. Adv Exp Med Biol 632:159–174

Yefenof E, Zehavi-Feferman R, Guy R (1990) Control of primary and secondary antibody responses by cytotoxic T lymphocytes specific for a soluble antigen. Eur J Immunol 20(8):1849–1853

Ying SC, Gewurz AT, Jiang H et al (1993) Human serum amyloid P component oligomers bind and activate the classical complement pathway via residues 14–26 and 76–92 of the a chain collagen-like region of C1q. J Immunol 150(1):169–176

Ytting H, Jensenius JC, Christensen IJ et al (2004) Increased activity of the mannan-binding lectin complement activation pathway in patients with colorectal cancer. Scand J Gastroenterol 39(7):674–679

Ytting H, Christensen IJ, Thiel S et al (2005) Serum mannan-binding lectin-associated serine protease 2 levels in colorectal cancer: relation to recurrence and mortality. Clin Cancer Res 11(4):1441–1446

Zhang M, Takahashi K, Alicot EM et al (2006) Activation of the lectin pathway by natural IgM in a model of ischemia/reperfusion injury. J Immunol 177(7):4727–4734

Zou W (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer 5(4):263–274

Zou W (2006) Regulatory T cells, tumour immunity and immunotherapy. Nat Rev Immunol 6(4):295–307

Zurlo JJ, Schechter GP, Fries LF (1989) Complement abnormalities in multiple myeloma. Am J Med 87(4):411–420

# Chapter 12
# Imaging Angiogenesis, Inflammation, and Metastasis in the Tumor Microenvironment with Magnetic Resonance Imaging

**Sébastien Serres, Emma R. O'Brien, and Nicola R. Sibson**

**Abstract** With the development of new imaging techniques, the potential for probing the molecular, cellular, and structural components of the tumor microenvironment in situ has increased dramatically. A multitude of imaging modalities have been successfully employed to probe different aspects of the tumor microenvironment, including expression of molecules, cell motion, cellularity, vessel permeability, vascular perfusion, metabolic and physiological changes, apoptosis, and inflammation. This chapter focuses on the most recent advances in magnetic resonance imaging methods, which offer a number of advantages over other methodologies, including high spatial resolution and the use of nonionizing radiation, as well as the use of such methods in the context of primary and secondary brain tumors. It also highlights how they can be used to assess the molecular and cellular changes in the tumor microenvironment in response to therapy.

**Keywords** Magnetic resonance imaging • Cancer • Angiogenesis • Vasculature • Inflammation • Metastasis • Tumour microenvironment

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## 12.1 Introduction

It has become increasingly apparent that cancer cells interact extensively with their environment, signaling to both stromal cells and the immune system, as well as the vasculature. It is critical to note that cancer cells seem to be able to exploit their microenvironment through these interactions to gain a growth advantage, from the induction of angiogenesis to shifts in metabolism (as illustrated in Fig. 12.1). The complex interactions between tumor cells and their surrounding environment are best studied in preclinical models, and considerable progress has been made in recent years in the development of new imaging techniques to probe the molecular and structural constituents of the tumor microenvironment (TME) *in vivo*. From cellular to more macroscopic changes, imaging techniques can provide information about the

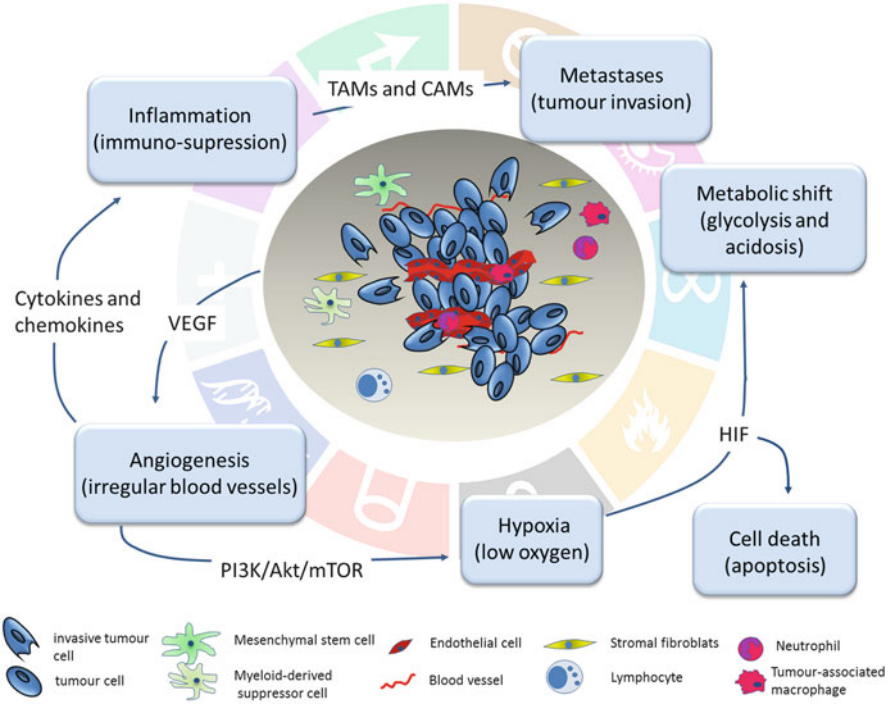


**Fig. 12.1** The tumor microenvironment (TME). The TME encompasses a complex interaction between cancer cells and other cell populations, including endothelial cells, stromal fibroblasts, tumor-associated macrophages, myeloid-derived suppressor cells, mesenchymal stem cells, lymphocytes, and neutrophils. During tumor growth, vascular endothelial growth factor is released and drives angiogenesis. The oncologic phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling pathway enables stabilization of hypoxia-inducible factors, which drives hypoxia, metabolic shift, and resistance to cell death. At the same time, formation of new blood vessels and release of chemokines and cytokines induce inflammation in the TME, which can drive tumor invasion and metastasis to distant organs. TAMs and cell adhesion molecules are pivotal in the formation of metastases. (Figure based on Hanahan and Weinberg 2011)

expression of molecules, cell motion, cellularity, vessel permeability, vascular perfusion, metabolic and physiological changes, apoptosis, and inflammation in the TME.

A multitude of imaging modalities have been successfully employed to probe the TME, including computed tomography, positron emission tomography (PET), single-photon emission computed tomography (SPECT), ultrasound, optical imaging, and magnetic resonance imaging (MRI). While all of these modalities can be used in either a preclinical or clinical setting to obtain insight into the TME, there are both advantages and limitations of each. For example, PET and SPECT provide high sensitivity, but they are limited by the use of radioisotopes and, compared to MRI, offer relatively low spatial resolution. Other modalities such as ultrasound and bioluminescence imaging have poor depth penetration, whereas invasive imaging modalities such as near-infrared light and fluorescence imaging are limited in their translation to the clinic (Brindle 2008; Weissleder and Pittet 2008). In comparison, magnetic resonance (MR) methodologies offer a number of advantages, including high spatial resolution and the use of nonionizing radiation. Thus, this chapter focuses on the development of MR-based techniques for imaging and probing the TME, with particular emphasis on inflammation, angiogenesis, and metastasis. We consider these approaches primarily in the context of the brain, although many are more widely applicable to the TME in general. Finally, we highlight how such modalities can be used to assess the molecular and cellular changes in the TME in response to therapy.

## 12.2 Magnetic Resonance Imaging

MRI is perhaps the best imaging method with which to probe the TME because of its large range of applications. MRI is noninvasive, does not use ionizing radiation, and provides the best soft-tissue resolution of all of the modalities mentioned above. MRI is limited by signal sensitivity; as a consequence it is generally limited to imaging water protons, which are abundant in living tissue. However, with the use of higher magnetic field strengths, contrast agents, and hyperpolarization of nuclei the signal can be significantly amplified, leading to multiple emerging proton- and non-proton-based applications for studying the TME.

Hydrogen atoms ($^1$H) have an inherent magnetic moment as a result of their nuclear spin, and when placed in a strong magnetic field, these magnetic moments tend to align with the magnetic field (or $z$-axis). Application of a radiofrequency pulse at the resonant frequency of the hydrogen nuclei can force the magnetic moments of the nuclei to partially or completely tip into a plane perpendicular to the applied field (transverse or x-y plane). Once in the transverse plane, the magnetic moments of the nuclei precess around the $z$-axis, and the energy produced by the rotating magnetic moment can be detected as the MR signal. The acquired signal may be manipulated in numerous different ways on the basis of the imaging sequence used and thus can yield a variety of different parameters related to structural, functional, and metabolic processes.

