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## The Role of Host-Tumor Interactions In Liver Metastasis of Colorectal Cancer

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THE ROLE OF HOST-TUMOR INTERACTIONS  
IN LIVER METASTASIS OF COLORECTAL CANCER

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Submitted in Partial Fulfillment of the Requirements

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University of South Carolina

2013

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## DEDICATION

This work is dedicated to:

My wife, Hanwen Xu

who will always be my soul mate, whenever, wherever, and whatever;

My daughter, Bonette Xu Zhang

whose beautiful presence has made the most charming project in my life;

My father, Chaoming Zhang

who always believe in me and allow me to stand on his shoulder;

My mother, Hua Zhao

who will always give her arms and everything to me;

My sister, Ren Zhang

who will always be proud of and protect her younger brother.

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## ABSTRACT

Colon cancer is the third most frequent cancer and the second leading cause of cancer deaths in the United States. Liver metastasis is the major cause of death in colon cancer. Successful metastases depend on productive collaborations between tumor cells and host-derived cells in the tumor microenvironment, target organ environments, and cells in the hematopoietic compartment.

To identify the host-tumor interactions promoting liver metastasis and their molecular and cellular mediators, an orthotopic mouse model of liver metastasis of colon cancer was established that recapitulates all stages of tumor growth and metastasis. A highly metastatic mouse carcinoma cell line CT26-FL3 was isolated from the CT26 colon adenocarcinoma cell line by *in vivo* selection. The CT26-FL3 cells were found to be more proficient in inducing a metastasis-promoting host environment as compared to the parental cell line. Using this mouse model, microarray analyses were utilized to determine the genetic signature of the highly metastatic CT26-FL3 cells and the genetic changes in the liver microenvironment in mice bearing tumors from CT26-FL3 cells before and during metastasis. The results showed CT26-FL3 induced immune responses and released numerous cytokines. Furthermore, Il33 and Lcn2 were selected from the genetic signature of cancer cells and liver environment respectively as target genes to verify their roles in promoting liver metastasis of colorectal cancer.

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# CHAPTER 1

## INTRODUCTION

### 1.1 COLON CANCER

Colon or colorectal cancer (CRC) is cancer that starts in the large intestine (colon) or the rectum (end of the colon) (Figure 1.1).

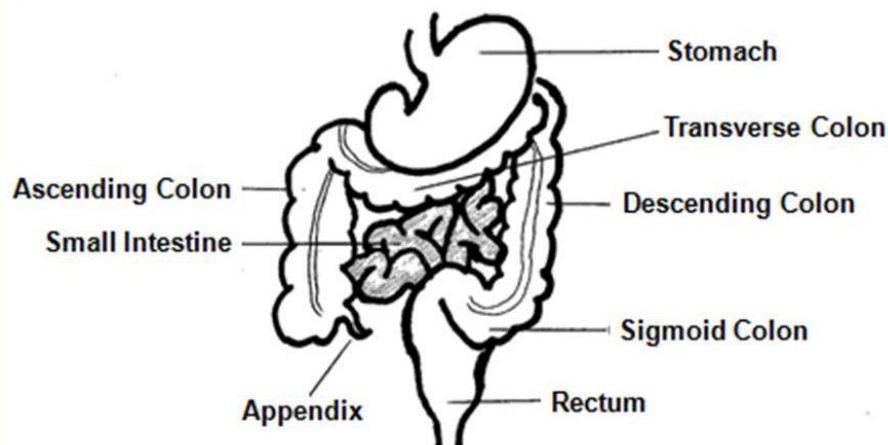


Figure 1.1 Diagram of large intestine. The large intestine is the portion of the digestive system. The indigestible residue of food from small intestine passes through the ascending, transverse, descending and sigmoid portions of the colon, and finally into the rectum for excreting. (Parry Medical Writing, Internet)

In most cases, colon cancer takes over several years to develop. Usually the tumor begins as a polyp which is a non-cancerous tissue, abnormally growing on the inner lining of the colon or rectum. Some polyps change into malignant tissue and develop into a cancerous tumor (cancer). The possibility of progressing into a colon cancer depends on

the type of polyp. The types of polyp that are commonly seen include adenomatous polyps, hyperplastic polyps, and dysplasia (American Cancer Society 2011). Adenomatous polyps (adenomas) begin in the cells of glandular structures lining the colon, and are most likely to develop into cancer. Therefore, most colon cancers are adenocarcinomas. Once the cancer forms and grows from a polyp, colon cancer cells can eventually break through the intestine wall of colon or rectum, spread into blood and lymph vessels, and travel to the lymph nodes and distant organs, such as the liver.

Colon cancer is the third most common cancer in the United States. According to the statistics from the American Cancer Society, 142,570 new cases will be diagnosed in the United States in 2013 (102,480 new cases of colon cancer; 40,340 new cases of rectal cancer) (American Cancer Society 2013). Although the application of polyp screening and improvements in treatment for colon cancer has led to a drop in the death rate (the number of deaths per 100,000 people per year) from colon cancer in last 20 years, an estimated 50,830 patients are still expected to die from it during 2013. This accounts for 9% of all cancer deaths, which makes it also the third leading cause of cancer-related death in the United States (American Cancer Society 2011; 2013). Many cases of colon cancer have no obvious symptoms. Most symptoms indicating colon cancer are not specific, including abdominal pain and tenderness in the lower abdomen, blood in the stool, diarrhea, constipation, or other changes in bowel habits, narrow stools, and weight loss with no known reason. Therefore, diagnosis mainly depends on screening tests which can detect colon cancer before symptoms develop (Cunningham, Atkin et al. 2010). Fecal occult blood test (FOBT) is used to identify small amounts of blood in the stool, suggesting the possibility of colon cancer. With colonoscopy, it is possible to observe the

entire colon, increasing the ability to diagnose colon cancer. In addition, CT or MRI scans of the abdomen, pelvic area, chest, or brain may be used to stage the cancer to discern if the cancer has spread (N.C.C. Network 2013). The staging is used to describe the extent of penetration of the cancer. Specifically in colon cancer, it is based on the depth by which the cancer has invaded into the intestine wall, whether or not it has reached nearby structures, and whether or not it has spread to the lymph nodes or distant organs. In the clinic, the stage of a cancer is viewed as the most important factor in determining the prognosis and treatments of patients (Smith, Cokkinides et al. 2012). Usually, there are two types of staging for colon cancer: clinical and pathologic stages, based on the physical exam, biopsy, and other tests. The pathologic stage is most often used, because it includes the results from surgery, and is more accurate (Cunningham, Atkin et al. 2010).

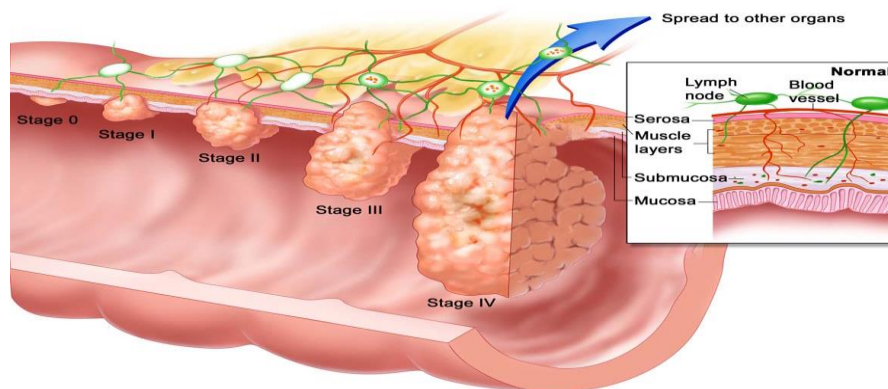


Figure 1.2 Pathologic stages of colon cancer. Stage 0: Very early cancer on the innermost layer of the intestine; Stage I: Cancer is in the inner layers of the colon; Stage II: Cancer has spread through the muscle wall; Stage III: Cancer has spread to the lymph nodes; Stage IV: Cancer has spread to other organs outside the colon. (The MetroHealth System, Internet)

Treatments of colon cancer depend on many factors, especially stage of the cancer during patients get diagnosis. Standard treatments include surgery to remove the tumor

lesion, chemotherapy to kill cancer cells, and radiation therapy to destroy cancerous tissue. For stages 0, I, II, and III cancer, colon resection and 6-8 month adjuvant chemotherapy is a typical therapeutic plan, sometimes combined with radiation therapy (Figure 1.2). For patients with stage IV disease that has distant metastasis, treatments directed at metastatic lesion also need be used. But the efficacy is typically very limited (American Cancer Society 2011).

Among the complications of colon cancer, metastasis is undoubtedly the leading cause of death. Most patients, whose cancer is detected at an early localized stage, can survive curative local resection of the primary tumor with approximately a 90% five-year survival rate; however, after metastasis has occurred, the survival rate drops to less than 12% (American Cancer Society 2013). The main reason is that the early symptoms are not specific and when metastases occur, patients have already missed the opportunity to be treated successfully through surgery or irradiation (Chambers, Groom et al. 2002). The liver is one of the most common sites of metastatic spread of colon cancer. Approximately 20-25% of patients with colon cancer present with liver metastasis at the time of diagnosis. However, autopsy results revealed that up to 70% of colon cancer patients had liver metastases (Schima, Kulinna et al. 2005). Given these colorectal cancer statistics, it is effortless to conclude that liver metastasis is the most major and direct cause of diminished survival in colon cancer patients.

## 1.2 CANCER MESTASTASIS

During tumorigenesis, cancer possesses six distinct biological capabilities. They include sustaining proliferative signaling, evading growth suppressors, resisting cell



death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg 2011). Metastasis is the spread of cancer cells from primary site to distant organs and is the final and most devastating step of cancer malignancy (Steeg 2006).

Although the genetic origins of the tumor are variable, the steps that lead to metastasis are generally similar: As a primary tumor grows, new blood vessels are developed to provide blood supply to satisfy the metabolic needs of tumor progression. This process is called angiogenesis. These new blood vessels also turn into a potential escape routes for cancer cells. Some of tumor cells acquire the ability to invade and penetrate the walls of lymphatic and blood vessels and enter into the circulatory system. Evading surveillance by the host immune system, these circulating cancer cells are able to survive and circulate through the blood stream and lymphatic system to other sites and tissues in the body. After the cancer cells arrest at another site, they extravasate into target organ through the vessel or walls, progress to proliferation and eventually a clinically detectable tumor is formed (Figure 1.3) (Woodhouse, Chuaqui et al. 1997).

In spite of its impact in clinical medicine, much remains to be studied about the biology of cancer metastasis due to the fact that it is an inherently secret process which occurs inside the body and is very difficult to record and observe. For more than a century, cancer biologists endeavored to understand the mechanism of metastasis to distant organs. Specific biological processes have been shown to be required for metastasis, such as angiogenesis, epithelial-to-mesenchymal transition, extracellular-matrix remodeling, and immune evasion among others (Chiang and Massague 2008). Moreover, certain genes needed at these individual processes have been identified. For

example, a loss of E-cadherin has been shown to lead to early metastasis (Perl, Wilgenbus et al. 1998); various members of the matrix metalloproteinase (MMP) family (e.g., MMP-2 and MMP-9) are implicated in cancer invasion (Egeblad and Werb 2002; Lopez-Otin and Matrisian 2007; Martin and Matrisian 2007); and vascular endothelial growth factor (VEGF) is involved in the angiogenic switch required for progression to metastasis (Xu, Cochran et al. 2006). To some extent, these discoveries account for the universal properties of cancer metastasis, such as increased capabilities in migration and invasion, but still do not elucidate the mechanisms underlying organ-specificity.

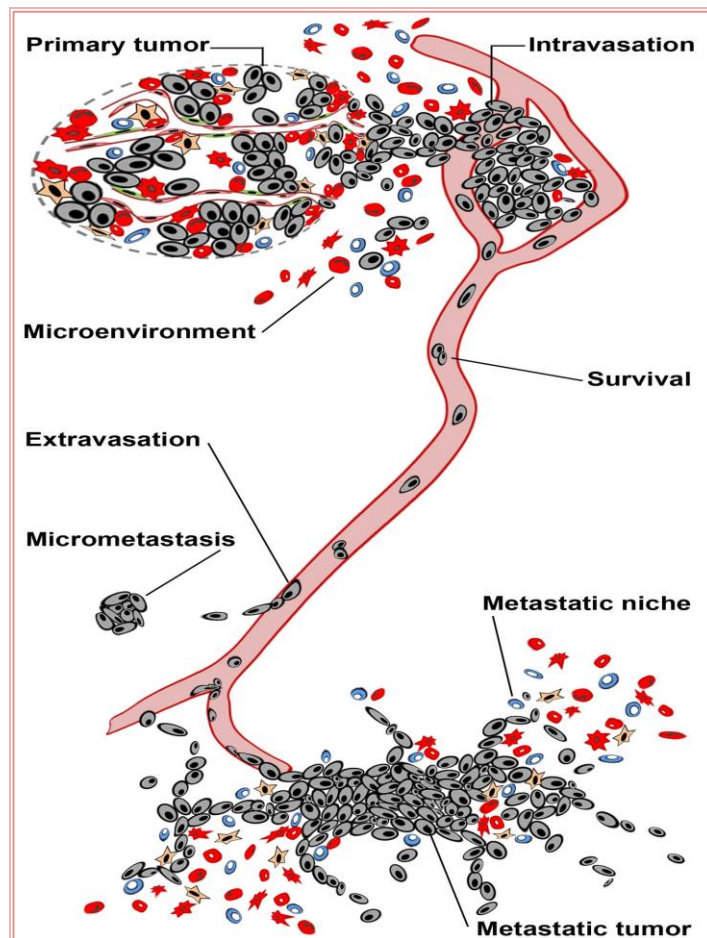


Figure 1.3 Stages of metastatic progression. Metastasis proceeds through the progressive acquisition of traits that allow malignant cells originating in one organ to disseminate and colonize a secondary site.

