MCPHATTER, LISA ANASTASIA. Characterization and Gene Expression of Gastrointestinal Stem Cell Markers and Experimental Therapeutics of Gastrointestinal-Colorectal Cancer. (Under the direction of Dr. Miles See and Dr. Bruce Klitzman.)

The extension of the tumor vascular network during angiogenesis is essential for tumor growth, and the persistent immaturity of the resulting neovessels facilitates metastasis. For these reasons and based on its peculiarities, disrupting tumor angiogenesis is a promising antitumor strategy. An efficient approach is to inhibit the proangiogenic activity of the vascular endothelial growth factor (VEGF), which can be done by means of Bevacizumab/rhuMab, a humanized anti-VEGF monoclonal antibody. Upon VEGF inactivation, the VEGF-activated promitogenic PI3K/AKT/mTOR axis is rescued by other tumor microenvironmental factors. Based on this observation, we reasoned that combining Bevacizumab to Rapamycin, an immunosuppressive drug binding to mTOR (mammalian target of rapamycin), will have superior antitumor activity compared with either agent alone. Utilizing an in vivo xenograft model of colon adenocarcinoma (HT29), we evidenced lower VEGF abundance in mouse tumors treated with the combination therapy versus each individual treatment. The combination significantly decreased angiogenesis (CD31 staining) and tumor cell proliferation (Ki67 staining) and increased tumor cell apoptosis (in situ caspase 3 activation assay). Furthermore, a decrease in tumor hypoxia (measured via CAIX staining) was observed. In tumor growth delay experiments, our results showed a more than additive effects (increased mean tumor regrowth delays) for animals treated with Bevacizumab + Rapamycin (3.4±0.6 days) versus Bevacizumab (1.4±0.8 days) or Rapamycin alone (0.2±0.5 days).
That stem cells are intimately involved in cancer causing mechanisms which encompass tumor establishment, progression, relapse, and metastasis, is becoming increasingly evident. Most human tumors derive from epithelial cells and colon adenocarcinoma presents as a cancer of epithelial cell origin. Gastrointestinal stem cells are pluripotent “master cells” which give rise to all cell lineages in the epithelium. Additionally, angiogenesis inhibitors have been shown to decrease putative cancer stem cell numbers. Discoveries in the identification of markers for gastrointestinal stem cells have formed the basis for focused research in the treatment of intestinal disease; surgical resection, in addition to antiangiogenic therapy in colorectal cancer, presents a therapeutic strategy for patients. Utilizing a mouse model, we sought to examine if there is a baseline difference between intestinal stem cell markers in the proximal (jejunum) when compared to the distal (ileum) after small bowel resection (SBR). We tested our hypothesis at time points using the selected intestinal stem cell markers: Musashi-1, Ascl2, and Olfm4, and Lgr5. Quantitative RT-PCR was performed to measure intestinal stem cell marker expression. Crypt dissociations and histological morphometrics were performed to compare post-resectional differences in the proximal (jejunum) versus the distal (ileum) intestine. Preliminary data showed Lgr5 mRNA in both cells and tissue in jejunum was higher versus ileum at same day time points. Furthermore, we evidenced significant augmentation of the jejunum when compared to the ileum for changes in histological morphometrics.

The studies presented here were undertaken to further the understanding of the characterization of gastrointestinal stem cells in the development of stem cell based therapy and the angiogenesis-dependent tumor vasculature as a target of antiangiogenic therapy in treatment of gastrointestinal cancer and disease. These studies have significance in patient
care, as gastrointestinal-colorectal cancer (CRC) is a leading cause of morbidity and mortality in the United States and worldwide with over a million new cases every year. Even more striking, the cost of cancer care in the United States alone is projected to increase from $125 billion in 2010 to $207 billion by the year 2020.
Characterization and Gene Expression of Gastrointestinal Stem Cell Markers and Experimental Therapeutics of Gastrointestinal-Colorectal Cancer

by
Lisa Anastasia McPhatter

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APPROVED BY:

___________________ _____________________
Dr. Miles See Dr. Bruce Klitzman
Committee Chair

___________________ _____________________
Dr. James Brown Dr. William Grant

___________________ _____________________
Dr. Charles Whisnant Dr. David Threadgill
DEDICATION

To Max, play with the Angels
BIOGRAPHY

Lisa Anastasia McPhatter is a native of North Carolina and was reared on the outskirts of Southern Pines, NC. She graduated with a Bachelor of Science from Western Carolina University and worked as a research assistant in the Department of Pathology at Duke University Medical Center where she co-authored a formative publication stemming from her research on neuropathological brain disease in children, published in the journal of Acta Neuropathologica. Lisa went on to earn a Master of Science in the interdisciplinary Nutrition program at North Carolina State University. She then performed research in the field of experimental cancer therapeutics under the guidance of the late Dr. Michael Colvin. She continued her studies in pursuit of a doctorate in Physiology under the co-mentorship of Dr. Miles See, Chair, in the Department of Animal Science at North Carolina State University, and Dr. Bruce Klitzman in the Department of Cell Biology at Duke University. While pursuing her doctoral studies Lisa was awarded a doctoral research assistantship from the National Cancer Institute.
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CHAPTER ONE: Literature Review of Gastrointestinal-Colorectal Cancer: Angiogenesis and Tumor Vasculature

Introduction

A History of Angiogenesis

In 1787, the term of angiogenesis was first utilized by British surgeon, John Hunter (Hall 2005). Angiogenesis is the formation of new blood vessels via remodeling and expansion of primary vessels. It is paramount in normal physiological processes such as tissue growth, wound healing, fetal development, and reproductive function, and arises from a series of complex interrelated steps. These steps involve specific and strong regulation of vascular proliferation and permeability, vasodilation, migration of endothelial cells, and survival (Herbst et al. 2005). In addition, angiogenesis is a central component in the transition of tumors from a benign state to a malignant one, and has led to the utilization of angiogenesis inhibitors in cancer therapy. Albeit, reports of tumor angiogenesis were rare until nearly 100 years later from John Hunter’s first use of the term and these were largely derived from anatomical studies. The vascular morphology of tumors was studied in substantial detail starting with observations in the 1860’s (Virchow 1863; Thiersch 1865); by 1907, the vascular network was visualized in both human and animal tumor specimens via intra-arterial injections of bismuth in oil (Goldman 1907). Moreover, the vascular morphology was studied in human and animal tumors during the first half of the twentieth century, and largely to ascertain whether vascular patterns could distinguish benign from malignant tumors and to comprehend the shedding of tumor emboli into the circulation or to clarify the delivery of active agents into tumors that were specific (Thiessen 1936; Wright
In the late 1930’s and early 1940’s a multitude of investigators had begun to study the developments of neovascularization in experimental tumors and not just from observations of anatomical specimens. Furthermore these reports for the first time, delineated the continuing neovascularization of tumors that were subcutaneously implanted or in transparent chambers, or that were in the hamster cheek pouch (Ide et al. 1939; Cowdry and Sheldon 1946; Lutz et al. 1950; Toolan 1951; Green 1952; Wood 1958; Day et al. 1951; Beirman et al. 1951; Gullino et al. 1962; Urbach and Graham 1962; Rubin and Casarette 1966). Ide et al. and Algire et al. were pioneers who began the experimental study on the formation of new blood vessels, or angiogenesis (Ide et al. 1939; Algire 1943; Algire et al. 1947). Experimental tumors were separated from host tissue with aid of a micropore filter in order to demonstrate that unknown diffusible substance was secreted from the tumor which could stimulate the growth of new blood vessels (Ehrmann and Knoth 1968). Onset of tumor neovascularization was explicated via several other reports (Gullino et al. 1962; Goldacre and Sylven 1962; Delarue et al. 1963; Day 1964; Goodall et al. 1965; Warren and Shubnick 1966; Tannock 1970; Folkman 2008). However, the cause of tumor neovascularization in these studies was unclear and was ascribed to inflammation, vasodilation, elevated tumor metabolism, overproduction of specific metabolites like lactic acid, or from tumors outgrowing their blood supply due to hypoxia (Folkman et al. 1962). Before 1970, that tumor angiogenesis was a side-effect of dying tumor cells, was predominantly regarded as true.

In 1971, Judah Folkman proposed the concept of angiogenesis as being fundamental in the growth of tumors and described them to be “hot and red and bloody” (Folkman 1971,
Furthermore, he predicted that by identifying principal molecular drivers in tumor angiogenesis, effective strategies to block it and thereby starve the tumor to death, could serve a promising approach. Folkman also showed preliminary evidence that tumors require neovascularization for growth beyond a 2-3mm diameter (Folkman 1971). During the last 40 years, Folkman’s seminal hypothesis, as well as the notable research demonstrated by past pioneers, has served to form the foundation for highly significant advances in the field tumor biology and angiogenesis.

Judah Folkman (1933-2008): The Father of Angiogenesis

Dr. Moses Judah Folkman, known as the father of angiogenesis, was a surgeon and biomedical researcher who founded a whole new field of basic and clinical research in angiogenesis as well as discovered a family of angiogenesis regulatory molecules that were not previously known. Folkman’s 1971 paper published in the *New England Journal of Medicine* was the first to propose the hypothesis of a novel form of tumor dormancy as a result of blocking angiogenesis. In this paper, he presented the concept of “antiangiogenesis” as a promising novel anticancer therapy. Folkman composed the following statement in his 1971 report, “If a tumor could be held indefinitely in the nonvascularized dormant state . . . it is possible that metastases will not arise” (Folkman 1971, p. 1184). That a therapeutic agent could be made against a putative tumor derived angiogenic factor was an outcome expressed by Folkman. He also deduced, “It has not been appreciated that the population of tumor cells and the population of capillary endothelial cells within a neoplasm may constitute a highly integrated ecosystem. In this ecosystem, the mitotic index of the two cell populations may depend on each other” (Folkman 1971, p.
Of note, Folkman’s hypothesis was first met with disregard by a majority of researchers in the field; however, he persevered with his work, and concepts he put forth are now broadly accepted and utilized in the treatment of cancer and disease. Dr. Folkman was known to be warm and humble, an inspirational teacher and mentor, extremely creative, and immensely knowledgeable.

**Tumor Angiogenesis and Pathophysiology**

Tumor angiogenesis can be described as the production of an increased number of blood vessels whose purpose is to supply the growing mass. Although the principal aim of tumor angiogenesis can be considered to maintain a cancer’s blood supply, the process occurs in a manner that is unmitigated and results in an extremely abnormal vascular network. Consequently, this presents a contradistinction to wound healing, whereby angiogenesis is tightly regulated (Chung et al. 2010; Dvorak 1986). Studies by Dvorak in angiogenesis led to a description of tumors as being wounds that do not heal. The extremely aberrant vasculature dramatically alters the tumor microenvironment and governs ways in which tumors develop and progress, as well as evade the host’s immune system, metastasize, and behave in anticancer therapy (Dvorak 1986).

**Aberrant Tumor Blood Vessel Structure and Function**

Normal microvessels comprise arterioles, capillaries, and venules, and structure a functioning architecture that is highly organized and controlled. By contradistinction, tumor vessels are dilated, saccular, tortuous, and heterogeneous in their spatial distribution (Jain 1988). Normal vasculature is characterized by dichotomous branching, whereas tumor
vasculature is unorganized and displays trifurcations and branching with uneven diameters, in addition to abnormal vessel wall structure (Chang et al. 2000; di Tomaso et al. 2005; McDonald and Choyke 2003). Furthermore, sizable inter-endothelial junctions, high numbers of fenestrations, vesicles, and vesicovacuolar channels, as well as a lack of normal basement membrane are frequently present within vessels of tumors (Dvorak et al. 2002; Winkler et al. 2004).

In diseased (or injured) cells several proangiogenic growth factors are released which include VEGF, basic fibroblast growth factors (bFGFs), platelet-derived growth factor (PDGF), tumor necrosis factor and keratinocyte growth factor (Motl 2005). These angiogenic factors are able to act on endothelial cells directly via the stimulation of their proliferation and migrational activities, or indirectly via the activation of other associated cells in angiogenesis. By contrast, antiangiogenic factors such as thrombospondin-1, angiostatin, endostatin, and tumstatin suppress the actions involved in vessel recruitment and growth (Midgley and Kerr 2005). Although a balance of pro- and antiangiogenesis is maintained during normal physiologic processes, this tightly regulated balance will shift in favor of proangiogenic activities during neoplastic growth.

The perivascular cells possess morphology that is abnormal along with a heterogeneous association with tumor vessels; the molecular mechanisms which result in these abnormal vascular architectures are poorly understood, although, the imbalance of proangiogenic and antiangiogenic factors is deemed a predominant contributor (Jain 2005). Also, a solid and mechanical stress generated by proliferating tumor cells will compress vessels in tumors (Padera et al. 2004; Roose et al. 2003). Consequently, both molecular and
mechanical factors combined might contribute to abnormal tumor vasculature. Within a vessel, extravasation of molecules from the bloodstream occurs by diffusion, convection, as well as transcytosis to a degree. The principal form of transvascular transport in tumors is recognized as diffusion (Jain 1987). A diffusive permeability of a molecule is dependent upon the size, shape, flexibility, and charge of the molecule, in addition to its transvascular transport pathway. In accordance with the aforementioned ultrastructural changes in the tumor vessel wall, vascular permeability in solid tumors is usually increased relative to normal tissues. Flow resistance is a function of the architecture of the vasculature and resistance in blood viscosity and rheology. In a vascular network, arterio-venous pressure difference as well as flow resistance will regulate the flow of blood, and abnormalities in the vasculature and viscosity, heighten resistance to tumor blood flow (Jain 1988).

There frequently exist focal leaks in tumor vessels, which might jeopardize the downstream blood flow as well. Due to this, the overall perfusion rate of blood flow per unit volume, i.e., perfusion, in tumors is more reduced than in normal tissues. Additionally, the average red blood cell velocity in tumor vessels can be an order of magnitude lower than in normal vessels. In contrast to normal vessels, red blood cell velocity is not dependent upon the diameter of tumor vessels. Moreover, blood flow in tumors does not have a uniform distribution and it can fluctuate with time. In addition, blood flow in tumors has the capability to reverse its direction within some vessels. Thus, regions having little or no perfusion whatsoever are observed routinely. The heterogeneity of blood flow in tumors results in an aberrant tumor microenvironment to curb delivery and efficacy of therapeutic agents to the tumor (Jain 2005).
Interstitial Fluid Pressure in Tumors

In normal tissue, the interstitial fluid pressure is about 0 mmHg, while tumors in both animals and humans demonstrate interstitial hypertension (Jain 2004). The aberrant architecture and function of blood and lymphatic vessels in tumors results in increased interstitial fluid pressure. Tumor vessels lack selectivity because of heightened vascular permeability and, as a result, the hydrostatic and oncotic pressures are nearly equivalent between the intravascular and extravascular spaces (Boucher and Jain 1992; Tong et al. 2004). There are equivalent increases and/or decreases in tumor interstitial fluid pressure in approximately ten seconds following the modification of microvascular pressure (Netti et al. 1995) and reduced transmural pressure gradients lower convection across tumor vessel walls. Also, interstitial fluid pressure is increased uniformly throughout a tumor and drops abruptly in the tumor margin (Boucher et al. 1990). Thus, the interstitial fluid slowly leaks out of the tumor into the surrounding normal tissue carrying angiogenic and lymphangiogenic growth factors as well as metastasizing tumor cells with it (Jain 2005). As a result, uniformly high interstitial fluid pressure weakens the delivery of therapeutic agents (drugs) both across the wall of the blood vessel and interstitum in tumors. Because interstitial fluid pressure represents a strong view of the comprehensive pathophysiology of tumors, it might be used for diagnosis as well as prognosis. The extreme increases in interstitial fluid pressure at the tumor periphery can be utilized to locate tumors during needle biopsy and enhance diagnosis of patients (Jain et al. 1995). Studies of cervical cancer, moreover, have shown that rises in tumor interstitial fluid pressure can predict a poor outcome of radiation therapy (Jain 2004). More studies are required in order to assess the prognostic importance of interstitial fluid
pressure in human tumors. Reducing vascular permeability may improve and restore the transmural pressure gradients and potentially reestablish blood flow in the nonperfused tumor areas; direct and indirect antiangiogenic therapies might normalize the tumor vasculature through this mechanism (Jain 2005). Interstitial fluid pressure can be reduced by antibodies against VEGF or VEGFR2 in fact (Tong et al. 2004; Willett et al. 2004).

**Abnormal Metabolic Environment in Tumors**

Hypoxia and acidosis are distinguishing characteristics of an abnormal metabolic environment in solid tumors (Harris 2002; Helmlinger et al. 1997; Tatum et al. 2006). A key role of the vasculature involves supplying adequate levels of nutrients and oxygen to the parenchymal cells as well the elimination of waste products. However, tumor vessels are deficient in this operation as a result of their aberrant architecture and their functioning. Because of imbalances in the vascular network development and proliferation of tumor cells, resultant hypovascular areas in tumors form. As the tissue diffusion oxygen limit is 100–200 μm, the areas farthest from blood vessels become chronically hypoxic (Krogh 1922). Moreover, the presence of blood vessels does not assure tissues will be oxygenated in tumors. Frequently the flow of blood in tumor vessels is sporadic, and, as a result, some regions of a tumor are periodically starved for oxygen, known as an acute hypoxic situation (Brown and Giaccia 1998). With the aid of high-resolution intravital microscopy and phosphorescence quenching microscopy, Helmlinger and colleagues showed there is no precise relationship between blood flow rate and oxygen tension (pO2) of individual vessels in tumors (Helmlinger et al. 1997). Of note, some perfused tumor vessel carry little to no oxygen. The transit time of the blood in tumors is extended as a result of the heterogeneous,
disorganized, dysfunctional vessel network in tumors, along with imbalances in the supply of oxygen and consumption, might cause tumor tissue hypoxia in spite of an existing blood flow.

Low extracellular pH is yet another outcome of the aberrant microcirculation tumors. An acidic extracellular pH interferes with the cellular uptake of weak base drugs such as Adriamycin, doxorubicin, and mitoxantrone, thus hindering their efficacy (Vukovic and Tannock, 1997). Subsequently, both lactic acid and carbonic acid are the known sources of H+ ions in tumors (Helmlinger et al. 2002; Pouyssegur et al. 2006). Lactic acid is the product of anaerobic glycolysis whereas carbonic acid is formed from CO2 and H2O by the enzyme carbonic anhydrase. Rises in the generation of H+ ions, as well as decreases in their removal, act to reduce the extracellular pH in tumors. Furthermore, reduced extracellular pH causes stress-induced alteration of gene expression, which encompasses the upregulation of VEGF and IL-8 in tumor cells in vitro (Xu et al. 2002). Both pO2 and pH are significant elements in the determination of tumor growth and metabolism, as well as the response to diverse therapies.

**Hypoxia**

Hypoxia upregulates a number of angiogenic growth factors that include angiopoietin (Ang) 2, platelet derived growth factor (PDGF), placenta growth factor (PlGF), transforming growth factor α (TGFα), interleukin (IL)-8, hepatocyte growth factor (HGF), as well as the vascular endothelial growth factor (VEGF) that will be further described (Harris 2002). The hypoxia inducible factor 1α (HIF1α) is acknowledged as the “master regulator” of oxygen homeostasis for its role in sensing and responding to hypoxic conditions (Semenza, 2003).
HIF1α is a transcription factor and it is upregulated in a multitude of human tumors (Harris 2002). HIF1α will attach to the hypoxia responsive element (HRE) in promoter hypoxia-responsive genes such as PDGF, TGFα and VEGF, and induce their expression (Harris 2002; Semenza 2003).

Oxygen is a paramount constituent of radiation therapy (Brown 1999). Hypoxia in solid tumors greatly reduces their sensitivity to radiation. In addition, tumor hypoxia is associated with resistance to some chemotherapeutics, such as bleomycin and neocarzinostatin (Brown 1999).

Immune cells targeting tumor cells are not fully operational under hypoxic (or acidic conditions) conditions thus giving tumors the opportunity to evade the host immune response, as well as therapies that are cell based. The exposure to hypoxia results in extremely invasive and metastatic tumor cells (Erler et al. 2006; Pennacchietti et al. 2003; Rofstad et al. 2006).

**Stromal Cells**

Neither the pathophysiology nor development of a tumor can be explained solely by the genes in tumor cells (Weinberg 2006). Host stromal cells greatly affect a number of steps in tumor progression which encompass tumor cell proliferation, invasion, angiogenesis, metastasis, and malignant transformation (Elenbaas and Weinberg 2001; Fukumura et al. 1998; Li et al. 2003; Liotta and Kohn 2001; Pollard 2004; Ruiter et al. 2001; Tlsty 2001). The interplay between various cell types within the tumor mass, by soluble factors as well as direct cell-to-cell contact, perform a significant function regarding the induction, selection, and expansion of neoplastic cells. Li and colleagues as well as others report that successful
tumor cells are those which have achieved the capacity to co-opt their normal neighbors by inducing them to release abundant fluxes of growth-stimulating signals (Li et al. 2003; Tlsty 2001; Weinberg 2006). Furthermore, co-implantation of fibroblasts augments the tumorigenicity of breast cancer cells in vivo (Noel et al. 1993). Consequently, fibroblasts associated with carcinomas will promote tumor growth and angiogenesis via the secretion of stromal-cell derived factor 1 or SDF-1 (Orimo et al. 2005). Inflammatory cells recruited to tumors, in addition to fibroblasts, might promote angiogenesis and subsequent growth of tumor cells (de Visser et al., 2006; Pollard, 2004). Intravital observation of tumors grown in a GFP reporter mouse showed that stromal fibroblasts express VEGF in tumors particularly in high amounts at the host-tumor interface (Fukumura et al. 1998). Moreover, stromal cells which express VEGF co-localize with the vasculature and surround tumor blood vessels deep inside the tumor (Brown et al. 2001). These data suggest that activated fibroblasts do indeed take part in angiogenesis and fortification of blood vessels, as well as the functioning of these vessels.

**Vascular Endothelial Growth Factor: VEGF**

The founding member of the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) family, and the most well-studied, is VEGF-A (isoform 164/5); VEGF-A was originally discovered on the basis of its robust capability to enhance vascular permeability (Dvorak et al. 1979; Senger et al. 1983; Dvorak 2006). VEGF-A is encoded by a single gene and is a highly conserved, disulfide-bonded dimeric glycoprotein. It is characterized as sharing important sequence homology with platelet derived growth factor (PDGF); however, as with PDGF, VEGF has cysteines that create integral inter-chain as well
as intra-chain bonds. Moreover, crystal structure show that the two chains composing VEGF-A are arranged antiparallel, with receptor binding sites located at either end (Muller et al. 1997). Subsequently, VEGF-A separates into its individual chains and loses biological activity entirely upon reduction (Dvorak 2003; Senger et al. 1993). Of note, mice without even one copy of the VEGF-A gene are embryonic lethals (Carmeliet 1996 et al. 1996; Ferrara et al. 1996). The human VEGF-A gene is located on the short arm of chromosome 6 and is differentially spliced in order to produce main isoforms which encode polypeptides of 206, 189, 165, 145, and 121 amino acids in human cells, whereas corresponding murine proteins are one amino acid shorter (Figure 1.1). In addition, 165b is another splice variant that has been located in normal kidneys and is an endogenous VEGF-A165 inhibitor; it is downregulated in renal tumors and thus might be considered antiangiogenic (Woolard et al. 2004). It is likely all VEGF-A isoforms with the exception of 165b possess the same fundamental activities in vitro but are especially different in the manner in which they bind to cells and matrices (Grunstein et al. 2000; Maes et al. 2002; Park et al. 1993; Yu et al. 2002; Ruhrberg 2003).