Because of the relaxation properties of the water protons following application of a radiofrequency pulse, two primary types of contrast are readily obtained. The first is generated by relaxation of the water protons from their excited state in the transverse plane to realign with the *z*-axis (longitudinal or $T_1$ relaxation). The second relies on the dephasing of nuclei from each other within the transverse plane due to the presence of small-field inhomogeneities causing the nuclei to precess at slightly different resonant frequencies (transverse or $T_2$* relaxation). Some of the effects of static inhomogeneities in the magnetic field can be compensated for using a specific type of imaging sequence called a spin-echo; in this case the resulting contrast is referred to as $T_2$ and reflects only the effects of randomly fluctuating changes in the magnetic field during spin dephasing. The relaxation rates of water in different tissues depends on both the interactions of the water molecules with other molecules in the tissue and the structure of the tissue. As a consequence, it is possible to generate tissue-specific contrast with appropriately $T_1$- or $T_2$-weighted imaging sequences. Because the constitution of tissues differs in healthy and pathological conditions, $T_1$- and $T_2$-weighted MRI can provide information about structural changes occurring during disease progression.

Beyond these basic MRI approaches, more elaborate methods can describe functional changes occurring in tissues, including those related to the macromolecular composition of the tissue (magnetization transfer MRI), tissue water diffusion (diffusion-weighted [DW] MRI), blood flow or volume (perfusion-weighted MRI), and vascular permeability, using intravenous paramagnetic contrast agents that alter either the $T_1$ or $T_2$* relaxation rates of nearby protons (dynamic contrast-enhanced [DCE] MRI). Although tumor volume is the conventional primary end point, as specified by Response Evaluation Criteria in Solid Tumors guidelines (Therasse et al. 2000), these other imaging modalities can provide important additional information on the TME. Finally, by targeting MRI contrast agents with an antibody or peptide that recognizes TME antigens, the emerging modality of molecular MRI enables accurate and precise visualization of molecules, receptors, and cells in the TME. In a related approach, tumor, inflammatory, and stromal cells can also be loaded with contrast agents to track their movements and interrogate their role in the TME (Kircher et al. 2011).

This chapter details the application of MRI to probe three of the hallmarks of cancer that are critical aspects of the TME: angiogenesis, inflammation, and tumor invasion, including metastasis (Hanahan and Weinberg 2011). It also provides an outlook for the future use of MRI methodologies that may lead to new insights in the role of these processes in the TME.

## 12.3 MRI Methods for Probing Angiogenesis and Tumor Vasculature

Angiogenesis is the formation of new blood vessels from the preexisting vasculature and is a vital component in many physiological processes (e.g., wound healing, embryogenesis) and numerous diseases (e.g., stroke, heart disease). The current

model for tumor growth considers angiogenesis a necessary component for malignancy (Folkman 1971), with de novo vessel formation facilitating the delivery of oxygen and nutrients to a larger tumor mass, as well as providing a route for metastasis. Tumor cells secrete pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor, and matrix metalloproteinases, stimulating endothelial and stromal cells to form new blood vessels. Such factors lead to the formation of abnormal blood vessels that are tortuous, leaky, and heterogeneous in size, as originally described by Virchow (Fokas et al. 2012). The apparent dependence of tumors on angiogenesis for growth has prompted the development of a number of drugs that aim to inhibit angiogenesis, either by targeting the angiogenic factors directly, as with VEGF-targeting bevacizumab, or indirectly via their receptors, as with drugs targeting the epidermal growth factor receptor (EGFR) (Plate et al. 1992; Brown and Wilson 2004). Such drugs have been successful in halting tumor angiogenesis and inducing tumor regression in preclinical models (Brown and Wilson 2004; Carmeliet and Jain 2000; Carmeliet 2005; Ferrara and Kerbel 2005). However, translation into the clinic has been disappointing (Fokas et al. 2012; Sitohy et al. 2012). Numerous hypotheses exist for the lack of therapeutic effect – tumor heterogeneity among them (Sitohy et al. 2012). In addition, it has been proposed that such therapies may also stabilize the vasculature and increase tumor invasiveness (Keunen et al. 2011). For these reasons, it is necessary to find noninvasive biomarkers that reflect the effects of such agents on the vasculature as well as monitoring response to treatment.

### *12.3.1 Dynamic Contrast-Enhanced Measurements of Tumor Vasculature*

MR modalities can provide multiple surrogate biomarkers of tumor vasculature. The uses of MRI, most notably dynamic contrast-enhanced (DCE) MRI, in the measurement of angiogenesis in the TME are numerous and well documented (Brown and Wilson 2004; Miller et al. 2005; Perini et al. 2008; Jackson et al. 2008). In DCE MRI, uptake of an MR contrast agent, such as gadolinium-diethylenetriaminepentacetate (DTPA), by tissue is measured over a short period of time using $T_1$-weighted MRI. This technique is sensitive to a combination of vascular perfusion, extracellular tumor volume, and vessel permeability. In the TME, this combination is altered relative to normal tissue and has atypical properties due to abnormal angiogenesis (Keunen et al. 2011; O'Connor et al. 2007). Together with the use of compartmental modeling approaches, it is possible to tease apart vascular parameters such as tumor blood flow, blood volume, and mean transit time (MTT) (Brown and Wilson 2004; Miller et al. 2005; Perini et al. 2008; Jackson et al. 2008). To be more precise, the conventional approach to modeling tumor perfusion with DCE MRI relies on the fact that (1) signal change is related to contrast concentration, (2) the integration of the contrast-time course curve is related to blood volume, and (3) the shape of the contrast curve is governed by the MTT through the tumor space. Hence, tumor perfusion affects how rapidly the contrast agent reaches the tumor, whereas tumor vascular permeability

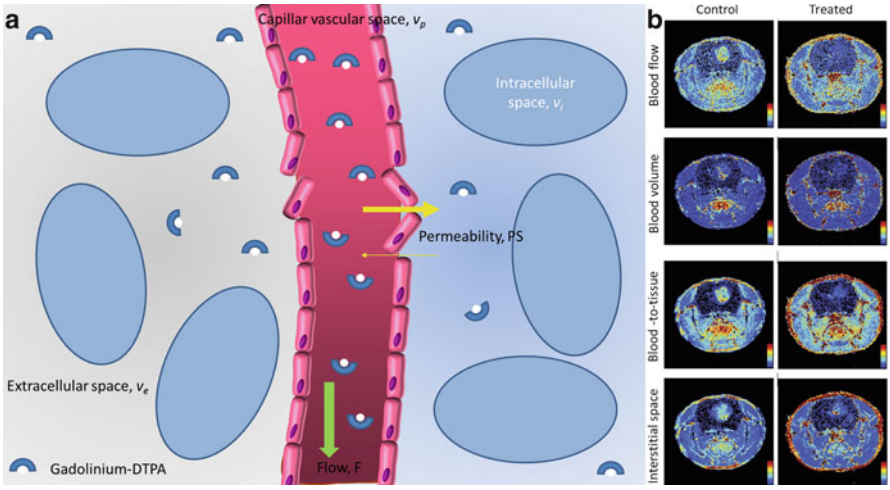


**Fig. 12.2** Imaging vascular changes in the tumor microenvironment (TME). (**a**) Schematic diagram illustrating compartmental modeling of the tumor microvasculature used to determine intracellular space ($v_i$), extracellular space ($v_e$), capillary vascular space ($v_p$), flow ($F$), and permeability ($PS$) parameters from dynamic contrast-enhance (DCE) magnetic resonance imaging (MRI) data. (**b**) Example of DCE MRI data from glioblastomas treated with bevacizumab. Tumor perfusion maps from control (*left*) and treated animals (*right*) show a significant reduction in tumor blood flow, blood volume per unit of tissue (*vb*), and blood-to-tissue extraction constant (Ktrans; reflecting change in permeability) after treatment. The interstitial space volume ($V_e$) per unit of tissue was not significantly modified. Colors range from *blue* (low values) to *red* (high values). (Figure adapted from Keunen et al. 2011)

affects the diffusion of the contrast agent into intracellular space of the tumor (Fig. 12.2). As a consequence, DCE MRI can be used as a tool to characterize tumor vasculature as well as response to treatment (Yankeelov et al. 2007; Keunen et al. 2011). MTT is of particular interest because it is considered to be inversely proportional to cerebral perfusion pressure and cerebral blood flow (CBF): CBF = cerebral blood volume (CBV)/MTT) (Warmuth et al. 2003). It has been hypothesized that a prolonged MTT reflects an increase in microvascular density in brain tumors and, hence, CBV (Aronen et al. 2000; Aronen et al. 1994). However, because of the immense heterogeneity of the tumor microvasculature, MTT may also decrease due to increased CBF, particularly at the tumor margins (Roberts et al. 2002).

### *12.3.2 Dynamic Susceptibility Contrast MRI Measurements of Tumor Blood Volume*

Similar approaches to DCE MRI exist to specifically measure tumor blood volume changes within the TME, such as dynamic susceptibility contrast (DSC) MRI, which uses gadolinium-DTPA or ultrasmall superparamagnetic particles of iron

oxide (USPIO) to induce signal changes on $T_2$- or $T_2$*-weighted images. In this case, images are acquired rapidly during bolus transit of the contrast agent, and the signal versus time curve is used to estimate relative blood volume, reflecting the changes in tumor vasculature.