Organ-specificity of cancer metastasis has been documented for a long time. Breast cancer normally disseminates to bone, liver and lungs; prostate cancer preferentially spreads to bone; and colon cancer frequently metastasizes to liver (Chambers, Groom et al. 2002). As early as 1889, Stephen Paget proposed the “seed and soil” hypothesis to explain organ specificity in metastasis. He proposed that organ specific patterns were derived from the “dependence” of the seed (the cancer cell) on a conducive soil (the secondary organ) (Paget 1989; Poste and Paruch 1989). Although this hypothesis was challenged by the circulatory pattern theory that proposed that organ-specific metastasis was due to the anatomy of circulation between a primary tumor and the secondary organ (Ewing 1928), it initiated the revolutionary idea that metastasis is a selective process for certain populations of cancer cells that involves numerous interactions between the tumor and its host, and that it is not sufficient to focus simply on the properties of the cancer cells themselves in order to elucidate the mechanisms involved in the process (Fidler 2003). Subsequently, experimental data from metastasis assays on laboratory mice and from human patient samples also support the concept that the compatibility of seed and soil contributes to organ-specific metastasis. Data identifying genetic factors within the metastatic cancer cells and activation of cytokines and proteases were collectively found to direct organ specific metastasis (Kaplan, Rafii et al. 2006). Genetic profiling of metastatic subpopulations of breast cancer cell lines have identified sets of genes that potentially can predict metastasis to lungs or bones (Gupta, Minn et al. 2005; Kang, He et al. 2005). More interestingly, an increasing body of evidence reveals the role of the host environment in cancer metastasis. Recent studies suggest that many tumor-associated stromal cells are bone marrow derived cells (BMDCs),

particularly the myeloid lineage, and are recruited by cancer cells to enhance their survival, growth, invasion, and dissemination (Joyce and Pollard 2009). In some cases of metastasis, certain tissue microenvironments that may be especially supportive for metastatic seeding and colonization by certain types of cancer cells are referred to as “niche” (Coghlin and Murray 2010; Peinado, Lavotshkin et al. 2011). A subset of VEGFR-1 expressing BMDCs is reported to be mobilized to the target organs in response to signals secreted by tumor cells in pre-metastatic lungs to create a fertile niche for tumor cells (Hiratsuka, Watanabe et al. 2006; Kaplan, Psaila et al. 2006). These studies provide compelling evidence that a supportive microenvironment in the secondary organ is required for disseminating tumor cells to engraft at distant sites. Furthermore, the notion has emerged that tumors are more than just a mass of transformed cells and that metastasis does not simply result from the interplay between wandering tumor cells and passive target tissues. A renewed whole picture of tumorigenesis supports the notion that random genetic and epigenetic alterations in cancer cells combined with a plastic and responsive host microenvironment promotes the metastatic evolution of tumors (Chiang and Massague 2008). This increases a tumor’s complexity, but also uncovers a new aspect that we can exploit and take advantage of – the tumor microenvironment (TME).

### 1.3 THE TUMOR MICROENVIRONMENT

The increasingly accepted importance of tumor microenvironment arose in the past decade. Now, the tumor microenvironment and its constituent “non-cancerous” cells have gained prominence and are the subject of intensive investigations. Its principal concept embodies the notion that cancer is not a monodrama orchestrated by cancer cells

alone, but that cancer cells recruit and persuade normal resident cell types in its host environment to serve as accessories in its progression (Hanahan and Coussens 2012).

In detail, the tumor microenvironment which is also called the tumor-associated stroma is composed of non-neoplastic cells such as fibroblasts, infiltrating immune cells, and endothelial cells, and structural components such as the extracellular matrix. They secrete special kinds of growth factors called chemokines and cytokines and chemicals like reactive oxygen species (Matrisian, Cunha et al. 2001). Cellular components are thought to be recruited by molecular signals from the cancer cells and are not just inert bystanders. On the contrary, they are influenced by cancer cells and cooperate with them to support tumor progression by not only enhancing the growth of the primary tumor but also facilitating its metastatic dissemination to distant organs. For example, endothelial cells have been shown to regulate angiogenesis (Ahmed and Bicknell 2009); tumor-infiltrating immune cells can promote invasion, metastatic dissemination, and seeding of cancer cells via their presence at the invading margins of the tumor (Mantovani 2010); while tumor-associated fibroblasts can also modulate tumor cell invasion and metastasis (Figure 1.4) (Xu, Rajagopal et al. 2010) .

All these stromal cells contribute in important ways to the biology of tumor; thus, the tumor is no longer regarded as just a mass of a single cancer cell type but an organ in which a heterogeneous collection of cancer cells collaborate with an equally heterogeneous collection of tumor stromal cells to fulfill its functions: initiation, proliferation, and invasion (Egeblad, Nakasone et al. 2010). The transition from normal to benign to metastatic is not just driven by events inside the tumor cell itself but also by events around it. Throughout this process, the tumor stroma is viewed as an integral part

in the course of multistep tumorigenesis. During the transformation of normal tissue into high-grade malignancies, both neoplastic cells and stromal cells around them change, and

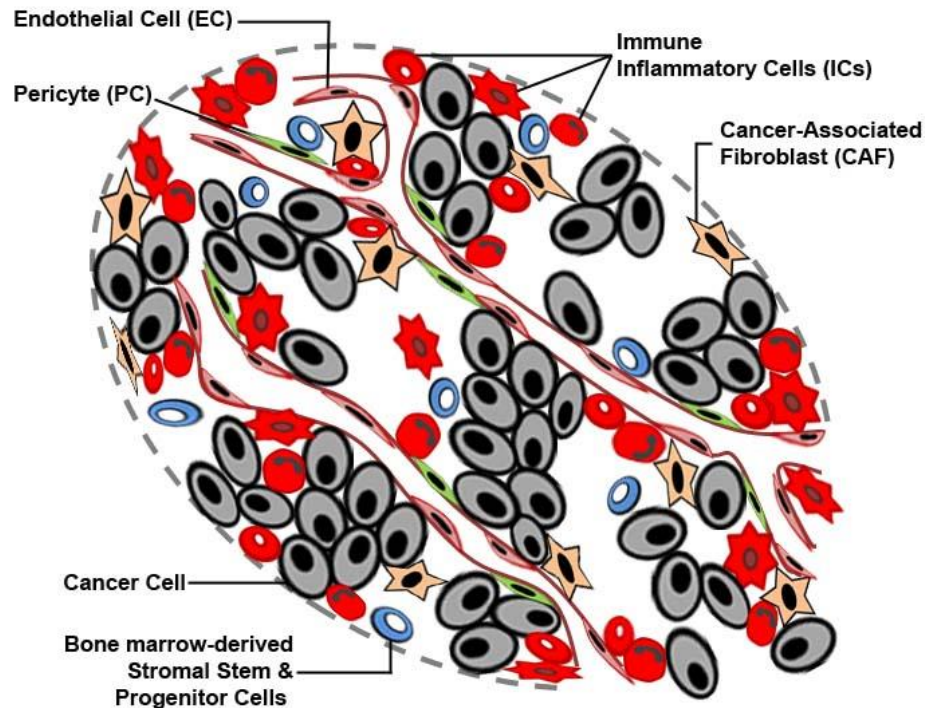


Figure 1.4 Cells in the tumor microenvironment. Tumors have increasingly been recognized as organs with specialized cell types within and the tumor microenvironment evolved during the course of multistep tumorigenesis from tumor growth to invasion and metastasis.

this histopathological progression must reflect underlying changes in heterotypic signaling between tumor parenchyma and stroma (Hanahan and Weinberg 2011). Cancer cells can send signals to stimulate normal cells within the supporting tumor-associated stroma, which reciprocate by supplying the cancer cells with various growth factors and cytokines (Cheng, Chytil et al. 2008). All these views outline a model of host-tumor interaction which depends on back-and-forth reciprocal heterotypic signaling between the cancer cells and supporting stromal cells during the stepwise progression of tumor. Incipient neoplasias begin the interplay by recruiting and activating stromal cell types

that assemble into an initial pre-neoplastic stroma, which in turn responds reciprocally by enhancing the neoplastic phenotypes of the nearby cancer cells. The cancer cells, which may further evolve genetically, again feed signals back to the stroma, continuing the reprogramming of normal stromal cells to serve the budding neoplasm; ultimately, signals originating in the tumor stroma enable cancer cells to invade normal adjacent tissues and disseminate (Hanahan and Weinberg 2011). Therefore, this reciprocal interaction between cancer and host environment appear like a dynamic rhythm with diverse chords composed by heterotypic signaling during tumor progression, and its climax and coda is metastasis. When viewed from this perspective, fully understanding the collaborative interactions between tumor and host environment becomes a new approach that will allow us to conquer cancer metastasis by disengaging the cancer cells from their multiple support networks in order to destroy them.

More recently, many studies with the goal to reverse the tumor-enhancing effects of the microenvironment and recreate suppressive host-tumor interactions in the process of metastasis are in progress. Although a wide range of agents are already available, at least in existing animal models, to target stromal cells to block the host-tumor interactions, like EGFR and CSF1R antagonists, VEGFA and VEGFR inhibitors, TNF- $\alpha$  inhibitors, S100 antibodies, protease inhibitors, anticoagulants and chemokine inhibitors, including CXCR4 antagonists (Hiratsuka, Nakamura et al. 2002; Shojaei, Wu et al. 2007; Hiratsuka, Watanabe et al. 2008), their effect in the translational trials are not very encouraging because of emerging instances of resistance. Moreover, it is unlikely that any of these strategies will work alone without the incorporation of a direct attack on the tumor cell itself. Even in some cases, therapy targeting the tumor stroma unexpectedly

resulted in increased metastasis (Joyce and Pollard 2009). Therefore, the studies focused on host-tumor interaction are still in adversity, and further cognition of this connection is an imperative.

#### 1.4 CANCER AND INFLAMMATION

With the increasingly appreciated importance of tumor microenvironment, the role of inflammation in carcinogenesis has been given fresh attention because its inflammatory component is present and has been shown to contribute to tumor proliferation, angiogenesis, metastasis, and resistance to chemotherapy (Wu and Zhou 2009). The association between the development of cancer and inflammation has long been recognized. Epidemiologic and clinical studies show that nearly 15 percent of cancer incidences is associated with microbial infection and approximately 25% of all human cancers in adults results from chronic inflammation (Kuper, Adami et al. 2000; Coussens and Werb 2002). This connection is also supported by the correlation between the use of anti-inflammatory agents and the reduced incidence of some cancers, such as colorectal and pancreatic cancer (Ulrich, Bigler et al. 2006).

Classically, the inflammatory response coordinates host defenses to microbial infection and mediates tissue repair and regeneration by manipulating all kinds of immune cells such as T cells, B cells, monocytes, etc. In this process, cancer and inflammation share many similarities in cellular behavior, signaling molecules, and gene expression. The cell proliferation, survival, and migration observed in tumor behavior also take place in wound healing in inflammation (Dvorak 1986). During tumor development, immune cells are recruited by cancer cells forming its tumor



microenvironment and exert some “tumor-associated” functions. For example, tumor associated macrophages are found to enhance angiogenesis and remodeling to promote tumor growth, and are a significant sign of poor prognosis (Bingle, Brown et al. 2002; Talmadge, Donkor et al. 2007). Neutrophil infiltration is increased at the invasive areas of the tumor, and have been implicated in enhancing angiogenesis and metastasis in animal models (Murdoch, Muthana et al. 2008). Mast cells are pivotal cells for maintaining immune response. Their increased numbers have been reported in many tumors and correlate with poor prognosis (Ribatti, Crivellato et al. 2004). Myeloid derived suppressor cells (MDSCs) are increased in almost all cancer patients and animal models, and have the unique ability to suppress T cells in order to interrupt immune-surveillance of cancer (Ostrand-Rosenberg 2008; Youn, Nagaraj et al. 2008). Even B cells, as important mediators of humoral immunity, have been shown to promote tumor malignancy (de Visser, Eichten et al. 2006).

During the construction of the tumor microenvironment, these infiltrating inflammatory cells secrete cytokines and growth factors that play an essential role in promoting tumor progression and metastasis (Wu and Zhou 2009). For example, TNF- $\alpha$  (tumor necrosis factor-alpha), a key inflammatory cytokine, is present in many malignant tumors and often associated with poor prognosis. Studies show that overexpression of TNF- $\alpha$  confers migratory and invasive properties of many cancer cell lines (Rosen, Goldberg et al. 1991). TNF- $\alpha$  can also stabilize NF- $\kappa$ B-mediated Snail to induce EMT (epithelial–mesenchymal transition), which is believed to be one of the most important mechanisms of cancer metastasis (Wu, Deng et al. 2009). Another pro-inflammatory cytokine interleukin 6 (IL-6) has been identified as inducer of EMT in breast cancer cells,

and can also promote tumor proliferation through the JAK/STAT3 cascade (Bromberg and Wang 2009). Interleukin 1 (IL-1) can stimulate inflammatory processes and augment metastasis. It accumulates in the tumor stroma and affects the whole process from tumor initiation to metastasis, and maintains the patterns of host-tumor interactions (Apte, Krelin et al. 2006). Metastasis is significantly reduced in mice when IL-1 is inhibited (Vidal-Vanaclocha, Fantuzzi et al. 2000).

Therefore, inflammation is a critical component of tumor progression. When the concept of tumor microenvironment was introduced, the role of the immune cells and their inflammatory factors in tumorigenesis became more apparent. They are an indispensable participant in the whole neoplastic process, fostering proliferation, promoting migration, boosting metastasis, orchestrating the host-tumor interactions all the way.

## 1.5 GOALS OF THE CURRENT STUDY

The overarching objective of the studies presented in this project is to elucidate the mechanisms of host-tumor interactions that drive liver metastasis of colon cancer. Accordingly, the main hypothesis is that tumors secrete molecules that direct the colonization and specific homing of metastatic cells to the target organ. Specifically, the goals in this study were to determine the host-tumor interactions that promote liver metastasis and identify markers for early diagnosis or targets to interrupt progression of liver metastasis. To accomplish these goals, we established a reliable mouse model system for studying host-tumor interactions during liver metastasis of colon cancer by orthotopic implantation of a mouse colon adenocarcinoma cell line into the cecum of a

syngeneic immunocompetent host strain. Characterization of the tumors and host microenvironment in tumor bearing mice showed that this model can recapitulate many hallmarks of human colorectal cancer development and progression to metastasis. Using this model, we determined the genetic signatures of cancer cells, derived from the parental cell line that had varying capabilities for liver metastasis and the host environment to reveal host-tumor interactions that promote liver metastasis of colon cancer. Finally, to begin to identify potential diagnostic markers and therapeutic targets at the early stage of liver metastasis, we characterized genes that are over-expressed in the tumor cells and in the target organ that are predicted to mediate the establishment of a metastasis-prone host microenvironment.

## CHAPTER 2

### DEVELOPMENT AND CHARACTERIZATION OF A RELIABLE MOUSE MODEL OF COLON CANCER METASTASIS TO THE LIVER

Colon cancer is the third most frequent cancer and the third leading cause of cancer deaths in the United States (American Cancer Society 2013). The major cause of death is metastasis and frequently, the target organ is the liver. Successful metastasis depends on acquired properties in cancer cells that promote invasion and migration, and on multiple interactions between tumors and host-derived cells in the microenvironment. These processes, however, occur asymptotically, thus, metastasis remains poorly understood and often diagnosed only at the final stage. To facilitate the elucidation of the mechanisms underlying these processes and identify the molecular regulators, particularly at the early stages, a mouse model of hepatic metastasis of colon cancer was established by cecal implantation of a mouse adenocarcinoma cell line in an immune competent host, which can reliably recapitulate all steps of tumor growth and metastasis within a defined period and is also especially suited to study host-tumor interactions essential for promoting the early stages of metastasis. By *in vivo* selection, a series of cells with increasing metastatic potential were isolated. The most highly metastatic CT26-FL3 cells produced liver metastasis as early as ten days after implantation in 90% of host mice. These cells expressed elevated levels of genes whose products promote invasion, migration, and mobilization of bone marrow derived cells (BMDCs). Sera from

mice bearing tumors from CT26-FL3 had elevated levels of OPN, MMP9, S100A8, S100A9, SAA3, and VEGF that promote invasion and BMDC mobilization, and showed BMDC recruitment to the liver where they established a pre-metastatic niche. This model provides an important platform to characterize metastatic cells and elucidate tumor-host interactions and mechanisms that drive liver metastasis of colon cancer. This chapter describes the development and characterization of this animal model.