Additional members of the VPF/VEGF family encompass VEGF-B, -C, and -D, as well as placenta growth factor (PIGF) and VEGF-E, a viral protein that is related to this family. VEGF-B has a function in coronary artery development (Joukov et al. 1997), and VEGF-B null mice present with smaller hearts, dysfunctional coronary vasculature, and recovery from cardiac ischemia which is impaired as well (Joukov et al. 1997; Nash et al. 2006). VEGF-C and -D are known as lymphangiogenic cytokines that have the ability to induce angiogenesis and enhance vascular permeability under suitable conditions (Oliver et
PIGF, as denoted by its name, was originally discovered in the placenta. While PIGF is not abundantly expressed in normal embryonic or adult tissues, it is expressed by a number of tumors as well as in other forms of pathological angiogenesis, where it is believed to both supplement and potentiate the activity of VEGF-A (Carmeliet et al. 2001; De Falco et al. 2002).

VPF/VEGF family members encompass three high-affinity receptor tyrosine kinases: VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (FLT-4) (Ferrara et al. 2003; Oliver et al. 2005; Olsson et al. 2006). VEGFR-1 and VEGFR-2 are broadly expressed by normal vascular endothelium in vivo and by cultured endothelial cells. Additionally, whereas VEGFR-2 is expressed in higher copy numbers than VEGFR-1, VEGFR-2 has an affinity for VEGF-A which is about tenfold lower. Subsequently, both VEGFR-1 and VEGFR-2 are upregulated in the vascular endothelium of tumors as well as in other examples of pathological angiogenesis that are VEGF-A-induced (Nagy et al. 2002; Dvorak 2003; Brown et al. 1997). The mechanisms which cause receptor overexpression are not completely comprehended; however, hypoxia, which stimulates the expression of VEGF-A, might upregulate Flt-1 while downregulating KDR (Detmar et al. 1997). Investigators originally thought that VEGFR-1 and VEGFR-2 were expressed exclusively on blood vascular or lymphatic endothelium. However, it is now clear that this is not the case. Both VEGFR-1 and VEGFR-2, and in particular, VEGFR-1 are established as being expressed on a broad range of normal and tumor cells, a number of which express VPF/VEGF family members in addition and thus provide opportunities for autocrine stimulation loops. Furthermore, the expression of VEGFR-2 on bone marrow and circulating endothelial cell progenitors
provides relevance from the context of angiogenesis (Raffi et al. 2002). Truncated forms of both VEGFR-1 and VEGFR-2 which are soluble have currently been found in the plasma; while not as much is known about VEGFR-2 (Shaked et al. 2005), the soluble form of VEGFR-1 is a result of alternative splicing, retains VEGF-A and PlGF binding activity, and has been recently discovered to play a role in pre-eclampsia (Maynard et al. 2003; Levine et al. 2006). Of note, two receptors which are not kinases include neuropilin-1 and -2 (Nrp-1, Nrp-2), also interact with members of the VPF/VEGF family in addition to the VEGF receptor tyrosine kinases (Guttmann-Raviv et al. 2006; Bielenberg et al. 2006). These receptors are expressed on the vascular endothelium as well as a wide variety of both normal and tumor cells (Figure 1.2).

**VEGF-A in Angiogenesis**

In normal physiology, VEGF-A is constitutively expressed at increased levels in several kinds of normal adult epithelium such as renal glomerular podocytes, adrenal cortex, breast, and lung epithelium in particular, as well as in macrophages, and cardiac myocytes (Brown et al. 1995; Berse et al. 1992; Maharaj et al. 2006). It is also expressed at increased levels in the physiological angiogenesis of development in regards to the angiogenesis associated with developing ovarian follicles and corpus luteum, and in the development of the endochondral bone (Beck et al. 1997; Yancopoulos et al. 2000; Folkman and D’Amore 1996; Carmeliet 2000; Berse et al. 1992; Phillips et al. 1990; Kamat et al. 1995; Gerber et al. 1999).

VEGF-A164/5 is considered to be representative of the key driver of pathological angiogenesis and is overexpressed in a wide range of solid human and animal carcinomas
(Dvorak 2002; Brown et al. 1997). The malignant cells express the majority of VEGF-A in solid tumors (Brown et al. 1997); however, tumor stromal cells are capable of VEGF-A synthesis (Fukumura et al. 1998). In addition, VEGF-A is overexpressed in sarcomas (Mentzel et al. 2001), hematological malignancies (Molica et al. 2004), multiple myeloma (Yasui et al. 2006), and malignant vascular tumors (Brown et al. 1996). Heightened expression of VEGFA and subsequent induction of angiogenesis is commonplace in a few premalignant lesions, i.e., precursor lesions of breast and cervical carcinomas (Guidi et al. 1997; Guidi et al. 1995); in these examples, VEGF-A expression levels are further increased, and this is in parallel with malignant progression. The function of VEGF-A and angiogenesis in benign tumors is less sure, principally because these tumors have not been studied as in depth. A few, such as pituitary adenomas and benign hemangiomas, seldom will overexpress VEGF-A; however, this overexpression is common in uterine leiomyomas (Brwon et al. 1996; Niveiro et al. 2005; Harrison et al. 1995). Moreover, VEGF-A164/5 is overexpressed in wound healing, chronic inflammation, as well as nearly all other kinds of pathological angiogenesis (Brown et al. 1997).

**Modulation of VEGF-A Expression**

Several factors regulate reduced level expression of VEGF-A in normal tissues as well as induce its overexpression in pathological angiogenesis. As mentioned, hypoxia has a key role in stimulating both VEGF-A transcription and mRNA stabilization (Claffey et al. 1996; Levy et al. 1997). As with other oxygen sensitive proteins, VEGF-A transcription is controlled by the hypoxia-inducible factor (HIF), which is characterized as a heterodimeric protein transcription factor. HIF-1α, described as a HIF-1 peptide, is promptly degraded

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under normoxic conditions via the ubiquitin pathway; but, when stabilized by hypoxia, HIF-1α dimerizes with HIF-1β, and the entire complex binds to and thus stimulates the hypoxia-responsive element in the VEGF-A promoter. Furthermore, the hypoxic modulation of VEGF-A expression has been shown in a number of tumors and may be important in wound healing, which is commonly ischemic. Low pH is a key feature of tumors and of ischemic tissues, and will upregulate VEGF-A, but performs this action independently of HIF-1 (Fukumura et al. 2001). Important mechanisms are thought to be mediated by oncogenes and tumor suppressor genes (Rak and Yu 2004; Mukhopadhyay et al. 2004). Furthermore, these genes initially considered to exert their effects exclusively on tumor cell growth properties, possess the added function of inducing the expression of VEGF-A, and thus angiogenesis.

**Experimental Cancer Therapeutics**

*The Anti-VEGF Agent: Bevacizumab (Trade name Avastin)*

In 2004, Bevacizumab (RhumabVEGF), the first angiogenesis inhibitor that targets vascular endothelial growth factor (VEGF), was approved by the US Food and Drug Administration (FDA). Because tumor cell lines will frequently overexpress VEGF, the action of bevacizumab reflects a prime targeting strategy in cancer therapy (Gerber and Ferrara 2005; Zondor and Medina 2004; Hicklin and Ellis 2005; Rosen 2002). In animal models, administration of bevacizumab blocked the growth of human tumor xenografts, thus decreasing both the size and number of metastases (Gerber and Ferrara 2005).

Over the 40 years since establishment of the concepts of angiogenesis-dependent tumor growth and metastasis, there has been substantial research effort in the field of
antiangiogenic therapy in regards to malignancy (Augustin 2003). Bevacizumab has been characterized as a humanized monoclonal immunoglobulin G antibody that is 93% human and 7% murine in protein sequence, and inhibits all active isoforms of VEGF to prevent tumor growth and proliferation (Gerber and Ferrara 2005). Bevacizumab is generated from recombinant biotechnology utilizing a Chinese hamster ovary cell line and has a molecular weight of approximately 149 kD (Genetech 2004). In addition, it maintains the high specificity and affinity of the parental antibody (murine VEGF monoclonal antibody A4.6.1) for VEGF-A, but with immunogenicity that is decreased and an extended biological half-life (Gerber and Ferrara 2005; Rosen 2005). The human region comprises antibody framework, while the murine component constitutes the complementarity-determining regions that attach to the vascular endothelial growth factor. Bevacizumab functions to neutralize the capability of the vascular endothelial growth factor to bind to the VEGF receptor (VEGFR), principally VEGF-1 (fit-1) and VEGF-2 (KDR/flk-1), on the surface of endothelial cells (Hicklin and Ellis 2005; Rosen 2002; Wang et al. 2004) VEGFR-1 and VEGFR-2 are described to be membrane bound tyrosine kinase receptors which cause specific downstream survival and proliferation pathways (Kabbinavar et al. 2005). As obtained from studies in vitro, VEGF-1 activation is responsible for cell proliferation, survival, and permeability, while VEGF-2 activation induces cell migration as well as generates tissue factors (Wang et al. 2004). Furthermore, the interaction of Bevacizumab prevents endothelial-cell mitogenic activity, vascular permeability-enhancing activity, in addition to other biologic functions of VEGF which boost angiogenesis (Hicklin and Ellis 2005; Rosen 2005). Consequently, the neutralization of VEGF has 2 cytostatic effects on tumor biology that include prevention of
neovasculature formation by limiting the blood supply, and normalization, i.e., pruning of the immature and aberrant blood vessels (Jain 2001). These effects lead to tumor endothelial cell death and reduce interstitial fluid pressure within the tumor, and this provides a much better capability for oxygen and chemotherapeutic drugs to reach specific targeted sites (Gerber and Ferrara 2005; Jain 2001).

The delivery of bevacizumab in preclinical xenograft models of colon cancer decreased microvascular growth as well as inhibited metastatic disease. Additional preclinical studies in mice reported the prevention of growth in various tumor types with anti-VEGF treatment (bevacizumab or murine parent antibody A4.6.1) that were compared to a control (Melnyk et al. 1999; Mesiano et al. 1998). Chen reported that the antitumor effect of VEGF-neutralizing antibodies is improved by combining them with cytotoxic chemotherapy, radiation, or other angiogenesis inhibitors (Chen 2004). For example, synergistic antitumor activity was found in a study in which bevacizumab was combined with the chemotherapeutic agent doxorubicin in breast cancer xenografts (Motl 2005; Borgstrom et al. 1999). Moreover, phase III trials showed a synergistic effect, thus demonstrating great benefit from combining anti-angiogenic therapy by use of bevacizumab with chemotherapy. Therefore, the mechanism for this effect might be that by inhibiting VEGF, a normalization of the tumor vessels may occur. Dickson and colleagues examined the effects of bevacizumab therapy in mice bearing two human neuroblastoma xenografts, both in ectopic and orthotopic areas whereby vessels were structurally more normal within 24 h of therapy, as well as showed decreased microvessel density and vessel length, diameter, and tortuosity (Dickson et al. 2007).
The VEGF-TRAP: Aflibercept

Aflibercept is a fusion protein with high VEGF affinity which can be attributed to binding sequences from the native receptors VEGFR1 and VEGFR2. Aflibercept monotherapy markedly diminishes tumor growth and prolongs survival in several orthotropic animal models, and has inhibited as well as reduced the growth of experimental choroidal neovascularization for example. The effects of aflibercept therapy on the architecture of tumor vessels were analyzed in a spontaneous murine model of pancreatic beta-cell derived tumors. Furthermore, the prevention of VEGF signaling in tumors that were established led to a decrease in neovascular sprouts and vascular regression within several days and vascular maturation with diminution of endothelial fenestration as well as tightened associations between endothelial cells and perivascular cells was indicated (Inai et al. 2004). In addition, ongoing phase III trials are evaluating the effectiveness of aflibercept combined with chemotherapy in patients with advanced carcinomas. The phase III VELOUR trial determined that patients receiving aflibercept with irinotecan/5-FU as second line chemotherapy for metastatic colorectal cancer experienced extended progression free survival and overall survival.

5-fluorouracil (5-FU)

Less than 20 years ago the sole chemotherapy treatment used for colorectal cancer (CRC) in the United States was 5-fluorouracil (5-FU). 5-FU kills cancer cells by interrupting their DNA-making abilities. However like many traditional chemotherapy drugs, 5-FU cannot discriminate between healthy and unhealthy cells, thus it damages many normal cells. Due to this, its side effects encompass low white blood cell count, diarrhea, as well as ulcers
of the mouth. 5-FU is nearly always administered in synergistic combination with leucovorin (LV) known as folinic acid (a Vitamin B derivative), to enhance its effectiveness and reduce its side effects.

*Camptosar (Irinotecan)*

In the 1990s, Camptosar was a new drug discovered as an alternative to 5-FU and was fully approved by the U.S. Food and Drug Administration (FDA) in 1998 for the treatment of colorectal cancer. Camptosar acts to block the enzyme topoisomerase-1 which prevents DNA from uncoiling and duplicating, but, like 5-FU it damages healthy cells and leads to diarrhea and low blood count side effects. Camptosar was integrated into standard colon cancer therapy in combination with 5-FU in a regimen known as IFL or the Saltz regimen.

Folinic acid (leucovorin), Fluorouracil (5-FU), and Irinotecan (Camptosar), comprise the chemotherapy regimen FOLFIRI which enhances efficacy while reducing side effects compared to the Saltz regimen.

*Capecitabine (Xeloda)*

In 2001, the FDA approved Capecitabine, an oral medication that functions similarly to 5-FU for the treatment of metastatic colon cancer. Capecitabine is used to treat metastatic colorectal cancer, cancer of the colon after surgery, as well as metastatic breast cancer. Capecitabine is characterized as a prodrug, and is enzymatically converted to 5-fluorouracil in the tumor, where it prevents DNA synthesis and reduces growth of tumor tissue.
**Eloxatin (Oxaliplatin)**

The FDA approved Eloxatin in 2002 for use in combination with 5-FU and leucovorin for patients presenting with treatment-resistant colorectal cancer. Eloxatin disrupts the cells’ DNA thus triggering apoptosis. Additionally, in 2004, Eloxatin in combination with 5-FU/leucovorin termed the FOLFOX regimen, was approved for the initial treatment of metastatic colorectal cancer. Of note, FOLFOX has been shown to be more effective than IFL and more effective than the combination of Camptosar and 5-FU (termed IROX) in clinical trials.

**Metastatic Colorectal Cancer**

Colorectal cancer (CRC) is a cancer of the gastrointestinal tract and is the second leading cause of death in the United States and affecting both men and women. In 2009 alone, 136,717 people were diagnosed with colorectal cancer and over 50,000 people died as a result of this disease. The general therapeutic strategy for most CRC patients includes surgical resection of the tumor as well as chemotherapeutic treatment. In adults younger than 50 years, the number of new colorectal cancer cases has steadily increased since 1998.

VEGF is known to be expressed in colorectal cancers (CRCs), and high expression has been correlated with decreased survival and increased distant metastases (Fernando and Hurwitz 2004). The use of bevacizumab represents a rational therapeutic approach in the treatment of metastatic CRC. Present treatment practice in patients with metastatic CRC supports the utilization of infusion-administered FU/LV combined with irinotecan (FOLFIRI) or oxaliplatin (FOLFOX) in place of bolus FU-based regimens due to decreased toxicity and improved survival (Meta-analysis Group in Cancer 1998). Bevacizumab is
presently added to these continuous-infusion regimens in patients with metastatic CRC in oncology practice. However, clinical trials of bevacizumab in which results have been fully published used traditional bolus FU therapies rather than continuous FU infusions (Kelly and Goldberg 2005). FU with LV was the standard treatment for metastatic CRC for several years. However, two additional chemotherapeutic agents, irinotecan and oxaliplatin, have been demonstrated to improve overall survival or progression free survival when combined with FU/LV-based therapies (de Gramont et al. 2000; Douillard et al. 2000; Goldberg and Gill 2004; Saltz et al. 2000). The median survival of patients with metastatic CRC was increased from 10 to 12 months with FU/LV alone to 14 to 16 months when either irinotecan or oxaliplatin was added to an FU-based regimen (Grothey et al. 2004; Meyerhardt and Mayer 2005). Less than 10 years ago, sequential or combination chemotherapy regimens comprising FU/LV, irinotecan, and oxaliplatin have prolonged median survival 20 to 21 months (Meyerhardt and Mayer 2005; Venook 2005; Tournigand et al. 2004). The development of selective targeted monoclonal antibodies such as bevacizumab (and cetuximab-erbitux), present with toxicity profiles which do not imbricate those of traditional chemotherapeutic agents, and this delineates the current combination strategy with use of FU/LV regimen.

**Rapamycin/mTOR**

Thirty years ago, rapamycin, a natural macrolide antibiotic derived from Streptomyces hygroscopicus, was discovered. Rapamycin is an inhibitor of the mammalian target of rapamycin (mTOR) and the subsequent elucidation of this target in yeast and mammalian cells has fundamentally contributed to further knowledge in the field of cancer
biology and therapy (Vezina et al. 1975). Rapamycin has been used for several years in the immunosuppression of patients following organ transplantation (Motzer et al. 2008) and relatively recently it has shown promise cancer therapy (Knox 2008) Rapamycin has been found to be antiangiogenic and it may possibly inhibit tumor cell growth (Guba et al. 2002).

The mammalian target of rapamycin (mTOR) is a central regulator of growth, in that it integrates diverse signals of growth factors, nutrients and energy sufficiency (Chiang and Abraham 2007; Wullschleger et al. 2006). mTOR is described as an atypical serine/threonine (S/T) protein kinase, which centrally regulates the cellular responses to multiple stimuli including amino acid availability and growth factor receptor signaling (Huang and Houghton 2003; Shamji et al. 2003). Increasing evidence suggests mTORs action as that of a ‘master switch’ of cellular anabolic and catabolic processes, which controls and modulates the rate of cell growth and proliferation by virtue of its capacity to sense mitogen, energy and nutrient levels (Dennis et al. 2001; Inoki et al. 2003). In cells that have adequate nutrients, mTOR relays a signal to the translational machinery leading to an enhanced translation of mRNAs encoding proteins essential for cell growth and cell cycle progression (Wullschleger et al. 2006; Inoki et al. 2002). Therefore, as a component of the mTORC1 complex, mTOR stimulates cell growth and protein synthesis through effects on mRNA translation and ribosome biogenesis (Guertin and Sabatini 2007).

mTOR occupies two distinctive multi-protein complexes: mTOR complex-1 (mTORC1) and mTOR complex-2 (mTORC2). The canonical rapamycin-sensitive mTORC1 is known for its highly important roles in the regulation of protein synthesis and growth. Furthermore, the PI3K/AKT pathway activates mTORC1 via phosphorylation and
inactivation of the tuberous sclerosis complex (TSC), a repressor of mTORC1. mTORC2, more recently discovered, phosphorylates and stimulates AKT which is known to be a regulator of cell growth, metabolism and survival. Moreover, the identification of mTOR as a vital downstream component of the PI3K/AKT signaling pathway, combined with evidence which supports the widespread dysregulation of this pathway in human cancer, gives a solid rationale for targeting mTOR as cancer therapy (Shor et al. 2009). Of note, the PI3K/Akt/mTOR axis is complex and extensive crosstalk occurs with other cellular signaling networks.

**Oncogenic Targets: PI3K/AKT/mTOR Signaling Pathway**

The phosphoinositide-3-kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) axis can be delineated as an important oncogenic pathway that might have direct as well as indirect affects the tumor vasculature. Moreover, effector molecules in this pathway have complex effects on tumor angiogenesis. Endothelial cell specific loss of certain PI3K catalytic subunits results in pronounced vascular hyperpermeability, and implicates PI3K in the maintenance of vascular integrity (Yuan et al. 2008). Additionally, mTOR signaling has a function in VEGF synthesis via the regulation of HIF-1α (Hudson et al. 2002) and through its own regulation by the tuberous sclerosis complex (Brugarolas et al. 2004; Brugarolas et al. 2003). Though PI3K/AKT lies upstream of mTOR, some of the antigenic properties are mTOR independent (Schnell et al. 2008). This includes PI3K/AKT- dependent activation of nitric oxide synthase (NOS) (Fulton et al. 1999), which when expressed in endothelial cells as endothelial NOS (eNOS), is a stimulator of angiogenesis as well as vascular permeability (Fukumura et al. 2001).
Pharmacological inhibition of differing components in the PI3K/AKT/mTOR axis has further clarified the role in tumor angiogenesis, and has shown that its inhibition can, by modulating downstream pro- and anti-angiogenic molecules, tip the balance towards equilibrium and thus normalize the vasculature. Studies which examined dual inhibitors of PI3K and mTOR demonstrate their capability to exert these effects; for example, one such inhibitor, NVPBEZ-235, effectively decreased vascular permeability and interstitial fluid pressure in orthtopically implanted breast cancer (Schnell et al. 2008). PI-103 is another dual PI3K/mTOR inhibitor. Its treatment of spontaneous and xenograft tumors showed normalized tumor vasculature both structurally, by reducing vessel tortuosity and increasing pericyte coverage, and functionally via improved tumor blood flow, perfusion and oxygenation, which normalized vessels within approximately five days of initiating therapy (Qayum et al. 2009). Inhibition of AKT signaling with the protease inhibitor nelfinavir, has also been demonstrated to normalize vessels structurally, in that it reduced irregularity and improved perivascular cell coverage, (Qayum et al. 2009) and functionally, in that it enhanced tumor blood flow and oxygenation (Pore et al. 2006; Qayum et al. 2009). As a result this benefit was observed when combined with radiation therapy (Pore et al. 2006). Of note, Pore et al. reported that these changes are anticipated to be related to downregulation of HIF-1α, thus VEGF production.

Vascular Normalization

The normalization hypothesis proposes that by correcting the abnormal architecture and functioning of tumor vessels, while not destroying these vessels completely, will normalize the tumor microenvironment and subsequent regulation of tumor progression as
well as an enhanced response to other therapies can occur (Jain 2005; Jain 2001). Furthermore, this may occur through differing physiological mechanisms. Firstly, increases in the homogeneity of functional vascular density and better organization in arrangement of vessels may decrease the heterogeneity in blood flow in different regions within a tumor. Secondly, improvement in connections between adjacent endothelial cells, increased proportion of perivascular cell-covered vessels, and a tighter connection between perivascular cells and endothelial cells would reduce vascular permeability, resulting in decreases in intratumoral interstitial fluid pressure (Jain 2005; Jain 2001). Although tumor vessels might not become fully “normal,” the effect of these changes is a more evenly distributed flow of blood within a tumor, and subsequent decreases in regions of hypoxia and acidosis. As a result, mitigation of the hypoxia-mediated rises in cancer cell metastatic potential, a more even delivery of systemically administered anti-cancer therapies within a tumor, and improved radiosensitivity of tumors would be expected (Jain 2001). Moreover, vessels which are normalized may curb the intravasation and extravasation of cancer cells, resulting in a diminution of metastasis.

**Molecular Mechanisms of Tumor Vascular Normalization**

In tissues which exhibit normality, cooperative action of differing proangiogenic factors such as VEGF, bFGF, and Ang-2, are in balance with the action of endogenous antiangiogenic and vascular stabilizing factors such as thrombospondin-1 (TSP-1) and Ang-1 (Jain 2003). However, in the abnormality of pathological angiogenesis, there is ongoing imbalance, which leads to unrelenting development of aberrant vessels. In addition, the spectrum of proangiogenic molecules expressed within a tumor will escalate with malignant
progression (Relf et al. 1997). To rectify this imbalance, there is a possibility to normalize tumor vessels; blockade of VEGF signaling is such an established mechanism of vascular normalization (Figure 1.3). VEGF is a factor that promotes the survival and proliferation of endothelial cells as well as a rise in vascular permeability. Its blockade can transiently reestablish the balance between pro- and anti-angiogenic signaling shifting it back towards equilibrium. Additionally this transient nature of vascular normalization following VEGF blockade may be due to the tumor expressing alternative pro-angiogenic factors (Jain 2001). Alternative angiogenic molecules and genes have been directly implicated in the development of an abnormal tumor vasculature and the normalization process. Summarily, genetic evidence has shown that a variety of genes which include those involved in vascular stability such as Ang-1/Ang-2, oxygen sensing (e.g., PHD2), and pericyte function such as Rgs5 (a regulator of G protein signaling), may each function as “master genes” and determine the tumor vessel maturation (Chae et al. 2010; Hamzah 2008; Mazzone et al. 2009; Winkler et al. 2004).