One caveat to this approach is that it is sometimes difficult to distinguish changes in blood volume from changes in vascular permeability. In the brain, for example, breakdown of the blood-brain barrier (BBB; the physical barrier formed by endothelial cells that separates blood from brain) caused by tumor growth can lead to inaccuracies in the estimate of CBV because of accumulation of the contrast agent in the brain tissue itself. Thus, CBV measurements can be difficult in brain tumors in which the BBB is compromised (Law et al. 2002, 2003a, b). However, substantial changes in CBV within the TME can be used to evaluate tissue perfusion in regions surrounding the tumor that are not affected by vascular permeability (Law et al. 2003b).

Increases in CBV are thought to reflect the effects of increased TME energy metabolism caused by substantial immune cell infiltration and local response to the enhanced energy demands of highly proliferative tumor cells. Combining perfusion MRI and MR spectroscopy for measuring metabolite concentrations in regions of increased CBV has helped to improve both the diagnostic accuracy and confidence of clinical findings in brain tumors (Law et al. 2003b). Similar approaches can be used to differentiate between necrosis and tumor recurrence in patients radiologically showing progression of cerebral metastases after treatment with stereotactic radiosurgery (Hoefnagels et al. 2009).

### *12.3.3 Arterial Spin Labeling Measurements of Tumor Blood Flow*

In addition to the DCE and DSC methods discussed above, other MRI methods exist to specifically probe tumor blood flow. Among them, arterial spin-labeling (ASL) MRI uses magnetically labelled endogenous blood water as a tracer (Detre et al. 1992; Williams et al. 1992; Alsop and Detre 1996). The longitudinal magnetization of arterial blood water must be manipulated so that it differs from the magnetization of tissue. In brief, a saturation pulse is applied to a slice outside of the tumor volume, which inverts the signal relative to that in the target tissue. As blood flows from the inverted slice into the target slice, a reduction in signal is observed. The difference between images acquired with and without ASL can be modeled to derive a calculated blood flow defining perfusion in milliliters per gram per minute per voxel (Buxton et al. 1998; Wong et al. 1998, 2006). Most ASL studies have been carried out in the brain because of the high tissue perfusion and well-defined arterial supply. ASL methods in particular benefit from high magnetic field strengths and the increased sensitivity provided by multicoil receivers (Wang et al. 2002), enabling clinical measurements of hyperperfusion in glioblastoma (Wang et al. 2005; Wolf et al. 2005). Measurement of CBF is also possible with the contrast agent methods described above, but it requires determination of the arterial input function, which

can be challenging (Warmuth et al. 2003). Nevertheless, such CBF measurements have been shown to correlate with tumor grade (Perkio et al. 2005).

### *12.3.4 Diffusion-Weighted MRI*

DW MRI recently has emerged as clinical biomarker for response to anti-angiogenesis treatment of tumors (Bauerle et al. 2011, 2012; Heijmen et al. 2012; Yankeelov et al. 2007). Unlike DCE MRI, DW MRI is not dependent on a contrast agent; instead it determines the apparent diffusion coefficient (ADC) of tissue water, which varies in the TME because of factors such as vasogenic edema and tumor hypercellularity (Yankeelov et al. 2007). For example, where edema accumulates, an increase in the ratio of less restricted extracellular water versus the more restricted intracellular water will increase the ADC in that region. Conversely, in hypercellular areas – for example, a highly proliferative tumor with extensive inflammatory infiltrate – the ratio will be increased in favor of the more restricted intracellular environment, and a reduction in ADC is consequently observed. For many years, DW MRI was relatively underused in the study of tumor treatment responses compared with DCE MRI. However, increased ADC in the TME has recently been shown to correlate significantly with positive treatment response (Heijmen et al. 2012), and it is now thought that DW MRI is superior to DCE MRI for early assessment of vascular tumor treatments (Moffat et al. 2005; Heijmen et al. 2012).

It is interesting to note that DW MRI can also be more sensitive than DCE MRI in detecting brain tumors and co-optive brain micrometastases in which the BBB remains intact (Leenders et al. 2003; Budde et al. 2012). Thus, with the recent development of BBB-permeable chemotherapeutics (Joyal et al. 2004; Goldschmidt et al. 2012), DW MRI seems to be a promising method to assess the efficacy of such drugs.

Despite these emerging and expanding applications of DCE, DSC, and DW MRI in monitoring the TME, it must be remembered that they are all indirect macroscopic measures of vascular changes and do not provide information about changes at the cellular scale. It is important, therefore, that methods to enable the direct detection of the molecular and cellular components of the TME also are developed, as will be discussed below.

## 12.4 Molecular and Cellular MRI of Inflammation, Tumor Invasion, and Angiogenesis

Poor tissue perfusion and high interstitial fluid pressure within the TME lead to hypoxia and inflammation. It is now well accepted that cancer-related inflammation is one of the hallmarks of cancer (Colotta et al. 2009; Hanahan and Weinberg 2011; Liu et al. 2004).Tumors are pathologically associated with infiltrating immune cells; in this way they mirror inflammatory conditions observed in nonneoplastic tissues

(Dvorak et al. 1986; Hanahan and Weinberg 2011). It has long been thought that inflammatory processes reflect the attempt of the immune system to eradicate cancer and thus could be harnessed in antitumoral therapies (Hanahan and Weinberg 2011). However, it also has been proposed that the tumor-associated inflammatory response may enhance tumor progression and invasiveness (DeNardo et al. 2010; Colotta et al. 2009; Hanahan and Weinberg 2011) through supply of growth factors and cytokines that promote angiogenesis and modify the extracellular matrix (Qian and Pollard 2010). It is interesting to note that molecular markers of angiogenesis share some similarities with those of inflammation and metastasis. For example, both metastatic invasion (Nguyen et al. 2009) and angiogenesis are linked to endothelial activation, a crucial element of the inflammatory response (Carbonell et al. 2009; Ferjancic et al. 2013; Serres et al. 2012; Laubli and Borsig 2010). Thus, development of imaging approaches that can probe the TME at the molecular and cellular levels may provide considerable insight into tumor progression and response to treatment.

Over the past 20 years, advances in molecular and cellular imaging have revolutionized the field of MRI (Weissleder et al. 1992) and cancer molecular imaging (Weissleder and Pittet 2008). The development of targeted MRI contrast agents that will recognize and bind to specific molecular targets, together with approaches for labeling specific cell populations with MRI-detectable contrast, has greatly enhanced the sensitivity and specificity of MRI to molecular and cellular processes. Such imaging agents have shown efficacy for the detection of early inflammation, metastasis, and angiogenesis and, in the long-term, have the potential to drive individualized treatment on the basis of molecular events.

### *12.4.1 In Vitro Labeling for Cell Tracking*

Cells can be labeled with a suitable contrast agent while in culture and subsequently introduced into animal models for cell tracking studies. Although gadolinium agents have been used quite widely to track cells such as lymphocytes, the MR signal caused by these agents is not sufficient for the detection of individual cells (Bhorade et al. 2000; Kircher et al. 2011). However, superparamagnetic iron oxide particles, such as USPIOs (10–50 nm) and superparamagnetic particles of iron oxide (SPIOs) (100–300 nm), have an inherently greater effect on MR relaxation times than gadolinium-based agents and now dominate the field of MRI-based cell tracking. These nanoparticles consist of an iron oxide core embedded in a dextran (Shen et al. 1993), silocan (Jung and Jacobs 1995), or polymer (Shapiro et al. 2005) shell that contains thousands of iron atoms, and therefore a smaller number of loaded cells are required for detection than in gadolinium-enhanced MRI. As discussed earlier, SPIOs shorten $T_2$ relaxation times and hence elicit hypointense signals on $T_2$- or $T_2$*-weighted images. Particles 10–100 nm in diameter can be taken up by cells either via phagocytosis, transfection agents, or translocation membrane peptides (Bell et al. 2011). Unmodified USPIOs were among the first contrast agents to be synthesized (Shen et al. 1993; Shen and Saunders 1993) and have been used to label

and image both glioma cells (Moore et al. 1997; Weissleder et al. 1997) and T cells (Sipe et al. 1999) *in vitro*. Similar approaches recently have been used to label metastatic cells *in vitro* for subsequent *in vivo* tracking, and the distribution of SPIO-loaded human metastatic breast cancer cells in either severe combined immunodeficiency mice (Sundstrom et al. 2013) or nude rats (Song et al. 2011) has enabled noninvasive determination of both the distribution and burden of brain metastases. In addition to contrast agents that alter the local relaxivity of tissue water, alternative nuclei can also be used for cell tracking by MRI. In particular, interest in fluorine-19 ($^{19}$F), which is second to $^{1}$H in terms of sensitivity (6 % lower sensitivity), has recently emerged. The *in vivo* abundance of $^{19}$F is very low; thus, this approach lends itself purely to the detection of tracers labeled by exogenous $^{19}$F. For example, labeling cells with perfluorocarbon monoparticles has been used to enable $^{19}$F cell tracking *in vivo* (Ahrens et al. 2005; Partlow et al. 2007).