## 2.1 REQUIREMENTS IN A MOUSE MODEL OF LIVER METASTASIS OF COLON CANCER

Elucidating the genetic and molecular mechanisms underlying the cross talk between the primary tumor and target organ environment at the early steps of metastasis requires a mouse model that can reliably recapitulate all stages from the growth of the primary tumor to proliferation in the secondary organ. Usually, two general strategies are pursued in mice to create a cancer model: genetically engineered models of cancer (GEM) and transplantable tumor model systems (xenografts). GEM can provide key insights into tumor initiation and early metastatic dissemination, but its metastatic patterns and occurrence is often restricted. For studies in colon cancer, many useful genetic models of benign adenomas have been developed, and in a number of models tumor can advance to the locally invasive stage (Kobaek-Larsen, Thorup et al. 2000; Heijstek, Kranenburg et al. 2005; Taketo and Edelmann 2009). However, none of these spontaneously progress to the invasive stage and metastasize to the target organs such as the liver, lymph nodes, and lungs (Taketo and Edelmann 2009). Moreover, some target genes in these GEM models have typically already been disrupted, which limit their value in identifying new genes

that promote metastasis. On the other hand, the xenograft models established by introducing mouse or human cancer cells into immunocompatible or immunocompromised mice are often the methods of choice to experimentally address metastatic dissemination and colonization of relevant organs. However, these models are also limited by sacrificing complete microenvironmental interface or by a limited range of highly metastatic mouse cell lines. For example, human colon cancer cell lines or tumor tissue fragments have been transplanted into nude or NOD-SCID mice either subcutaneously or in the cecum (Kobaek-Larsen, Thorup et al. 2000; Alencar, King et al. 2005; Cespedes, Espina et al. 2007). Although convenient, subcutaneous injection does not give rise to metastases in the liver or other organs.

Metastasis to the liver has been studied by injection of cancer cell lines into the spleen, portal vein, or directly into the liver in either immunocompromised or syngeneic mouse models (Taketo and Edelmann 2009; Hackl, Man et al. 2013) (Figure 2.1). However, these models not only ignore the primary anatomical structure around colon but also skip the early steps of tumor growth and establishment of the pre-metastatic niche (PMN). Thus, the character of the microenvironment and genetic changes therein during tumor progression are thoroughly disregarded, thereby missing the opportunities for identifying molecular and genetic factors that facilitate the cross-talk between the primary tumor and target organ environment at the early steps of metastasis. Furthermore, immunocompromised mice lack an intact immune system from which many of the cells that mediate these interactions are derived. Although liver metastases occurred in some cases when human or mouse cancer cell lines or tissues were implanted into the cecum or

rectum of immunocompromised or syngeneic host strains, it was observed in only 10-20% of the hosts (Bresalier, Hujanen et al. 1987).

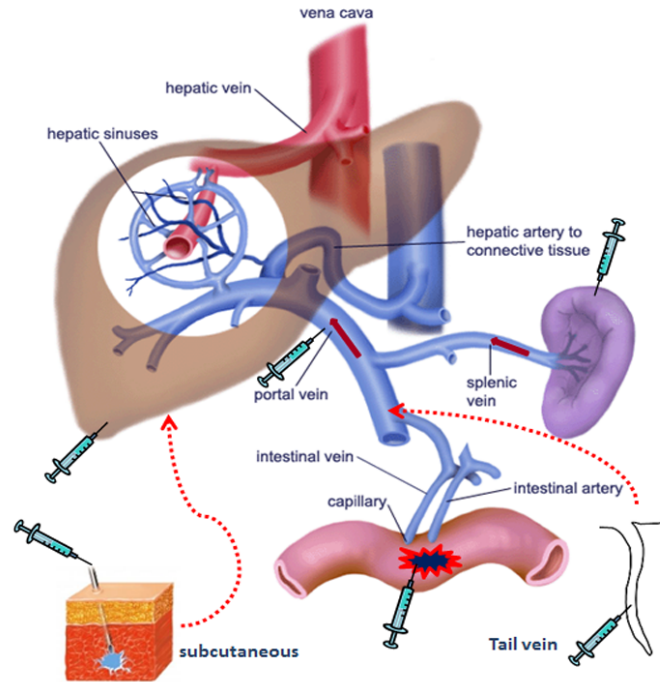


Figure 2.1 Transplatable tumor models of colon cancer. Metastasis to the liver has been established by injection of cancer cell lines into the spleen, portal vein, tail vein, subcutaneous, cecum or directly into the liver in either immunocompromised or syngeneic mouse models.

In conclusion, these available mouse models have limitations in determining the mechanisms of interaction between tumor and host microenvironment during the early stages of liver metastasis. Therefore, based on the specific aims in this study, the ideal mouse model must meet the following requirements: 1) The host mice should have an intact immune system as the natural host environment; 2) The method to establish the colon cancer should recapitulate the correct organ environment, anatomical structure of the primary tumor, all stages of tumor growth and development, and induce spontaneous

liver metastasis; and 3) The incidence of liver metastasis should be high enough to ensure the reliability of the model.

## 2.2 ESTABLISHMENT OF A MOUSE MODEL OF COLON CANCER BY CECUM IMPLANTATION

In order to satisfy the above requirements, a surgical orthotopic homograft was used to establish mouse model of colon cancer in Balb/cByJ mice, an immunocompetent mouse strain which ensures an intact and natural host environment. CT26 colon carcinoma cells, which are syngeneic to Balb/cByJ mouse strains, were harvested, and  $2 \times 10^6$  cells were suspended in 10  $\mu$ L PBS. A midline incision was made in eight-week old Balb/cByJ mice anesthetized with 2% isoflurane to exteriorize cecum. Cells were injected subserosal into the cecum which was then returned to the abdominal cavity. The incision was closed by absorbable suture in two layers (Figure 2.2).

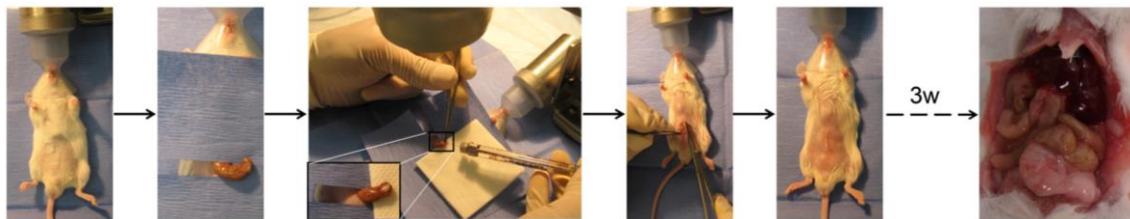


Figure 2.2 Cecum implantation surgery to establish a mouse model of colon cancer. Mice were anesthetized. An abdominal incision was made to exteriorize the cecum. Two million CT26 cells were injected subserosal into cecum, and the incision was sutured in two layers. Three weeks after surgery a visible single nodule of tumor formed in the cecum of mouse.

After 4 weeks, mice were sacrificed and tissues isolated for histological analysis. Results showed that all the injected mice developed primary colonic tumors, appearing as small white neoplasms within one week, but only 8% of the mice (2 out of 25) developed



spontaneous liver metastases after 4-8 weeks of tumor growth. The kinetics of primary tumor progression in the cecum was similar in all mice. In addition, typical clinical symptoms associated with advanced disease were observed such as weight loss, splenomegaly, cramping pain, internal hemorrhage, and cachexia that are consistent with the pathology of colon cancer in human patients. However, only in about 8% of the mice was tumor growth observed in the liver that were subsequently identified as metastases by pathological analyses.

These results confirmed that orthotopic cecal implantation of CT26 colon cancer cells resulted in the consistent development of a primary tumor in Balb/cByJ mice. Utilization of Balb/cByJ mice preserves an intact immune system that can be used for further studies examining host-tumor cell interactions. Orthotopic implantation preserves the colonic microenvironment and anatomical structure for the primary tumor, and all the stages of liver metastasis, particularly the early stages. However CT26 cells possess a limited tendency for liver metastasis in this model, with approximately only 8% of mice developing metastatic lesions. Therefore, increasing the incidence of spontaneous liver metastasis in this model became the next pivotal goal.

### 2.3 ISOLATION OF CELLS WITH HIGH INCIDENCE OF SPONTANEOUS LIVER MEASTASIS BY IN VIVO SELECTION

In the former study, a mouse model was established by cecal implantation of a well characterized mouse colon cancer cell line, CT26 to mimic the development of colon cancer in immune-competent syngeneic hosts, the Balb/cByJ mice. However, it only gave rise to liver metastases in less than 10% of host mice. Therefore, a strategy of *in vivo*

selection was adopted to obtain highly metastatic cell lines that will reliably metastasize to the liver. *In vivo* selection is a process designed to use the natural physiological environment of an intact animal to select and generate certain target cells with specific capabilities or behaviors, (Vendrov and Deichman 1986; Morikawa, Walker et al. 1988). In mouse models of colon cancer, it has been used to increase the metastatic frequency of colon cancer cell lines by serial passaging in both immune-deficient or syngeneic host mice (Bresalier, Hujanen et al. 1987; Morikawa, Walker et al. 1988; Lin, Cheng et al. 1991). In this study, *in vivo* selection was applied to derive highly liver metastatic colon cancer cells from the CT26 cell line to increase the frequency and reliability of liver metastasis in our orthotopic model of colon cancer.

$2 \times 10^6$  CT26 cells were first injected subcutaneously into Balb/cByJ mice. After 2 weeks, recipient mice were sacrificed; tumor tissues were excised and treated with digestive enzymes (collagenase, deoxyribonuclease, and hyaluronidase) to obtain a single cell suspension. After temporary culture in medium to remove cell debris and red blood cells, the purified cells, named CT26-F1 were implanted into the cecum of Balb/cByJ mice. After four weeks, primary cecal tumor growth was observed in all mice, and 40% (10 out of 25) of the mice developed liver metastases. Tumor tissues were excised from the metastatic lesions in the liver, digested to obtain a single cell suspension, grown in culture, and then injected into the cecum of new recipient mice. This cycle was repeated three times as shown in Figure 2.3a. After three rounds of *in vivo* selection, a liver, highly-metastatic colon cancer cell line named CT26-FL3 was obtained, which gave rise to 90% frequency of liver metastasis (23 out of 25 mice), approximately 10-fold higher compared to that in mice injected with the parental CT26 cell line (Figure 2.3b). During

autopsy, a single nodular tumor localized in the cecum was observed in animals implanted with CT26 or CT26-FL3 cells (Figure 2.3c); while no tumor growth was detected in mice injected with PBS into the cecum in sham surgery controls. In mice injected with CT26, few nodules (2-4) were observed in the liver of mice with metastasis within 4-6 weeks after cecal implantation (Figure 2.3b, e). On the other hand, multiple nodular tumors were found in the liver of mice implanted with CT26-FL3 (Figure 2.3f, g) within 4 weeks of cecal implantation.

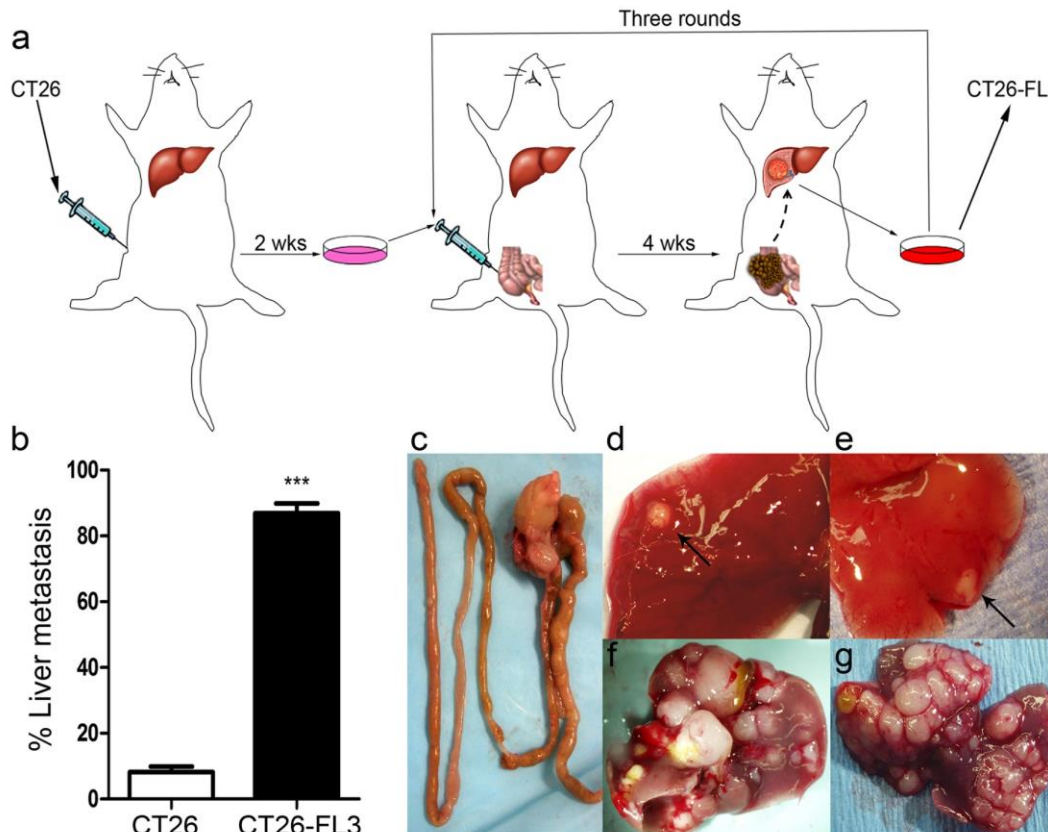


Figure 2.3 Establishment of an orthotopic mouse model of colon cancer with high frequency of spontaneous liver metastasis by *in vivo* selection. a. The procedure of *in vivo* selection, b. Tumors from CT26 and CT26-FL3 cells gave rise to 8 and 90% frequency of liver metastasis, respectively, c. Representative intestinal section showing a single primary tumor in the cecum at four weeks post-implantation, d. and e. few metastatic lesions are observed in liver of mice bearing tumors from CT26 cells, f. and g. multiple metastatic nodules were observed in liver of mice bearing tumors derived from CT26-FL3 cells.

Thus, mice implanted with CT26-FL3 had a higher frequency of metastasis and a higher number of metastatic lesions in the liver within a defined period after cecal implantation.

In order to characterize the properties of the tumors derived from CT26-FL3 cells, the histopathology of tissues from the primary tumor in the cecum and metastatic lesions in the liver of mice implanted with CT26-FL3 were analyzed by staining formalin-fixed, paraffin-embedded sections with hematoxylin and eosin (H&E) and the results are shown in Figure 2.4.