In contrast to the genetic studies, vascular normalization that has been induced pharmacologically, is transient and described by a “time window” which refers to the time period following the onset of anti-angiogenic therapy during which vessels demonstrate features of the normalization phenotype (Jain 2005). Studies of murine and human tumors have identified the onset of normalization, at approximately 1 to 2 days after beginning therapy, and this is followed by an eventual “closure” of the normalization window, at which point features of normalization are lost (Jain 2005). In addition, this might correlate to either extremely increased or prolonged dosing of antiangiogenic therapy, which tips the balance
past equilibrium to favor anti-angiogenic molecules, and leads to vascular pruning and regression, or to the development of resistance via stimulation of alternative proangiogenic pathways/mechanisms in the acquisition of new vessels (Jain 2005). Furthermore, Jain and colleagues proposed that vascular normalization will take place solely in tumor regions where the imbalance between pro- and anti-angiogenic molecules has been corrected, therefore wise judgment in the dosing of anti-angiogenic therapies is necessary to induce and maintain normalization for as long a time as possible (Jain 2005; Jain 2001). Cancer cells have increased vulnerability to cytotoxic drug therapies especially while the normalization window remains open; therefore, determining its timing in various circumstances is crucial.

**Cancer Prevention**

Cancer has been established by the United Nations as a non-communicable disease presenting a global health problem with substantial economic consequences (Bloom et al. 2011; Mariotto et al. 2011). The cost of cancer care in the United States alone is projected to increase from $125 billion in 2010 to $207 billion by the year 2020 (Mariotto et al. 2011). Though much advancement has been made in decreasing the mortality from cardiovascular disease as well as other non-communicable diseases through preventive efforts, cancer continues to be treated at advanced, often metastatic, disease stages (Albini et al. 2007). Screening methods have shown improvement in prognosis for some cancers; (Qaseem et al. 2012) but, early detection is not yet possible for most malignancies, and the advantage of screening has been challenged for some tumors (Nishizawa et al. 2009). Targeted therapies are being utilized; however, treating all patients with these expensive agents is not economically sustainable (Shih et al. 2011; Whyte et al. 2010). As the world population
exceeds 7 billion, cancer prevention is emerging a compelling aim. A number of cancers are thought to be preventable by lifestyle changes, such as avoiding tobacco use, excessive UV exposure, infectious agents, poor dietary habits and obesity (Lippman and Hawk 2009). However, studies of behavior indicate that promotion of healthy dietary habits and exercise has shown only moderate success (Willyard 2011). Thus, cancer prevention remains problematic. It is predicted that approximately half of men and a third of women will be diagnosed with a cancer in their lifetime. Autopsy studies have demonstrated that approximately 40% of women between 40 and 49 years old have occult breast cancers (Nielson et al. 1987) Similarly, in situ prostate cancer has been indentified postmortem in 24% of men aged 60 to 70 years (Sanchez-Chapado et al. 2003). Microscopic thyroid cancers are predicted to exist in 98% of individuals by the age of 70 years. However, these cancers are still diagnosed in only 2%, 8%, and <0.5%, respectively, in these age groups (Black and Welch 1993; Harach et al. 1985).

Angioprevention in Cancer

Reduction of caloric intake and energy restriction has been shown to reduce cancer risk, thus is involved in angioprevention (Hursting et al. 2010). The mTOR pathway regulates protein metabolism jointly with AMPK and AKT, and over-activation of mTOR induces VEGF-mediated angiogenesis (Phoenix et al. 2009). The antidiabetic drug, Metformin, an AMPK stimulator, inhibits mTOR and demonstrates antiangiogenic activity in vitro and in vivo (Phoenix et al. 2009; Xavier et 2010; Esfahanian et al. 2010). Metformin targets glucose metabolism and is extensively used in the clinic for the treatment type 2 diabetes. It is sufficiently tolerated and associated with limited side effects in addition.
From Currie et al. and Goodwin et al., retrospective population studies showed a 21% reduced risk and improved survival for all cancers in patients with type 2 diabetes receiving metformin (Currie et al. 2009; Goodwin et al. 2011). These results were significant even after adjusting for body mass index (BMI), which is a risk factor of cancer. In contrast, diabetics treated with non-metformin regimens showed an increased (1.36–1.42 fold) risk for cancer that (Currie et al. 2009). Yet another study with patients taking metformin showed a 62% risk reduction for pancreatic cancer (Li et al. 2009). Furthermore, Metformin was found to upregulate circulating levels of the antiangiogenic extracellular matrix molecule thrombospondin-1 (TSP-1) in insulin-resistant obese women, presumably a protective mechanism of action (Tan et al. 2009). Studies on the long term use of aspirin (or NSAIDs) uncovered a reduced risk of colon, breast, prostate and lung cancer (Harris 2009). Colon cancer and colon polyp formation have undergone comprehensive analysis, and because they present frequent lesions, higher risk groups can be identified and treated, and regular colonoscopy can be utilized to formulate end points which are precise for both colon polyps and cancer. Many studies have demonstrated that aspirin and NSAIDs, as well as selective COX-2 inhibitors, reduce colonic adenoma formation (William et al. 2009; Cole 2009). Green tea consumption is associated with a decreased risk of colon, prostate, lung, and other cancers (Albini and Sporn 2007; Cao and Brakenheilm 2002). Abnormal matrix remodelling by unchecked protease activity provides a segue for sprouting vessels, breaking the extracellular matrix and promoting detachment, attachment, and spatial organization of endothelial cells. Targeting this remodeling is one mechanism of action accredited to the antiangiogenic activity of green tea, (by inhibiting the MMP-2 and MMP-9 matrix
metalloproteinases) (Cao 1999). The organosulfur compound phenethyl isothiocyanate, is naturally present in dietary cruciferous vegetables, such as broccoli and brussel sprouts. One primary target of phenethyl isothiocyanate is protein kinase C (PKC), which leads to downstream inhibition of MMP-2 and MMP-9 (Yang et al. 2010). Curcumin and quercetin are antioxidant molecules and when used in combination decreased aberrant crypt foci by 40% in a phase II a trial (Carroll 2011), and also decreased the incidence of colon adenomas in patients with familial adenomatous polyposis.139 (Cruz-Correa et al. 2006).

Angioprevention is coming to light as a clinical reality that further changes how oncological disease is approached, and employs strategies appropriate for healthy individuals as well as those at a high risk for cancer. Four distinct levels of angioprevention have been proposed: level (I) for the general ‘healthy’ population exhibiting low risk of developing cancer; level (II) for those with conditions associated with increased cancer risk and includes healthy individuals with genetic abnormalities that can be connected with a higher cancer risk, occupational and lifestyle exposure (such as asbestos, toxins, tobacco, or heavy alcohol consumption), immunosuppression and metabolic disease; level (III) for treatment of preneoplastic lesions; and level (IV) for preventing the recurrence of cancer in patients who have achieved remission status. While traditional chemoprevention is directed at cancer cells, angioprevention may serve to augment anticancer defense mechanisms as well as provide protection against a wide range of neoplasms.
Concluding Remarks and Prospectus

Angiogenesis is the formation of new blood vessels via the remodeling and expansion of primary vessels. Over 40 years ago, Folkman’s seminal paper proposed the hypothesis of angiogenesis-dependent tumor growth and metastasis. Moreover, he proposed that angiogenesis is required for tumor growth beyond a 2-3 mm diameter and for metastasis. In the absence of new blood vessels and a functional vasculature, tumors are limited in their capability to grow, and tumor cell proliferation will give way to tumor cell death. Since Folkman’s theories, there has been enormous research effort in the area of antiangiogenic therapy and malignancy. Additionally, the concept of normalizing the vasculature as a therapeutic strategy directed against tumors was first proposed by Jain et al. in 2001 and is thus still relatively young. Consequently, significant insight into molecular mechanisms has emerged during the last few years based on these cutting edge suppositions. As knowledge is acquired about the individual contributions of pro- and anti-angiogenic factors, tumor/stromal cells, and hypoxic impact, as well as various signaling networks (PI3K-AKT-mTOR axis), further elucidation of the tumor vascular and advances in cancer therapies can be realized.

VEGF is expressed in colorectal cancer (CRC) with increased expression resulting in decreased survival and increased distant metastasis and has been validated as a therapeutic target. Moreover, anti-VEGF agents and chemotherapeutics have significantly enhanced the treatment of cancer. The emergence of Bevacizumab (Avastin), the first angiogenesis inhibitor to have been approved by the FDA, has shown beneficial effects in cancer treatment. Bevacizumab has been demonstrated in the clinic to delay time of recurrence and
extends life expectancy by 4 to 5 months in patients with advanced colon cancer. Further Phase III trials have shown it to be effective in other forms of cancer as well. The beneficial effects of bevacizumab can be attributed to its effects on the blood vessels of tumors. Recent findings that VEGF receptors are also expressed on some tumor cells, indicates that bevacizumab could be directly affecting tumor cells in addition.

In 2006, Berenson reported in the New York Times, the typical cost of a year of bevacizumab treatment for colorectal cancer (CRC) is over $50,000 (Berenson 2006). Thus, the cost of cancer care comes with considerable expense. A number of cancers are thought to be preventable by lifestyle changes, such as reducing tobacco and alcohol use as well as obesity. As a result, angioprevention is emerging as a clinical reality and presents an alternative approach in the treatment of oncological disease.

Targeted therapeutics are being utilized in antiangiogenesis for the treatment of cancer. Combining bevacizumab with chemotherapy and/or novel molecular targeted agents such as mTOR, presents a rational approach which might improve efficacy and curtail nonselective toxicity.

Disrupting tumor angiogenesis is a promising antitumor strategy. An approach is to inhibit the vascular endothelial growth factor (VEGF) pathway with bevacizumab, a humanized anti-VEGF monoclonal antibody. We reasoned in our study in chapter two, that by combining bevacizumab with rapamycin, an immunosuppressive drug binding to mTOR, we would have superior antitumor activity than via the action of either agent alone.
Figure 1.1: The Human VEGF-A structure with commonly expressed isoforms
In above schematic, VEGF-A189 is comprised of exon 1 through exon 5, and contains a portion of exon 6 (exon 6a), exon 7, and exon 8. VEGF-A165, the most commonly expressed isoform, lacks all of exon 6. VEGF-A121 lacks exon 6 as well as exon 7. VEGF-A145, also shown here, lacks both exons 6b and 7. Of note, murine isoforms have one amino acid less, for example, mouse VEGF-A164 when compared to human VEGF-A165. (Figure adapted by author from Yukata et al. 2005.)
Figure 1.2: VEGF receptors and their ligands
Vascular endothelial growth factor (VEGF) ligands modulate their angiogenic effects by binding to specific VEGF receptors (VEGFRs), leading to receptor dimerization and signal transduction. VEGFR-1 and VEGFR-2 are primarily engaged with angiogenesis while VEGFR-3, is known to be associated with lymphangiogenesis. The neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) receptors are thought to function in the binding affinity of the various VEGF ligands to these primary receptors. Proteolytically processed forms (proc) of VEGF-C and VEGF-D exclusively bind to VEGFR-2. (Figure adapted by author from Google Images.)
Tumor vasculature presents as structurally and functionally aberrant and interstitial fluid pressure (IFP) is increased resulting in difficulty for antitumor agents to leave the blood stream and enter the tumor. It is proposed that antiangiogenic therapies (VEGF blockade) will initially normalize the architecture and function of tumor vessels. Prolonged or aggressive antiangiogenic treatment may eventually prune away these vessels, resulting in a vasculature that is resistant to further therapy and no longer adequate for drugs or oxygen delivery. (Figure adapted by author from Jain 2005.)
References


CHAPTER TWO: Combination Therapy of Bevacizumab and Rapamycin in Antitumor Activity

Abstract

Purpose: Bevacizumab/Avastin®, a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody, is used in the clinics to disrupt tumor angiogenesis and repress tumor growth. However its efficacy as a monotherapy in humans is less than predicted from preclinical evaluations. Resistance to treatment can be due to the rescue of the VEGF-activated mTOR promitogenic pathway by VEGF-independent oncogenic factors. We therefore reasoned that combining bevacizumab to rapamycin, a drug inactivating mTOR, would have superior antitumor activity than either agent alone.

Experiment design: We tested our hypothesis in the HT29 model of human colon adenocarcinoma in nude mice. Size-matched HT29 flank tumors received the following treatments: (1) saline, (2) rapamycin alone (1.5 mg/Kg/day), (3) bevacizumab alone (three weekly injections of 100 µg/Kg/), or (4) bevacizumab + rapamycin. We determined tumor growth delays from caliper measurements, and characterized key molecular changes in response to tumor treatments by using Western blotting, ELISA, and immunohistochemistry.

Results: The combination therapy retarded HT29 tumor growth 2.5-fold and 15-fold more efficiently than bevacizumab and rapamycin monotherapies, respectively. Although early histological changes were not apparent, we substantiated increased mTOR inhibition in tumors when combining both treatments in vivo.

Conclusions: Our findings demonstrate a significant therapeutic gain when combining bevacizumab with rapamycin for cancer treatment. Improved tumor growth retardation does not result from early tissue changes in tumor, but rather finds a rationale in a
mutual potentialization in which VEGF blockade improves rapamycin tumor delivery and mTOR inhibition secures bevacizumab-induced DNA damage, resulting in late cellular responses.

**Introduction**

Angiogenesis, the extension of the vascular network, plays a key role in cancer progression. It supports the survival of cancer cells, promotes sustained tumor growth, and facilitates the shedding of tumor cells into the blood stream, a process accounting for the onset of distant metastases (Rak et al. 1995; Karayiannakis et al. 2003). Angiogenesis is regulated by several molecules released by cancer cells and by nonmalignant host cells within the tumor microenvironment, especially in response to hypoxia. Amongst pro-angiogenic factors, the vascular endothelial growth factor (VEGF) family comprises VEGF165, a diffusible glycoprotein that has been identified to be extremely potent at stimulating tumor angiogenesis (Ferrara 1995; Carmeliet 2000). High expression of VEGF has been observed in most human malignancies and is directly correlated with increased tumor neovascularisation (3). In addition, a high level of VEGF in the serum of patients is a poor prognostic factor in a variety of cancer types (see Toi et al. 2001 for review). The pleiotropic effects of VEGF make it an attractive protein to target in strategies aimed at disrupting tumor angiogenesis.

At the endothelial cell surface, the binding of VEGF to its tyrosine-kinase receptor 2 (VEGF-R2) initiates downstream cascades that promote vascular permeabilization and fenestration, and endothelial cell proliferation and migration. It also constitutes a chemotactic stimulus that orients sprouting vessels towards cells demanding oxygen and nutrients. At the
molecular level, the engagement of VEGF-R2 leads to receptor dimerization and relocalization in the caveolar signaling platforms. In these microdomains of the plasma membrane, the phosphorylated receptor triggers the phosphoinositol-3 kinase (PI3K) pathway. The cascade of phosphorylations ultimately activates factors involved in cell survival (AKT/PKB), vasodilation, vascular disruption, migration/invasion (endothelial nitric oxide synthase, eNOS), and promote cell multiplication (mammalian target of rapamycin, mTOR). Because all these downstream effectors participate in tumor angiogenesis, a particularly attractive antitumor strategy consists of inhibiting VEGF itself or its binding to VEGF-R2 rather than downstream events.

Several agents were developed that block VEGF-induced angiogenesis and suppress tumor growth in vivo (Kim et al. 1993). Recently, bevacizumab/Avastin, a recombinant humanized monoclonal antibody directed against VEGF, has emerged as the leading angiogenesis inhibitor used in the clinics (Veronese and O’Dwyer 2004). As a single agent, its range of applicability is lower than what had been predicted in preclinical trials. This constitutes a rationale that fosters the search for adjuvant approaches. One of the explanations for the limited efficiency of bevacizumab may reside in the existence of alternative pathways that can rescue part of the VEGF signal upon VEGF neutralization. In this study, we focused on mTOR, a member of the ataxia-telangectasia-mutated (ATM) family of kinases. It functions as a G1 checkpoint (Podsypanina et al. 2001) activated by the VEGF/PI3K/AKT cascade but also by several other promitotic pathways (Sekulic et al. 2000). Previous studies have shown that these alternative pathways can maintain mTOR activity upon VEGF blockade. Based on these observations, we reasoned that the antitumor
efficiency of bevacizumab could benefit from the inhibition of mTOR. To test this hypothesis, we delivered bevacizumab, the mTOR inhibitor rapamycin, or a combination of both drugs to cohorts of tumor-bearing mice. We tracked changes in tumor growth by repeated caliper measurements, and studied key molecular determinants of the responses by using Western blotting, ELISA and immunohistochemistry on tumor biopsies.

**Materials and Methods**

**Mouse tumor model and treatment.** Six to eight week-old athymic female Nude NcR mice (Charles River Laboratories, Wilmington, MA) were allowed to adapt to the laboratory environment for 7 days before experiments. Human colorectal adenocarcinoma HT29 cells (American Type Culture Collection, ATCC, Manassas, VA) were routinely cultured in Dulbecco’s Modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). One million cells in balanced salt solution were injected subcutaneously into the right flank of mice. Tumor long (L) and short (W) diameters were measured every 2–3 days with a caliper, and the tumor volume was calculated using the formula $V=L \times W^2 \times \pi/6$ (Teicher et al. 2001). When tumors reached a volume of 200–300 mm$^3$, mice were randomly assigned to a treatment group. Mouse treatment started at Day 0. Groups of 10 mice received saline (control), bevacizumab alone, rapamycin alone, or the combination of bevacizumab and rapamycin. In control mice, saline was injected intraperitoneally (i.p.) once a day. Bevacizumab (100ug/kg) was administered i.p. every 3 days from Day 0. Rapamycin (1.5 mg/kg) was administered every day by gavage. When the tumor volume reached 5x the initial treatment volume x mm$^3$, the animals were sacrificed and the tumors were biopsied.
for further processing. Animal care and all experimental procedures were approved by
Institutional Animal Care and Use Committee.

**Immunohistochemistry.** Immunohistochemistry was made on tumor cryoslices as
previously shown (Carson 1997; Carson FL. 1997 Histotechnology: A Self-Instructional Text
(2nd ed) Chicago: ASCP Press.). Primary antibodies were: rabbit polyclonal anti-VEGF
(Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-carbonic anhydrase-9
(CA-IX; gift from Dr. Oosterwijk), rabbit monoclonal anti-cleaved caspase 3 (Cell Signaling
Technology, Danvers, MA), rat monoclonal anti-CD31 (BD Pharmingen, San Jose, CA), and
rabbit polyclonal anti-Ki67 (Vector Laboratories, Burlingame, CA). Omission of the primary
antibody served as negative control. Biotinylated donkey anti-rabbit or anti-mouse secondary
antibodies were used where relevant. The ABC kit and NovaRed solution (Vector
Laboratories) were used according to manufacturer’s recommendations. Slides were
counterstained with Harris’ hematoxylin. The relative expressions of VEGF and CA IX in
treated versus control tumors were calculated from the quantification of positively-stained
area (dark brown staining) and total area per low power field using Adobe Photoshop.
Microvessel density was calculated at lower magnification (x 100) and CD31-positive
vessels were counted in each tumor xenograft from 4-5 random fields. Vascular hot spots
were also identified, and individual microvessel counts were made on a 100X field. Any
highlighted endothelial cell or cell group occurring closely together and distinctly apart from
microvessels, tumor cells, or other near-lying connective tissue morphology, was defined as a
singular microvessel count (Weidner et al. 1996). Results were expressed as the highest
number of microvessels in any single 100 X field. Tumor cells with intranuclear staining
were considered positive for Ki67 and activated caspase-3. After the slides were scanned at low magnification (40 x), positive cells were counted in 4-5 random fields at a magnification of 400x (NIH Image J). The total number of cells in the respective fields was also determined to derive mitotic and apoptotic indexes.

**Tumor extracts.** Tumor samples were weighed, minced, and placed into lysate matrix tubes with 500μl to 1ml of lysis buffer containing a protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN). The tissue was homogenized using Fast Prep 120 (Q-Biogene, Carlsbad, CA). Supernatants were repeatedly passed through a 20G needle.

**ELISA and Western blotting.** The concentration of human VEGF was determined from tumor lysates by using the QuantiQuine kit from R&D Systems (Minneapolis, MN) according to manufacturer’s protocol. The optical density of each well was determined at 450 nm using a microplate reader (Bio Kinetics Reader, Bio-Tek Instruments Inc., Winnooski, VT). Western blotting was done as previously reported (Kim et al. 2005). Briefly, equal amounts of proteins from each tumor sample were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Primary antibodies were a rabbit polyclonal, anti-mTOR (#2972 Cell Signaling Technologies, Danvers, MA), and an anti-phospho-mTOR (#2971 Cell Signaling Technologies, Danvers, MA). Following incubation with horseradish peroxidase-conjugated secondary antibodies, specific bands were visualized using the SuperSignal WestPico chemiluminescence kit (Pierce, Rockford, IL) and autoradiography.

**Statistics.** Results are expressed as means ± SE. Student’s t test and one-way ANOVA (Bonferroni’s post-hoc test) were used where indicated. P<0.05 was considered statistically significant.
Results

The combination of bevacizumab with rapamycin has more than additive antitumor effects. To identify a potential additive antitumor effect, mice bearing HT29 tumors were treated with bevacizumab alone, rapamycin alone, bevacizumab + rapamycin, or received saline (controls) from Day 0. Tumor growth was determined from repeated caliper measurements and plotted over time (Figure 2.1A). The inhibition of tumor growth in response to the combination of bevacizumab and rapamycin was more pronounced than either treatment alone or the control group, which received repeated saline injections. We quantified the antitumor effects of each treatment by determining tumor growth delays, i.e., the delay for the tumor volume to double in a treated group versus the control (Figure 2.1B). The combination treatment significantly delayed tumor growth by 3.4±0.6 days when compared to saline (P<0.01, one-way ANOVA). It contrasted with the absence of a statistically significant tumor response when mice received bevacizumab alone (1.4±0.8 days) or rapamycin alone (0.2±0.5 days) (P>0.05 versus saline). Statistical analyses further showed that the antitumor effect of bevacizumab and rapamycin was more than additive, revealing that both drugs act at least partially on the same pathway.

We then determined the level of mTOR phosphorylation/activation in size-matched whole tumor samples from control mice, mice treated with rapamycin alone, bevacizumab alone, or the drug combination. Figure 2.2 shows that mTOR activation (expressed as P-mTOR/total mTOR) in HT29 tumors were equally repressed by ~25% after the administration of rapamycin or bevacizumab as single agents. The combination treatment induced a decrease of ~50% of mTOR phosphorylation, revealing additive effects at the
mTOR activity level. This experiment also confirmed that both rapamycin and bevacizumab reached a therapeutic availability in HT29 tumors after systemic in vivo delivery.