In human, such cell tracking approaches have been used to label phagocytotic cells, including dendritic cells (de Vries et al. 2005) and pancreatic islets cells (Saudek et al. 2010), for assessing cell transplantation in patients with melanoma and hepatic tumors, respectively. However, although multiple studies detailing the ability of cells to be labeled with USPIOs and SPIOs have been published, how such labeling techniques can be used to monitor disease and treatment has yet to be fully determined (Unger 2003). In addition, despite the successful development of cell tracking MRI and initial reports showing that labeling mesenchymal cells and HeLa cells with SPIOs has no toxicity (Arbab et al. 2003), recent studies report that SPIO labeling may significantly influence the metabolism and function of these cells (Schafer et al. 2010). To overcome this potential issue, micron-sized microparticles of iron oxide (MPIOs) have been developed to increase MRI detection of the particles at cell-sized resolution (Shapiro et al. 2004, 2005) on the premise that by reducing the number of labeling particles required per cell, the consequent effect on cell function would also be decreased. As a result, the use of MPIOs has become popular for cell tracking with *in vitro* cell loading, and it has been shown that the fate of a single metastatic cell can be tracked with this approach after intracardiac injection into severe combined immunodeficiency mice. Heyn et al. (2006a, b) demonstrated that *in vitro* loading of the human breast cancer metastatic cell line MDA-MB-231BR with MPIOs enables the visualization of both the delivery and distribution of solitary cells within the brain, with no effect on tumor cell function. It was recently shown that MPIO-labeling of stem cells can be used to determine their tropism toward glioblastoma; MPIO-labeled human mesenchymal stem cells and fetal neural stem cells both showed localization first at the tumor margins and subsequently within the tumor mass (Chaumeil et al. 2012). These findings suggest that such labeling approaches may enable not only determination of pathotropism and dissemination in stem cell therapy studies but also more fundamental studies of tumor stem cell dynamics *in vivo* (Boulland et al. 2012). Finally, this approach may hold promise for the noninvasive detection of therapy delivery through the use of immune cells as vectors for chemotherapeutics (Gao et al. 2013). Monocyte/macrophages loaded with MPIOs have been detected in glioma weeks after intravenous

injection; thus, this approach might allow noninvasive tracking of the delivery of vectorized therapy to brain tumors (Valable et al. 2007).

### *12.4.2 In Vivo Labeling for Cell Tracking and Detection*

*In vivo* labeling of immune cells was first reported in the 1980s and has facilitated the detection of pathologies in the spleen, liver, bone marrow, and lymph nodes (Weissleder et al. 1987a, b; Hahn et al. 1988; Weissleder et al. 1988a, b). Subsequent to intravenous injection, USPIOs rapidly accumulate in phagocytic cells within normal, but not metastatic, lymph nodes (Weissleder et al. 1990), allowing the detection of nodal metastases in patients with primary cancers (Harisinghani et al. 2003; Harisinghani and Weissleder 2004). Such nanoparticles are also thought to be rapidly phagocytosed by immune cells following introduction into the bloodstream, thus enabling tracking of these labeled cells to sites of injury or disease. Immune cell recruitment has been imaged with this approach in multiple pathologies, including cerebral ischemia (Rausch et al. 2002), animal models of multiple sclerosis (Pirko et al. 2003; Oude Engberink et al. 2010; Serres et al. 2009), and amyotrophic lateral sclerosis (Bataveljic et al. 2011). By extension, infiltration of immune cells in the TME could be used both as an imaging surrogate for tumor presence and as a means to quantify and interrogate the role of recruited immune cells in tumor progression. Indeed, *in vivo* uptake of USPIOs by microglia allows demarcation of glioma and characterization of the immunological response in primary brain tumors (Fleige et al. 2001). In a small clinical study, $T_1$ signal enhancement was observed after the administration of iron oxide particles in glioma patients, correlating with astrocyte and microglial activation and enabling the detection of tumors not observed with the current gold-standard gadolinium detection technique (Neuwelt et al. 2004). However, it should be noted that, despite these findings, there is little direct *in vivo* evidence for the uptake of such particles by circulating immune cells.

Although the BBB represents a significant barrier to iron oxide particles (because of its impermeability to molecules >30 kd in size), some groups have investigated the potential for labeling cells within the brain itself using iron oxide–based contrast agents. USPIOs have been shown to facilitate accurate delineation of glioma margins in the rat at a point when the tumor BBB is compromised as a result of phagocytosis by tumor cells (Zimmer et al. 1995b). Moreover, other studies have investigated experimental approaches to osmotically disrupt the BBB (Zimmer et al. 1995a) or facilitate intracerebral delivery of USPIOs. Although these approaches have been applied in rat brain, they would be arguably less easily translated into clinical use. However, it is plausible that if selective permeabilization strategies restricted to tumor sites alone were developed, the MPIO-labeling approach might yield an important route for accurate determination of tumor margins for surgical resection.

### *12.4.3 Targeted Contrast Agents for Molecular Imaging*

Although the approaches described above allow cellular imaging, iron oxide particle labeling with targeting ligands enables molecular imaging, as pioneered by Cerdan et al. in 1989). In that study, monoclonal antibodies against surface antigens on the HT-29 colon cancer cell line were conjugated to iron oxide particles and shown to enable tumor xenograft detection with MRI. However, conjugation of biological ligands to nano-sized particles is challenging and has the inherent limitations of low target valency and long half-life of blood, which reduces binding-specific contrast effects. Thus, such agents have achieved relatively little success. On the other hand, the use of larger MPIOs has proved more practical for antibody and peptide conjugation, permitting a broader range of TME hallmarks to be probed with such molecularly targeted agents. In particular, inflammatory markers that may be upregulated early in tumor growth can be targeted with a diverse range of conjugates. For example, antibodies against vascular cell adhesion molecule-1 (VCAM-1), the endogenous selectin ligand sialyl-Lewis$^x$, and the cognate ligand to the activated platelet glycoprotein IIb/IIIa receptor or ligand induced binding site all have been conjugated to MPIOs and used to detect early inflammatory processes before the onset of clinical symptoms (McAteer et al. 2007; von Zur Muhlen et al. 2008; Hoyte et al. 2010; Serres et al. 2009, 2011, 2012; van Kasteren et al. 2009).

In the context of imaging the TME, such tools hold great promise for the early detection of metastasis, especially in the brain, where diagnosis is currently only possible at later stages of tumor progression. Cancer cell extravasation across the BBB, and subsequent proliferation in the perivascular niche, leads to "activation" of the luminal endothelial membrane and the expression of cell adhesion molecules (CAMs) (Serres et al. 2012). Such CAMs provide an accessible tag for the detection of acute inflammatory events within the brain, without the need to either cross the BBB or accumulate the agent within the brain itself. The current imaging modality for clinical detection of brain metastasis is gadolinium-enhanced MRI. However, because accumulation of this nontargeted contrast agent in the tumor is dependent on BBB breakdown, this technique is sensitive only to larger tumors (0.5–1.0 cm in diameter) with a permeable BBB and at a time when therapeutic options are limited. In contrast, we have recently shown that VCAM-1 is upregulated on blood vessels associated with early micrometastases in the brain (Serres et al. 2012) and that this CAM can be used to detect the presence of metastases by MRI in conjunction with MPIOs conjugated to anti-VCAM-1 antibodies (Serres et al. 2012) (Fig. 12.3). At clinical imaging resolutions, this approach is likely to enable the detection of metastases two to three orders of magnitude smaller than that allowed by current clinical methods. Similar early upregulation of VCAM-1 in lung metastasis (Ferjancic et al. 2013) further suggests the possibility of translating this approach to other metastatic sites.