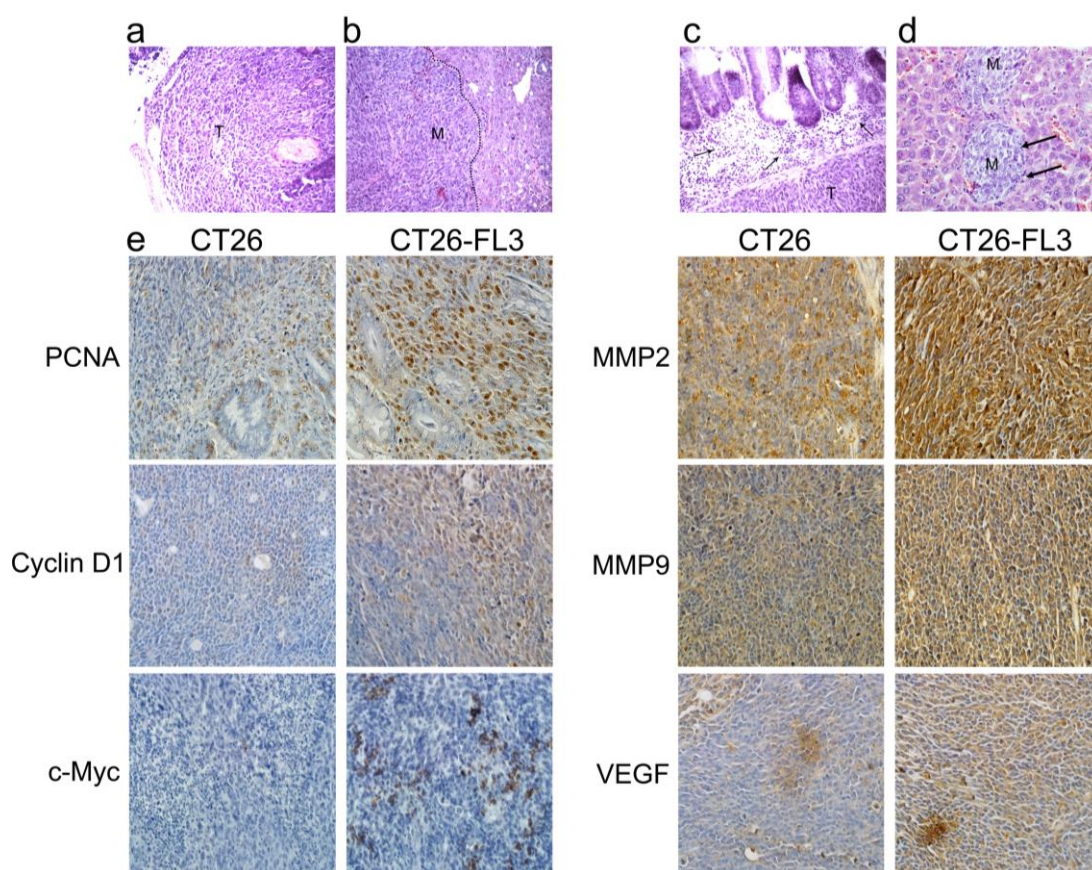


Figure 2.4 Histopathological analyses of primary tumor from the cecum and metastatic tumors from the liver. H & E stained sections from a. primary tumor (T) in the cecum, b. metastatic tumor in the liver (M), c. abundant leukocyte infiltration (indicated by arrow) at the invasive front of the primary tumor and in d. micrometastasis in liver, e. Immunohistochemical stained primary tumor sections from mice bearing tumors from CT26 (left panel) or CT26-FL3 (right panel) cells with antibodies against PCNA, Cyclin D1, c-Myc, MMP2, MMP9, and VEGF. (Shown at 200× magnification)

Histopathological analyses showed a typical, hyper-cellular solid carcinoma with high grade atypia and frequent mitosis in the tumor cells in both primary (Figure 2.4a, indicated by T) and hepatic metastatic tumors (Figure 2.4b, indicated by M). Interestingly, a prominent infiltration of leukocytes or BMDCs (indicated by black arrows) was observed at the invasive margin of the primary tumor or metastatic lesion (Figure 2.4c and d). In addition to the visible nodules, micrometastatic lesions were detected in the liver (Figure 2.4d). Very few (2-4) metastatic lesions were observed in mice implanted with the parental CT26 cell line that had liver metastasis. Sections from primary cecal tumors derived from CT26 or CT26-FL3 cells were examined by immunohistochemistry for expression of biomarkers associated with proliferation, invasion, and angiogenesis such as PCNA, Cyclin-D1, c-MYC, MMP9, MMP2, and VEGF. The results in Figure 2.4e show that these proteins were more highly expressed in primary tumors from CT26-FL3 as compared to those from CT26 cells. Collectively, these data indicated that by *in vivo* selection, a predictable mouse model of colon cancer has been established, which has a high frequency of hepatic metastasis within a defined time-frame, and two isolated isogenic cell lines, CT26-F1 and CT26-FL3, have increasing potentials for hepatic metastasis as compared to the parental cell line CT26.

#### 2.4 COMPARISON OF PROLIFERATION, INVASION, AND MIGRATION OF CT26 AND CT26-FL3 CELL LINES

To define the character of highly metastatic colon cancer cell line CT26-FL3 isolated from the parental CT26 cell line by *in vivo* selection, its growth rate *in vitro* in cell culture and *in vivo* by subcutaneous injection into the flank of Balb/cByJ mice were

compared to CT26 cells. The results showed that the CT26 cells grew faster when grown in tissue culture (Figure 2.5a). On the other hand, when equal numbers of cells were injected into the flank of Balb/cByJ mice, tumor growth from the CT26-FL3 was faster as compared to that from CT26 cells (Figure 2.5b). Then the invasive properties of the two cell lines were compared by using a matrigel transwell invasion assay.

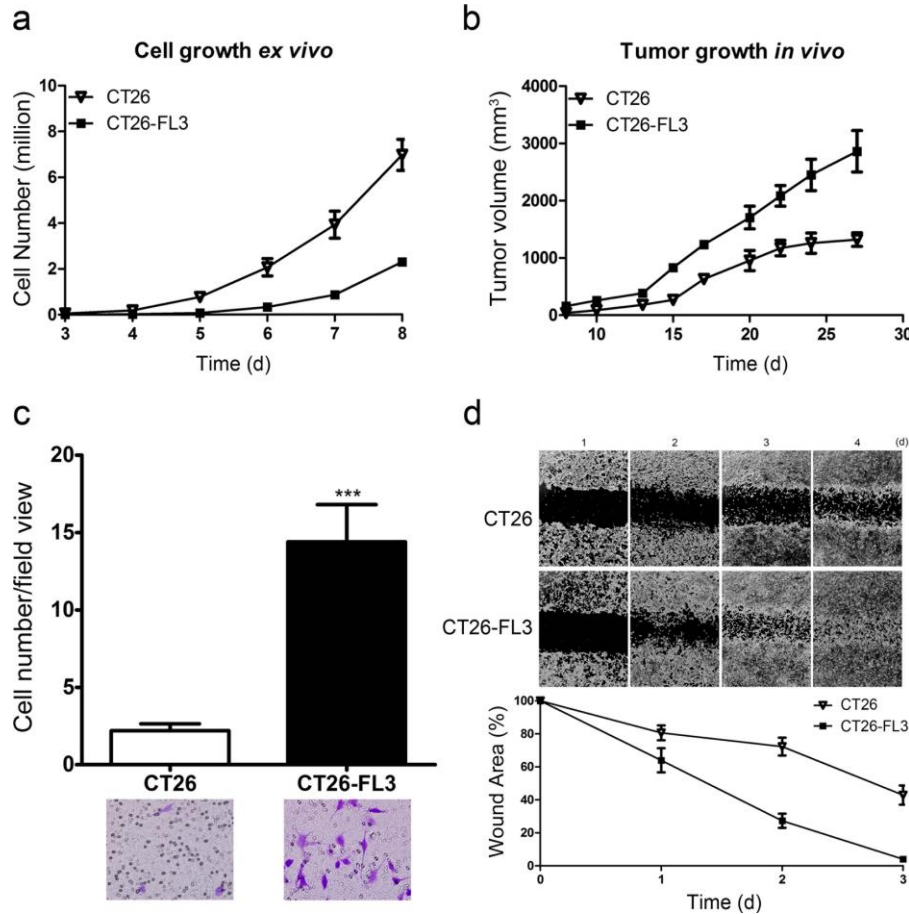


Figure 2.5 Assessment of proliferation, invasion, and migration of CT26 and CT26-FL3 cells. a. Proliferation of CT26 and CT26-FL3 in tissue culture, b. Growth of tumors derived from  $2 \times 10^6$  CT26 or CT26-FL3 cells injected into the flank of Balb/cByJ mice, c. Invasion of CT26 and CT26-FL3 cells through matrigel-coated transwells, d. Migration of CT26 and CT26-FL3 cells in a wound healing assay.

The results showed that CT26-FL3 cells were approximately five-fold more invasive as compared to CT26 cells (Figure 2.5c). In a wound healing assay, CT26-FL3

had a higher ability for migration compared to CT26 cells (Figure 2.5d). In summary, these results indicate that the CT26-FL3 cells have enhanced capabilities for proliferation, migration, and invasion that most likely account for its enhanced ability to metastasize to the liver in host mice. Its faster growth *in vivo* also suggests that the CT26-FL3 cells can better adapt to the surrounding microenvironment, possibly as a consequence of serial *in vivo* passaging, due to enhanced capabilities for interacting with cells in the host microenvironment as compared to CT26 cells.

A critical step during the invasive phase of metastasis is the activation of embryonic transcription programs that enable epithelial cancer cells to convert to cells with mesenchymal properties (Kalluri and Weinberg 2009; Prabhu, Korlimarla et al. 2009). This epithelial to mesenchymal transition (EMT) allows the cells to undergo biochemical changes that result in reduced intercellular adhesion, loss of polarity, enhanced migratory capacity and invasiveness, as well as resistance to apoptosis and enhanced production of extracellular matrix components (Kalluri and Weinberg 2009). Since EMT is accompanied by loss of epithelial markers and acquisition of mesenchymal cell markers. The CT26 and CT26-FL3 cell lines and primary tumors from these cells were examined for expression of E-cadherin, an epithelial cell marker, and fibronectin, vimentin, and  $\beta$ -catenin, markers that are associated with mesenchymal cells, as well as differences in cell morphology. The results showed that when grown in cell culture, there are no differences in the cell morphologies of CT26 and CT26-FL3 cells, and that both cell lines are in constitutive EMT, expressing all the markers examined at elevated levels (Figure 2.6, columns a and b). In contrast, tumors from CT26-FL3 expressed much



higher levels of markers associated with mesenchymal cells (Figure 2.6, columns c and d), supporting data indicating their enhanced migratory and invasive properties.

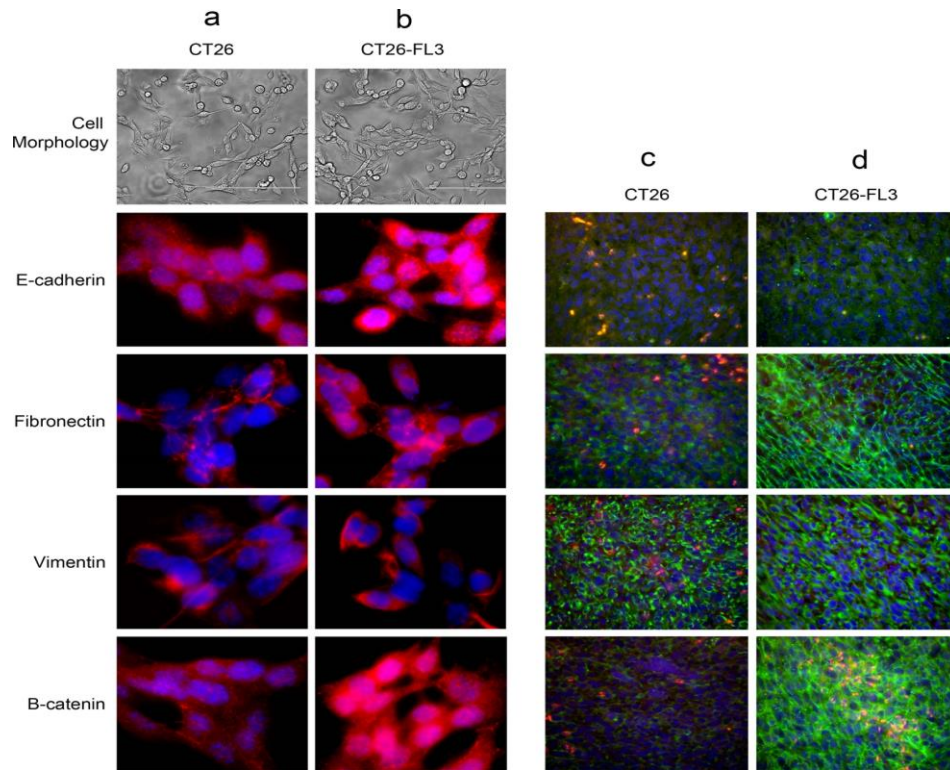


Figure 2.6 Assessment of epithelial to mesenchymal transition markers in CT26 or CT26-FL3 cells and tumors. Cells were grown in slide chambers and examined for morphology (a and b). Primary tumor sections were taken from cecum of mice implanted with CT26 or CT26-FL3 cells (c and d). Cells and tumor sections were stained with antibodies against E-cadherin, an epithelial cell marker or Fibronectin, Vimentin and  $\beta$ -catenin, markers found in mesenchymal cells. Column a. CT26 cells, Column b. CT26-FL3 cells; Red=protein marker, Blue=DAPI. Column c. primary tumor from CT26 cells Column d. primary tumor from CT26-FL3 cells; Red=Tumor cells, Green=protein marker, Blue=DAPI.

## 2.5 TUMORS FROM CT26-FL3 INDUCE SECRETION OF PROTEINS THAT PROMOTE METASTASIS

To determine the influence of tumors originating from the CT26 or CT26-FL3 cell lines on the host environment, blood serum from tumor bearing mice were harvested



to assess the levels of proteins that are typically associated with invasion, signaling, angiogenesis, or establishment of the PMN such as MMP9, OPN, VEGF, the chemokines S100A8 and S100A9, and SAA3 protein (van Kempen and Coussens 2002; Hiratsuka, Watanabe et al. 2008; Tomonari, Fukuda et al. 2011; Yamada, Yamaguchi et al. 2012). Protein levels were determined by Western blotting using albumin (ALB) as an internal loading control. The results showed that the levels of these proteins were higher in sera obtained from mice bearing tumors from CT26-FL3 cells (Figure 2.7a). Interestingly, the sera from these mice contained 27.5-fold higher levels of S100A8, a chemokine that has been shown to promote the establishment of the PMN and to activate critical genes and pathways that promotes tumor growth and metastasis (Ichikawa, Williams et al. 2011).

To determine the source of these proteins, total protein extracts from CT26, CT26-F1, and CT26-FL3 cells were used to detect their expression levels. Visual examination of immunoblots indicated that the relative intracellular levels of these proteins did not change in cancer cells with increasing metastatic potential (Figure 2.7b). Because these proteins are secreted, mRNA expression levels in these cells were measured by qRT-PCR. The results showed that consistent with their increased serum levels, intracellular mRNA levels of *Mmp9*, *Opn*, *Vegf-a*, and *Saa3* increased by approximately 5- to 8-fold between CT26 and CT26-FL3 cells, with intermediate expression levels in CT26-F1 (Figure 2.7c). In contrast, mRNA levels of S100A8 and S100A9 remain unchanged as metastatic potential increased in spite of the 27- and 3-fold increase in serum levels, respectively (Figure 2.7c). These data suggest that MMP9, OPN, VEGF-A, and SAA3 are in part, secreted by the highly metastatic tumors into circulation, while S100A8 and S100A9 are most likely derived from host cells infiltrating into the

tumor. Immunohistochemical analyses showed that tumors from CT26-FL3 are more highly infiltrated by cells expressing S100A8 or S100A9 (Figure 2.7d), as compared to tumors from CT26 cells.