**In contrast to bevacizumab, rapamycin does not decrease the availability of VEGF in tumors.** In view of the additive antitumor effects of the combination treatment, we sought to determine whether rapamycin influenced VEGF levels in the tumor microenvironment. In control experiments, we first checked whether the binding of bevacizumab to VEGF resulted in lower measurable VEGF levels in tumor cryoslices. To do so, tumors from untreated animals were sliced and incubated overnight in the presence of bevacizumab or saline. Anti-VEGF immunostaining showed that VEGF was not detectable after exposure to bevacizumab (Figure 2.3A). By comparison, high amounts of VEGF were stained in the untreated sections. To check that VEGF depletion by bevacizumab also occurred in our tumor model in vivo, size-matched tumors were analyzed for VEGF expression after the treatment of the animals with saline, bevacizumab, rapamycin, or the combination treatment. Using ELISA, we detected similar amounts of VEGF in whole tumor lysates of rapamycin and saline-treated mice (Figure 2.3B, P>0.05, one-way ANOVA). Treatment with bevacizumab (alone or in combination with rapamycin) was associated with an almost complete absence of VEGF detection in the corresponding tumor lysates. The decreases were highly significant (P<0.005 for bevacizumab versus saline and for bevacizumab + rapamycin versus saline, one-way ANOVA), confirming the VEGF-neutralizing activity of bevacizumab. The delivery of rapamycin to bevacizumab-treated mice did not further decrease measured VEGF levels in tumors (P>0.05, one-way ANOVA).
Changes in VEGF levels in whole tumor lysates corresponded to changes in tumor cryoslices (Figure 2.3C). Less VEGF was always observed in tumors from mice treated with bevacizumab (± rapamycin) than with saline or rapamycin alone. Quantification of VEGF in randomized low-power fields (Figure 2.3D) confirmed that rapamycin did not significantly decrease VEGF labeling (P>0.05 versus saline), whereas bevacizumab (P<0.05) or the combination treatment (P<0.05) equally reduced VEGF staining by ~ 2-fold (one-way ANOVA). ELISA results independently verified that rapamycin did not further modulate the effects of bevacizumab on VEGF (P>0.05, one-way ANOVA), indicating that the drug acts on the VEGF pathway downstream of VEGF itself.

**Rapamycin does not increase the antiangiogenic effect of bevacizumab.** In the clinics, bevacizumab is used for its antiangiogenic properties. By inhibiting mTOR, rapamycin could further enhance this effect. We evaluated the antiangiogenic potential of rapamycin (alone or in combination with bevacizumab) by determining the microvascular density (MVD) in control and treated tumors. Mice were administered with saline (negative control) or bevacizumab alone. Immunostaining against the endothelial cell marker CD31 (Figure 2.4A) showed a high density of microvessels in saline and rapamycin-treated tumors. This contrasted with the lower microvascular density that we detected in the tumors after bevacizumab or bevacizumab + rapamycin treatments. The quantification of individual CD31-positive clusters confirmed the difference (Figure 2.4B). We observed a significant decrease in MVD when comparing tumors treated with saline (224±15) versus bevacizumab (136±7, P<0.005) or with saline versus bevacizumab + rapamycin (154±7, P<0.01). MVD in
tumors treated with saline was not different than after treatment with rapamycin alone (199±16; P>0.05, one-way ANOVA).

Repression of tumor cell proliferation and higher apoptosis index upon anti-VEGF treatment are not modulated by rapamycin. In addition, to its pro-angiogenic effect, VEGF is known to activate survival pathways in tumor cells and in tumor-coopted host cells. By inhibiting the VEGF signal, bevacizumab could thus tip the proliferation:apoptosis balance in favor of cell death, and rapamycin could further modulate this balance in an angiogenesis-independent way. To test this hypothesis, we first determined to which extent bevacizumab, rapamycin, and the combination treatment affected cell proliferation. Tumor cryoslices were immunostained against the nuclear proliferation marker Ki67 (Figure 2.5A). Rapamycin exerted no effect on cell proliferation when compared to saline. By contrast, Ki67-stained cells were less abundant upon bevacizumab treatment and this decrease in cell proliferation appeared to be independent of mTOR inhibition by rapamycin. To objectify the changes, we counted cells with Ki67-positive nuclei in random fields for each treatment arm. Quantification confirmed that, compared to saline (315±37), bevacizumab significantly reduced cell proliferation (152±1, P<0.005), and this effect was preserved but not reinforced by the concomitant delivery of rapamycin (107±5.31, P<0.005 versus saline but P>0.05 versus bevacizumab alone, one-way ANOVA). As a single agent, rapamycin did not affect cell proliferation in tumors (316.5±8, P>0.05 versus saline).

We next checked the other side of the proliferation:apoptosis balance by labeling cells for activated caspase 3 (aCASP-3), a marker of apoptosis, on slices from treated tumors (Figure 2.5B). Interestingly, these stainings mirrored Ki67 stainings. Increased abundance of
apoptotic cells after bevacizumab treatment was observed compared to saline or rapamycin alone. Furthermore, rapamycin did not seem to improve the pro-apoptotic effect of bevacizumab. The lack of pro-apoptotic activity of rapamycin in tumors was confirmed by the quantification of cells showing a positive nuclear staining for aCASP-3 (Figure 2.5B, bottom panel). To further determine the target range of each treatment, we counted the total number of cells on slides and sorted them into 3 subpopulations: proliferating cells (positive for Ki67), apoptotic cells (positive for aCASP-3), and quiescent cells (negative for both markers). No cell showed simultaneous Ki67 and caspase 3 staining. Figure 2.5C definitely confirms that bevacizumab tips the proliferation:apoptosis balance in favor of apoptosis. It contrasts with the lack of effect of rapamycin, even upon VEGF blockade. Overall, ~ 40% of the cells within the tumors were quiescent and remained in that state whatever the treatment regimen.

**Bevacizumab increases tumor oxygenation, an effect which is not modulated by rapamycin.** Our aforementioned results reveal two different profiles of the therapeutic antibodies at the doses that we tested. On one hand, bevacizumab potently inhibited tumor angiogenesis and promoted tumor cell apoptosis, but it did not result in tumor shrinkage or growth delay. On the other hand, rapamycin has no antitumor activity per se but, in combination to bevacizumab, retards tumor growth without any obvious effect at the histological level for the endpoints that we assessed. We therefore reasoned that rapamycin could trigger tumor cell death by apoptosis-independent mechanisms (such as senescence or mitotic catastrophe), or that it could inhibit tumor cell metabolism. In the absence of vascular remodeling (our aforementioned observation), these events should lower cell oxygen
consumption, resulting in higher tumor pO2 upon rapamycin delivery. To address this eventuality, we tested whether our treatments affected tumor hypoxia, which we quantified by determining the abundance of the endogenous hypoxic marker carbonic anhydrase 9 (CA-IX) in tumor cryoslices (Kim et al. 2005). Unexpectedly, gross examination of the stainings showed no effect of rapamycin on CA-IX tumor expression when compared to saline (Figure 2.6A). It strikingly contrasted with the apparent decrease in CA-IX abundance after bevacizumab treatment. This robust decrease in tumor hypoxia was not further modulated by rapamycin. We then quantified overall tumor hypoxia by calculating the ratio of the CA-IX positive surface divided by the total surface of the slices in the treatment groups (Figure 2.6B). Compared to saline (32.9±3.6%) or to rapamycin (27.2±4.0%), bevacizumab (14.1±4.5%) induced a ~ 2-fold decrease in tumor CAIX abundance, which was highly significant (P<0.05, one-way ANOVA). Rapamycin showed no intrinsic effect on tumor CA-IX abundance (P>0.05 versus saline), and did not modulate the decrease in CA-IX achieved by bevacizumab (12.2±1.1%) in the combination treatment (P>0.05 versus bevacizumab alone).

**Discussion**

Although bevacizumab was recently accepted by the Food and drug administration for clinical practice under the name of Avastin® (Ferrara et al. 2004), its use is currently restricted to few tumor types that include metastatic colorectal cancer, non-small cell lung cancer and metastatic renal cancer. Such clinical profile profoundly contrasts with much more potent antitumor effects that were identified in preclinical settings (Kim et al. 1993). This prompted us (the present study) and others (Hurwitz et al. 2004; Jain et al. 2006) to test
bevacizumab in combination with other antitumor treatments, the rationale of our approach being a utilization of novel combinations of antiangiogenic compounds which have potential additive effects. mTOR is an important checkpoint protein that promotes cell proliferation after VEGF binding to its VEGF-R2 or in response to the activation of other oncogenic signaling pathways. This redundancy of stimuli makes it very attractive to inhibit mTOR to reinforce the antitumor efficacy of VEGF inhibitors and/or to decrease resistance to such treatment.

The aim of the present study was thus to identify a potential antitumor therapeutic gain by combining the delivery of the VEGF neutralizing antibody bevacizumab with the mTOR inhibitor rapamycin to tumor-bearing mice, and to characterize the underlying determinants of such effect. Using a model of human colorectal adenocarcinoma, we documented additive effects when the animals were administered both treatments concomitantly. These effects were attributable, at least in part, to a greater inhibition of mTOR phosphorylation/activity by the combination treatment versus each agent used as a monotherapy. Interestingly, the modulating activity of rapamycin turned out to be independent of any anti-angiogenic, antiproliferative, or pro-apoptotic effects. Moreover, it did not affect the availability/release of VEGF within tumors or the state of tumor oxygenation, suggesting that rapamycin does not cause early tumor cell death or metabolic adaptation to an extent greater than bevacizumab. As a single treatment, rapamycin had no significant antitumor activity and left tumor histology and pO2 (as determined with CA-IX staining) unaffected. By contrast to rapamycin, bevacizumab had strong intrinsic anti-angiogenic and pro-apoptotic effects associated to a decrease in tumor cell proliferation. It
potently reduced tumor hypoxia. Despite these dramatic effects, it failed to induce tumor
growth retardation on its own.

In the last decade, there has been an expansion of experimental and clinical
substantiation collocated on integrating novel agents which target the VEGF pathway in
anticancer treatment. VEGF intracellular signaling has indeed repeatedly been shown to be a
limiting factor in the pathology of cancer because it drives angiogenesis, regulates apoptosis
and, as a consequence, promote tumor growth and metastasis (Byrne et al. 2005). As single
agents, VEGF inhibitors such as bevacizumab proved strong efficiency in abating tumor
growth in a vast array of preclinical tumor models and in a more limited number of clinical
tumors (Ranieri et al. 2006). Several studies have shown that the combination of
bevacizumab with standard first line chemotherapy resulted in improved tumor growth
control and patient survival. For instance, in a phase III trial by Hurwitz et al. (Hurwitz et al.
2004), bevacizumab interfaced with combination chemotherapy resulted in significantly
improved overall survival in colorectal cancer patient. A rationale behind these observations
could reside, at least in part, in the fact that, besides its direct antiangiogenic effects,
bevacizumab may allow better drug accessibility to the tumor by pruning immature and
inefficient tumor blood vessels, coinciding with an increase in tumor perfusion and
oxygenation. This “vascular normalization” theory initially formulated by Rakesh Jain in
2001 (Jain 2001) is supported by several studies having shown that VEGF blockade in
particular and antiangiogenic treatments in general lead to improved tumor oxygenation (Lee
Rapamycin could also benefit from the antiangiogenic effects of bevacizumab leading to
increased rapamycin delivery and bioavailability within tumors. This eventuality deserves further investigations. The apparent lack of histological or molecular potentiation of the known effects of bevacizumab by rapamycin could find a further explanation in that the lesions in DNA caused by bevacizumab remain unrepaired due to the blocking mTOR by rapamycin (Bjornsti et al. 2004). The securization of DNA damage by rapamycin would cause cells to degenerate over periods of times longer that the one that we assayed in our study. These salient cellular effects may thus not be detected in our endogenous markers of proliferation, apoptosis and oxygenation immunostainings at the end of treatments.

**Conclusion**

To summarize, our study reports unprecedented antitumor benefit in combining mTOR inhibition by rapamycin to VEGF neutralization by bevacizumab. Interestingly, the improved repression of tumor growth by the combined treatment is not explained by a rapid modulation of any of the known biological consequences of VEGF blockade, which suggests the existence of late cellular response(s) consecutive to combined VEGF blockade and mTOR inhibition that retard(s) tumor growth.

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Figure 2.1: The combination of bevacizumab with rapamycin has more than additive antitumor effects

A: The growth of HT29 tumors was determined by measuring tumor diameters in randomized mice treated with the combination of bevacizumab and rapamycin (τ, n=9), bevacizumab alone (ν, n=9), rapamycin alone (σ, n=10), or saline (□, n=10). Treatments started at Day 0. Bevacizumab (100ug/kg) was then administered every 3-days, and rapamycin (1.5 mg/kg) or saline every day until the end of the experiment. For convenience, tumor volumes are expressed as % of Day 0.

B: Growth delays were determined when tumors reached twice of their initial volume (dashed line).

*P<0.01 versus saline.
Figure 2.2: Combining bevacizumab with rapamycin results in improved mTOR inhibition

HT29 tumor-bearing mice were untreated, treated with bevacizumab, rapamycin, or the combination thereof as in Fig 1. Whole tumor lysates were probed with antibodies against phosphorylated mTOR (P-mTOR), and total mTOR tumor content was also determined. The upper panel shows representative immunoblots. The lower graph shows P-mTOR/mTOR protein ratio (indicative of mTOR activity) for each treatment.
n=3, *P<0.05, **P<0.01 versus saline; #P<0.05 versus rapamycin.
Figure 2.3: In contrast to bevacizumab, rapamycin does not induce a decrease in the availability of VEGF in tumors

A: Cryoslices from untreated HT29 tumors were incubated overnight in saline (control, left panel) or bevacizumab (right panel), and immunostained against VEGF.

B: Mice bearing size-matched HT29 tumors were treated with saline, bevacizumab alone, rapamycin alone, or bevacizumab + rapamycin. After biopsy, VEGF was detected from whole tumor lysates using ELISA. n=10.

C: Cryoslices of the treated tumors were also immunostained to detect VEGF distribution.

D: For each treatment, the area positive for VEGF staining on slices was quantified in random fields, and expressed as % of the whole surface of the sample. n=4, *P<0.05, **P<0.005 versus saline (one-way ANOVA).

Abbreviations: S = saline; B = bevacizumab; R = rapamycin; B+R = bevacizumab + rapamycin.
Figure 2.4: Rapamycin does not improve the antiangiogenic response to bevacizumab

HT29-bearing mice were treated with saline, rapamycin alone, bevacizumab alone, or the combination of bevacizumab and rapamycin. Tumor cryoslices were stained against the endothelial marker CD31.

A: Shown are representative pictures of the stainings.

B: The microvascular density within treated tumor cryoslices was quantified by counting CD31-positive clusters in random fields. n=12, **P<0.01, ***P<0.005 versus saline (one-way ANOVA).

Abbreviations: S = saline; R = rapamycin; B = bevacizumab; B+R = bevacizumab + rapamycin; MVD = mean vessel density.
Figure 2.5: Rapamycin does not affect the antiproliferative and pro-apoptotic effects of bevacizumab

Groups of HT29 tumor-bearing mice received repeated injections of either saline, bevacizumab, rapamycin, or both bevacizumab and rapamycin.

A: The representative pictures show tumor cryoslices stained against the proliferation marker Ki67. Proliferating cells expressing Ki67 in the nucleus were counted in random fields of stained tumor slices (bottom panel).

B: We further identified how the different treatments affected apoptosis by staining the activated caspase 3 on the tumor cryoslices. Representative pictures are shown (upper panel), as well as the quantitative results obtained by counting positively stained cells in random fields (bottom panel).

C: Cells in random fields were sorted as undergoing proliferation (black bars), apoptosis (gray bars), or remaining unaffected (white bars) by the treatment. This allowed us to determine the relative contribution of each subpopulation to the whole tumor cell population. n=4, **P<0.01, ***P<0.005 versus saline (one-way ANOVA).

Abbreviations: S = saline; R = rapamycin; B = bevacizumab; B+R = bevacizumab + rapamycin.
Figure 2.6: VEGF blockade by bevacizumab increases tumor oxygenation independently of mTOR activity
Tumor bearing mice received saline, bevacizumab, rapamycin, or bevacizumab + rapamycin treatments.
A: Cryoslices from the treated tumors were immunostained against carbonic anhydrase 9 (CA-IX), a robust marker of hypoxia.
B: In each slice, areas with positive CA-IX staining were identified. Their cumulative surface was quantified and expressed as % of the total surface of the slice. Bars show the average CA-IX staining for each treatment regimen. n=4. *P<0.05, **P<0.01 versus saline (one-way ANOVA).
Abbreviations: S = saline; R = rapamycin; B = bevacizumab; B+R = bevacizumab + rapamycin.
References


CHAPTER THREE: Literature Review of Gastrointestinal Stem Cells

Introduction

There have been great advances in the field of intestinal stem cell biology. The intestinal tract is made up of the small intestine and colon which are anatomically and functionally distinct organs (Gregorieff and Clevers 2005). The morphology of the epithelium that lines the lumen is strikingly dissimilar between the small intestine and colon and matches the clear-cut function of each. The surface area of the small intestine epithelium predominately functions in absorption. This role is greatly maximized via abundant finger-like protrusions called villi and numerous invaginations known as crypts of Lieberkühn which are found at the base of those villi. In contrast the colon epithelium does not have villi and presents a flat surface morphology with deep cavities of crypts of Lieberkühn penetrating into the underlying mucosa. Thus, the colon is better adapted to majorly function in compacting stool rather than in the role of absorption. In mice large intestine the mucosa of the cecum is thin and contains short crypts of Lieberkühn.

Intestinal stem cells located near the crypt base give rise to proliferating progenitor or transit amplifying cells which migrate up the crypt-villus axis while continuously dividing and differentiate into the four primary epithelial cell types. Three differentiated lineages populate the villus epithelium and include enterocytes, goblet cells, and enteroendocrine cells. Absorptive enterocytes comprise 90% of the villus-associated cells and are highly ubiquitous at the proximal end of the small intestine. These are cells which are highly polarized and contain a basal nucleus as well as an intricate apical brush border bearing the glycocalyx. Enterocytes have tight junctions and function in secretory and absorptive roles.
on the villus (and in crypts) which are essential for the gut’s very existence. The goblet cells are functionally responsible for secreting mucin which protect and lubricate the mucosa. These cells are so termed as they are shaped like that of a goblet, with an apical component formed as a cup, because it is distended by a large number of mucinogen granules, and a basal component shaped like a stem, because it is narrowed due to a lack of these granules. In addition, goblet cells are found at the distal bowel end where their lubricating role becomes further important. Enteroendocrine cells secrete various hormones which control physiological conditions along the intestinal tract. They are sometimes referred to as neuroendocrine cells and have abundant dense core neurosecretory granules which contain peptide hormones. The fourth epithelial lineage known as Paneth cells however, are largely restricted to the base of the crypt of Lieberkühn (Cheng et al. 1969) whereby these cells regulate the microbial environment as well as secrete stem cell niche signals (Sato et al. 2011). Paneth cells contain large secretory granules, and express several proteins that include lysozyme, tumor necrosis factor, and small molecular weight peptides known as cryptins in the mouse. These small molecular weight peptides are related to defensins in having antimicrobial action. In addition, Paneth cells have phagocytic properties, and their features illustrate a role in maintaining a sterile crypt milieu. Of note, although Paneth cells are normally restricted to the small intestine, they are occasionally observed in the proximal colon, and in some inflammatory syndromes are conspicuous in the colon as Paneth cell metaplasia (Wright 2000).

With these four paramount epithelial cell types, essential functions of the intestine are accomplished. Other cell types that are not as well-known include deep crypt secretory cells
which may typify the colon counterparts of Paneth cells (Rothenberg et al. 2012).

Tuft/brush/caveolated cells and cup cells exist also in the intestinal epithelium however represent extremely rare populations whose function and importance are little known. M cells are another less prominent cell type which are found on lymphoid peyer’s patches and transport antigens from the gut lumen to the underlying lymphoid tissue (de Lau et al. 2012).

The epithelium of the small intestine (and of the colon) exhibits a self-renewal that can be described as exceptional and most likely driven by the harsh operating environment of the intestine from continual physical, biological, and chemical insult. The entire epithelium is consequently replenished every 3-5 days (Leblond and Stevens 1948). This massive proliferation which drives the self-renewal process originates at the crypt base and is confined to crypts. Individual crypts make up approximately 250 cells and generate a number close to this in new cells each day. The resident stem cells located at the crypt base continuously divide to generate highly proliferative progenitors called transit amplifying (TA) cells. Through successive rounds of division, TA cells rapidly expand and begin differentiation as they migrate up toward the crypt-villus border and subsequently exit the crypt onto the villus over a period of 2 days (Heath et al. 1996). They have fully matured at the time of their crypt exit and will subsequently carry out their specialized functions as they continue their ascent toward the villus tip, where they die and are shed into the lumen. The replenishing work of up to ten crypts is needed in order to compensate cell shedding at each villus tip. However paneth cells by contrast, avoid this upwardly mobile migration and instead move down to occupy the crypt base where they survive for 6-8 weeks (Ireland et al. 2005). Finally, the morphology of the crypt-villus unit combined with the profound process
of self-renewal give the intestinal tract a distinct appeal as a model for the study of intestinal stem cell biology (Figure 3.1).

Historical Studies on Intestinal Stem Cells

Adult intestinal stem cells can be fundamentally characterized as having longevity in that stem cells persist for the lifetime of their owner, and multipotency in that stem cells are capable of generating all cell types of the tissue in which they belong (Barker and Clevers 2007).

The evidence of resident stem cells in the adult intestinal crypt has been indisputable for more than 30 years yet identifying their phenotype and localization has been challenging due to their relative scarcity and a lack of specific stem cell markers. However two candidate intestinal stem cell models have been vigorously deliberated and a unifying theory recently formulated. These include the stem cell zone model and the +4 model. The stem cell zone model was originally developed when nearly 40 years ago electron microscopy studies from Cheng and Leblond revealed that Paneth cells at the crypt base were interspersed with slender immature cycling cells referred to as crypt base columnar cells (CBC) (Cheng and Leblond 1974). These cells were sensitive to tritiated-thymidine exposure and many CBC cells died and were subsequently phagocytosed by surviving CBC cells. The resulting radioactive phagosomes, initially confined to CBC cells were later observed within more differentiated cells thus facilitating a rudimentary lineage tracing analysis. At later time points, radiolabeled phagosomes were observed in all four lineages of differentiated epithelial cells therefore providing powerful evidence for the CBC cell as a common cell of origin. When chemical mutagenesis was subsequently used to mark intestinal epithelial cells
at random, Bejerknes and Cheng observed both long-lived and short-lived clones of marked cells. However, only the long-lived clones encompassing all four primary cell lineages consistently included a marked CBC cells. Although indirect, this was perceived as further evidence for CBC cells as the self-renewing and multipotent stem cells (Bejerknes and Cheng 1999, 2002). The stem cell zone model states that the CBC stem cells reside in a “stem cell permissive” environment (Bjerknes and Cheng 1981a, 1981b, 1999). These cycling stem cells routinely produce progeny that will then exit the niche and traverse the “common origin of differentiation” around position +5 where they commit towards their distinct individual epithelial lineages. Progenitors will mature as they migrate upward onto the villus whereas maturing Paneth cell progenitors move downward, and the oldest Paneth cells will occupy at the very base of the crypt.