CAMs such as VCAM-1 (Klemke et al. 2007), intercellular adhesion molecule-1 (Roland et al. 2007), and activated leukocyte cell adhesion molecule (Wiiger et al. 2010; Ihnen et al. 2011) have been proposed to be important in the proliferation of metastases; therefore, monitoring the expression of these molecules *in vivo* may

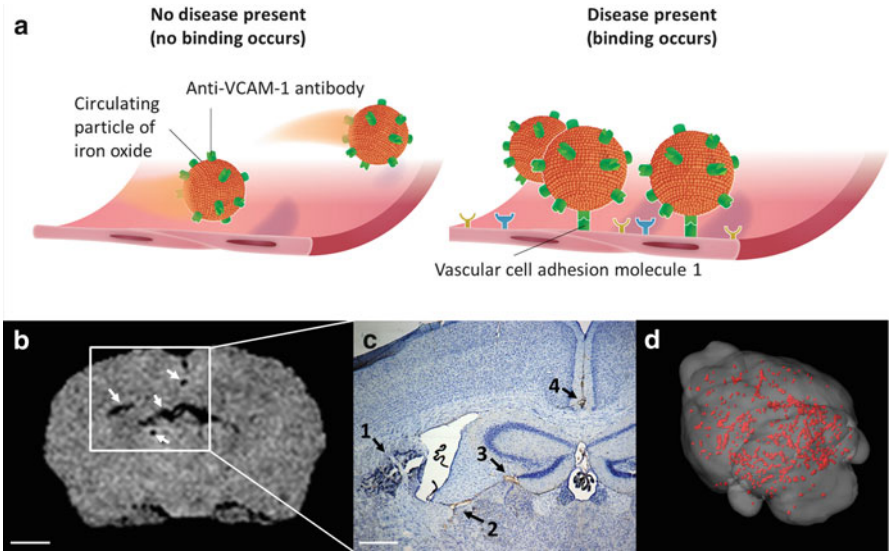


**Fig. 12.3** Molecular imaging of inflammation and metastasis. (**a**) Magnetic resonance imaging (MRI) can be used to image specific molecules expressed on endothelial cells using appropriate targeting antibodies conjugated to microparticles of iron oxide (MPIOs) (e.g., vascular cell adhesion molecule [VCAM]-MPIO targeting endothelial VCAM-1). When disease is absent, no binding occurs and the agent is rapidly cleared from the circulation. When disease is present and the molecule being targeted is expressed, specific binding of the contrast agent occurs, giving rise to potent and specific contrast changes at that site. (Figure courtesy of Franks and Franks.) (**b**) Example of VCAM-MPIO binding and detection of metastasis in a mouse model of brain metastasis. Selected $T_2$*-weighted images from a three-dimensional (3D) dataset 21 days after intracardiac injection of MDA231BR-GFP; cells show focal hypointense areas (*black*) corresponding to VCAM-MPIO binding (scale bar: 1 mm). (**c**) Colocalization of the MRI hypointense signals (*arrows* in **b**) with VCAM-1 expression (*brown*) and metastases (*arrows* in **c**). (**d**) 3D reconstruction showing the spatial distribution of VCAM-MPIO binding (*red*). (Figure adapted from Serres et al. 2012)

provide reliable assessment of anti-inflammatory therapies in metastasis. Molecular imaging approaches using intercellular adhesion molecule-1 conjugated to gadolinium (Geelen et al. 2012; Paulis et al. 2012) or paramagnetic liposomes (Deddens et al. 2013) have shown efficacy in detecting vascular endothelium activation, but these are yet to be translated into the field of cancer research. Similar approaches can also be used to probe enzyme activity, such as a myeloperoxidase conjugated gadolinium-based agent targeting the inflammatory enzyme myeloperoxidase. This agent has been shown to selectively detect intra- and peritumoral inflammation, as well as monitor glioma response to treatment by differentiating between tumor mass and associated inflammation (Kleijn et al. 2011).

Tumor receptor expression is a further potential target for molecular imaging approaches. EGFR is overexpressed in numerous cancers (Milanezi et al. 2008; Domingo et al. 2010; Saif 2010), including glioma (Sauter et al. 1996; Schwechheimer et al. 1995; Geelen et al. 2012; Paulis et al. 2012). Using a pretargeting approach in an

*in vivo* glioma study, an EGFR monoclonal antibody conjugated to horseradish peroxidise was initially administered and followed by a substrate for horseradish peroxidase conjugated to gadolinium, di(tyramido)-DTPA(Gd). Conversion of the substrate into an active compound only occurred at the site of EGFR, resulting in enhanced contrast retention in the EGFR-expressing tumors. Because EGFR is the signature of highly aggressive gliomas, this technique could identify patients who would benefit from anti-EGFR therapy, thus enabling patient stratification (Shazeeb et al. 2011).

Finally, because of the preclinical promise of anti-VEGF/VEGF receptor therapy, interest is growing in molecular imaging techniques that monitor the vascular response to anti-angiogenic therapies. To that end, MRI contrast agents have been synthesized to directly probe angiogenic vessels. Increased expression of certain integrins, such as $\alpha_v\beta_3$, has been linked to angiogenic vessels, and expression of $\alpha_v\beta_3$ has been shown to correlate positively with tumor grade. The peptide arginine-glycine-aspartic acid (RGD) has a high and specific affinity for $\alpha_v\beta_3$, and RGD-labeled agents have been developed for SPECT, PET, and near-infrared fluorescence imaging of angiogenic vessels (Gao et al. 2013). However, each of these approaches has limitations, as described in the Introduction, and so $\alpha_v\beta_3$-targeted agents able to be detected by MRI have recently been developed based on RGD-targeting of USPIOs (Zhang et al. 2007; Combes et al. 2013). Insights from preclinical models, such as the detection of nascent vessels in a mouse xenograft model of human melanoma, suggest that this approach may have merit for stratifying patients by identifying tumors that have undergone an "angiogenic switch" and hence are more aggressive (Schmieder et al. 2005). Moreover, in a glioblastoma model the use of RGD-coupled USPIOs for noninvasive monitoring of the tumor response to anti-VEGF therapy has been demonstrated and found to be more sensitive to treatment effect than conventional anatomical approaches based on tumor volume measurements (Zhang et al. 2012).

## 12.5 Outlook and Conclusion

Research over the past two decades has revolutionized our view of cancer pathology; tumors cannot be considered neoplastic cells alone, but rather must be defined in the context of a multicellular microenvironment. By monitoring the interactions between tumors and surrounding stromal cells, it has been possible to better understand tumor progression. Using this knowledge, new anticancer therapies that target elements of the TME have been developed to complement traditional treatments that directly target cancer cells, such as radiotherapy and chemotherapy. In parallel, new MR-based imaging modalities also have been developed to target different elements of the TME, which can in turn be used not only for more sensitive diagnosis and/or monitoring but also for a more informational treatment strategy. This chapter has reviewed MRI modalities that are currently used to monitor and probe microscopic (molecules and cells) and macroscopic (tissue and organ) changes occurring in the TME. Although MRI holds great promise for clinically monitoring the TME, there are still improvements to be made, including standardization of methodologies

and data analysis as well as increases in sensitivity, particularly at clinical field strengths. Nevertheless, with such developments, imaging of the TME using MRI approaches would be well placed to become routine in clinical practice and a valuable platform for patient diagnosis, monitoring, and treatment.

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## References

Ahrens ET, Flores R, Xu H, Morel PA (2005) In vivo imaging platform for tracking immunotherapeutic cells. Nat Biotechnol 23:983–987

Alsop DC, Detre JA (1996) Reduced transit-time sensitivity in noninvasive magnetic resonance imaging of human cerebral blood flow. J Cereb Blood Flow Metab 16:1236–1249

Arbab AS, Bashaw LA, Miller BR, Jordan EK, Lewis BK, Kalish H, Frank JA (2003) Characterization of biophysical and metabolic properties of cells labeled with superparamagnetic iron oxide nanoparticles and transfection agent for cellular MR imaging. Radiology 229:838–846

Aronen HJ, Gazit IE, Louis DN, Buchbinder BR, Pardo FS, Weisskoff RM, Harsh GR, Cosgrove GR, Halpern EF, Hochberg FH et al (1994) Cerebral blood volume maps of gliomas: comparison with tumor grade and histologic findings. Radiology 191:41–51

Aronen HJ, Pardo FS, Kennedy DN, Belliveau JW, Packard SD, Hsu DW, Hochberg FH, Fischman AJ, Rosen BR (2000) High microvascular blood volume is associated with high glucose uptake and tumor angiogenesis in human gliomas. Clin Cancer Res 6:2189–2200

Bataveljic D, Stamenkovic S, Bacic G, Andjus PR (2011) Imaging cellular markers of neuroinflammation in the brain of the rat model of amyotrophic lateral sclerosis. Acta Physiol Hung 98:27–31

Bauerle T, Komljenovic D, Merz M, Berger MR, Goodman SL, Semmler W (2011) Cilengitide inhibits progression of experimental breast cancer bone metastases as imaged noninvasively using VCT, MRI and DCE-MRI in a longitudinal in vivo study. Int J Cancer 128:2453–2462

Bauerle T, Komljenovic D, Semmler W (2012) Monitoring molecular, functional and morphologic aspects of bone metastases using non-invasive imaging. Curr Pharm Biotechnol 13:584–594

Bell LK, Ainsworth NL, Lee SH, Griffiths JR (2011) MRI & MRS assessment of the role of the tumour microenvironment in response to therapy. NMR Biomed 24:612–635