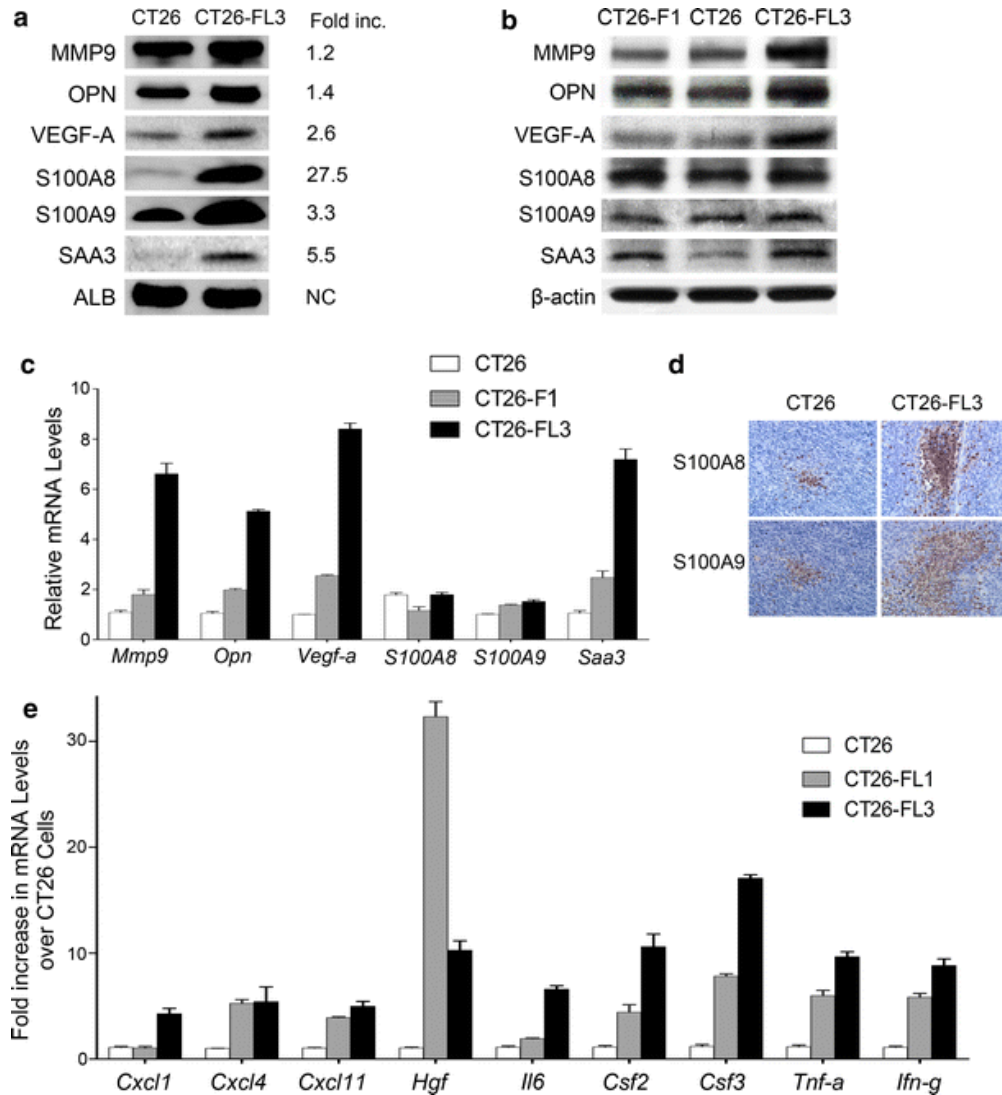


Figure 2.7 Expression of pro-metastatic proteins and genes in CT26, CT26-F1, and CT26-FL3 cells. a. Sera taken from mice bearing tumors from CT26 or CT26-FL3 cells at four weeks after cecal implantation were analyzed by Western blotting. b. Total protein extracts from CT26, CT26-F1, and CT26-FL3 cells were analyzed by Western blotting. c. mRNA levels of pro-metastatic genes were measured by qRT/PCR. d. Immunohistochemical analysis of sections from primary cecal tumors derived from CT26 and CT26-FL3. e. mRNA expression levels of *Hgf*, *Il6*, *Tnf-a*, *Ifn-g*, *Csf2*, *Csf3*, *Cxcl1*, *Cxcl4*, and *Cxcl11* were measured by qRT/PCR.

The creation of a permissive microenvironment requires the ability to recruit non-neoplastic host derived cells to the tumor stroma where they play an important role in promoting tumor growth and progression to metastasis (Kaplan, Psaila et al. 2006; Kaplan, Rafii et al. 2006). These include BMDCs such as neutrophils, monocytes, macrophages, and other leukocytes. The abilities of CT26, CT26-FL1 and CT26-FL3 derived tumors to recruit BMDCs to the primary tumor and target organ microenvironment were compared by measuring the mRNA levels of a number of cytokines and growth factors that are thought to mediate the crosstalk between neoplastic cells in the primary tumor and stromal cells in the microenvironment. As shown in Figure 2.7e, CT26-FL3 cells expressed significantly higher levels of the Hgf, Il-6, Tnf- $\alpha$ , Ifn- $\gamma$ , Csf 2 and 3, and the cytokines Cxcl1, Cxcl4, and Cxcl11 as compared to CT26 and CT26-FL1. These data suggest that CT26-FL3 may be more proficient in mobilizing stromal cells that promote a pro-metastatic host environment as compared to CT26 cells.

It should be noted that the CT26-FL1 and CT26-FL3 cells used in these analyses were obtained from metastatic lesions in the liver after one or three sequential passages through the liver. The tumors were debulked into single cell suspensions and briefly grown in culture to remove any contaminating stromal cells. Interestingly, CT26-FL1 expressed 35- and 3-fold higher levels of Hgf as compared to CT26 and CT26-FL3, respectively, after a single passage through the liver. It is tempting to speculate that CT26-FL1 might require higher levels of HGF for specific homing to the liver in the first round of metastasis, but enhanced expression of other genes in the highly metastatic CT26-FL3 might not necessitate the same levels of HGF after repeated passaging through the liver.

## 2.6 BMDCs ARE RECRUITED TO THE LIVER MICROENVIRONMENT PRIOR TO METASTASIS

It was previously shown that prior to the arrival of metastasizing melanoma and Lewis lung carcinoma (LLC) cells, BMDCs are recruited to the lung microenvironment to create a PMN where arriving metastatic cells can attach and proliferate (Kaplan, Riba et al. 2005; Kaplan, Rafii et al. 2006). In the previous section, CT26-FL3 cells have been shown to over-express cytokines and growth factors that are known to induce the mobilization of a variety of BMDCs. Here, the goals were a) to determine if a PMN is established in the liver prior to the arrival of metastatic colon cancer cells, b) to examine the proficiency of tumors derived from CT26 and CT26-FL3 cells in recruiting BMDCs to the liver, and c) to enhance the mouse model so that it can be used to facilitate the characterization of interactions between tumor cells and BMDCs that are essential for invasion and metastasis.

The cecal implantation was therefore combined with transplantation of BM cells expressing enhanced Green fluorescent protein (eGFP) (Figure 2.8a). Interactions between tumor cells expressing the mCherry RFP by stable transfection (Figure 2.8b) and BMDCs expressing eGFP can then be visualized by confocal microscopy or quantitated by flow cytometry. Recipient Balb/cByJ mice were lethally irradiated and transplanted with whole BM from donor Balb/cByJ-UBC-GFP mice. Analyses of peripheral blood by flow cytometry at 4 weeks post-transplant showed that the transplanted marrow successfully engrafted, with approximately 86 to 98 percent of leukocyte cells expressing eGFP (Figure 2.8a).

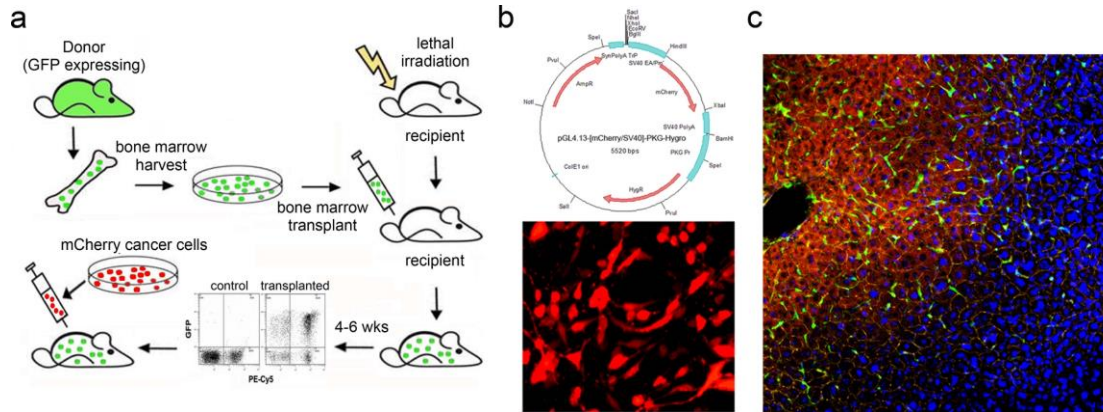


Figure 2.8 Scheme for combining cecal implantation with BMT to visualize interactions between tumor and BMDCs. a. Bone marrow from transgenic mice expressing GFP was transplanted into lethally irradiated 4-week old recipient Balb/cByJ mice. Six weeks after BMT, when transplanted marrow was fully engrafted, CT26 or CT26-FL3 cells stably transfected with the mCherry RFP were injected into the cecum and allowed to grow and metastasize, b. Plasmid map of vector expressing mCherry-RFP (upper panel) and representative confocal microscopy image of stably transfected CT26 cells (lower panel), c. Representative confocal microscopy image of an established metastatic tumor in the liver after invading the hepatic lobule and colonizing the central vein. Red = CT26-FL3 cells expressing mCherry-RFP, Green = GFP positive BMDCs, Blue = DAPI ( $\times 100$  magnification).

Similar results were observed when leukocytes were stained with antibodies that specifically detect B lymphocytes, monocytes, or macrophages (data not shown). A typical image of a metastatic lesion in the liver showed that tumors (red) were abundantly infiltrated by BMDCs (green) indicating an active interaction between these cells (Figure 2.8c). Thus, combining cecal implantation and BMT can be used to track the interactions between cancer cells and host-derived BMDCs at various stages of colon cancer metastasis to the liver.

To determine if tumors derived from CT26 and CT26-FL3 cells can induce the recruitment and mobilization of BMDCs to the liver prior to the arrival of metastatic cells, two million RFP-labeled cancer cells were implanted into the cecum of Balb/cByJ mice

transplanted with eGFP-expressing BM. Liver sections were examined by confocal microscopy for the presence of eGFP-positive BMDCs at weekly time points after tumor implantation. A representative result taken from mice transplanted with CT26-FL3 cells is shown in Figure 2.9a, upper panel. One week after cecal implantation, very few green cells were observed in the liver sections. However, the number of GFP positive infiltrating BMDCs increased between two to three weeks after tumor implantation before the establishment of metastatic lesions (Figure 2.10). At around three weeks post tumor cell implantation, RFP-expressing tumor cells were first detected in the liver. After four weeks, metastatic lesions (red) were formed and numerous eGFP-positive BMDCs were observed infiltrating and around the invasive front of the lesion. After five weeks, when the metastatic lesions were fully established, numerous eGFP-positive BMDCs were mostly observed at the invading front of the lesions. In general, BMDC infiltration was observed as early as seven days, while red fluorescent tumor cells had been detected as early as 10 days post implantation of CT26-FL3 cells.

On the other hand, BMDC infiltration in mice implanted with CT26 cells was typically observed after two weeks, and tumor cells were detected after three to four weeks (Figure 2.9a, middle panel). Development of metastatic lesions occurred after four to as much as eight weeks post CT26 implantation and was found in only 8% of implanted mice. No obvious BMDCs were observed in the liver of control mice that had undergone surgery but were injected with PBS into the cecum in place of tumor cells (Figure 2.9a, lower panel). These results not only confirm that the primary tumor can affect the host liver microenvironment, but they also indicate the enhanced ability of the



CT26-FL3 cells to recruit BMDCs to initiate the establishment of what is potentially the PMN in the liver prior to the arrival of metastasizing tumor cells.

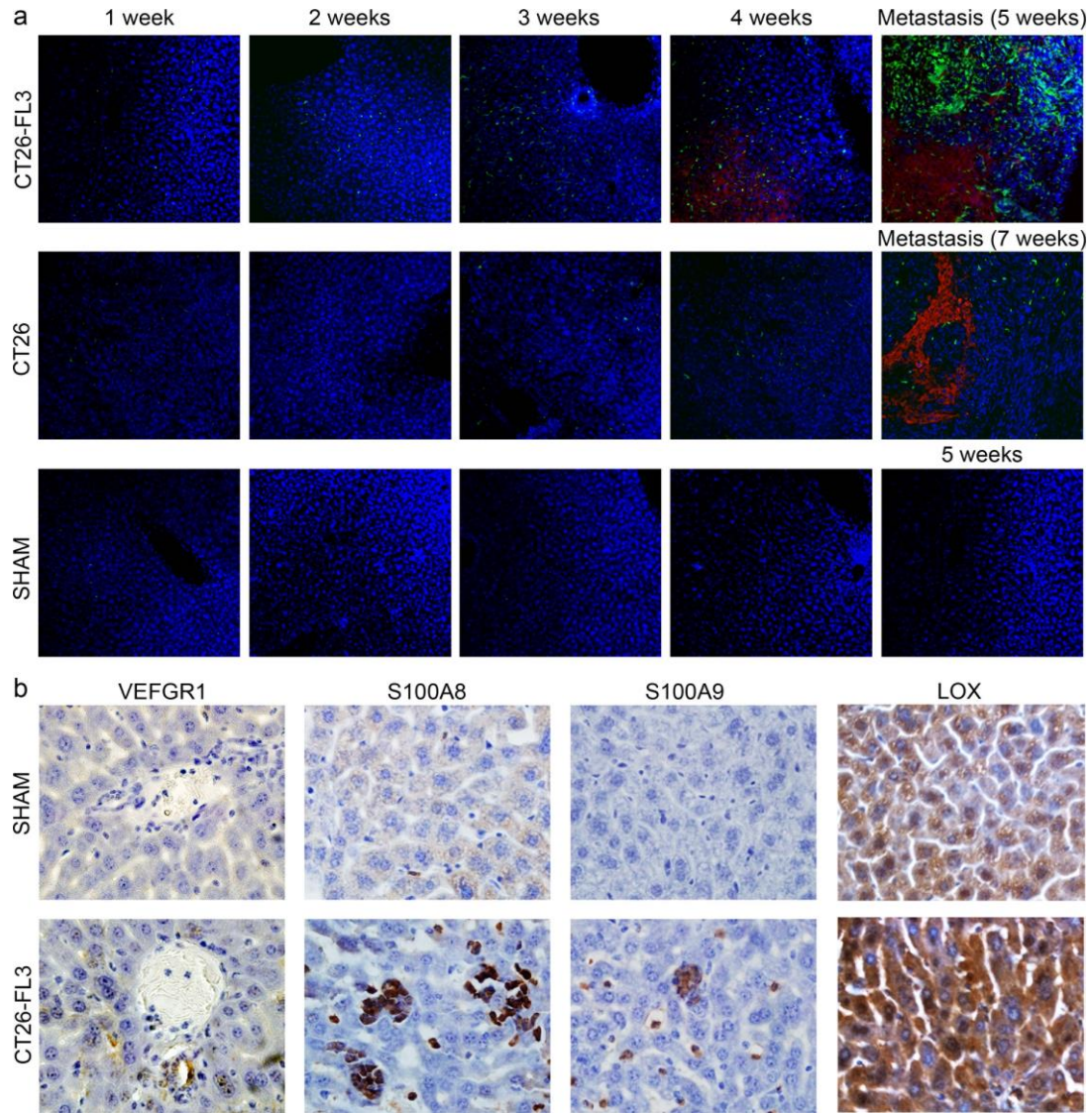


Figure 2.9 Migration of BMDCs and cancer cells into the liver. a. BMDCs migrated into the liver after cecum implantation before the arrival of CT26-FL3 tumor cells (upper panel) or CT26 tumor cells (middle panel), sham injected animals at the same time points (bottom panel). Red= mCherry-RFP, Green=eGFP positive BMDCs, blue=DAPI ( $\times 100$  magnification) b. Immunohistochemical analysis of liver sections from mice bearing CT26-FL3 derived tumors. Sections were taken at 2.5 weeks after cecal implantation and stained with VEGF-R1, S100A8, S100A9, and LOX ( $\times 400$  magnifications).