In 1965, the +4 model was originally introduced by Cairnie et al. when early cell tracking experiments predicted a common cell origin at position 4–5, directly above the differentiated Paneth cell compartment (Cairnie et al. 1965). Approximately 12 years later it was reported that radiation-sensitive label-retaining cells (LRCs) inhabit immediately above the uppermost Paneth cell most notably at positions from +2 to +7 however on average at the +4 position (Potten 1977). Because it prevented the accumulation of detrimental genome changes the sensitivity to radiation was deemed as a benefit for stem cells. Retention of DNA labels is universally regarded as a reliable surrogate stem cell trait signifying quiescence under physiological conditions. Though not known to many in the field of stem cell biology Potten et al. gave an account of label-retaining +4 cells undergoing active proliferation with a cell cycle time of 24 hours similar to CBC cells. From a statement by Potten and Loeffler:
“In a mouse, it divides approximately once a day probably under the influence of circadian factors, and hence during the animal’s full potential life span (in the laboratory for example), may undergo a thousand cell divisions” (Potten and Loeffler, 1990, p 1017). The LRC phenotype was instead however, suggested to have arisen from asymmetric segregation of old (labeled) and new (unlabelled) DNA strands into stem cells and their daughters, respectively (Potten et al. 2009). This rare occurrence of the “immortal strand” would protect the stem cell genome from accumulating mutations (Cairnes 1975).

**Intestinal Stem Cell Markers**

There has been a lack of reliable markers for the identification and study of intestinal stem cells. Although previous experimental approaches have reported stem cell number and positional information of marker expression, definitive identification of intestinal stem cells is still debated. However, through the recent discovery of several putative candidate markers for intestinal stem cell populations, the field of intestinal stem cell biology has progressed considerably. Notably, the complete physiological function and role in intestinal stem cell homeostasis of many of these markers remain elusive.

*Lgr5: Leucine-rich-repeat-G-protein coupled receptor 5*

The first marker to be discussed here and that has been arduously examined is Lgr5, a specific CBC cell marker. The Lgr5 gene encodes an orphan G protein-coupled receptor, and is identified by its extensive leucine-rich extracellular domain. Additionally, Lgr5 is closely akin to those receptors having glycoprotein hormone ligands, like TSH, FSH, and LH receptors. Lgr5 is also known to be expressed in stem cells of the hair-follicle, another self-
renewing structure regulated by Wnt (Morris et al. 2004). The Lgr5 gene is under Wnt signal control and was first established as a Wnt target gene in colon cancer (van de Wetering et al. 2002; van der Flier et al. 2007). Moreover, Lgr5 encodes a facultative component of the receptor complex of Wnt (Carmon et al. 2012; de Lau et al. 2011). Lgr5 is a 7TM protein and acts as the receptor for a small family of Wnt pathway agonists termed R-spondins (Carmon et al. 2011; de Lau et al. 2011; Glinka et al. 2011). The generation of an Lgr5-EGFP-ires-CreERT2/Rosa26RlacZ mouse model allowed visualization of live CBC cells including their in vivo lineage tracing (Barker et al. 2007). Lgr5+ cells are delineated as having a very uniform morphology, invariably touching Paneth cells, and dividing daily in a uniform fashion. These cells do not retain DNA labels (Escobar et al. 2011; Schepers et al. 2011). Individual crypts will harbor approximately 15 of these cells, with approximately 10% residing at the +4 position. One day after stochastic induction of Lgr5 locus-controlled Cre activity via single tamoxifen pulse, lacZ expression was observed, and was restricted to single CBC cells. At later time points, lacZ stained cells of all epithelial lineages were observed extending from crypt base to villus tip (i.e. throughout the crypt-villus axis) demonstrating both longevity and multipotency of the originally labeled Lgr5+ CBC cells. In addition, Lgr5+ cells at the colonic crypt base were established as adult stem cells (Barker et al. 2007, 2008b). This was the first study which consensually identified the Lgr5+ CBC as a self-renewing, multipotent stem cell belonging to the intestinal crypt. Of note, lacZ-labelled crypt-villus units continued during the lifetime of the mice. These in vivo observations of stemness were substantiated by functional in vitro assays. In the presence of well-established growth factors of the intestinal epithelium, particularly EGF (Dignass and Sturm 2001),
Noggin (Haramis et al. 2004) and the Wnt agonist R-spondin (Kim et al. 2005), mouse crypt preparations have been demonstrated to undergo continuous budding events, that lead to the formation of an organoid structure that keeps crypt-villus architecture (Sato et al. 2009). The organoids are comprised of a multitude of crypts that are organized around a central lumen lined with an epithelium that is villus-like. They can be reproduced (> 8 months) and not lose discernible characteristics. Exclusively, isolated Lgr5+ cells were capable of efficiently generating organoids consisting of all epithelial lineages that were compatible with a stem cell identity under comparable setting. Analogous observations were made when Lgr5+ cells at the bottom of colonic crypt were traced, with the marked distinction that colonic stem cells seemed more quiescent than their counterparts of the small intestine (Barker et al. 2007). Furthermore, colon epithelium can be expanded in culture utilizing the organoid model, and culture-expanded Lgr5+ colon-stem cells have recently been demonstrated to be transplantable and functional donor cells (Yui et al. 2012). This finding by Yui et al. may also serve as an important segue for the future of colon stem cell therapy.

*Ascl2: Achaete Scute-Like 2*

In the search for other putative CBC markers, the transcriptional profile of CBC cells was subsequently acquired by fluorescence activated cell sorting (FACS) of the LGR5-EGFP+ crypt population (van der Flier et al. 2009). As anticipated the CBC stem cell signature constituted several previously identified Wnt-dependent genes, that notably include Ascl2 (Van der Flier et al. 2007; 2009) thus confirming Ascl2 expression in the intestinal epithelium as Wnt-dependent. The Ascl2 gene encodes a basic helix-loop-helix transcription factor with an unusual restricted pattern of expression (Guillemot et al. 1994) in addition.
Although in situ hybridization proved that expression of these Wnt-target genes in Lgr5+ cells was increased, decreased expression levels were detected in cells of the TA compartment, thereby reducing their capability to serve as specific stem cell markers. However, the Wnt-target gene Ascl2 was an exception, and its expression was confined to the slender CBC cell (Van der Flier et al. 2009). Notably, as Ascl2−/− animals die around E10.5, conditional deletion of Ascl2 in the adult was performed utilizing an inducible Cre driven by the CYP1A1 promoter, AhCre mouse (Ireland et al. 2004), which is active from crypt base to villus tip. Total recombination of the floxed Ascl2 alleles was observed at early time points after induction and this resulted in the disappearance of CBC cells from the stem cell compartment over a relatively short period of time. Eight days post-induction, the minority of remaining escapee Ascl2+ stem cells had begun to repopulate recombined crypts as speculated through a mechanism of crypt fission, and two weeks after induction only wild-type epithelium existed (Van der Flier et al. 2009). This work demonstrated Ascl2 as a master regulator of stem cell function.

**Olfm4: Olfactomedin-4**

The aforementioned work also identified Olfm4 which is a Wnt-independent gene, to be a robust and highly specific marker for the CBC stem cell population. Moreover, this confirms the existence of stem cell specifiers that are not Wnt associated (van der Flier et al. 2009). From Zhang et al, the Olfm4 gene encodes a molecule secreted whose function is not known and that was originally cloned from human myeloblasts (Zhang et al. 2002). It has also been shown that Xenopus ONT1 of the Olfm4 family, acts as a bone morphogenetic protein (BMP) antagonist (Inomata et al. 2008). Though the expression of Olfm4 appears to
be confined just to Lgr5+ cells of the small intestine in mice, crypts of the human colon are enriched for Olfm4 (Kosinski et al. 2007). Moreover, human Olfm4 in colonic crypts is expressed in a comparable pattern to Olfm4 in mouse small intestine and this expression is confined to CBC (Barker et al. 2007; van der Flier et al. 2009). Although murine Olfm4 is not expressed in the colon, the detection of a specific signal was demonstrated in human colonic epithelium evocative of Lgr5 expression in the colon (Barker et al. 2007). In addition, Olfm4 was highly expressed in a subset of cells within colorectal carcinomas and expression in these tumor cells was far more increased than the expression that was observed in wild-type crypt base columnar cells at flanking crypt bottoms (van der Flier et al. 2009). Olfm4 expression has been speculated as a useful way to identify stem cells in human small intestine and in colon. Preceding studies involving human adenocarcinomas of the colon signify that these tumors house tumor-initiating cells or purported cancer stem cells (Dalerba et al. 2007; O’Brien et al. 2007; Ricci-Vitiani et al. 2007; Vermeulen et al. 2008). These studies have prompted speculation for potentially further research work utilizing Olfm4 as a marker of cancer cells with stem-like properties, in colon adenocarcinomas.

*Msi-1: Musashi-1*

Musashi protein was originally identified as an RNA-binding protein necessary for two successive asymmetric divisions of sensory organ precursor cells in Drosophila (Nakamura et al. 1994). In wild-type animals, the sensory organ precursor cell divides into a non-neural precursor cell and a neural precursor cell, but in musashi mutants, two non-neural precursor cells are generated instead. The symmetrically divided non-neural precursor cells differentiate to hair-forming cells which lead to a double-bristle phenotype rather than the
single-hair wild-type phenotype. Based on this double-hair form, the Musashi-1 gene derived the name “Mushashi” from a brave and famous Japanese swordsman named Musashi Miyamoto (A.D. 1584-1645), who fought with two swords (Horisawa and Yanagawa 2004; 2009).

Musashi-1 is a mouse neural RNA-binding protein with sequence comparable to Drosophila Musashi. Members of the Musashi family comprise Drosophila Musashi, Xenopus laevis nervous system-specific RNP protein-1 (Richter 1990), and mouse Musashi-1 (Sakakibara 1996). Sakakibara et al. speculated mouse Musashi-1 to be necessary for asymmetric distribution of intrinsic determinants in the developing mammalian nervous system (Sakakibara et al. 1996). All members of the Musashi family have two tandem RNA recognition motifs (RRMs) (Good et al. 1998). Furthermore, Msi-1 is highly expressed in neural precursor cells capable of generating both neurons and glia during embryonic CNS development (Kaneko 2000). The human musashi homolog gene is found within human chromosome 12q24.1-q24.31 and is extremely similar to mouse Msi-1 (Good et al. 1998). The Musashi gene is well conserved in most species and Musashi-1 was the first Musashi homolog identified in mammals (Sakakibara et al. 1996). Through immunohistochemical (IHC) analysis, Musashi-1 protein expression has been observed in neural precursor cells of the developing central nervous system and these undifferentiated, proliferative cells produce many different types of cell types which include neurons, astrocytes, and oligodendrocytes (Kaneko et al. 2000).

Notably, Musashi was the first such marker reported as a putative candidate stem cell marker within the intestinal epithelium of mice (Booth et al. 2000; Kayahara et al. 2003;
Potten et al. 2003) and the expression of Msi-1 in stem cells of the small intestinal crypts of mice was identified under steady-state conditions. Furthermore, Msi-1-positive cells were found throughout the entire crypt, from bottom to top, after irradiation and this may indicate the potential use of Msi-1 as a marker of stem cells within the crypt epithelium of the intestine (Booth et al. 2000). That Msi-1 positive cells are actually stem cells within normal human colon crypts has been indicated, albeit the mechanism governing Musashi-1 protein expression in colon crypt cells has still not been ascertained. Potten et al. observed Msi-1 expression in neonatal, adult, and post irradiation samples of mouse small intestine and colon, as well as small intestinal adenomas of Min+/- mice, and they described Msi-1 expression patterns consistent with the predicted number and distribution of early lineage cells including functional stem cells, and early dysplastic crypts and adenomas that were robustly Msi-1 positive. But, their studies on human small and large bowel showed patterns that were not as robust or reproducible (Potten et al. 2003). In addition, there has been consensus that the role of Msi-1 in the regulation of epithelial differentiation within intestinal crypts has the potential for further examination. Of note, Msi-1 has been shown to potentiate the transcriptional factor Hes-1’s promoter activity, indicating Msi-1 and Hes-1 are closely associated (Imai et al. 2001). Still others have indicated the expression of Msi-1 and Hes-1 in the small bowel (Sakakibara et al. 1996; Jensen et al. 2000). It has been shown that Musashi-1 (He et al. 2007) and Prominin-1 (Zhu et al. 2009), a gene which localizes to membrane protrusions and is frequently expressed on stem cells, both also mark crypt base columnar cells; however, their expression may extend into the subjacent transit amplifying cell compartment (Snippert et al. 2009). Musashi-1 endures as an intriguing candidate stem cell
marker primarily because its protein localization is congruous with that of intestinal stem cells and because it plays a key role in the maintenance of neuronal stem cells (Kayahara et al. 2003; Potten et al. 2003).

Other Markers

An alternative stem cell population was presented when classical cell tracking experiments proposed that an ancestral cell resided at position +4/+5 above the floor of the crypt (Carinie et al. 1965) This concept was supported by subsequent studies which showed that cells displaying DNA label retention and a great susceptibility to X- and γ-radiation, attractive features of stem cells, were found mostly at this +4 location (Potten et al. 1977). Furthermore, they reasoned that because label-retaining cells in the intestine were also shown to be cycling, long-term label retention was a result of asymmetric DNA segregation instead of the quiescence customarily associated with stem cells (Potten et al. 1974; 2002).

Moreover, experiments performed using tritiated-thymidine to identify template strands in the stem cells and the thymidine analogue BrdU, to label newly synthesized strands were true to type with the idea of an immortal strand, whereby the template strands were kept in the putative stem cells, while daughter cells inherited newly synthesized DNA (Potten et al. 2002). In 1975 this mechanism had been suggested to preserve long-term DNA integrity in the stem cell genome (Cairnie et al. 1975). A plethora of new molecular markers based on their expression patterns have been proposed to identify the +4 stem cells; still, the majority of these are not exclusive to the +4 cells and a plethora of studies of the +4 population do not have the functional demonstration of stemness (Sato et al. 2009; van der Flier et al. 2009; Snippert et al. 2009; Zhu et al. 2009; Formeister et al. 2009; Gracz et al. 2010; Mori-
Akiyama et al. 2007; Potten et al. 2003; May et al. 2009; Gerbe et al. 2009; Demidov et al. 2007; Gregorieff et al. 2005; von Furstenberg et al. 2011). In reference to the many new markers, mouse telomerase reverse transcriptase (mTERT) and Bmi1 have been confirmed by lineage tracing. Telomerase activity is known to augment resistance to senescence in spite of multiple rounds of division, and is a general quality of adult stem cells. Thus, this aroused investigation of mTERT as a candidate marker for the label-retaining cells at the +4 position and with the aid of an mTERT-GFP transgenic model, it was reported that a minority subset of cells at the +4 position were expressing mTERT with a report of 1 GFP+ cell in 150 crypts (Breault et al. 2008). A short pulse-chase experiment with BrdU showed these cells nearly quiescent, and this was subsequently validated as the proliferation marker, Ki67, demonstrated an absence of expression (Breault et al. 2008; Montgomery et al. 2011). Notably, lineage tracing experiments validated that all four intestinal lineages could be generated by cells expressing mTERT, still the near low frequency of labeled crypts indicated that only a subset of stem cells were identified using the mTERT reporter transgene (Montgomery et al. 2011). Though it’s unchallenged that the marked mTERT+ cells depict a subset of the multipotent intestinal stem cells, the paucity of labeled mTERT+ crypt cells restricts utilizing mTERT for use as general marker of the +4 population. However, notably, Bmi 1 has conclusively emerged as the most broadly accepted marker of the +4 stem cell population, and as such, was the first +4 stem cell marker investigated by lineage tracing (Sangiorgi and Capecchi 2008). The Bmi 1 gene presents as a polycomb ring finger oncogene that has previously been suggested to have a regulative role in the self-renewal of hematopoietic as well as neural stem cells (Park et al. 2004). It was shown that Bmi1 could
mark rare cells at the +4 cell position singularly in the proximal small bowel. This was demonstrated using mRNA in situ hybridization. In vivo lineage tracing with the aid of a Bmi1-ires-CreER/Rosa26RlacZ mouse model produced ribbons (of labeled progeny) under conditions that were not injurious and were somewhat similar to those achieved in the Lgr5 model. The ribbons of labeled progeny were observed to originate from the crypt to the villus and they encompassed all cell lineages of the intestinal epithelium suggesting that perhaps some of the originally labeled Bmi 1+ cells located at the +4 position were self-renewing, multipotent stem cells (Sangiorgi et al. 2008).

Moreover, ablation of the Bmi1-Cre+ population using targeted expression of diphtheria toxin resulted in crypt death, loss of the stem cell compartment and subsequent mouse death. A study implemented later, supported the idea that the Bmi1+ cell population has distinction from the Lgr5+ population because they are extremely resistant to radiation plus quiescent, and capable of mediating injury-induced regeneration; whereas Lgr5+ cells mediate homeostatic self-renewal (Yan et al. 2012). Yet another promising new marker called Hopx, recently discovered to identify +4 cells, and also confirmed via lineage tracing, was proposed by Epstein and associates. Hopx is described as an atypical homeobox (in that there is a DNA sequence found within genes in the regulation of morphogenesis) protein (Takeda et al. 2011). Examination and determination of Hopx-LacZ knock-in mice demonstrated a strong expression of β-galactosidase largely at the +4 position along the intestine, and was observed to mark label-retaining cells under conditions of normalcy after irradiation (Takeda et al. 2011). A Hopx-CreER mouse was created in an effort to mark the predetermined path of Hopx+ cell. That Hopx descendants were persistent plus lived long
with the capability to repopulate the entire crypt-villus unit, served to conclusively prove Hopx as a new biomarker for stem cells at the +4 spot. Importantly, this was confirmed by lineage tracing. Of note, in regard to the candidate +4 markers, exact positioning along the crypt axis, has proved challenging to determine. Several other proposed putative markers of the +4 population have been identified; however, this recognition has been on the basis of their expression pattern being limited mainly to the +4 position alone. Thus, they will require confirmation via functional lineage tracing experiments to be established as +4 markers of intestinal stem cell populations.

**Constituents of Wnt Signaling at the Cell Membrane**

The name “Wnt” (pronounced wint) was made known 20 years ago and combined the names of two orthologous genes: Wingless (Wg), a Drosophila segment polarity gene (Sharma et al. 1976) and Int-1, a mouse protooncogene (Nusse and Varmus 1982; Rijsewijk et al. 1987). The Wnt family is immense and is a group of secreted glycoproteins with at least 19 known human members that are expressed in species ranging from Drosophila to man and majorly operates in cell fate specification, CNS patterning, and control of asymmetric cell division (Miller 2002). And too, transcription of the family of Wnt genes presents as being developmentally regulated in a fashion that is explicitly spatial as well as temporal. Notably, signaling is triggered after the Wnt ligand binding to a member of the Fz family of seven-span transmembrane receptors (Bhanot et al. 1996; Wang et al. 1996; He et al. 1997; Dale 1998) combined with the co-receptors LRP-5 or LRP-6, that are members of the low-density lipoprotein receptor related protein family (LRP) (Wehrli et al. 2000). Canonical Wnt signaling is facilitated only when both Fz and LRP are intricately complexed to Wnt (Wehrli
et al. 2000; Tamai et al. 2000; Pinson et al. 2000). Moreover, the majority of Wnt proteins can attach to several Fzs and they can attach in reverse order, indicating repetition (in vivo). There is a notion that soluble Fz–FzB structures will function as antagonists, and suppress Wnt before it can attach to Fz bound at the membrane (Leyns et al. 1997; Wang et al. 1997; Finch et al. 1997). Similar to FzBs, Dickkopf (Dkk) proteins are secreted inhibitors, which differ structurally from Wnt proteins (Fedi et al. 1994; Glinka et al. 1998). They are powerful inhibitors of Wnt signaling and are engaged in head induction in Xenopus embryogenesis; moreover, Dkk will obstruct Wnt signaling by binding LRP-6 in such a manner as to sterically hinder Wnt binding (Mao et al. 2001). Kremen2 a transmembrane protein subsequently creates a ternary complex structure with Dkk and LRP-6, which promotes a hasty endocytosis and removal of the LRP-6 Wnt receptor from the cell membrane (Mao et al. 2002).

The Wnt Signaling Pathway and Colorectal Cancer

It has been projected by the American Cancer Society that more than 50,000 people in the United States will die from colorectal cancer (CRC) each year (The American Cancer Society. www.cancer.org). Furthermore, CRC is a leading cause of morbidity and mortality in the United States and worldwide. Extraordinary features about CRCs are the uniform molecular mechanisms which underlie practically all cases. Notably, above 90% of all CRCs will have an activating mutation of the canonical Wnt signaling pathway which will ultimately give way to both stabilization and accumulation of β-catenin within the cell nucleus. Nuclear β-catenin is highly indicative of an active canonical Wnt pathway; moreover, the presence of nuclear β-catenin is apparent in even the least detectable lesions as
a consequence of Wnt mutations (Kongkanunt et al. 1999). Furthermore, the regularity of mutations at any portion of the Wnt signaling pathway in CRC causes this cancer to become an appealing model for molecular intervention. Orford et al. reported that when the Wnt signal was omitted, unstimulated cells regulate β-catenin levels by a multiprotein complex which phosphorylates β-catenin marking it for subsequent ubiquitination and degradation (Orford et al. 1997). The adenomatous polyposis coli (APC) tumor suppressor protein, axin, and the glycogen synthase kinase, GSK3β comprise this β-catenin degradation complex. The Wnt ligand will dock onto its Frizzled (Fz) receptor, and subsequently a cascade of events is relayed that will destabilize the degradation complex, causing unphosphorylated β-catenin levels to accumulate then translocate to the nucleus where β-catenin operates as a cofactor for transcription factors of the T-cell factor/lymphoid enhancing factor (TCF/LEF) family. The genetic program that is triggered by β-catenin and TCF/LEF transcription factors designates the transcription of a specific subset of genes, which will predominately decide cell fate, and modulate proliferation. Signaling through this pathway exists during embryogenesis, where it has been demonstrated in the modulation of a multitude of episodes involving patterns of development in organisms that extend from worm to man. The formation of the dorsal–ventral axis in the developing embryo relies on action of Wnt signaling and deregulation of Wnt signaling results in developmental defects. Similar to other major pathways which regulate morphogenesis in early embryogenesis, research work shows a high occurrence of mutations that constitutively activate Wnt/β-catenin signaling in specific human tumors. Plus, cancer’s origins have been understood to be involved in this pathway (Polakis et al. 2000). Vogelstein and colleagues delineated the early pathways to CRC tumorigenesis:
Tumorigenesis is triggered when a single colorectal epithelial cell acquires a mutation in the tumor suppressor APC gene that controls the Wnt/β-catenin signaling pathway. Loss of function of APC is seen in most of sporadic cases of CRC at approximately 80% and too, is responsible for familial adenomatous polyposis (FAP), the inherited form of colon cancer (Groden et al. 1991; Kinzler et al. 1991). Mutations in the KRAS and BRAF genes facilitate growth into an adenoma that has a diameter higher than 1cm and is clinically significant. Additional mutational hits in TGF-β, PIK3CA, and TP53 continue to drive clonal expansion and transformation from a benign adenoma to a carcinoma of which the potential for invasion and metastasis exists (Jones et al. 2008).

**Theory of the Cancer Stem Cell**

It has been well acknowledged that tumors comprise a heterogeneous population of cells that contain various levels of cellular differentiation and morphologic features; however, there is a belief most tumors are monoclonal in origin (Fiaklow et al. 1976; Volgelstein et al. 1985). This supports the idea that the originating tumor must have the ability of producing various cell types which compose the tumor. Of note, for many years selection of mutant subpopulations originating from a common progenitor- clonal evolution, in addition to microenvironmental impact, have been the main reasons for how a complex and heterogeneous tumor develops from a single cell. Moreover, there is the notion that these selective pressures will supply the impetus for tumor growth and progression (Nowell et al. 1976). Of late, parts of this model have been challenged by increasing evidence that tumor growth and progression are supported by a small population of tumor cells possessing stem-like properties, thus invoking the cancer stem cell (CSC) theory. While most normal tissues
are supported by a small population of slowly cycling and self-renewing stem cells, the CSC theory suggests the existence of a similar tumor cell hierarchy with a CSC residing at the apex (Dalerba et al. 2007). In this model, the self-renewing CSC divides to produce tumor cell subpopulations (with more limited replicative ability) which generally compose the bulk of tumor mass. Due the difference in replicative capacity, there is the notion tumorigenic supporting abilities are exclusive to the CSC, while tumor growth and expansion are ascribed to the rapidly dividing progeny. This critical point marks the departure from prior models of tumorigenesis that substantiate the notion that each tumor cell should have the ability to form the tumor mass (Clarke et al. 2006).