Bhorade R, Weissleder R, Nakakoshi T, Moore A, Tung CH (2000) Macrocyclic chelators with paramagnetic cations are internalized into mammalian cells via a HIV-tat derived membrane translocation peptide. Bioconjug Chem 11:301–305

Boulland JL, Leung DS, Thuen M, Vik-Mo E, Joel M, Perreault MC, Langmoen IA, Haraldseth O, Glover JC (2012) Evaluation of intracellular labeling with micron-sized particles of iron oxide (MPIOs) as a general tool for in vitro and in vivo tracking of human stem and progenitor cells. Cell Transplant 21:1743–1759

Brindle K (2008) New approaches for imaging tumour responses to treatment. Nat Rev Cancer 8:94–107

Brown JM, WILSON WR (2004) Exploiting tumour hypoxia in cancer treatment. Nat Rev Cancer 4:437–447

Budde MD, Gold E, Jordan EK, Frank JA (2012) Differential microstructure and physiology of brain and bone metastases in a rat breast cancer model by diffusion and dynamic contrast enhanced MRI. Clin Exp Metastasis 29:51–62

Buxton RB, Frank LR, Wong EC, Siewert B, Warach S, Edelman RR (1998) A general kinetic model for quantitative perfusion imaging with arterial spin labeling. Magn Reson Med 40:383–396

Carbonell WS, Ansorge O, Sibson N, Muschel R (2009) The vascular basement membrane as "soil" in brain metastasis. PLoS One 4:e5857

Carmeliet P (2005) Angiogenesis in life, disease and medicine. Nature 438:932–936

Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257

Cerdan S, Lotscher HR, Kunnecke B, Seelig J (1989) Monoclonal antibody-coated magnetite particles as contrast agents in magnetic resonance imaging of tumors. Magn Reson Med 12:151–163

Chaumeil MM, Gini B, Yang H, Iwanami A, Sukumar S, Ozawa T, Pieper RO, Mischel PS, James CD, Berger MS, Ronen SM (2012) Longitudinal evaluation of MPIO-labeled stem cell biodistribution in glioblastoma using high resolution and contrast-enhanced MR imaging at 14.1 tesla. Neuro Oncol 14:1050–1061

Colotta F, Allavena P, Sica A, Garlanda C, Mantovani A (2009) Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. Carcinogenesis 30:1073–1081

Combes S, Jacob S, Combes N, Karam N, Chaumeil A, Guy-Moyat B, Treguer F, Deplagne A, Boveda S, Marijon E, Albenque JP (2013) Predicting favourable outcomes in the setting of radiofrequency catheter ablation of long-standing persistent atrial fibrillation: a pilot study assessing the value of left atrial appendage peak flow velocity. Arch Cardiovasc Dis 106:36–43

de Vries IJ, Lesterhuis WJ, Barentsz JO, Verdijk P, van Krieken JH, Boerman OC, Oyen WJ, Bonenkamp JJ, Boezeman JB, Adema GJ, Bulte JW, Scheenen TW, Punt CJ, Heerschap A, Figdor CG (2005) Magnetic resonance tracking of dendritic cells in melanoma patients for monitoring of cellular therapy. Nat Biotechnol 23:1407–1413

Deddens LH, van Tilborg GA, van Der Toorn A, van Der Marel K, Paulis LE, van Bloois L, Storm G, Strijkers GJ, Mulder WJ, de Vries HE, Dijkhuizen RM (2013) MRI of ICAM-1 upregulation after stroke: the importance of choosing the appropriate target-specific particulate contrast agent. Mol Imaging Biol 15(4):411–422

Denardo DG, Andreu P, Coussens LM (2010) Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metastasis Rev 29:309–316

Detre JA, Leigh JS, Williams DS, Koretsky AP (1992) Perfusion imaging. Magn Reson Med 23:37–45

Domingo G, Perez CA, Velez M, Cudris J, Raez LE, Santos ES (2010) EGF receptor in lung cancer: a successful story of targeted therapy. Expert Rev Anticancer Ther 10:1577–1587

Dvorak HF, Galli SJ, Dvorak AM (1986) Cellular and vascular manifestations of cell-mediated immunity. Hum Pathol 17:122–137

Ferjancic S, Gil-Bernabe AM, Hill SA, Allen PD, Richardson P, Sparey T, Savory E, Mcguffog J, Muschel RJ (2013) VCAM-1 and VAP-1 recruit myeloid cells that promote pulmonary metastasis in mice. Blood 121(16):3289–3297

Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:967–974

Fleige G, Nolte C, Synowitz M, Seeberger F, Kettenmann H, Zimmer C (2001) Magnetic labeling of activated microglia in experimental gliomas. Neoplasia 3:489–499

Fokas E, Mckenna WG, Muschel RJ (2012) The impact of tumor microenvironment on cancer treatment and its modulation by direct and indirect antivascular strategies. Cancer Metastasis Rev 31:823–842

Folkman J (1971) Tumor angiogenesis: therapeutic implications. N Engl J Med 285:1182–1186

Gao Z, Zhang L, Hu J, Sun Y (2013) Mesenchymal stem cells: a potential targeted-delivery vehicle for anti-cancer drug, loaded nanoparticles. Nanomedicine 9:174–184

Geelen T, Yeo SY, Paulis LE, Starmans LW, Nicolay K, Strijkers GJ (2012) Internalization of paramagnetic phosphatidylserine-containing liposomes by macrophages. J Nanobiotechnology 10:37

Goldschmidt P, Degorge S, Benallaoua D, Batellier L, Di Cave D, Chaumeil C (2012) Rapid detection and simultaneous molecular profile characterization of Acanthamoeba infections. Diagn Microbiol Infect Dis 74:137–141

Hahn PF, Weissleder R, Stark DD, Saini S, Elizondo G, Ferrucci JT (1988) MR imaging of focal splenic tumors. AJR Am J Roentgenol 150:823–827

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674

Harisinghani MG, Weissleder R (2004) Sensitive, noninvasive detection of lymph node metastases. PLoS Med 1:e66

Harisinghani MG, Barentsz J, Hahn PF, Deserno WM, Tabatabaei S, van de Kaa CH, de la Rosette J, Weissleder R (2003) Noninvasive detection of clinically occult lymph-node metastases in prostate cancer. N Engl J Med 348:2491–2499

Heijmen L, Verstappen MC, Ter Voert EE, Punt CJ, Oyen WJ, de Geus-Oei LF, Hermans JJ, Heerschap A, van Laarhoven HW (2012) Tumour response prediction by diffusion-weighted MR imaging: ready for clinical use? Crit Rev Oncol Hematol 83:194–207

Heyn C, Ronald JA, Mackenzie LT, Macdonald IC, Chambers AF, Rutt BK, Foster PJ (2006a) In vivo magnetic resonance imaging of single cells in mouse brain with optical validation. Magn Reson Med 55:23–29

Heyn C, Ronald JA, Ramadan SS, Snir JA, Barry AM, Kmackenzie LT, Mikulis DJ, Palmieri D, Bronder JL, Steeg PS, Yoneda T, Macdonald IC, Chambers AF, Rutt BK, Foster PJ (2006b) In vivo MRI of cancer cell fate at the single-cell level in a mouse model of breast cancer metastasis to the brain. Magn Reson Med 56:1001–1010

Hoefnagels FW, Lagerwaard FJ, Sanchez E, Haasbeek CJ, Knol DL, Slotman BJ, Vandertop WP (2009) Radiological progression of cerebral metastases after radiosurgery: assessment of perfusion MRI for differentiating between necrosis and recurrence. J Neurol 256:878–887

Hoyte LC, Brooks KJ, Nagel S, Akhtar A, Chen R, Mardiguian S, Mcateer MA, Anthony DC, Choudhury RP, Buchan AM, Sibson NR (2010) Molecular magnetic resonance imaging of acute vascular cell adhesion molecule-1 expression in a mouse model of cerebral ischemia. J Cereb Blood Flow Metab 30:1178–1187

Ihnen M, Kilic E, Kohler N, Loning T, Witzel I, Hagel C, Holler S, Kersten JF, Muller V, Janicke F, Milde-Langosch K (2011) Protein expression analysis of ALCAM and CEACAM6 in breast cancer metastases reveals significantly increased ALCAM expression in metastases of the skin. J Clin Pathol 64:146–152

Jackson A, O'Connor J, Thompson G, Mills S (2008) Magnetic resonance perfusion imaging in neuro-oncology. Cancer Imaging 8:186–199

Joyal CC, Pennanen C, Tiihonen E, Laakso MP, Tiihonen J, Aronen HJ (2004) MRI volumetry of the vermis and the cerebellar hemispheres in men with schizophrenia. Psychiatry Res 131:115–124

Jung CW, Jacobs P (1995) Physical and chemical properties of superparamagnetic iron oxide MR contrast agents: ferumoxides, ferumoxtran, ferumoxsil. Magn Reson Imaging 13:661–674