To further establish the creation of the PMN, liver sections taken at 2.5 weeks after cecal implantation of CT26-FL3 cells were analyzed by immunohistochemistry to determine the presence of molecules that have been implicated in its formation such as VEGF-R1, S100A8, S100A9, and LOX (Erler, Bennewith et al. 2009; Spano and Zollo 2012; Yamada, Yamaguchi et al. 2012). The results indicated that BMDCs expressing VEGF-R1, as well as S100A8, S100A9, and LOX aggregated in the liver prior to the arrival of CT26-FL3 cells (Figure 2.9b, lower panel). These molecules were not detected in liver sections taken from control, sham injected mice (Figure 2.9b, upper panel).

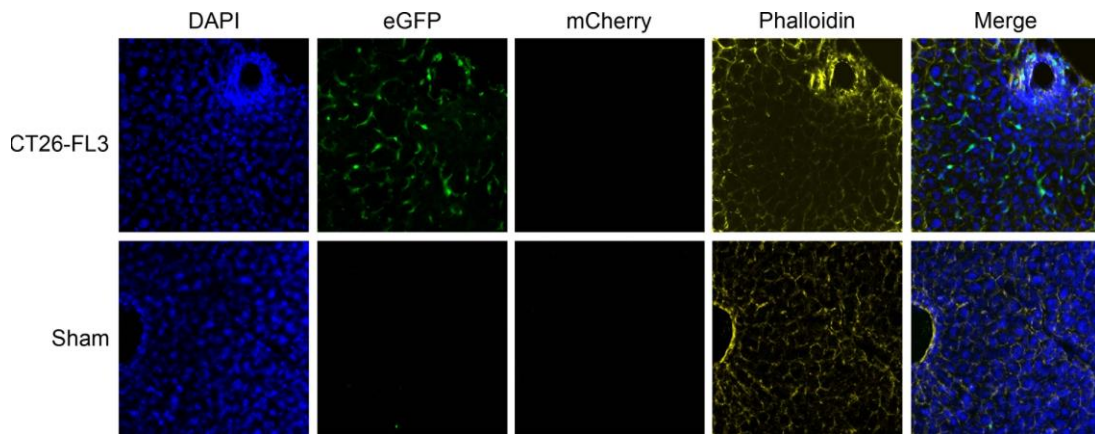


Figure 2.10 Migration of eGFP positive BMDCs into the liver prior to the arrival of mCherry-RFP positive tumor cells at three week post cecal implantation. Cells implanted with CT26-FL3 showed the presence of BMDCs and the absence of tumor cells (upper panel) while both green and red fluorescence were absent in control Sham injected animals (lower panel). Phalloidin (actin) and DAPI (blue). ( $\times 200$  magnification)

Co-localization studies in liver from tumor bearing mice transplanted with eGFP-expressing BM showed that VEGFR1, S100A8 and S100A9 are expressed by infiltrating BMDCs (Figure 2.11). In contrast, we found diffused basal levels of LOX in normal hepatocytes, very high levels in liver of tumor bearing mice, and its expression was not associated with infiltrating BMDCs. Together, these data confirm that prior to the arrival



of cancer cells, the primary tumor can direct the recruitment of BMDCs to the liver, and that the CT26-FL3 cells are more proficient than CT26 cells in this process.

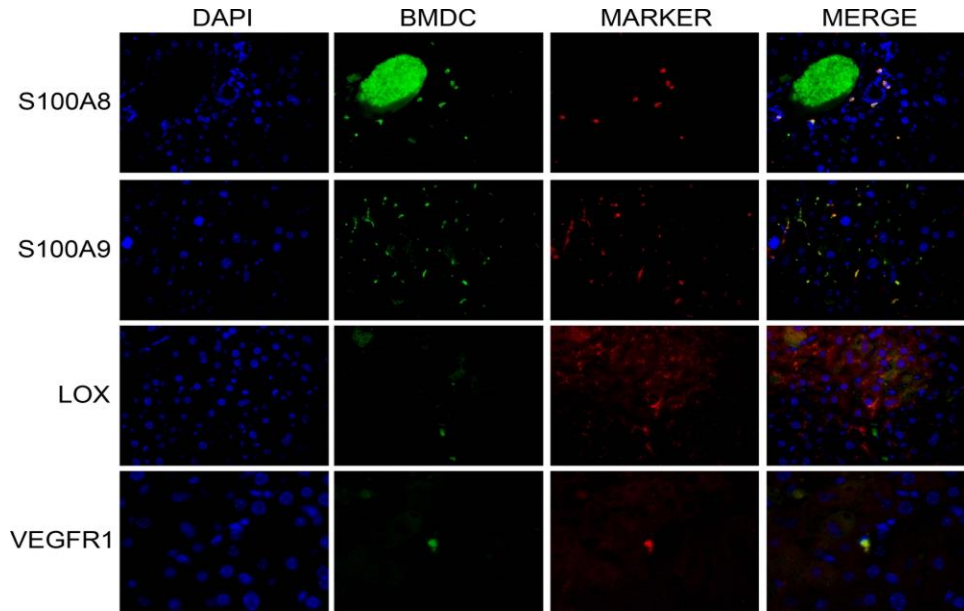


Figure 2.11 Co-localization of markers associated with pre-metastatic niche formation with BMDCs infiltrating the liver. Balbc/ByJ mice were transplanted with BM from transgenic mice expressing eGFP prior to implantation of CT26-FL3 cells into the cecum. Liver sections were taken 2.5 weeks after implantation, stained with antibodies against S100A8, S100A9, LOX, and VEGF-R1, then examined for the presence of BMDCs (Green), and counterstained with DAPI (Blue). Red = Positive staining for protein specific antibodies. Images were the merged to determine co-localization of the protein markers with BMDCs.

## 2.7 SUMMARY AND DISCUSSION

Understanding the molecular, cellular, and genetic factors that promote the metastasis of colon cancer to the liver requires a mouse model that can reproducibly recapitulate all steps, from the growth of the primary tumor to the development of metastatic lesions. In this chapter, a mouse model of colon cancer with a high frequency of liver metastasis within a defined time frame in a host with an intact immune system

was developed. The CT26 mouse colon adenocarcinoma cell line was injected into the cecum of syngeneic Balb/cByJ mice to establish an orthotopic model of hepatic metastasis of CRC in an immune competent host. Although all implanted mice developed a primary tumor in the cecum, only 8% developed liver metastasis. By applying a sequential method of *in vivo* selection, isogenic cell lines with increasing metastatic potential were isolated, which increased the frequency of liver metastasis by 10-fold to 90%. The most highly metastatic cell line, CT26-FL3 gave rise to micrometastatic lesions as early as 10 days after cecal implantation, thus providing a predictable model that can be used to study various aspects and stages of liver metastasis.

To begin to characterize the CT26-FL3 cells, and to ensure that the model reflects known mechanisms of metastasis, the expression levels of proteins that enhance proliferation, invasion, and angiogenesis. such as c-Myc, CCND1, VEGF, MMP9, MMP2, and PCNA were determined in tumors derived from these cells (Partin, Schoeniger et al. 1989; Silletti, Paku et al. 1998) (Otte, Schmitz et al. 2000; van Kempen and Coussens 2002; Deryugina and Quigley 2006; Malkas, Herbert et al. 2006; Loges, Mazzone et al. 2009). Data from immunohistochemical analyses of tissue sections from primary tumors revealed that these molecular markers were expressed at higher levels in tumors derived from the CT26-FL3 cell line as compared to tumors from the parental CT26 cell line. These findings are supported by the significantly higher abilities for invasion and migration of CT26-FL3 cells compared to CT26 cells, as measured by the matrigel-coated Boyden Chamber and wound healing assays. Although both CT26 and CT26-FL3 cells undergo constitutive EMT when grown in culture (Huber, Maier et al.

2010), tumors derived from CT26-FL3 expressed higher levels of mesenchymal cell markers, further underscoring their enhance capabilities for migration and invasion.

In an unbiased analysis of mRNA transcripts expressed in both cell lines, HGF mRNA levels were 10-fold higher in CT26-FL3 cells. HGF was thought to be expressed primarily by mesenchymal tissue such as fibroblasts and mononuclear cells (Kammula, Kuntz et al. 2007). It interacts with c-Met, a tyrosine kinase receptor and an oncogene in cancer cells in a paracrine fashion to activate genes involved in tumor progression, indicating that a reciprocal relationship between the tumor and cells in the microenvironment is critical for tumor invasion and metastasis (Silletti, Paku et al. 1998). However, consistent with our observations, Kammula et al quantitatively showed that HGF was also highly expressed in primary colon cancer tissues and that elevated levels of both proteins correlated with an advanced invasive stage and metastatic disease as well as poor prognosis (Grivennikov and Karin 2011).

In addition, increased mRNA levels of pro-tumorigenic cytokines IL-6, TNF- $\alpha$ , and IFN- $\gamma$ , IL-6 and TNF- $\alpha$  were found. These are critical regulators of tumor-associated inflammation (Balkwill 2009), and promote cancer development by activating oncogenic transcription factors such as NF- $\kappa$ B, AP-1 (TNF), and STAT 3 (IL-6) in epithelial cells (Bromberg, Wrzeszczynska et al. 1999; Naugler and Karin 2008; Balkwill 2009). High levels of IL-6 in sera of cancer patients and tumor-bearing mice correlate with poor prognosis (Grivennikov, Karin et al. 2009). While it is mostly produced by hematopoietic-derived stromal cells at the early stage of colon cancer development (Grivennikov, Kuprash et al. 2006), it has also been shown to be produced in sporadic colon cancer where it can act by autocrine mechanisms to enhance STAT3 signaling

(Balkwill 2009). In addition, IL-6 can promote the differentiation of Th17 cells, survival of T cells, inactivation of regulatory T cells, control the trafficking and recruitment of myeloid cells and neutrophils, as well as differentiation of myeloid-derived suppressor cells (MDSCs) (Balkwill 2009), indicating a role in BMDC mobilization. TNF is critical in maintaining chronic inflammation and promotes tumorigenesis by activating signaling pathways that stimulate cell proliferation and survival such as those driven by AP-1 and NF- $\kappa$ B (Matthews, Colburn et al. 2007; Popivanova, Kitamura et al. 2008). The TNF receptor is expressed in BMDCs rather than in epithelial cells (Zaidi and Merlino 2011), suggesting that TNF might play a role in mobilizing these cells to the tumor microenvironment (Grivennikov, Kuprash et al. 2006). IFN- $\gamma$  is a pleiotropic cytokine that promotes cytotoxic, cytostatic, and antitumor effects in adaptive immune response and has been used to treat various malignancies (Zaidi and Merlino 2011). However, it also enhances proliferation, and through autocrine signaling, promotes metastasis by conferring increased resistance to natural killer (NK) cells (Lollini, Bosco et al. 1993; Gorbacheva, Lindner et al. 2002). It can induce an inflammatory cascade by recruiting immune cells such as macrophages, NK cells, and CTLs (Zaidi and Merlino 2011) to create a pro-tumorigenic environment at the site of oncogenesis. In addition, mRNA levels of the cytokines CSF2, CSF3, and chemokines CXCL1, CXCL4, and CXCL11 were found over expressed by four to eight folds in CT26-FL3 cells. CSF2 and CSF3 control the differentiation, production, and functions of granulocytes and/or macrophages (Metcalf, Begley et al. 1986; Smith 1990). They are typically produced by immune cells such as macrophages, mast cells, T cells, and NK cells as well as endothelial cells and fibroblasts. Recently, CSF2 was shown to be over-expressed in more than one-third of

human colorectal tumors due to aberrant DNA demethylation of its promoter; simultaneous overexpression of its receptor correlated with prolonged survival making them useful prognostic markers for cancer immunotherapy (Urduingio, Fernandez et al. 2013). CXCL1 and CXCL11 act as chemoattractants for the recruitment of neutrophils and activated T cells, while CXCL11 can interact with angiogenic growth factors such as fibroblast growth factor and VEGF to promote angiogenesis (Scapini, Morini et al. 2004; Berencsi, Meropol et al. 2007; Acharyya, Oskarsson et al. 2012).

Collectively, these data indicate that enhanced metastasis by CT26-FL3 cells is due, in part, to the elevated expression of genes whose products not only confer growth advantage and invasiveness, but also mediate tumor interactions with host derived cells, particularly the immune cells, in the microenvironment and stimulate their mobilization either to the primary tumor or the secondary organ environment. Here, the study showed that sera from mice bearing CT26-FL3-derived tumors had elevated levels of MMP9, OPN, VEGF-A, the pro-inflammatory calcium-binding cytokines S100A8 and S100A9, and SAA3. OPN secreted by tumor cells has been shown to activate BMDCs causing their migration to sites of tumorigenesis (McAllister, Gifford et al. 2008; Elkabets, Gifford et al. 2011). The last three proteins were shown by Hiratsuka, et al. to be critical for the establishment of the PMN in lungs (Hiratsuka, Watanabe et al. 2008). Secretion of VEGF-A, TNF- $\alpha$ , and TGF- $\beta$  by tumor cells induced the expression of S100A8 and S100A9 in pre-metastatic lung where they promoted the recruitment of macrophage antigen-1 (Mac-1)-expressing myeloid cells as well as the expression of SAA3 which acted as a positive feedback regulator for further secretion of chemo-attractants that in turn promoted tumor cell migration (Hiratsuka, Watanabe et al. 2008). SAA3 can induce

the expression of NF- $\kappa$ B through the TLR4 receptor, providing a link to an inflammatory-like response in the formation of the PMN. Although these responses were studied in the pre-metastatic lung, Ichikawa et al. (Ichikawa, Williams et al. 2011) further showed that S100A8/A9 secreted by MDSCs residing in the primary tumor and at sites of metastasis created an autocrine pathway for further recruitment of more MDSCs. In colon tumor cells, they induced the secretion of several genes whose products promote tumor cell migration, angiogenesis, the recruitment of leukocytes, and the formation of a PMN in distant organs (Ichikawa, Williams et al. 2011). The data from this study showed that serum levels of S100A8 in CT26-FL3 tumor bearing mice was elevated by approximately 27-fold suggesting that these cells might be highly proficient in establishing the PMN. In all, these results indicate that the cancer cells with a high propensity for metastasis are better able to manipulate the surrounding microenvironment and recruit BMDCs to the primary tumor or secondary organ.