Although there is increasing evidence substantiating that the CSC exists, the origins of this cell are still not certain. There is the possibility of genetic and/or epigenetic differences contributing to a normal tissue stem cell becoming cancerous, or contributing to stemness of a progenitor or differentiated cell (Huntly et al. 2005). It is due to this uncertainty, the designation of ‘cancer-initiating cell’, or ‘tumor-initiating cell’ are frequently used reciprocally with the term of ‘cancer stem cell’. However, a genuine definition of a CSC will be based on the function (of having the particular ability for self-renewal and to give rise to the heterogeneous lineages of cancer cells which compose a tumor mass) (Clarke et al. 2006). Though the CSC theory of tumorigenesis has garnered much recognition of late, this theory was founded on ideas and concepts that have existed for more than 150 years. In 1855, Rudolph Virchow put forth the idea that tumors develop from residual embryonic nests and this idea has been frequented again and again over the past century (Huntly et al. 2005). During the 1960s, evidence which substantiated the concept that all tumor cells do not
possess an equal capability for tumorigenesis was a focal point in regards to the quantitative tumor autotransplantation assays. In the 1990s, J. Dick et al, first identified cancer stem cells from blood of patients with acute myelogenous leukemia (AML) (Bonnet et al. 1997; Lapidot et al. 1994). They demonstrated utilizing xenotransplantation assays in NOD/SCID mice that tumorigenic potential existed with just a small subset of leukemic cells, which was characterized by increased CD34 and decreased CD38 expression at the cell surface. Moreover, upon transplantation of these leukemic cells into immunocompromised mice, they developed AML that was phenotypically very much like the subtype of AML existent in the patient from which the cells originated. Clarke et al, many years later were the first to potentially identify cancer stem cells in a solid tumor (Al-Hajj et al. 2003). Utilizing comparable xenotransplantation assays, they identified a breast cancer cell population characterized by increased CD44 and decreased CD24 expression which reiterated the original tumor phenotype, and this was developed from as little as 100 transplanted cells. A multitude of studies have since been published characterizing CSC populations across a broad range of solid organ tumors, that include colorectal cancers, as well as cancers of the CNS, pancreas, and head and neck (Dalerba et al. 2007; Hermann et al. 2007; Prince et al. 2007; Singh et al. 2003).

Traditional cancer therapies normally target the rapidly dividing tumor cell population and, as proposed by increasing evidence, may preferentially spare the CSC part of the tumor (Bertolini et al. 2009; Dylla et al. 2008). This could provide a reason for the frequently encountered clinical situation by which a tumor will show complete volumetric tumor reduction that is followed by a subsequent local recurrence. Importantly, the CSC
theory has the potential for expansive clinical significance as well as adding to the knowledge base of tumor biology. Comparable to their normal tissue counterparts, CSCs have been demonstrated to exhibit high chemoresistance and radioresistance (Diehn et al. 2009; Bao et al. 2006; Bertolini et al. 2009; Chang et al. 2009; Dylla et al. 2008; Hong et al. 2009). By its very nature, the CSC theory suggests the identification and characterization of cancer stem cells may serve as a segue for novel experimental therapeutics. What’s more much endeavor has unfolded to discover biomarkers for cancer stem cells that will aid in their delineation as well as selective targeting.

**Intestinal Stem Cells as the Origin of Colorectal Cancer**

Colorectal cancer (CRC) follows a sequence that has been amply described in both sporadic and hereditary forms. This sequence of tumorigenesis is characterized by histopathological changes with early hyperplasia of the crypt and the development of the adenoma into an invasive carcinoma (Fearon and Vogelstein 1990). That the colorectal tumor derives from a series of mutations leading to the activation of oncogenes and inactivation of tumor suppressor genes, culminating in unregulated growth, has contributed our prehension of colon tumor biology. Moreover, mutations in many genes are necessary for malignant transformation, while benign tumor growth depends upon fewer changes (Fearon and Vogelstein 1990). Furthermore, that stochastic acquisition of mutations within various combinations of signaling pathways can result in cancer, proposes that acquisition of CRC is an imminent and temporally dependent event (Jones et al. 2008). The assimilation of this concept into the CSC model implies these mutations to take place within the long-lived stem cell, which result in an accumulation of several mutations over time (Ricci-Vitiani et al.
The mutated stem cell has the capacity to generate additional mutated stem and progenitor cells via symmetric and asymmetric division thereby seeding tumor growth with mutated, transformed and heterogeneous cells alike. By this means the CSC is capable of nurturing its own microenvironmental niche, because the survival of its diverse population is selected by the surrounding tumor stromal cells. To substantiate this notion, it was demonstrated that establishment and maintenance of the CRC stem cell niche relies upon the Wnt signaling pathway coordinated by myofibroblasts. This suggests microenvironmental cues being as urgently important for the molecular diversity of tumors, as mutations (Vermeulen et al. 2010). Of note, metastatic spread of disease is also compatible with the CSC theory. Independent subclonal populations within the tumor are enabled with distinct functional properties; however, just selected clones have the potential to metastasize to distant organs (Dalerba et al. 2007). In further explanation of this model, the metastatic cells may arise from a monoclonal expansion of the original clonal cell population; however, over the course of time, additional genetic mutations will occur. Thus, responsiveness to environmental signals and acquisition of metastatic properties will be implemented; particularly the capacity to invade the surrounding region, intravasate through vasculature, evade the immune system and extravasate at a distant site (Dalerba et al. 2007). Moreover, as substantiated by this diversity that has been achieved, metastatic tumors have the potential for major morphologically divergence from the primary tumor. The evidence of late from gene-expression microarrays, support metastases of epithelial tumors which encompass colorectal tumors, in regards to the CSC theory (Ricci-Vitiani et al. 2009).
The acute loss of the APC gene throughout the intestinal epithelium in mice is adequate to induce rapid development of intestinal adenomas, and thereby, contributing a model that can be of benefit in the study of CRC (Shibata et al. 1997). Direct evidence still lacks for the origin of CSCs in human cancer. Experiments by Clevers and associates were performed in mice explicitly to focus on the operation of stem cells as adenoma-initiators. Thus, an inducible Lgr5-EGFP-IRES-creERT2 cassette was utilized to delete floxed APC alleles solely in CBC stem cells. In a period of days, (following tamoxifen dispense), β-catenin accumulation was found in isolated Lgr5-EGFP+ stem cells and these transformed cells rapidly became associated with clusters of β-catenin-expressing progeny, upwardly migrating crypts (Barker et al. 2009). Consequently zealous expansion of Wnt-transformed cells quickly led to the development of macroadenomas in the conjoined villus stroma culminating in a lethal tumor load within weeks. Of note, transformed Lgr5+ cells appeared in colon crypts, albeit their expansion was not as quick. The reduced rate of formation in colon adenomas is likely due to the generally slower tissue renewal kinetics in the colon organ. In contrast, when Apc was deleted in the more differentiated TA cell compartment, there was no development of macroadenomas. Thus, these studies utilizing a murine model indicate the transformation of Lgr5+ cells is the predominant means by which the adenoma is formed, therefore establishing intestinal stem cells as the cells of origin in intestinal cancer. (Barker et al. 2009). Attendant with this work, intestinal dysplasia is observed when a form of β-catenin capable of being activated, is expressed in Bmi1+ or Prominin1+ intestinal cell populations, both of which have been confirmed by lineage tracing to have stem cell properties (Sangiori et al. 2008).
Intestinal Stem Cells as Identifiers of Colorectal Cancer Stem Cells

Cancer relapse is the cause of a major proportion of CRC deaths. Furthermore the tumors which recur are the result of cancer cells that not only have disseminated, but have resisted chemotherapeutics. These cancer cells express resiliency in that they are capable of reestablishing the cellular organization of the primary tumor by renewing all tumor populations, thus as defined, they behave as cancer stem cells. Targeting these speculated CSCs may potentially contribute to novel therapeutics; however selective biomarkers for CSCs have yet to be discovered. Candidate CSC populations have been traditionally characterized by their capability to reconstitute tumors after transplantation in immunodeficient mice. Moreover, fluorescence activated cell sorting (FACS) is regularly used to separate putative CSC populations from the tumor mass on the basis of stem cell specific expression of surface markers.

CD133 was first suggested as a candidate biomarker of CRC stem cells by virtue of its utility to identify brain tumor-initiating cells (O’Brien et al. 2007; Ricci-Vitiani et al. 2007). The isolated CD133+ cell population was discovered to be enriched in tumorigenic cells, with the capacity to reestablish the original tumor in xenotransplantation models as well as form colonic organoids in vitro (O’Brien et al. 2007; Ricci-Vitiani et al. 2007). However, the expression pattern of CD133 in normal epithelial tissue crucially impedes its therapeutic application, and further studies report that CD133- cells can initiate tumors in addition. CD44 and EpCAm are alternative CSC markers that were identified utilizing xenografts and profiling of this sorted population has yielded CD166 as a new and original putative comarker (Dalerba et al. 2007). Merlos-Suarez et al, put forth the hypothesis that the
intestinal stem cell program could also define the cancer stem cell niche in colon cancer, given the proposed connection between normal intestinal stem cells and cancer stem cells. The tyrosine kinase receptor EphB2 is expressed in a gradient that is decreased from the crypt base to the villus in normal crypts, and too, is conducive for the positioning of the various cell types along the axis of the crypt (Battle et al. 2002). To characterize their respective transcriptional profiles, populations enriched in ISCs, early and late TA cells were sorted according to their differential expression of EphB2 (Merlos-Suarez et al. 2011). van der Flier and colleagues found only 54 genes that were specifically enriched in EphB2\textsuperscript{hi} cells of the ISC-enriched fraction, and this includes the previously described intestinal stem cell genes Lgr5 and Ascl2 (van der Flier et al. 2009). Examination of a group of 340 patients showed that a ‘humanized’ version of the mouse ISC signature correlated with the more aggressive poorly differentiated CRC tumors, as well as being a reliable predictor of disease relapse. Poor prognosis is frequently the result of high numbers of CSCs in the primary tumor that might propagate the cancer. Thus, a proposed interpretation for the compelling association between the ISC gene signature and disease relapse, is that these aggressive tumors have an increase in the numbers of CSCs. Therefore a presupposition that the ISC gene signature could be directly probed for CSC markers exists. In an effort to identify and localize the ‘evasive’ cancer stem cells, genes identified within the ISC program have been utilized as surrogate markers to identify tumor cells showing an ISC-phenotype (Merlos-Suarez et al. 2011) and Lgr5 is widely detected in CRC tumors with its expression frequently localized at the tumor–host interface (Takahashi et al. 2011). It has been described that tumors which present with crypt-like invaginations into the surrounding stroma have Lgr5+
cells which reside at basal positions that are comparable in location with the ISCs in normal colon crypts (Merlos-Suarez et al. 2011). Expression of Lgr5 in nearly all tumors is proposed to be strongly associated to increased EphB2 surface levels, and this implies that an EphB2 gradient is in addition, maintained across the axis of the crypt-like tumor structures. This histopathology observed substantiates the notion in which tumors maintain the hierarchical organization of their normal tissue counterparts. Therefore, in all likelihood, ISC-like CSCs are culpable for cancer relapse (Merlos-Suarez et al. 2011). Elevated Lgr5 expression has been reported in advanced metastatic CRC cell lines, and corresponds with an ill-fated prognosis in patients (Uchida et al. 2010).

From Merlos-Suarez and colleagues, the ISC-like tumor cells identified using ISC markers had the capacity to reestablish the populations and architecture of the primary tumor after transplantation in immunodeficient mice, which supports a CSC operation for this subpopulation. Moreover, though Lgr5 presents a notable marker of CSCs, there has been hardship in producing dependable monoclonal antibodies against it, and has thus far restricted its application for the study of human CRC. Therefore more thorough analysis of the ISC-gene signature will be required in order to identify readily exploitable human CSC surface markers. Subsequently, the use of CSCs for prognostic and predictive tools is still not yet certain in resected and broadly metastatic disease (Artells et al. 2010; Saigusa et al. 2009; Li et al. 2009; Horst et al. 2008).

In conformity with aberrant Wnt activation serving as the initiator of CRC, the forced transformation of intestinal epithelial cells from normal tissue to adenoma can then be observed via the buildup of nuclear β-catenin in affected tissue, and this occurs with a
considerable increase in many target genes of Wnt, that include the Lgr5 intestinal stem cell marker. However there is a marked downregulation of these Wnt-target genes which occurs in a majority of patients once the tumor progresses to an invasive stage, in an absence of any decreases in staining of nuclear β-catenin (de Sousa E Melo et al. 2011). This inconsistency was explicated when it was shown that a multitude of the Wnt-target genes, which encompass Lgr5, underwent methylation-dependent regulation and that these relative methylation levels could be predictive in an immense way for recurrence of disease (de Sousa E Melo et al. 2011). It has been proposed that though the ISC-derived predictive profiles provide a strong tool to aid in prognosis, they can be deemed a reflection of the general state of the tumor and are not exact gauges of the portion of CSC which exists. There is only a subset of genes characterized by these profiles which will be useful as biomarkers of cancer stem cells. Furthermore the signature for intestinal stem cell like genes is apt to signify a general immaturity of the tumor however specific phenotypic shifts of CSCs as the tumor develops, is not taken into account. In vitro studies involving the downregulation of Lgr5 propose that this phenotypic shift in CSCs is not merely a reflection of the tumor progression but could have functional connotations as well. From Walker et al, modulation of Lgr5 expression in colorectal cell lines was discovered to control tumorigenicity, and inhibition of Lgr5 was implicated in increased Wnt-signaling, upregulation of epithelial-mesenchymal transition (EMT) genes and higher invasiveness, culminating in increased tumor formation rates upon examination in xenografts (Walker et al. 2011). Segregated CSC expression profiles may need to be determined for each stage of the tumor and various combinations of biomarkers will be required to target a phenotypically developing stem cell
in distinct stages of cancer progression. In addition, it has been proposed that potentially disrupting the tumor stem cell niche, may serve as an alternative strategy for targeting these disease cells that recur, by virtue of the challenges in identifying reliable markers.

Considering the likenesses between normal intestinal stem cells and cancer stem cells which have been proposed, the plausibility that the homeostasis of stem cells involve the same signaling pathways, has been suggested.

Drug development strategies have focused in particular on inhibiting the Wnt-signaling cascade, because of its prevalent function in normal niche regulation, and evidence that dysregulated Wnt signaling is acknowledged as being the driver in CRC initiation (Barker et al. 2006; Takahashi-Yanaga et al. 2010; de Sousa EM et al. 2011). It was found that high Wnt activity was not only an intrinsic cell property to describe CSCs, but that Wnt-signaling could be modulated in addition, to reestablish tumorigenicity in more differentiated cells (Vermeulen et al. 2012). Of note, the favorable outcome in forming long-term cultures of human colonic stem cells and the characterizing conditions to produce human colonic organoids, is thought to provide an up-and-coming screening platform for drug development (Sato et al. 2011; Jung et al. 2011).

**Cancer Therapeutics: Targeting Wnt**

There has been vigorous enthusiasm in the pharmaceutical and biotechnology sectors in developing effective Wnt pathway inhibitors (Korinek et al. 1997; Morin et al. 1997; Rubinfeld et al. 1997). This work began in the late 1990s when aberrant Wnt pathway activation was initially connected to colon cancer. A number of therapeutics, which may in addition, directly, or indirectly, target the Wnt pathway and which could be adapted for
cancer treatment include for example, NSAIDS, select vitamins, antibody-based therapeutics, and small-molecule inhibitors.

**NSAIDS: Nonsteroidal Anti-inflammatory Drugs**

The traditional NSAIDS (pronounced en-saids), encompass aspirin, sulindac and indomethacin, and are utilized globally for the treatment of fever, pain, and inflammation. Recently, NSAIDS have been able to allure substantial attention as promising anticancer drugs. What’s more many epidemiological studies have highlighted the benefits of using aspirin regularly and utilizing other NSAIDS to aid in decreasing both the incidence and severity of various human cancers (Dubois et al. 1996; Giovannucci et al. 1994). This chemoprotective function is especially apparent for familial and hereditary forms of colon cancer in which unregulated signaling of Wnt is believed to be a major driver (Thun et al. 1997, 2002). In addition, the efficacy of NSAIDS as anticancer agents can be ascribed to several effects which span from preventing cancer cell proliferation and induction of apoptosis to repression of the cancer cell invasion. The exact mechanism of action for NSAIDs is thought complicated and likely differs for each class of drugs. Without a doubt suppression of heightened action of the cyclooxygenase (COX) enzyme in cancer cells is a principal element in the anticancer activity of a multitude of NSAIDS (Maier et al. 2004). This is not the only segue of action, however, because NSAIDS act efficiently against tumor cells that do not have COX activity and, conversely, a few NSAIDS lacking COX activity have anticancer action in vivo (Zhang et al. 1999; Smith et al. 2000). Furthermore, it has become even more discernible that a uniting point of issue regarding the action of NSAID on human cancers, may lie in the suppression of dysregulated Wnt signaling. This was initially
suggested upon NSAID therapy being effective in reversing polyp growth in patients with
Familial adenomatous polyposis (FAP) and curbing polyp formation in the mouse model of
FAP (Giardiello et al. 1993; Labayle et al. 1991).

Approximately 4% of patients using NSAIDS suffer from severe intestinal bleeding,
kidney damage, or both, thus it is impractical to have extensive prescription of these drugs as
anticancer agents. More safely and effectively developed NSAID derivatives exist as a result;
these maintain and increase their anticancer actions, as well as restrict toxicity in their side
effects. They consist of the following selective COX2 inhibitors: celecoxib (Celebrex;
Pfizer), rofecoxib (Vioxx; Merck), as well as nitric oxide-releasing NSAIDS (NONSAIDS).
In addition, NO-releasing aspirin (NO-ASA) is a derivative of aspirin and hailed several
thousand-fold more effective in stopping human colon cancer cell growth than aspirin in
vitro and has much less toxicity (Williams et al. 2001; Rigas et al. 2002; Fiorucci et al.
2003). These NSAID derivatives considerably reduce Wnt signaling in colon cancer cell
lines by disrupting the nuclear Tcf–β-catenin complex formation (Williams et al. 2004; Gao

*Vitamins A and D*

Retinoids are derived from the conversion of vitamin A in the body. As evidenced
from epidemiological studies, clinical trials, rodent cancer models and in vitro cellular
models, there is mounting support employing retinoids and their synthetic derivatives as
pharmacological agents in cancer therapy and cancer prevention (Soprano et al. 2004). Some
skepticism pertaining to the therapeutic promise in utilizing retinoids to treat Wnt addicted
cancers were made by a study that recorded increased intestinal tumour growth in APC\textsuperscript{min}
mice treated with retinoic acid (Mollersen et al. 2004). However, several studies suggest that these retinoid activated receptors interact with β-catenin in direct competition with Tcf factors as well (Shain et al. 2003; Xiao 2003). The resultant decrease of the Tcf–β-catenin complex formation may assist in inhibiting colon cancer growth in vitro and in animal models observed after retinoid treatment (Hoosein 1988; O’Dwyer 1987).

Studies in epidemiology indicate that stimulation of vitamin D synthesis in the organ of skin by sunlight exposure has protective effects against a variety of cancers, which include breast, prostate, and cancer of the colon (Giovannucci et al. 2005). Additionally, the physiologically active form of vitamin D, 1α,25- dihydroxy vitamin D3 (1α,25[OH]2D3), and synthetic derivatives prevent the growth of a range of cancer cells in vitro by inhibiting cell proliferation and inducing differentiation, as well as show chemopreventive action in animal models of breast and of colorectal cancer (Akhter et al. 1997; VanWeelden et al. 1998; Harris et al. 2004).

Antibody-based Therapeutics

A variety of human cancers which lack mutations in APC, Axin or β-catenin may yet use an aberrantly activated signaling pathway of Wnt via elevated expression of Wnt ligands (such as Fzd receptors and Dvl), which are the more upstream pathway components. Thus, this will introduce the opportunity to develop antibodies against the overexpressed Wnt and Fzd proteins as potential cancer therapeutics effecting blocking of Wnt signaling or either the recruitment of immune effectors to the cancer cells (You et al. 2006). In cancers of the head and neck, a range of Wnts and also the FZD2 receptor are over-expressed frequently (Rhee et al. 2002). As a proof of concept, treatment of a head and neck cancer cell line over-
expressing WNT1 with a WNT1 monoclonal antibody effectively curbed the signaling of Wnt, blocked proliferation and induced apoptosis (Rhee et al. 2002). WNT1 is expressed substantially in non-small-cell lung cancer (NSCLC) primary tumors and cell lines in addition (He et al. 2005). Again, treatment with the WNT1 antibody that was the same, elicited apoptosis and effectively blocked tumor growth in mice (He et al. 2004). Additional cancers encompassing gastric, colon, melanoma, mesothelioma and non-small cell lung carcinoma (NSCLC) express elevated levels of WNT2 (Katoh 2001; Milovanovic et al. 2004; You et al. 2004; Holcombe et al. 2002; Vider et al. 1996). Moreover, treatment of NSCLC, melanoma and mesothelioma cells with a WNT2 monoclonal antibody induces apoptosis in vitro, thereby again accentuating the potential of therapeutic antibodies directed against components of Wnt (Mazieres et al. 2005; You et al. 2004). FZD1 and FZD2 receptors, known to be expressed substantially in breast cancers as well as poorly differentiated colon cancers relative to normal tissue, serve as other, different, targets for antibody-based therapeutics (Milovanovic et al. 2004; Holcombe et al. 2002).

**Small-Molecule Inhibitors**

There are many routes human cancers use to aberrantly activate and dysregulate the Wnt signaling pathway. A characteristic that is shared by and which encompasses all these cancers is continuous Tcf–β-catenin complexes in their nuclei. This thereby results in chronic activation of a genetic program which mediates cancer establishment by triggering cell growth, preventing cell death, as well as to alter the cell movement. It has been found artificial disruption of Tcf–β-catenin complex formation in colon cancer cells can effectively act to block target gene activation as well as prevent their in vitro growth (Tetsu et al. 1999;
van de Wetering et al. 2002). Drugs that are designed to mimic disrupting Tcf binding to β-catenin in vivo are anticipated to have much, much, promise in treating a variety of Wnt dependent cancers. Achievements made from the last several years in developing effective small-molecule inhibitors of protein complexes reestablished enthusiasm in the potential therapeutic potential regarding this approach (Berg et al. 2002; Chen et al. 2002). This resulted in the Tcf–β-catenin protein complex becoming a high interest target for small-molecule inhibitor development in the pharmaceutical and biotechnology sectors. Three natural compounds which include PKF115-584, PKF-222-815, and CPG049090 were discovered via High-throughput screening (HTS) of natural compounds and consistently ranked as effective inhibitors of Tcf–β-catenin binding in secondary assays. In addition, their capability to prevent axis duplication induced by artificial stimulation of Wnt signaling in Xenopus embryos (Molenaar et al. 1996) as well as to selectively stop growth of colon cancer cell lines with constitutively active Wnt signaling substantiates even more, the proposal that these compounds are bona fide Tcf–β-catenin inhibitors possessing cancer therapeutic potential (Miyaki et al. 1994).