Keunen O, Johansson M, Oudin A, Sanzey M, Rahim SA, Fack F, Thorsen F, Taxt T, Bartos M, JIRIK R, Miletic H, Wang J, Stieber D, Stuhr L, Moen I, Rygh CB, Bjerkvig R, Niclou SP (2011) Anti-VEGF treatment reduces blood supply and increases tumor cell invasion in glioblastoma. Proc Natl Acad Sci U S A 108:3749–3754

Kircher MF, Gambhir SS, Grimm J (2011) Noninvasive cell-tracking methods. Nat Rev Clin Oncol 8:677–688

Kleijn A, Chen JW, Buhrman JS, Wojtkiewicz GR, Iwamoto Y, Lamfers ML, Stemmer-Rachamimov AO, Rabkin SD, Weissleder R, Martuza RL, Fulci G (2011) Distinguishing inflammation from tumor and peritumoral edema by myeloperoxidase magnetic resonance imaging. Clin Cancer Res 17:4484–4493

Klemke M, Weschenfelder T, Konstandin MH, Samstag Y (2007) High affinity interaction of integrin alpha4beta1 (VLA-4) and vascular cell adhesion molecule 1 (VCAM-1) enhances migration of human melanoma cells across activated endothelial cell layers. J Cell Physiol 212:368–374

Laubli H, Borsig L (2010) Selectins promote tumor metastasis. Semin Cancer Biol 20: 169–177

Law M, Cha S, Knopp EA, Johnson G, Arnett J, Litt AW (2002) High-grade gliomas and solitary metastases: differentiation by using perfusion and proton spectroscopic MR imaging. Radiology 222:715–721

Law M, Teicher N, Zagzag D, Knopp EA (2003a) Dynamic contrast enhanced perfusion MRI in mycosis fungoides. J Magn Reson Imaging 18:364–367

Law M, Yang S, Wang H, Babb JS, Johnson G, Cha S, Knopp EA, Zagzag D (2003b) Glioma grading: sensitivity, specificity, and predictive values of perfusion MR imaging and proton MR spectroscopic imaging compared with conventional MR imaging. AJNR Am J Neuroradiol 24:1989–1998

Leenders W, Kusters B, Pikkemaat J, Wesseling P, Ruiter D, Heerschap A, Barentsz J, de Waal RM (2003) Vascular endothelial growth factor-A determines detectability of experimental melanoma brain metastasis in GD-DTPA-enhanced MRI. Int J Cancer 105:437–443

Liu Y, Karonen JO, Vanninen RL, Nuutinen J, Koskela A, Soimakallio S, Aronen HJ (2004) Acute ischemic stroke: predictive value of 2D phase-contrast MR angiography–serial study with combined diffusion and perfusion MR imaging. Radiology 231:517–527

Mcateer MA, Sibson NR, von Zur Muhlen C, Schneider JE, Lowe AS, Warrick N, Channon KM, Anthony DC, Choudhury RP (2007) In vivo magnetic resonance imaging of acute brain inflammation using microparticles of iron oxide. Nat Med 13:1253–1258

Milanezi F, Carvalho S, Schmitt FC (2008) EGFR/HER2 in breast cancer: a biological approach for molecular diagnosis and therapy. Expert Rev Mol Diagn 8:417–434

Miller JC, Pien HH, Sahani D, Sorensen AG, Thrall JH (2005) Imaging angiogenesis: applications and potential for drug development. J Natl Cancer Inst 97:172–187

Moffat BA, Chenevert TL, Lawrence TS, Meyer CR, Johnson TD, Dong Q, Tsien C, Mukherji S, Quint DJ, Gebarski SS, Robertson PL, Junck LR, Rehemtulla A, Ross BD (2005) Functional diffusion map: a noninvasive MRI biomarker for early stratification of clinical brain tumor response. Proc Natl Acad Sci U S A 102:5524–5529

Moore A, Weissleder R, Bogdanov A Jr (1997) Uptake of dextran-coated monocrystalline iron oxides in tumor cells and macrophages. J Magn Reson Imaging 7:1140–1145

Neuwelt EA, Varallyay P, Bago AG, Muldoon LL, Nesbit G, Nixon R (2004) Imaging of iron oxide nanoparticles by MR and light microscopy in patients with malignant brain tumours. Neuropathol Appl Neurobiol 30:456–471

Nguyen DX, Bos PD, Massague J (2009) Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer 9:274–284

O'connor JP, Jackson A, Parker GJ, Jayson GC (2007) DCE-MRI biomarkers in the clinical evaluation of antiangiogenic and vascular disrupting agents. Br J Cancer 96:189–195

Oude Engberink RD, Blezer EL, Dijkstra CD, van Der Pol SM, van Der Toorn A, de Vries HE (2010) Dynamics and fate of USPIO in the central nervous system in experimental autoimmune encephalomyelitis. NMR Biomed 23:1087–1096

Partlow KC, Chen J, Brant JA, Neubauer AM, Meyerrose TE, Creer MH, Nolta JA, Caruthers SD, Lanza GM, Wickline SA (2007) 19F magnetic resonance imaging for stem/progenitor cell tracking with multiple unique perfluorocarbon nanobeacons. FASEB J 21:1647–1654

Paulis LE, Jacobs I, van Den Akker NM, Geelen T, Molin DG, Starmans LW, Nicolay K, Strijkers GJ (2012) Targeting of ICAM-1 on vascular endothelium under static and shear stress conditions using a liposomal Gd-based MRI contrast agent. J Nanobiotechnology 10:25

Perini R, Choe R, Yodh AG, Sehgal C, Divgi CR, Rosen MA (2008) Non-invasive assessment of tumor neovasculature: techniques and clinical applications. Cancer Metastasis Rev 27:615–630

Perkio J, Soinne L, Ostergaard L, Helenius J, Kangasmaki A, Martinkauppi S, Salonen O, Savolainen S, Kaste M, Tatlisumak T, Aronen HJ (2005) Abnormal intravoxel cerebral blood flow heterogeneity in human ischemic stroke determined by dynamic susceptibility contrast magnetic resonance imaging. Stroke 36:44–49

Pirko I, Ciric B, Johnson AJ, Gamez J, Rodriguez M, Macura S (2003) Magnetic resonance imaging of immune cells in inflammation of central nervous system. Croat Med J 44:463–468

Plate KH, Breier G, Weich HA, Risau W (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature 359:845–848

Qian BZ, Pollard JW (2010) Macrophage diversity enhances tumor progression and metastasis. Cell 141:39–51

Rausch M, Baumann D, Neubacher U, Rudin M (2002) In-vivo visualization of phagocytotic cells in rat brains after transient ischemia by USPIO. NMR Biomed 15:278–283

Roberts HC, Roberts TP, Ley S, Dillon WP, Brasch RC (2002) Quantitative estimation of microvascular permeability in human brain tumors: correlation of dynamic Gd-DTPA-enhanced MR imaging with histopathologic grading. Acad Radiol 9(Suppl 1):S151–S155

Roland CL, Harken AH, Sarr MG, Barnett CC Jr (2007) ICAM-1 expression determines malignant potential of cancer. Surgery 141:705–707

Saif MW (2010) Colorectal cancer in review: the role of the EGFR pathway. Expert Opin Investig Drugs 19:357–369

Saudek F, Jirak D, Girman P, Herynek V, Dezortova M, Kriz J, Peregrin J, Berkova Z, Zacharovova K, Hajek M (2010) Magnetic resonance imaging of pancreatic islets transplanted into the liver in humans. Transplantation 90:1602–1606

Sauter G, Maeda T, Waldman FM, Davis RL, Feuerstein BG (1996) Patterns of epidermal growth factor receptor amplification in malignant gliomas. Am J Pathol 148:1047–1053

Schafer R, Bantleon R, Kehlbach R, Siegel G, Wiskirchen J, Wolburg H, Kluba T, Eibofner F, Northoff H, Claussen CD, Schlemmer HP (2010) Functional investigations on human mesenchymal stem cells exposed to magnetic fields and labeled with clinically approved iron nanoparticles. BMC Cell Biol 11:22

Schmieder AH, Winter PM, Caruthers SD, Harris TD, Williams TA, Allen JS, Lacy EK, Zhang H, Scott MJ, Hu G, Robertson JD, Wickline SA, Lanza GM (2005) Molecular MR imaging of melanoma angiogenesis with alphanubeta3-targeted paramagnetic nanoparticles. Magn Reson Med 53:621–627

Schwechheimer K, Huang S, Cavenee WK (1995) EGFR gene amplification–rearrangement in human glioblastomas. Int J Cancer 62:145–148

Serres S, Anthony DC, Jiang Y, Broom KA, Campbell SJ, Tyler DJ, van Kasteren SI, Davis BG, Sibson NR (2009) Systemic inflammatory response reactivates immune-mediated lesions in rat brain. J Neurosci 29:4820–4828