Therefore, the cecal implantation model was combined with transplantation of HSCs expressing eGFP to assess the mobilization of BMDCs to the liver microenvironment in tumor bearing mice. After engraftment, implantation of tumor cells labeled with mCherry RFP allowed visualization of the tumor and stromal cells by confocal microscopy. The data showed that the highly metastatic CT26-FL3 was very proficient at mobilizing the recruitment of eGFP-positive BMDCs to the liver microenvironment. Immunohistochemical staining of liver tissues from tumor bearing mice revealed the presence of molecular and cellular markers associated with the PMN such as VEGF-R1-positive cells (Kaplan, Riba et al. 2005), S100A8 and S100A9 (Hiratsuka, Watanabe et al. 2006; Hiratsuka, Watanabe et al. 2008), and LOX (Erler,

Bennewith et al. 2006; Erler and Giaccia 2006; Erler, Bennewith et al. 2009). While representing only a small fraction of the participants, these molecules play key roles in establishing the PMN. Further studies need to be undertaken to enumerate the full complement of BMDCs and molecules that comprise the hepatic PMN as well as the molecular signals that direct their organ specific migration.

## CHAPTER 3

### INTEGRATED EXPRESSION PROFILING REVEALS GENE SIGNATURES OF HOST-TUMOR INTERACTIONS PROMOTING LIVER METASTASIS IN COLON CANCER

With decades of cancer research, the concept of cancer has evolved from simply a “mutation” into an organized “organ”. The stromal cells originating from the host are recruited by cancer cells and exert multiple functions in tumorigenesis as parts of this “organ”, and a series of dynamic and energetic interactions between cancer cells and the host directs each step in forming this organ. Convincingly, metastasis is much more dependent on a harmonious and supportive host-tumor interaction to successfully fulfill the dissemination inside the host and relocation of cancer cells in distant organ. With this new concept of cancer, it is pivotal to take the tumor stroma and host-tumor interactions into consideration in seeking information about the mechanisms and molecular basis of metastasis.

The microarray technique has been widely used in expression profiling to identify molecular factors which contribute the tumorigenesis. However, its applications in metastasis are still limited by the inherent complexity of metastasis and the numerous factors involved. Specifically, one of critical limitations is due to the difficulty in choosing appropriate samples for comparison. In many cases, samples used to acquire target molecule information are not specific enough and lack well-defined controls resulting in a flawed comparison. For example, most genes have been identified by



comparing cell lines with different metastasis tendencies but were developed by using different chemicals to induce mutations (Flatmark, Maelandsmo et al. 2004; Kao, Salari et al. 2009) or tumor tissue from patients with different prognosis for metastasis (Seike, Yanaihara et al. 2007). In each of these cases, the diversity of genetic background among the samples was high enough to obscure the designed intention which was to find the genes involved in metastasis. To add to the complexity, the interaction between tumor and host microenvironment is composed of dynamic and heterotypic signaling, so that make the opportunities to locate the genes which are specifically involved in metastasis-related host-tumor interactions, especially detect signals in pre-metastasis and early stage metastasis for diagnosis and early intervene more faint and inefficient. Also, logically it seems like impossible to adopt a pair of gene comparison to understand all information about metastasis in dynamic course and different organs.

In the study from last chapter, a mouse model of colon cancer mouse liver metastasis was developed, which can reliably give rise to spontaneous metastases in the liver of immune-competent syngeneic hosts and recapitulate information in all stages of metastasis. The CT26-FL3 cell line obtained through *in vivo* selection and its parental CT26 cell lines is a unique pair of isogenic cell lines with different capabilities for disseminating to the liver, providing well-controlled samples in comparative studies for microarray analyses, because the only genetic alterations that may have occurred in the transition from CT26 to CT26-FL3 potentially resulted from the interactions between tumor cells and host environment, and contribute to a higher incidence of liver metastasis in CT26-FL3. Therefore, these cell lines form a valuable platform for identifying crucial genes that mediate the mechanisms involved in liver metastasis of colon cancer.

### 3.1 MICROARRAY DESIGN AND SAMPLE COLLECTION

With the appropriate research platform developed by study in last chapter, a two-pronged approach was designed to identify genetic changes within the cancer cell (the seed) or in the target organ environment (the soil) that are necessary for liver metastasis of colon cancer by using microarray technique. Two groups of comparison were performed:

Group 1 - The seed: CT26 vs. its highly metastatic derivative CT26-FL3 cells. A comparison of the genetic signatures of these cell lines will reveal the genes expressed in cancer cells that promote metastasis.

Group 2: The soil: normal liver tissues vs. pre-metastatic liver tissue vs. metastatic liver tissues.

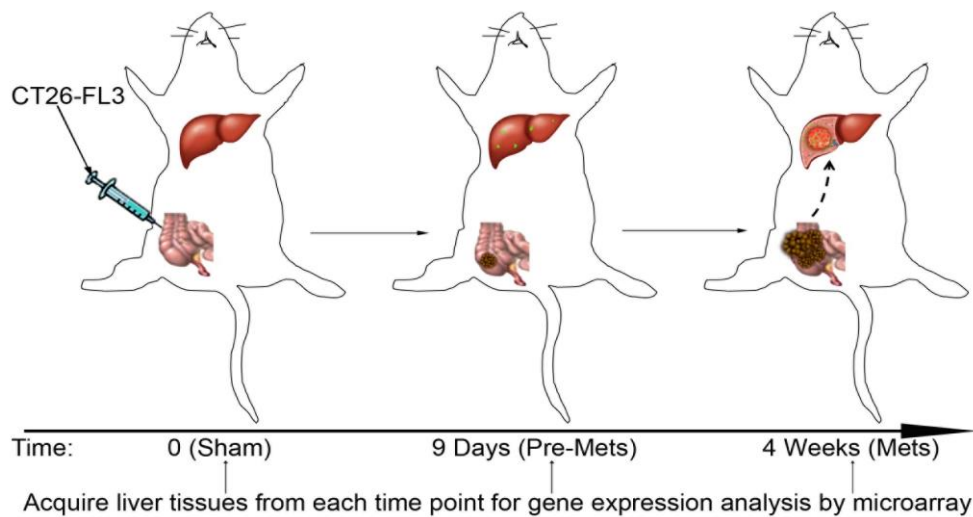


Figure 3.1 Design of microarray experiments on liver tissue. Liver tissues from mice bearing CT26-FL3 tumors were collected to isolate total RNA at three time points, 0 day, 9 days, and 4 weeks.

In this group, a comparison of liver tissue from non-tumor bearing mice compared to tumor bearing mice prior to the establishment of metastasis will disclose genes that are

involved in setting up the pre-metastatic niche, while a comparison of the liver tissue prior before and after the establishment of metastasis will allow the identification of genes whose products may be required to maintain metastatic growth. The experimental design is outline in Figure 3.1. Balb/cByJ mice were implanted with CT26-FL3 expressing mCherry RFP into the cecum to initiate the primary colon tumor. Liver tissue samples were harvested from sham injected control mice which underwent the surgical procedure but injected with PBS instead of cancer cells in the cecum; pre-metastatic liver tissue samples were harvested from mice bearing CT26-FL3 in the cecum at 9 days post implantation prior to the arrival of cancer cells, while metastatic liver tissue samples were harvested from mice bearing visible metastatic CT26-FL3-derived lesions in the cecum at 4 weeks post implantation. In order to avoid the possibility that minor metastatic lesions were present in the liver tissues, a part of homogenate were used to isolate DNA and analyzed by PCR using primers specific to the mCherry red fluorescent protein gene that was stably transfected into the CT26-FL3 cells to detect the cancer cells. The absence of the mCherry-specific PCR product indicated the liver samples were not contaminated with genetic material from metastasizing cancer cells.

The liver tissues and cell pellets were placed in dry ice, then homogenized and stored in RNeasy lysis buffer at -20°C. RNA was extracted and the quality and quantity were evaluated in an Agilent 2100 Bioanalyzer using RNA Pico chips. RNA samples with an RNA integrity number (RIN) greater than 8 were stored at -80°C for microarray analyses (Table 3.1). At least five samples were taken for each time point or cell line as replicates. Microarray studies were performed by the Microarray Core Facility of the South Carolina.

College of Pharmacy, using the Agilent 4x44K whole mouse genome gene expression microarray kits.

Table 3.1 Total RNA samples used in gene expression profiling.

Group	Sample ID	Mouse Label	Type	Concentration (ng/μl)	RIN	Volume (μl)	Total RNA (μg)
Sham Liver	SHAM 165	ZY 191	LIVER	472	9.30	50.00	23.60
	SHAM 166	ZY 192	LIVER	530	9.40	50.00	26.50
	SHAM 167	ZY 193	LIVER	299	9.10	50.00	14.95
	SHAM 168	ZY 194	LIVER	1065	9.40	50.00	53.25
	SHAM 169	ZY 195	LIVER	614	9.20	50.00	30.70
	SHAM 170	ZY 196	LIVER	740	9.10	50.00	37.00
Pre-Mets Liver	9 DAYS 85	ZY 237	LIVER	1701	8.50	50.00	85.05
	9 DAYS 86	ZY 238	LIVER	1420	8.50	50.00	71.00
	9 DAYS 88	ZY 240	LIVER	1068	8.40	50.00	53.40
	9 DAYS 89	ZY 241	LIVER	1634	8.50	50.00	81.70
	9 DAYS 90	ZY 242	LIVER	989	8.40	50.00	49.45
	9 DAYS 91	ZY 243	LIVER	1016	8.40	50.00	50.80
Post-Mets Liver	METS 69	ZY 206	LIVER	1347	9.40	30.00	40.41
	METS 72	ZY 209	LIVER	2046	9.50	30.00	61.38
	METS 158	ZY 211	LIVER	1039	9.40	50.00	51.95
	METS 159	ZY 212	LIVER	1676	9.40	50.00	83.80
	METS 162	ZY 221	LIVER	1359	9.40	50.00	67.95
	METS 163	ZY 224	LIVER	1251	9.20	50.00	62.55
CT26	CT26 119	N/A	CELL	2089	10.00	40.00	83.56
	CT26 120	N/A	CELL	2318	10.00	40.00	92.72
	CT26 121	N/A	CELL	2696	10.00	40.00	107.84
	CT26 122	N/A	CELL	2633	10.00	40.00	105.32
	CT26 171	N/A	CELL	910	9.90	45.00	40.95
CT26-FL3	FL3 123	N/A	CELL	2502	10.00	40.00	100.08
	FL3 124	N/A	CELL	2629	10.00	40.00	105.16
	FL3 125	N/A	CELL	2753	10.00	40.00	110.12
	FL3 126	N/A	CELL	3206	10.00	40.00	128.24
	FL3 173	N/A	CELL	872	9.70	45.00	39.24

### 3.2 RESULTS FROM MICROARRAY ANALYSIS

After background correction and normalization of raw data at the Microarray Core Facility, RNA expression levels from microarray analysis were uploaded and analyzed by the Gene Sifter software (Geospiza). Considering the small number of

samples in groups, the nonparametric Wilcoxon rank-sum test was utilized to determine the differentially expressed genes, because other available t-test statistics assume that the underlying distribution of data values for two groups is normal distribution, which is usually used to estimate large number of samples. All the comparisons were computed and applied by the Benjamini and Hochberg correction. Genes with fold changes (FC) in expression greater or equal to 2 (up or down) and p-value smaller than 0.05 were considered to be statistically and differentially expressed, respectively.

Using these cut-off values, 1177 genes were found to be differentially expressed in Group 1, amongst which 487 were up-regulated and 690 were down-regulated in the CT26-FL3 cell line as compared to the CT26 cell line. In Group 2 three pairwise comparisons were set up: Pairwise A (Sham Liver vs. Pre-Metastatic Liver), Pairwise B (Pre-Metastatic Liver vs. Metastatic Liver), and Pairwise C (Sham Liver vs. Metastatic Liver). In Pairwise A, 659 genes were found to be differentially expressed, amongst which 615 were up-regulated and 44 were down-regulated. In Pairwise B, 2095 were found to be differentially expressed, 977 of them were up-regulated and 1118 were down-regulated. In Pairwise C, 2987 genes were found to be differentially expressed, 2323 of them were up-regulated and 664 were down-regulated (Table 3.2).

Table 3.2 Statistics of differentially expressed genes. Changes in gene expression with p-values smaller than 0.05 and fold changes (FC) greater or equal to 2 (up or down) were considered to be statistically significant and differentially expressed.

Group	Sample	Pairwise	Total	Up	Down
1	Cell pellet	CT26 vs. CT26-FL3	1177	487	690
2	Liver tissue	Sham vs. 9 Days	659	615	44
		9 Days vs. Mets	2095	977	1118
		Sham vs. Mets	2987	2323	664

A scatter plot of the average expression ratio showing the differentially expressed genes in each comparison is presented in Figure 3.2.

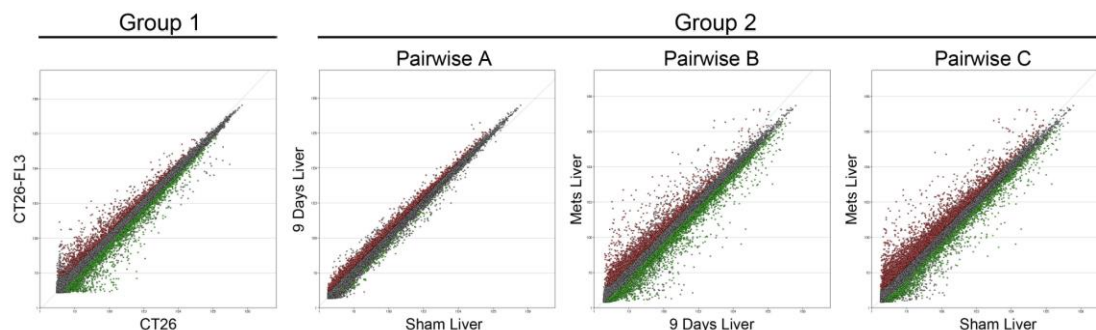


Figure 3.2 Scatter plots of average expression ratios. Differentially expressed genes were shown in CT26 cells vs. CT26-FL3 cells comparison and liver tissue comparisons at tumor bearing mice during liver metastasis. Green spots represent down-regulated genes and red spots represent up-regulated genes in Y-axis as compared to X-axis samples.