**Stem Cell Therapy for Gastrointestinal Disorders**

Discovery in the identification of markers for intestinal stem cells will serve to provide an invaluable tool for research endeavor which focuses on treatment of intestinal disorders. Gastrointestinal disorders encompass disease whereby impairment in regenerating and functioning within the small intestine or colon exists. These disorders include short bowel syndrome, inflammatory bowel disease such as Crohn’s disease, ischemic bowel, or impairment after radiation.
Surgical resection or small bowel resection (SBR) is performed to treat conditions such as Crohn’s disease or Necrotizing enterocolitis in infants, as well as cancer, and results in short bowel syndrome. Crohn’s disease is characterized by chronic inflammation whereby surgical intervention is necessary to close fistulas or remove parts of the intestine where inflammation is severe. Cancer of the small intestine (and colon) occurs when malignant cells are observed within these tissues. Adenocarcinoma, lymphoma, sarcoma, and carcinoid tumors account for the majority of intestinal cancers and surgery to remove the cancer is the most common form of treatment. Thus, when the tumor is large, removal of the small bowel segment containing the cancer is usually indicated. Another condition requiring surgery is that of ulcers. Ulcers are crater-like lesions on the mucous membrane of the small bowel caused by an inflammatory, infectious, or malignant condition frequently necessitating surgery and bowel resection in some cases. Yet another condition a small bowel resection may be performed to treat is intestinal obstruction. This condition involves a partial or complete blockage of the bowel whereby intestinal contents cannot pass through and may result in intestinal failure.

These diverse human intestinal disorders are likely to be the first targets for novel therapies. Presently, stem-cell based therapies for gastrointestinal disorders have focused primarily on the use stem cells originating from bone marrow and mesenchymal stem cells as enhancers of epithelial repair and treatment.

**Concluding Remarks and Prospectus**

Stem cells are the cells that lay down the many cell lineages which comprise the tissues and organs. Furthermore, they are paramount in the continuous generation of cells in
tissues whereby constant renewal is a distinct characteristic. Remarkable progress has been made in identifying the stem cell niche in the small intestine through the years. The intestinal epithelium consists of a rapidly proliferating as well as continuously differentiating epithelium with the principal functional unit of the small bowel known as the crypt-villus axis. It is quite established the epithelium is maintained by intestinal stem cells located in the crypts of Lieberkühn.

Epithelial stem cells are of great importance, as much of the body’s surfaces are covered and cavities lined by epithelial cells. Furthermore, nearly all human tumors arise from epithelial tissues. There is increased evidence which substantiates the presence of a cancer stem cell or tumor initiating cell as being responsible for tumor establishment, progression, relapse and metastasis. Although identification of the origin of the cancer stem cell is still elusive in human colorectal cancer, there have been in advances in utilizing mouse models of intestinal cancer. That transformation of gastrointestinal stem cells is at the origin of adenoma development, and that stem cell niches are thus established within the tumor, has been proposed in a robust manner. The exact operation of the cancer stem cell in these tumorigenic steps of colorectal cancer, which encompasses dysregulated signaling pathways, such as aberrantly activated Wnt, yet is not clear. Thus, discerning a therapeutic regiment which will effectively target aberrant Wnt activation while having the least possible effect on normal stem cell operations remains a challenge. A number of potential therapeutic drugs have been proposed as Wnt pathway inhibitors. In addition, the robust connection which exists between normal and cancer stem cells suggests that much of the knowledge of gastrointestinal maintenance can be directly translated to cancer stem cell biology.
Stem cells have great promise for regenerative medicine, but they are elusory in many tissues, including the small bowel and colon. The crucial transition from studies of animal models to studies involving humans remains impeded by a lack of established stem cell specific markers. Lgr5-driven models have provided a helpful aid in the pursuit of novel selective biomarkers. Thus, in recent years significant gain in the comprehension of the self-renewal process (and homeostasis) within the gastrointestinal tract, can been attributed to advances achieved in techniques of cell tracking, and to the discovery of several putative stem cell markers, such as Lgr5. Importantly, the recent breakthroughs in identifying intestinal stem cell markers will most certainly serve to drive efforts in novel research which spotlight stem cell based therapy in the treatment of cancer and diseases whereby impairment of the small bowel and colon exists.

Novel advances in the characterization of intestinal stem cells have been made with the identification of several putative candidate stem cell markers. Importantly, the physiological behavior of many of these markers remains unknown and there is no present universal consensus in regards to the quintessential intestinal stem cell marker. In our preliminary study in chapter four, we sought to examine if there is a baseline difference between intestinal stem cell markers in the proximal (jejunum) when compared to the distal (ileum) following small bowel resection (SBR).
Figure 3.1: Cellular structure of the intestinal crypt epithelium
A schematic of self-renewal along the crypt-villus axis. (A). Epithelial homeostasis is propelled by cycling Lgr5+ crypt base columnar cells (CBC) located at the crypt base which generate the 4 principal epithelial cell lineages that include absorptive, goblet, endocrine, and paneth cells (B). These CBC stem cells generate rapidly cycling transit amplifying (TA) cells (amplification compartment) which migrate up the crypt-villus axis and differentiate over a span of 2 days. They have matured fully by the time of crypt exit, and will exert their function another 3-5 days while continuing their ascent. At the villus tip cells experience apoptosis and are subsequently shed. Paneth cells evade this upward motion and migrate downward to the base of the crypt, surviving approximately 6-8 weeks. Immunohistochemical detection demonstrates the 4 major cell lineages of the small bowel (upper Panel B). The upper panel B shows complete crypt-villus axis while the lower panel B shows enlarged magnifications of crypts. The epithelium undergoes continuous stem cell renewal along the crypt-villus axis and dead cells are shed into the lumen. (Figure adapted by author from Pinto and Clevers 2005.)
References


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CHAPTER FOUR: Intestinal Stem Cell Marker Expression and Morphometric Differences in the Proximal (Jejunum) and Distal (Ileum) Following Small Bowel Resection

Abstract

Purpose: Novel advances in the characterization of intestinal stem cells have been made with the identification of several putative candidate stem cell markers. Importantly, the physiological behavior of many of these markers remains unknown and there is no present universal consensus in regards to the quintessential intestinal stem cell marker. We sought to examine if there is a baseline difference between intestinal stem cell markers in the proximal (jejunum) when compared to the distal (ileum) following small bowel resection (SBR).

Experimental design: We tested our hypothesis at time points beginning on the 3rd postoperative day using selected markers that include Musashi-1, Lgr5, Ascl2, and Olfm4, along with smooth muscle actin (SMA), and the mesenchymal marker, Vimentin in both intestinal cells and tissue of wild-type C57BL/6J mice that underwent small bowel resection (SBR) (n=5/time point). Quantitative RT-PCR was carried out to measure intestinal stem cell marker expression. Crypt dissociations and histological morphometrics were performed in addition to compare post-resectional differences in the jejunum versus the ileum small bowel.

Results: Intestinal stem cell marker expression was augmented in the jejunum when compared to ileum. Our data showed P<0.05 for Lgr5 mRNA in both cells and tissue in jejunum versus ileum at same day time points. Furthermore, histological morphometric comparison demonstrated statistically significant augmentation of the jejunum when compared to ileum. A 38% difference between jejunum versus ileum was shown for crypt depth whereas villus height differed between jejunum and ileum at 31%. This trend of greater
stem cell marker expression in jejunum as well as morphometric differences when compared to ileum was consistently evidenced throughout the study.

**Conclusions:** Our findings report significant augmentation of the jejunum when compared to the ileum for changes in stem cell marker expression and for histological morphometrics after SBR which could provide further insight into the therapeutic benefits of characterizing stem cells and in the adaptive response for short bowel syndrome (SBS) resulting from massive loss of the intestine due to gastrointestinal tumors and other intestinal maladies.

**Introduction**

The structure and physiology of the small intestine as well as the large intestine or colon are adaptive and dynamically meet ever-changing absorptive and digestive requirements. The small intestine is separated into three proximal and distal segments that include the duodenum, jejunum, and ileum. Though jejunum and ileum are histologically tantamount, they differ in their finger-like protrusions known as villi and the presence of paneth cells that are principally found within the jejunum of the mouse. The villi of the jejunum are tall and cylindrical, while they are short and cylindrical in the ileum. The absorptive surface area of the small intestine is remarkably increased by numerous villi and invaginations into the submucosa that are known as the crypts of Leiberkühn (Barker et al. 2008). The epithelium of the villi is continuously supplied by these crypts of Leiberkühn that are the proliferative compartments of the small intestine. The number and depth of crypts are known to increase during the second and fourth week of postnatal life and is based on an increase in intestinal circumference and length (Cheng et al. 1985; Herbst and Sunshine
1969). Crypt fission, also known as crypt branching and bifurcation, modulate this increase. Of note, a model of crypt growth was developed which demonstrates the bifurcation of crypts upon doubling their volume. Implicit in this model is the prediction of crypt fission being driven via a doubling in the number of stem cells (Totafurmo et al. 1987).

Nearly 40 years ago pioneering work of Cheng and Leblond established that the small intestinal epithelium is maintained by a population of tissue-specific stem cells (Cheng and Leblond 1974). Intestinal stem cells dwell near the crypt base furnishing immature progeny which migrate up the crypt while continuously dividing. They then differentiate and give rise to any of the four types of intestinal epithelial cells that include absorptive enterocytes which are 90% of the villus-associated cells and highly ubiquitous at the proximal end of the small intestine, goblet cells found more distally, enteroendocrine cells, and paneth cells (Pinto and Clevers 2005). Furthermore intestinal stem cells capably replenish their own population. Characteristically, paneth cells migrate to the crypt bottom and are especially found within the jejunum in the mouse. Under normal physiological conditions it is believed this process occurs by asymmetric division whereby each stem cell divides and gives rise to one stem cell and one progenitor cell (Scoville et al. 2008). Under perturbed conditions such as chemotherapy or radiation the intestinal stem cells are subjected to symmetric division and replenish their own population by undergoing division thus giving rise to two daughter cells (Booth et al. 2000). Of note, increased proliferation of intestinal stem cells into enterocytes is highly necessary for adaptation (Loran et al. 1960).

Small intestinal adaptation is an actuality which occurs after a massive amount of intestinal length has been removed or resected. Via this process the remaining intestine
compensates for the loss of absorptive area either macroscopically by way of increased villus height, crypt depth, and intestinal lengthening, or microscopically by way of augmented cellular protein and DNA content per unit length (O’Brien et al. 2001). Animal models taking into account the adaptive response after intestinal resection have shown that sustained increases in crypt depth and villus height are detected, indicating that steady-state set points for these parameters are augmented after resection (O’Brien et al. 2001). Patients with short bowel syndrome are in a state of maldigestion and malabsorption after extensive loss of the small bowel (Hoellwarth 1999; Williamson 1984). In the rodent model of small bowel resection, the residual small intestine of both the jejunum and/or the ileum undergoes a series of adaptive processes leading to a significant increase in intestinal absorptive surface area, the exact mechanisms of which are still poorly understood (Nygaard 1967; Bristol and Williamson 1988; Weale et al. 2005).

Present-day advances in characterizing intestinal stem cells have led to the identification of several putative candidate stem intestinal stem cell markers. Importantly, the physiological role and function of many of these markers remain unascertained. Furthermore, there’s been an absence of specific markers for intestinal stem cells and Musashi-1 is the first such marker identified in the small intestine (Kayahara et al. 2003; Potten et al. 2003). The recent discovery of Lgr5 as an intestinal stem cell marker has served as a significant advancement in stem cell biology. Moreover, recent work has discovered a new intestinal stem cell marker, Ascl2, demonstrated to be enriched in Lgr5+ (positive) stem cells (Van der Flier et al. 2009).
In this preliminary study, we selected several putative candidate intestinal stem cell markers to examine if there is a baseline difference between intestinal stem cell markers in proximal (jejunum) cells versus those cells in distal (ileum) small intestine using nonoperated wild-type C57BL/6J mice. These putative markers include *Musashi-1, Lgr5, Ascl2*, and *Olfn4*, along with smooth muscle actin (SMA), and the mesenchymal marker, *Vimentin*. We again utilized these same markers to examine if there is a baseline difference between intestinal stem cell markers in cells and in tissues of the proximal (jejunum) and the distal (ileum) small intestine using wild-type C57BL/6J mice that underwent small bowel resection (SBR). Crypt dissociations were performed to isolate and collect viable single epithelial cells from intestinal crypts. Quantitative RT-PCR assay was used to measure the degree of enrichment of mRNA for stem cell markers and morphometric analysis of intestinal histology was performed on nonoperated and SBR mice. These methods were utilized in an effort to compare post-resectional differences between stem cell markers in the proximal (jejunum) and distal (ileum) small bowel which could provide potential insight into the adaptive response.

**Materials and Methods**

**Animals.** All animal work was performed after protocol approval by the Institutional Animal Care and Use Committee. Adult male and female wild-type mice (WT, C57BL/6J mice) ranging in weight approximately 20-25g were obtained from The Jackson Laboratory (Bar Harbor, ME) and were housed in groups of 5 at 21°C and kept on a 12 hour light-dark schedule with wood chip bedding and were allowed to acclimate to their environment for a least 7 days before being randomly assigned to undergo either no resection
(sham surgery) or small bowel resection (SBR). A liquid diet (Micro-Stabilized Rodent Liquid Diet LAD 101/101A, Purina Mills, St. Louis, MO) was administered approximately 1-2 days prior to surgeries. Nonoperated SBR (unresected sham) control animals underwent excision at the same location as SBR animals with no removal of intestine (n=15). These control animals were similarly adapted to the liquid diet 1-2 days prior to tissue harvest. Operations on mice were carried out under sterile conditions whereby the abdomen was clipped, cleansed with a broad spectrum antiseptic and immediately covered with a sterile drape. An operating microscope with X 7 magnification was utilized and 2% isoflurane and 90% oxygen was the administered anesthesia. Mice underwent SBR procedure (n=15) as previously described (Helmrath et al. 1996). Briefly, resection of the small bowel between the point 8 to 10cm distally to the ligament of Treitz and 2 to 3 cm proximal to the ileocecal junction was performed. Intestinal continuity was then restored with an end-to-end, single-layered, interrupted anastomosis using 8-0 monofilament suture. Mice received warm intraperitoneal saline to hydrate, and the abdomen closed with a running suture. Figure 4.1 shows a representative cartoon depicting standard operative procedures. Liquid diet was promptly administered after surgery and this diet was sustained for the duration of the animal work. The mice which died or appeared ill and languid at the time of sacrifice were excluded from further study.

**Tissue Harvest.** Using the cervical dislocation method mice were euthanized while under 2% isoflurane anesthesia at postoperative time points (3-5 days and 6-8 days). In addition, mice were administered an intraperitoneal injection of bromodeoxyuridine (BrdU; 120 mg/kg) 90 min prior to sacrifice. The intestine was flushed with Hanks Balanced Salt
Solution- HBSS (GIBCO, Life technologies, Grand Island, NY). The first centimeter proximal and distal to the anastomosis was discarded and the remaining small bowel was divided in half with 1 cm proximal (jejunum) and 1cm distal (ileum) being fixed in 10% buffered zinc formalin (Fisher Scientific, Kalamazoo, MI) for histology. A subsequent 1 cm proximal and also distal was minced then placed in FastPrep-24 Lysing Matrix D tubes (MP Biomedicals, Solon, OH) containing approximately 600 m of RLT lysis buffer, an item component of the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). The above mentioned samples were snap frozen in liquid nitrogen and stored at -80°C isolation for RNA. Tissue samples for nonoperated mice were harvested in the same manner.

**Crypt Dissociation.** Tissue samples of proximal (jejunum) and distal (ileum) were harvested for cell isolation in addition. Two approximately 5cm lengths for jejunum and ileum were each cut into two pieces and placed in cold EDTA solution, then transferred to warm EDTA at 37°C and manually shaken for crypt removal at intermittent timed intervals. The jejunum and ileum tissue were removed, pinned flat to a wax bottom dissecting pan, and fixed for histology in 10% buffered zinc formalin (Fisher Scientific, Kalamazoo, MI). The remaining cell suspension was pelleted then reconstituted and subjected to gentle enzymatic digestion at 37°C and briefly shaken. FBS was added to a concentration of 5% and the cells filtered through 100µm, 70µm, and 40µm filters successively. 10µL -100µL was removed and a dilution made for cell counting. The remaining cells in solution were pelleted and stored at -80°C. Briefly, using trypan blue staining, also characterized as the dye exclusion method, cells were counted to assess viability.
**Histology and morphometric analysis.** Routine histology was performed and histologic sections were stained with hematoxylin and eosin (H and E) and analyzed for intestinal morphology. In addition, immunohistochemistry (IHC) was used to determine BrdU incorporation. Following BrdU IHC, proliferative index, was determined by calculating the ratio of BrdU (+) positive nuclei to total cells within at least 10 intact crypts. Intestinal morphometric analysis was carried out in a blinded approach with the use of digital images acquired utilizing an Axio Imager microscope (Zeiss, Thornwood, NY). Crypt depth (CD) and villus height (VH) were determined from at least 10 well-oriented crypt-villus units measured per animal. The number of crypts undergoing crypt fission (CF) was determined by observing numerous (over 50) crypts per animal presenting with a bifurcating crypt with a bisecting fissure creating at least 2 flask-shaped bases at a shared single crypt-villus axis (Fritsch et al. 2002). Utilizing this method the percent CF per animal was determined. Measurements of circumference were obtained from the submucosa using AxioVision 4.6 as described previously (Dekaney et al. 2007). The number of crypts per circumference was counted in intact sections.

**RNA isolation and quantitative real time PCR (qRT-PCR) on collected tissue and cells.** Total RNA was isolated from tissue samples using the Qiagen RNeasy Mini Kit per the manufacturer’s instructions after addition of 6 mL β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) to the lysing tubes. Total RNA was isolated from cell samples using TRIzol (GIBCO BRL, Gaithersburg, MD) and chloroform reagents. Complimentary DNA was then created using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR was performed on cDNA samples in triplicate using an
Applied Biosystems StepOne Plus system. Taqman individual probes for Lgr5 (Mm00438890_m1), Ascl2 (Mm01268891_g1), Olfm4 (Mm01320260_m1), Msi-1 (Mm00485224_ml), Vimentin (Mm00449208_ml), and SMA (Mm01546133_ml Acta 2 (SMA) from Applied Biosystems were used and data analyzed using a relative standard curve method normalized to β-actin (Mm00607939_s1). β-actin was used as the internal control gene for sample comparison.

Statistics. Results are presented as means ± SE. Values were analyzed by t-test to compare group means. A Probability (P) value of .05 or less was considered as statistically significant.

Results and Discussion

We sought to examine if there is a baseline difference between intestinal stem cell markers in the proximal (jejunum) versus the distal (ileum) small bowel utilizing a murine model. While there is currently no direct method of isolating stem cells, we performed initial crypt dissociations on wild-type nonoperated mice to isolate and collect viable single epithelial cells from intestinal crypts and to ascertain reproducible cell populations for proximal (jejunum) and distal (ileum) small bowel. Total cell counts were much greater in the jejunum when compared to ileum cells (P<0.005) (Figure 4.2). We next carried out quantitative RT-PCR assay to measure the degree of enrichment of mRNA for intestinal stem cell markers, Olfm4, Lgr5, Ascl2, and Msi-1 in cells of jejunum and ileum along with smooth muscle actin and the mesenchymal marker vimentin. Interestingly we consistently found increased expression in the jejunum for all stem cell markers when compared to ileum
These results prompted us to further query this comparison in a model of small bowel resection (SBR).

The relative expression of mRNA for stem cell markers in wild-type (WT) mouse cells is augmented in the jejunum when compared to ileum following SBR. Quantitative RT-PCR was used to measure the degree of enrichment of mRNA for markers of smooth muscle actin and mesenchymal vimentin, along with the putative intestinal stem cell markers, Olfm4, Lgr5, Ascl2, and Msi-1 in cells of jejunum and ileum following small bowel resection at time points. Groups of five mice per time point (3-5 days and 6-8 days) underwent analysis. The third postoperative day time point was selected as other pioneering studies in mouse small intestine have shown measurable changes in proliferation by approximately 3-5 days (Cheng and Leblond 1948; 1974 and Wright and Irwin 1982). All expression values were normalized to β-actin expression. Relative expression is given as expression of each individual marker relative to a nonoperated control. Smooth muscle actin (SMA) expression was substantially reduced in cells in both jejunum and ileum at all of the featured time points (days) and (P<0.05) when jejunum was compared to ileum at same time point (Figure 4.4) Vimentin, a mesenchymal marker showed decreased expression in the small bowel cells as did SMA and (P<0.01) when jejunum and ileum were compared at same time point. Though reduced when compared to the jejunum, ileum cells showed a slight increase in expression at 6-8 days although this was not statistically significance (Figure 4.5) Olfactomedin-4 (Olfm4) cells suggested a trend of increased expression in the jejunum when compared to the ileum across all time points. No statistical significance was found between jejunum and ileum however (Figure 4.6). In leucine-rich repeat-G-protein coupled receptor 5
(Lgr5) expression, mRNA from cells displayed an increase in the jejunum versus the ileum at each time point; however, the probability value was less than 0.05 (P<0.05) in jejunum cells versus ileum cells at same time point (Figure 4.7). As with Lgr5, Achaete Scute-Like 2 (Ascl2) mRNA displayed an increase in the jejunum versus the ileum at each time point. Ascl2 cells showed heightened expression in the jejunum when compared to the ileum at the same time point (P<0.005) (Figure 4.8). Musashi-1 (Msi-1) cells demonstrated higher levels of expression when jejunum was compared to the ileum at both same time points (P<0.05) (Figure 4.9).

*The relative expression of mRNA for stem cell markers in wild-type (WT) mouse tissue is augmented in the jejunum when compared to ileum following SBR.* Quantitative RT-PCR was used to measure the degree of enrichment of mRNA for markers of smooth muscle actin and mesenchymal vimentin, along with the putative intestinal stem cell markers, Olfm4, Lgr5, Ascl2, and Msi-1 in tissue of jejunum and ileum following small bowel resection at different time points. Groups of 5 mice per time point (3-5 days and 6-8 days) underwent analysis. All expression values were normalized to β-actin expression. Relative expression is given as expression of each individual marker relative to a nonoperated control. Though showing slightly higher increases than in cells, smooth muscle actin (SMA) expression was substantially reduced in tissue in both jejunum and ileum at all of the time points (days) and (P<0.01) when jejunum was compared to ileum at both same points (Figure 4.10). Though not statistically significant, the mesenchymal marker, Vimentin, suggested a trend of higher expression in jejunum tissue when compared to ileum across all time points (Figure 4.11). Olfm4 tissue showed a trend of increased expression in the jejunum when
compared to the ileum across all time points. As with Olfn4 cells, no statistical significance was found between jejunum and ileum (Figure 4.12). In Lgr5 expression, mRNA from tissue presented an increase in the jejunum when compared to ileum with (P<0.05) at same time point of 3-5 days (Figure 4.13). Interestingly, as with Lgr5, Ascl2 mRNA displayed increased levels when jejunum tissue was compared to that of ileum and Ascl2 showed the larger expression in jejunum versus ileum at the same time point of 3-5 days (P<0.01).

Present-day progress in intestinal stem cell biology has demonstrated this new marker Ascl2, to be an enriched gene of Lgr5 (Vander der Flier et al. 2009) (Figure 4.14). Musashi-1 (Msi-1) tissue showed increases in the level of expression in jejunum versus ileum, and higher levels of expression were shown when jejunum (P<0.05) was compared to the ileum at 6-8 days (Figure 4.15).