Serres S, Mardiguian S, Campbell SJ, Mcateer MA, Akhtar A, Krapitchev A, Choudhury RP, Anthony DC, Sibson NR (2011) VCAM-1-targeted magnetic resonance imaging reveals subclinical disease in a mouse model of multiple sclerosis. FASEB J 25:4415–4422

Serres S, Soto MS, Hamilton A, Mcateer MA, Carbonell WS, Robson MD, Ansorge O, Khrapitchev A, Bristow C, Balathasan L, Weissensteiner T, Anthony DC, Choudhury RP, Muschel RJ, Sibson NR (2012) Molecular MRI enables early and sensitive detection of brain metastases. Proc Natl Acad Sci U S A 109:6674–6679

Shapiro EM, Skrtic S, Sharer K, Hill JM, Dunbar CE, Koretsky AP (2004) MRI detection of single particles for cellular imaging. Proc Natl Acad Sci U S A 101:10901–10906

Shapiro EM, Skrtic S, Koretsky AP (2005) Sizing it up: cellular MRI using micron-sized iron oxide particles. Magn Reson Med 53:329–338

Shazeeb MS, Sotak CH, Deleo M 3rd, Bogdanov A Jr (2011) Targeted signal-amplifying enzymes enhance MRI of EGFR expression in an orthotopic model of human glioma. Cancer Res 71:2230–2239

Shen JF, Saunders JK (1993) Double inversion recovery improves water suppression in vivo. Magn Reson Med 29:540–542

Shen T, Weissleder R, Papisov M, Bogdanov A Jr, Brady TJ (1993) Monocrystalline iron oxide nanocompounds (MION): physicochemical properties. Magn Reson Med 29:599–604

Sipe JC, Filippi M, Martino G, Furlan R, Rocca MA, Rovaris M, Bergami A, Zyroff J, Scotti G, Comi G (1999) Method for intracellular magnetic labeling of human mononuclear cells using approved iron contrast agents. Magn Reson Imaging 17:1521–1523

Sitohy B, Nagy JA, Dvorak HF (2012) Anti-VEGF/VEGFR therapy for cancer: reassessing the target. Cancer Res 72:1909–1914

Song HT, Jordan EK, Lewis BK, Gold E, Liu W, Frank JA (2011) Quantitative T2* imaging of metastatic human breast cancer to brain in the nude rat at 3 T. NMR Biomed 24:325–334

Sundstrom T, Daphu I, Wendelbo I, Hodneland E, Lundervold A, Immervoll H, Skaftnesmo KO, Babic M, Jendelova P, Sykova E, Lund-Johansen M, Bjerkvig R, Thorsen F (2013) Automated tracking of nanoparticle-labeled melanoma cells improves the predictive power of a brain metastasis model. Cancer Res 73(8):2445–2456

Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG (2000) New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 92:205–216

Unger EC (2003) How can superparamagnetic iron oxides be used to monitor disease and treatment? Radiology 229:615–616

Valable S, Barbier EL, Bernaudin M, Roussel S, Segebarth C, Petit E, Remy C (2007) In vivo MRI tracking of exogenous monocytes/macrophages targeting brain tumors in a rat model of glioma. Neuroimage 37(Suppl 1):S47–S58

van Kasteren SI, Campbell SJ, Serres S, Anthony DC, Sibson NR, Davis BG (2009) Glyconanoparticles allow pre-symptomatic in vivo imaging of brain disease. Proc Natl Acad Sci U S A 106:18–23

von Zur Muhlen C, Sibson NR, Peter K, Campbell SJ, Wilainam P, Grau GE, Bode C, Choudhury RP, Anthony DC (2008) A contrast agent recognizing activated platelets reveals murine cerebral malaria pathology undetectable by conventional MRI. J Clin Invest 118:1198–1207

Wang J, Alsop DC, Li L, Listerud J, Gonzalez-AT JB, Schnall MD, Detre JA (2002) Comparison of quantitative perfusion imaging using arterial spin labeling at 1.5 and 4.0 Tesla. Magn Reson Med 48:242–254

Wang J, Rao H, Wetmore GS, Furlan PM, Korczykowski M, Dinges DF, Detre JA (2005) Perfusion functional MRI reveals cerebral blood flow pattern under psychological stress. Proc Natl Acad Sci U S A 102:17804–17809

Warmuth C, Gunther M, Zimmer C (2003) Quantification of blood flow in brain tumors: comparison of arterial spin labeling and dynamic susceptibility-weighted contrast-enhanced MR imaging. Radiology 228:523–532

Weissleder R, Pittet MJ (2008) Imaging in the era of molecular oncology. Nature 452:580–589

Weissleder R, Hahn PF, Stark DD, Rummeny E, Saini S, Wittenberg J, Ferrucci JT (1987a) MR imaging of splenic metastases: ferrite-enhanced detection in rats. AJR Am J Roentgenol 149:723–726

Weissleder R, Stark DD, Compton CC, Wittenberg J, Ferrucci JT (1987b) Ferrite-enhanced MR imaging of hepatic lymphoma: an experimental study in rats. AJR Am J Roentgenol 149:1161–1165

Weissleder R, Hahn PF, Stark DD, Elizondo G, Saini S, Todd LE, Wittenberg J, Ferrucci JT (1988a) Superparamagnetic iron oxide: enhanced detection of focal splenic tumors with MR imaging. Radiology 169:399–403

Weissleder R, Stark DD, Elizondo G, Hahn PF, Compton C, Saini S, Wittenberg J, Ferrucci JT (1988b) MRI of hepatic lymphoma. Magn Reson Imaging 6:675–681

Weissleder R, Elizondo G, Wittenberg J, Lee AS, Josephson L, Brady TJ (1990) Ultrasmall superparamagnetic iron oxide: an intravenous contrast agent for assessing lymph nodes with MR imaging. Radiology 175:494–498

Weissleder R, Bogdanov A, Papisov M (1992) Drug targeting in magnetic resonance imaging. Magn Reson Q 8:55–63

Weissleder R, Cheng HC, Bogdanova A, Bogdanov A Jr (1997) Magnetically labeled cells can be detected by MR imaging. J Magn Reson Imaging 7:258–263

Wiiger MT, Gehrken HB, Fodstad O, Maelandsmo GM, Andersson Y (2010) A novel human recombinant single-chain antibody targeting CD166/ALCAM inhibits cancer cell invasion in vitro and in vivo tumour growth. Cancer Immunol Immunother 59:1665–1674

Williams DS, Detre JA, Leigh JS, Koretsky AP (1992) Magnetic resonance imaging of perfusion using spin inversion of arterial water. Proc Natl Acad Sci U S A 89:212–216

Wolf RL, Wang J, Wang S, Melhem ER, O'Rourke DM, Judy KD, Detre JA (2005) Grading of CNS neoplasms using continuous arterial spin labeled perfusion MR imaging at 3 Tesla. J Magn Reson Imaging 22:475–482

Wong EC, Buxton RB, Frank LR (1998) A theoretical and experimental comparison of continuous and pulsed arterial spin labeling techniques for quantitative perfusion imaging. Magn Reson Med 40:348–355

Wong EC, Cronin M, Wu WC, Inglis B, Frank LR, Liu TT (2006) Velocity-selective arterial spin labeling. Magn Reson Med 55:1334–1341

Yankeelov TE, Lepage M, Chakravarthy A, Broome EE, Niermann KJ, Kelley MC, Meszoely I, Mayer IA, Herman CR, Mcmanus K, Price RR, Gore JC (2007) Integration of quantitative DCE-MRI and ADC mapping to monitor treatment response in human breast cancer: initial results. Magn Reson Imaging 25:1–13

Zhang C, Jugold M, Woenne EC, Lammers T, Morgenstern B, Mueller MM, Zentgraf H, Bock M, Eisenhut M, Semmler W, Kiessling F (2007) Specific targeting of tumor angiogenesis by RGD-conjugated ultrasmall superparamagnetic iron oxide particles using a clinical 1.5-T magnetic resonance scanner. Cancer Res 67:1555–1562

Zhang F, Huang X, Zhu L, Guo N, Niu G, Swierczewska M, Lee S, Xu H, Wang AY, Mohamedali KA, Rosenblum MG, Lu G, Chen X (2012) Noninvasive monitoring of orthotopic glioblastoma therapy response using RGD-conjugated iron oxide nanoparticles. Biomaterials 33:5414–5422

Zimmer C, Weissleder R, O'Connor D, Lapointe L, Brady TJ, Enochs WS (1995a) Cerebral iron oxide distribution: in vivo mapping with MR imaging. Radiology 196:521–527

Zimmer C, Weissleder R, Poss K, Bogdanova A, Wright SC Jr, Enochs WS (1995b) MR imaging of phagocytosis in experimental gliomas. Radiology 197:533–538

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