**Jejunum is augmented in morphometric comparison of intestinal histology after SBR.** Histological and morphometric comparisons of jejunum and ileum were analyzed at 4-5 days and 6-7 days as they demonstrated intact well oriented morphology after histological processing, moreover these time points correspond within same postoperative time points at tissue harvest. Crypt depth and villous height were ascertained from approximately ten well oriented crypts-villus units measured per animal. Although ileum shows an increase across time points, measurements of crypt depth demonstrate that the jejunum is significantly augmented when compared to the ileum at 4-5 days (P<0.01) and 6-7 days (P<0.05). Of note, both jejunum and ileum were slightly increased in crypt depth at the latter time point (Figure 4.16A). Increases in measurements of villous height in jejunum over that of ileum occurred within 4-5 days (P<0.005) as well as 6-7 days (P<0.01) following SBR (Figure 4.16B) while
measurements of submucosal circumference was more pronounced in jejunum versus ileum 6-7 days after SBR (Figure 4.16C). BrdU immunohistochemistry (IHC) was used to compare proliferative index in jejunum versus ileum. BrdU + (positive) cells showed increases in percentage of cells for both jejunum and in ileum 4-5 days following SBR and jejunum showed a significant difference at 6-7 days when compared to ileum (P<0.05) (Figure 4.17). Proliferative index was determined by calculating the ratio of BrdU + cells to total nuclei. A representative immunostaining exposing BrdU positive (brown reacting) cells in mouse jejunum is demonstrated in (Figure 4.18). Crypt fissioning was augmented in the jejunum when compared to ileum 4-5 days (P<0.05) and 6-8 days (P<0.005) after SBR (Figure 4.19). The number of crypts undergoing fission was determined by observing crypts presenting with a bifurcating crypt with a bisecting fissure that creates at least 2 flask-shaped bases. Figure 4.20 shows an H&E stained jejunum from a SBR mouse which demonstrates an example of crypt fission. Figures 4.21 A, B, and C show percent changes in crypt depth, villus height, and submucosal circumference between jejunum and ileum, as each calculated from their corresponding preoperative measurements. Jejunum showed the largest percent difference in crypt depth at 38% 6-7 days after SBR, whereas villus height differed between jejunum and ileum at 31% 4-5 days after SBR, and finally, submucosal circumference showed a change of 17% at 6-7 days when jejunum was compared to ileum following SBR. Although jejunum showed the more substantial percentage changes across these end points measured, total submucosal circumference in ileum was nearly equal to jejunum at 4-5 days with just a 4% change.
With these aforementioned results, we have shown presence of stem cell markers strongly expressed in the small bowel. Furthermore, these results consistently demonstrate a distinct trend of the jejunum being augmented in expression of mRNA for stem cell markers when compared to ileum following SBR in cells and in tissue. What’s more, extensive morphometric comparison of intestinal histological end points revealed a distinct trend of augmentation in the jejunum above that of ileum.

**Conclusion**

Advances in characterizing intestinal stem cells (ISCs) in the field of stem cell biology, have led to the identification of several putative intestinal stem cell markers. Musashi-1 (Msi-1) was the first such marker identified (Kayahara et al. 2003; Potten et al. 2003). Furthermore, Lgr5, initially identified as a Wnt target gene expressed in CRC (van de Wetering et al. 2002), has demonstrated an impressive recent advancement in the field.

Elucidating the precise identity of the intestinal stem cells has demonstrated controversy over the last 30 years with two opposing models governing the literature. These include the “+4 position” model originally introduced by Cairnie and colleagues in 1965 (Cairnie et al. 1965), and a more recent, albeit less well-accepted, “stem cell zone” model proposed by Cheng and Leblond in the early 1970s (Cheng and Leblond 1974). Conclusive evidence for either model has proven elusive due to the lack of specific markers for these important pluripotent cells. To date, there continues debate in regards to the quintessential marker and no single marker appears to universally identify ISCs.

This study was performed to examine if there are base line differences between unique intestinal stem cell markers in the jejunum compared to ileum after small bowel
resection in the mouse, and reports significant augmentation of the jejunum versus ileum for all end points that were analyzed. These findings could potentially provide further insight into the therapeutic benefit for patients via developing the methodology to identify, isolate, expand, and ultimately transplant ISCs into a damaged host. The utilization of transgenic mice expressing ISC markers should provide a valuable tool. Thus precisely characterizing stem cell behavior, as well as the adaptive response concerning short bowel syndrome (SBS) resulting from massive intestinal loss due to gastrointestinal cancers, intestinal disease and/or intestinal failure has the capacity for great therapeutic promise.
Figure 4.1: Cartoon depicting surgical procedures
Jejunum and Ileum segments were studied from:
A: Mice that underwent no resection, i.e., nonoperated SBR (sham surgery), or
B: Mice that underwent small bowel resection (SBR)
(Figure drawn by L. McPhatter, 2013.)
A. Unresected (nonoperated SBR or sham surgery)

B. Small Bowel Resection (SBR)
Figure 4.2: Quantification of total Cell Counts of Jejunum versus Ileum
Total cell counts were higher in the jejunum when compared to ileum cells. H& E staining of jejunum and ileum (in upper panel) demonstrating as well as confirming the isolation and removal of single epithelial cells from crypts. ***P<0.005, n=10
Figure 4.3: Quantitative RT-PCR assay
qRT-PCR was performed to measure the degree of enrichment of mRNA for intestinal stem cell markers SMA (A), Vimentin (B), Olfm4 (C), Lgr5 (D), Ascl2 (E), and Msi-1 (F), in cells of jejunum and ileum from WT nonoperated mice. Increased expression was found in the jejunum for all markers when compared to ileum.
A

SMA CELLS

FOLD EXPRESSION

0 0.0005 0.001 0.0015 0.002 0.0025 0.003 0.0035 0.004 0.0045 0.005

Jejunum Ileum

WT Cells after SMA
B

VIMENTIN CELLS

FOLD EXPRESSION

WT Cells after Vimentin

Jejunum

Ileum
C

OLFM4 CELLS

FOLD EXPRESSION

Jejunum

Ileum
**Figure 4.4: Relative expression of Smooth Muscle Actin (SMA) mRNA in WT mouse cells**

SMA mRNA levels in jejunum and ileum cells at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. *P < 0.05 when jejunum and ileum were compared at same time points. Relative expression is given as SMA expression relative to a nonoperated standard. Error bars = SE, n=5/time point.
Figure 4.5: Relative expression of Vimentin mRNA in WT mouse cells
Vimentin mRNA levels in jejunum and ileum cells at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. *P < 0.01 when jejunum and ileum were compared at same time points. Relative expression is given as Vimentin expression relative to a nonoperated standard. Error bars = SE, n=5/time point.
Figure 4.6: Relative expression of Olfactomedin-4 (Olfm4) mRNA in WT mouse cells
Olfm4 mRNA levels in jejenum and ileum cells at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. Expression in jejunum suggests a trend of increase versus the ileum across all time points. Relative expression is given as Olfm4 expression relative to a nonoperated standard.
Error bars = SE, n=5/time point.
Figure 4.7: Relative expression of Leucine-rich-repeat-G-protein coupled receptor 5 (Lgr5) mRNA in WT mouse cells
Lgr5 mRNA levels in jejunum and ileum cells at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression.
*P < 0.05 when jejunum and ileum were compared at same time points.
Relative expression is given as Lgr5 expression relative to a nonoperated standard.
Error bars = SE, n=5/time point.
**Figure 4.8: Relative expression of Ascl2 (Achaete Scute-Like 2) mRNA in WT mouse cells**

Ascl2 mRNA levels in jejunum and ileum cells at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression.

*P < 0.005 when jejunum and ileum were compared at same time points.

Relative expression is given as Ascl2 expression relative to a nonoperated standard.

Error bars = SE, n=5/time point.
Figure 4.9: Relative expression of Musashi-1 (Msi-1) mRNA in WT mouse cells
Msi-1 mRNA levels in jejunum and ileum cells at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. *P < 0.05 when jejunum and ileum were compared at both same time points. Relative expression is given as Msi-1 expression relative to a nonoperated standard. Error bars = SE, n=5/time point.
Figure 4.10: Relative expression of Smooth Muscle Actin (SMA) mRNA in WT mouse tissue
SMA mRNA levels in jejunum and ileum tissue at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. *P < 0.01 when jejunum and ileum were compared at both same time points. Relative expression is given as SMA expression relative to a nonoperated standard. Error bars = SE, n=5/time point.
Figure 4.11: Relative expression of Vimentin mRNA in WT mouse tissue
Vimentin mRNA levels in jejunum and ileum tissue at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. Expression in jejunum suggests a trend of increase versus the ileum across all time points. Relative expression is given as Vimentin expression relative to a nonoperated standard.
Error bars = SE, n=5/time point.
Figure 4.12: Relative expression of Olfactomedin-4 (Olfm4) mRNA in WT mouse tissue
Olfm4 mRNA levels in jejunum and ileum tissue at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. Expression in jejunum suggests a trend of increase versus the ileum across all time points. Relative expression is given as Olfm4 expression relative to a nonoperated standard.
Error bars = SE, n=5/time point.
Figure 4.13: Relative expression of Leucine-rich-repeat-G-protein coupled receptor 5 (Lgr5) mRNA in WT mouse tissue
Lgr5 mRNA levels in jejunum and ileum tissue at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression.
*P < 0.05 when jejunum and ileum were compared at same time point. Relative expression is given as Lgr5 expression relative to a nonoperated standard.
Error bars = SE, n=5/time point.
Figure 4.14: Relative expression of Ascl2 (Achaete Scute-Like 2) mRNA in WT mouse tissue
Ascl2mRNA levels in jejunum and ileum tissue at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. *P < 0.01 when jejunum and ileum were compared at same time point. Relative expression is given as Ascl2 expression relative to a nonoperated standard. Error bars = SE, n=5/time point.
Figure 4.15: Relative expression of Musashi-1 (Msi-1) mRNA in WT mouse tissue
Msi-1 mRNA levels in jejunum and ileum tissue at time points following SBR (small bowel resection) were measured by quantitative RT-PCR. All expression values were normalized to β-actin expression. *P < 0.05 when jejunum and ileum were compared at same time point. Relative expression is given as Msi-1 expression relative to a nonoperated standard. Error bars = SE, n=5/time point.
Figure 4.16: Morphometric comparison of intestinal histology in WT mice following small bowel resection
Comparison of crypt depth, villus height, and total submucosal intestinal circumference in the jejunum versus ileum. **P<0.01 when crypt depth (CD) within the jejunum and ileum were compared at 4-5 days, and *P<0.05 when jejunum and ileum CD were compared at same time point of 6-7 days (A). ***P<0.005 when villus height (VH) within jejunum and ileum were compared at 4-5 days, and **P<0.01 when VH within jejunum and ileum were compared at 6-7 days (B). *P<0.05 when submucosal circumference (SC) within jejunum and ileum were compared at same time point (C).
Error bars= SE, n=4 per time point.
**Figure 4.17: Comparison of Proliferation Index (PI) in WT mice**

Percent of bromodeoxyuridine (BRdU) labeling in crypts within the jejunum and ileum in WT mice at 4-5 days, and 6-7 days, following small bowel resection. *P < 0.05 when jejunum versus ileum were compared at 6-7 days. Error bars= SE, n=4/time point.
**Figure 4.18: BrdU immunohistochemistry**
Representative BrdU immunostained jejunum from a nonoperated mouse. Arrows indicate areas of BrdU (+) positive cells.
Figure 4.19: Comparison of crypt fission in WT mice

Percent of crypts undergoing fission within the jejunum versus ileum in WT mice at 4-5 days, and 6-7 days, after small bowel resection. Significant differences in influence of time are indicated as *P < 0.05 when jejunum and ileum were compared at 4-5 days, and ***P<0.005 when jejunum and ileum were compared at latter time point of 6-7 days.

Error bars = SE, n=4 per time point.
Figure 4.20: Crypt fission
The arrow denotes crypt fission bifurcation within an H&E stained jejunum from a small bowel resected mouse.
Figure 4.21: Morphometric comparison of intestinal histology from small bowel resected WT mice indicating percent change as calculated from preoperative measurements

Jejunum showed the largest percent difference in Crypt depth (38%) at 6-7 days (A), Villus height (31%) at 4-5 days (B), and Submucosal Circumference (17%) at 6-7 days (C), when compared to Ileum following SBR. n=4 animals per time point.
B

VH % change over preop

% Change

\[ \begin{align*}
4-5d & : 
\text{Jejunum} & \quad 80 \\
\text{Ileum} & \quad 50 \\
6-7d & : 
\text{Jejunum} & \quad 40 \\
\text{Ileum} & \quad 30
\end{align*} \]

Time After SBR
Circ % change over preop

Time After SBR

% Change

Jejunum
Ileum
References


CHAPTER FIVE: A Summation and Concluding Remarks

Summation

The solid tumor will create an organ-like entity that is composed of neoplastic cells and non-transformed host stromal cells embedded in an extracellular matrix. Like normal tissues, blood vessels nourish cells residing in tumors. However, unlike normal blood vessels, the tumor vasculature presents with an aberrant organization, structure, and function. Tumor vessels are leaky and flow of blood flow is heterogeneous and very often unable to function normally. Vascular-hyperpermeability along with the absence of functional lymphatic vessels within tumors is responsible for increased interstitial fluid pressure in solid tumors (Jain 2004). Consequently, each one of these abnormalities forms a physiological barrier to the delivery of therapeutic agents to tumors. In addition, abnormal microcirculation in tumors results in a hostile microenvironment characterized by hypoxia and acidosis, which impede the effectiveness of antitumor treatments such as radiation therapy and chemotherapy (Fukumura et al. 2001). Furthermore, host-tumor associations modulate expression of pro and anti-angiogenic factors, and are partly responsible for their imbalance as well as the pathophysiological characteristics of the tumor. As proposed by Jain and colleagues, a reestablishment of pro- and anti-angiogenic balance in tumors may “normalize” the tumor vasculature thereby improving function (Jain 2001). More than 40 years ago, in 1971, Judah Folkman proposed his seminal hypothesis that solid tumors needed angiogenesis for their growth and metastasis. In that report, Folkman showed preliminary evidence that tumors could not grow beyond a 2-3 mm diameter without recruiting new capillary blood vessels, or
microvessels. Since the introduction of these concepts, antiangiogenic therapy has been widely studied in both preclinical and clinical settings (Folkman, 2000).

The addition of bevacizumab to combination chemotherapy demonstrates an effective and typically well tolerated first-line choice for advanced colorectal cancer (CRC). Combining bevacizumab with chemotherapy and/or novel molecular targeted agents represents a rational undertaking that might improve efficacy and curb nonselective toxicity. Indeed targeting angiogenic signaling axes such as provided by VEGF, which is overexpressed in the majority of solid tumors, can be an enthusiastic strategy to reverse some of the pathology. Neutralizing antibodies against VEGF and VEGFR-2 have been shown to improve the tumor vasculature and microenvironment, i.e., pruning some of the aberrant tumor vessels and remodeling the remaining vasculature in human colon cancer xenografts so that it more closely resembles normal vasculature.

Bevacizumab/Avastin® has been identified as a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody, which is utilized in the clinics to disrupt tumor angiogenesis and to suppress the growth of tumors. However its efficacy as a monotherapy in humans has been less than predicted from preclinical evaluations. This resistance to treatment may be attributed to the rescue of the VEGF-activated mTOR promitogenic pathway by VEGF-independent oncogenic factors. Thus, we reasoned that combining bevacizumab to rapamycin, a drug which inhibits mTOR, would have superior antitumor activity than either agent alone. We utilized a mouse model of human colon adenocarcinoma and discerned tumor growth delays from caliper measurements, as well as identified prime molecular changes in response to tumor treatments using Western blotting, ELISA, and
immunohistochemistry (IHC). We evidenced a reduction in microvessel density (MVD) and in hypoxia. Furthermore, cell proliferation was decreased while cell death was increased. Interestingly, our findings (derived from these phenotypic tumor changes), demonstrated a significant therapeutic gain when combining bevacizumab with rapamycin for cancer treatment. Thus, we concluded combining bevacizumab with rapamycin has more than additive antitumor effects in vivo and that rapamycin could benefit from the antiangiogenic effects of bevacizumab, hence a vascular normalization effect resulting in an increased delivery of rapamycin.

Colorectal cancer (CRC) is a cancer of the gastrointestinal tract and is the second largest cause of death in the United States affecting both men and women. Moreover cancer has been identified by the United Nations as a global health danger with substantial economic repercussions (Bloom et al. 2011; Mariotto et al. 2011). The cost of cancer care in the United States by itself is currently over $100 billion (Mariotto et al. 2011). In addition, lifestyle changes that include a refrain from consuming a poor diet, tobacco and alcohol use, as well as a reduction in obesity may serve to prevent cancer (Lippman and Hawk 2009). Angiogenesis is a hallmark of cancer and this includes CRC. In addition angiogenesis has an early permissive role in tumorigenesis. Although epithelial cells that harbor mutations retain distinct, organ specific phenotypes, endothelial cells are generally untransformed and a common target across many cancers. As aforementioned, Judah Folkman suggested that tumor dormancy could be maintained by preventing neovascularization of microscopic cancers (Folkman 1971). Although antiangiogenic therapy has contributed to an improvement in the standard of care for some cancers, the overall clinical efficacy as a
monotherapy is modest for some patients and primarily observed in the form of prolonged progression free survival (Ebos et al. 2011; Jayson et al. 2012); thus, combining bevacizumab with chemotherapy and novel molecular targeted agents has been shown as a promising approach to improve efficacy while restricting the customary nonselective toxicities. In the setting of heavy disease burden, most patients treated with antiangiogenic agents eventually experience disease progression, however. In colorectal cancer a major proportion of deaths are the result of recurrent tumors or cancer relapse. Moreover the tumors that recur are the result of cancer cells which not only have disseminated, but have resisted chemotherapeutics. Beyond microscopic tumors, cancer stem cells, also known as cancer initiating cells, reside in, and are dependent on the tumor microvasculature (Albini et al. 2011). Angiogenesis inhibitors have been shown to decrease putative cancer stem cell numbers, suggesting that angiopreventive treatments could not only inhibit tumor growth but may also restrict cancer stem cell expansion (Albini et al. 2011). These cancer cells are resilient in that they have the ability to reestablish the cellular organization of the primary tumor by renewing all tumor populations, therefore behaving as cancer stem cells.

That stem cells are intimately involved in cancer causing mechanisms which encompass tumor establishment, progression, relapse, and metastasis, is becoming increasingly evident (Dalerba et al. 2007). Most human tumors derive from epithelial cells and colon adenocarcinoma presents as a cancer of epithelial cell origin. Gastrointestinal stem cells are shown to be pluripotent and can be described as “master cells” which give rise to all cell lineages in the epithelium (Barker and Clevers 2007). Following damage, gut stem cells generate reparative cell lineages and promote regeneration and healing. An increase in stem
cell number is considered to induce crypt fission as well as result in higher numbers of crypts, which also occurs in the adult (Totafurno et al. 1987); it is also believed to be the mode of spread of mutated clones in the colorectal mucosa.

Discoveries in the identification of markers for gastrointestinal stem cells have formed the basis for focused research endeavor in the treatment of intestinal disorders. Gastrointestinal disorders encompass cancer and diseases whereby impairment in regenerating and functioning within the small intestine and colon exists. Surgical resection as well as chemotherapeutic and/or antiangiogenic treatment in colorectal cancer presents a therapeutic strategy for patients.

Novel advances in the characterization of intestinal stem cells have been made with the identification of several putative candidate stem cell markers. Utilizing a mouse model, we sought to examine if there is a baseline difference between intestinal stem cell markers in the proximal (jejunum) when compared to the distal (ileum) after small bowel resection (SBR). We tested our hypothesis at time points using selected markers that include Musashi-1, Lgr5, Ascl2, and Olfm4, along with smooth muscle actin (SMA), and the mesenchymal marker, Vimentin in both intestinal cells and tissue of wild-type mice that had undergone small bowel resection. Quantitative RT-PCR was performed to measure intestinal stem cell marker expression. Crypt dissociations and histological/morphometrics were performed in addition to compare post-resectional differences in the jejunum versus the ileum small bowel. Our preliminary findings report significant augmentation of the jejunum when compared to the ileum for changes in stem cell marker expression and for histological morphometrics following SBR. This could provide further insight into the therapeutic benefits of
characterizing stem cells and in the adaptive response for gastrointestinal disorders such as short bowel syndrome (SBS), caused by massive loss of the intestine due to gastrointestinal tumors and other intestinal maladies.

In the pathophysiological conditions of gastrointestinal cancer and disease, homeostasis is disrupted whereby intestinal stem cell niches may no longer facilitate stem cell homeostasis and self-renewal. Moreover, dysregulation of various signaling axes, and a dysfunctional microenvironment as well as tumor vasculature, in gastrointestinal cancer, ensues. Colorectal cancer (CRC) is one of the most frequent cancers worldwide with over a million new cases every year. This dissertation has reviewed the current advances toward 1) explicating the angiogenesis-dependent tumor vasculature as a target of antiangiogenic therapy in gastrointestinal cancer, and 2) characterizing gastrointestinal stem cells and their markers, as well as reports our novel research studies on both 1 and 2.

Future Directions 1

While our findings demonstrated significant therapeutic gain when combining bevacizumab with rapamycin for cancer treatment, improved tumor retardation did not result from early tissue changes in tumor. We propose this finds a rationale in the mutual potentialization whereby VEGF blockade improves rapamycin tumor delivery and mTOR inhibition secures bevacizumab-induced DNA damage, resulting in late cellular responses. Further experiments will include targeted combination treatments in mouse groups employing our same or different experimental therapeutics, as well as utilizing different tumor models. As discussed, antiangiogenic therapies such as blockade of VEGF can cause vascular normalization, leading to a window of increased tumour oxygenation. In addition we
will elucidate potential compensatory signaling pathways upon antiangiogenic targeting. We propose these additional studies in an effort to demonstrate efficacy and contribute to abrogating tumor progression in patients suffering from CRC as well as other cancer types. Targeted therapeutics have the ability to alter the interaction between tumor cells and their microenvironment, leading to enhanced tumor cell killing and increased probability of a cure.

Future Directions 2

While we were able to strongly demonstrate that intestinal stem cell marker expression was augmented in the jejunum when compared to the ileum after small bowel resection (SBR) in mice, we propose further research employing the surgical resection model in operative and nonoperative mouse groups. We will also utilize the Lgr5-EGFP (Enhanced Green Fluorescent protein sequence) mouse and side population sorting analysis to study the response of intestinal stem cells. We will employ a damage model involving irradiation or with use of an antitumor or other agent. In addition targeting stem cells termed putative cancer stem cells (CSCs) that are known to resist traditional therapeutics, thus causing tumor recurrence, utilizing FACS (Fluorescence Activated Cell Sorting), will be performed in further investigation. Furthermore, we will elucidate signaling pathways that may be implicated in intestinal development such as the abberant Wnt axis that upregulates VEGF. We propose these additional experiments in an effort to identify and isolate ISCs, to further understand stem cell functioning and expansion. Ultimately, characterizing ISCs has the potential to be clinically applicable via developing the methodology for transplanting ISCs into a damaged host. Thus, acquiring precise methods to characterize the behavior of gastrointestinal stem cells holds great promise for therapeutic benefit in patients suffering
from massive intestinal damage and loss due to gastrointestinal cancer and disease. Lgr5-driven models have provided highly important tools in this pursuit.

**Concluding Remarks**

Recent breakthroughs in identifying intestinal stem cell markers will most assuredly serve to continue driving efforts in novel research for stem cell characterization and highlight stem cell based therapy in the treatment of disorders of the gastrointestinal tract that include gastrointestinal tumors. It is important to understand the biology of the tumor vasculature as the target of antiangiogenic therapeutics for enhanced efficacy and the increased probability of a cancer cure. There is much more to be elucidated in gastrointestinal stem cell and cancer pathophysiology; thus, exciting times lie ahead for these investigations.
References