### 3.3 COMPARISON OF GENE EXPRESSION IN CT26-FL3 VERSUS CT26 CELLS

Comparison of the genetic signatures of CT26-FL3 and CT26 cells were designed to identify genetic changes that confer the properties needed for invasion and metastasis by cancer cells. Because it is derived from the parental CT26 cells, CT26-FL3 maintains some degree of genetic homology with CT26 but presents a much higher tendency for liver metastasis. Previous studies have shown that even within the same tumor, cancer cells display substantial heterogeneity in virtually all distinguishable phenotypic features such as cellular morphology, gene expression, metabolism, motility, and angiogenic, proliferative, immunogenic, and metastatic potential (Marusyk and Polyak 2010). This diversity can endow metastatic cancer cells with genetic properties that direct their unique organ specificities which have been elegantly shown by Massague et al (Gupta, Minn et al. 2005) in breast cancer as well as different capabilities to construct its tumor stroma and manipulate their relationship with their host. It is therefore possible that the process of *in vivo* selection resulted in the sorting and enrichment of a subgroup of cells within the parental CT26 cell line that have at least two special qualifications: the

capability to manipulate the host-tumor interactions to promote metastasis and enhanced homing and adaptability to the liver. Thus, comparing CT26-FL3 with CT26 could identify the altered genes that support those two properties in CT26-FL3 cells.

After determining the significantly differential genes by pairwise comparison of CT26 and CT26-FL3, a KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis using GeneSifter was performed to understand the biological processes involved in the alteration from CT26 to CT26-FL3. Z-scores indicate whether a pathway is hit more or less frequently than expected by chance and were calculated in GeneSifter using the following formula:  $z\text{-score} = [r - n(R/N)] / \sqrt{[v((n(R/N))(1 - R/N)(1 - ((n - 1)/(N - 1))))]}$ ; where R = total number of genes meeting selection criteria, N = total number of genes measured, r = number of genes meeting selection criteria with the specified gene ontology (GO) term, and n = total number of genes measured with the specific GO term. Z-scores greater than or equal to +2.0 or less than or equal to -2.0 are suggestive of biological significance, indicating that the expression of more (z = positive no.) or fewer (z = negative no.) genes in a particular KEGG/GO pathway were altered than would be expected by random occurrence (Nijland, Schlabritz-Loutsevitch et al. 2007).

Based on the z-score report, the top 10 affected pathways in this comparison are shown in Table 3.3. Among these ten most significantly affected pathways, five resulted from up-regulated genes (z-score up) and five resulted from down-regulated genes (z-score down). Interestingly, the pathway most affected by up-regulated genes is that involved in cytokine-cytokine receptor interaction. The Jak-STAT signaling pathway which is the principal signaling mechanism for a wide array of cytokines and growth factors is also highly affected. The other three pathways that are involved in Toll-like

receptor signaling, cytosolic DNA-sensing, and African trypanosomiasis include genes that are mainly involved in inflammation and activation of innate immunity, but the immune responses induced by these pathways include the induction of cytokines that induce other cytokines such as TNF, IFN, and interleukins. Therefore, compared with

Table 3.3 Top 10 affected pathways between CT26 vs. CT26-FL3 cells based on the Z-score by KEGG pathway analyses using GeneSifter microarray analysis software.

KEGG Pathway	List	Up	Down	Gene Set	z-score (Up)	z-score (Down)
Cytokine-cytokine receptor interaction	60	37	23	247	<b>8.22</b>	0.72
Toll-like receptor signaling pathway	27	20	7	97	<b>7.8</b>	-0.32
Cytosolic DNA-sensing pathway	14	13	1	51	<b>7.33</b>	-1.61
African trypanosomiasis	10	9	1	30	<b>6.82</b>	-0.96
Jak-STAT signaling pathway	31	22	9	147	<b>6.28</b>	-0.88
Steroid biosynthesis	13	1	12	18	0.23	<b>9.13</b>
Focal adhesion	54	12	42	197	1.15	<b>6.93</b>
Terpenoid backbone biosynthesis	8	0	8	14	-0.81	<b>6.74</b>
ECM-receptor interaction	25	2	23	84	-0.92	<b>6.53</b>
Amoebiasis	38	13	25	114	3.65	<b>5.47</b>

CT26 cell, the biological processes and phenotypic features mainly affected by the up-regulated genes in CT26-FL3 cells are related to induction of inflammation and immune responses, particularly, cytokine expression. Cytokines and growth factors are usually viewed as signaling molecules in mammals, but they are also used by cancer cells to interact reciprocally with the supporting tumor-associated stroma.

The next five most affected pathways result from the down-regulation of genes involved in focal adhesion and ECM-receptor interactions that mediate cell communication with the extracellular environment. These genes play essential roles in cell proliferation, cell motility, and adhesion. Steroid biosynthesis and terpenoid backbone biosynthesis are involved in metabolism, while genes in the amoebiasis pathway typically relate to pathogen infection and induce a series of immune responses to release various pro-inflammatory factors such as TNF and IL-6.



Since many genes encoding cytokines and cytokine receptors were found to be significantly affected, unsupervised agglomerative hierarchical cluster analysis was conducted on the differentially expressed genes between the CT26 and CT26-FL3 cells. The goal of this analysis was to determine if the differentially expressed genes had the capability for classifying the CT26 and CT26-FL3 cell samples into two groups. To perform this analysis, the microarray results were calculated using the Cluster software for clustering, and then graphically visualized and examines using the software TreeView.



Figure 3.3 Unsupervised hierarchical cluster analysis of gene expression in CT26 cell and CT26-FL3 cell samples. Red = up-regulated, Green = down-regulated, and Black = unchanged.

The result showed that all eight cell samples were separated into two groups, one group encompassing the 4 samples from CT26 cells and the other group formed by the 4

samples from the CT26-FL3 cells (Figure 3.3). The correct classification of the samples into two outcome groups by cluster analysis when utilizing the differentially expressed genes in cytokine-cytokine receptor interaction pathway showed the significant disparity between CT26 and CT26-FL3 cells with respect to this biological process. Because cancer cells release cytokines and other signals to induce host-tumor interactions and immune responses, the expression of the up-regulated genes in this pathway was validated by real-time PCR (Figure 3.4). The outcomes verify the results observed in the microarray analyses.

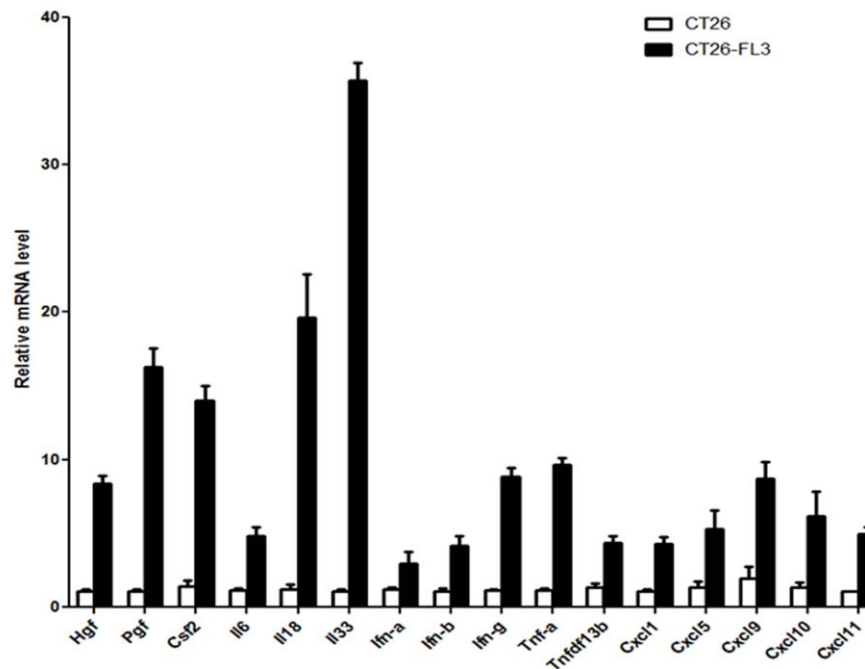


Figure 3.4 Significantly up-regulated genes encoding cytokines in CT26-FL3 cells compared to the CT26 cells.

In summary, KEGG pathway analysis of the results from the pairwise comparison the gene expression signatures of CT26 and CT26-FL3 cells revealed that the principal transitions in biological processes from CT26 to CT26-FL3 lie in the capacity to communicate and interact among the cancer cells, tumor stroma, ECM and host. In

particular, CT26-FL3 cells express genes that can promote activation of immune responses and release of cytokines which can activate various signaling pathways. Thus, CT26-FL3 cells might be more active and better manipulators of the host environment to facilitate its metastasis to the distant organ.

Furthermore, pairwise comparison the gene expression signatures of CT26 and CT26-FL3 cells also disclosed the most significantly differential genes. The top 10 most significantly up-regulated and down-regulated genes are listed in Table 3.4 and Table 3.5. Interestingly, a number of the genes listed in these tables were recently found to play important roles in carcinogenesis and metastasis.

Table 3.4 Top 10 most significantly up-regulated genes in CT26-FL3 as compared to CT26 cells.

ID	Gene Name	Ratio	Pathway
Raly1	RALY RNA binding protein-like	80.7	Nucleotide binding
Bcl11b	B-cell leukemia/lymphoma 11B	44.21	Lymphocyte Signaling
Grin3b	Glutamate receptor, ionotropic	38.14	Glutamic acid signaling
Il33	Interleukin 33	34.41	Cytosolic DNA-sensing pathway
Cck	Cholecystokinin	17.41	Rhodopsin-like receptors
Tnc	Tenascin C	16.56	ECM-receptor interaction
Acs16	Acyl-CoA synthetase long-chain family member 6	15.65	PPAR signaling pathway
Ttc12	Tetratricopeptide repeat domain 12	14.85	n/a
Ctss	Cathepsin S	14.74	Antigen processing and presentation
Ido1	Indoleamine 2,3-dioxygenase 1	13.58	African trypanosomiasis

Table 3.5 Top 10 most significantly down-regulated genes in CT26-FL3 as compared to CT26 cells.

ID	Gene Name	Ratio	Pathway
Csf2ra	colony stimulating factor 2 receptor, alpha, low-affinity	90.76	Cytokine-cytokine receptor interaction
Chst7	Carbohydrate sulfotransferase 7	48.43	Glycosaminoglycan biosynthesis
Pdgfb	Platelet derived growth factor, B polypeptide	33.32	MAPK signaling pathway
Lilrb4	Leukocyte immunoglobulin-like receptor, subfamily B, member 4	24.77	Osteoclast differentiation
Akr1c14	Aldo-keto reductase family 1, member C14	23.5	Steroid hormone biosynthesis
Runx1t1	Runt-related transcription factor 1; translocated to 1	21.16	Acute myeloid leukemia
Ccl9	Strain SJL/J small inducible cytokine A10	19.58	Cytokine-cytokine receptor interaction
Tnnt2	Troponin T Type 2	18.47	Cytoskeletal Signaling
Pmp22	Peripheral myelin protein 22	15.34	Neural Crest Differentiation
F11r	F11 receptor	13.86	Cell adhesion molecules

For example, tenascin C (Tnc) was reported in breast cancer as a metastatic niche component for colonization of the lungs (Oskarsson, Acharyya et al. 2011). Cathepsin S (Ctss) was proven to mediate gastric cancer cell migration and invasion via a putative network of metastasis-associated proteins (Yang, Lim et al. 2010). Indoleamine 2,3-dioxygenase 1 (Ido1) was identified as a nodal pathogenic driver of lung cancer and metastasis development (Smith, Chang et al. 2012), while peripheral myelin protein 22 (Pmp22), found as a significantly down-regulated gene, was reported as an independent prognostic factor for disease-free overall survival in breast cancer patients (Tong, Heinze et al. 2010). These discoveries serve as indirect evidence to support the reliability of the microarray result, and its potential value in identifying pivotal genes in liver metastasis of colon cancer.

### 3.4 ANALYSIS OF CHANGES IN GENE EXPRESSION IN THE LIVER MICROENVIRONMENT AT DIFFERENT STAGES OF COLON CANCER DEVELOPMENT AND METASTASIS

The comparison of gene expression in liver tissue at different stages of colon cancer progression was aimed at determining the genetic changes in the target organ of metastasis using mouse model of colon cancer liver metastasis (the soil in the “seed & soil” hypothesis). Since CT26-FL3 cells reliably gives rise to considerable liver metastasis in this model, liver tissues from mice bearing CT26-FL3-derived tumors in cecum were used to optimize the ability to detect changes in gene expression in the liver during cancer progression. In particular, the pre-metastatic livers from tumor bearing mice before the arrival of cancer cells can be trusted as valuable resource that might

provide important information on the genetic and cellular changes in the early stage of liver metastasis. This is significant because very little is known about the genetic, molecular, and cellular mechanisms at this critical stage of metastasis when early diagnosis and intervention can be very beneficial to the patient

Unlike the comparison of CT26 and CT26-FL3 cells, the gene identified in the liver tissue do not necessarily come from one type of cells and the expression of any particular gene represents the overall level in the liver tissue. The differentially expressed genes from the microarray comparisons could be derived from hepatocytes, immunocytes, endothelial cells, or any other cell types present in the liver tissues, this made pathway analysis used in cell comparison inadequate. Therefore, in this study pairwise comparisons of the gene expression levels in the liver were performed at the following time points: Pairwise A: Normal liver tissue vs. Pre-metastatic liver tissue (0 day vs. 9 days); Pairwise B: Pre-metastatic liver tissue vs. Metastatic liver tissue (9 days vs. 28 days); and Pairwise C: Normal liver tissue vs. Metastatic liver tissue (0 day vs. 28 days). Pairwise A was used to identify genes that potentially regulate the establishment of the pre-metastatic niche at the early stage of metastasis. Pairwise B and C were used to identify genes that might be required to maintain metastatic lesions upon establishment or promote re-metastasis of cells to other organs.

The results showed that the number of significantly changed genes found by pairwise comparison of liver tissues at various stages of colon cancer metastasis was high and belonged to a variety of biological pathways. The top 10 most significantly up-regulated and down-regulated genes are listed in Table 3.6.

Table 3.6 Top 10 most significantly altered genes in the liver at different stages of metastasis.

Liver: Sham vs. 9 Days (Pre-metastatic liver)

ID	Gene Name	Ratio	Direction	Pathway
Prph	Peripherin	7.31	Up	Cytoskeleton remodeling Neurofilaments
Rab11fip4	MKIAA1821 protein	5.87	Up	Endocytosis
Fgf21	Fibroblast growth factor 21	5.22	Up	MAPK signaling pathway
Il18r1	Interleukin 18 receptor 1	4.95	Up	Cytokine-cytokine receptor interaction
Nr1d1	Nuclear receptor subfamily 1,D1	4.82	Up	NF-kappaB Signaling
Ki67	Mki67	4.40	Up	Cell Cycle / Checkpoint Control
Crot	Carnitine O-octanoyltransferase	4.34	Up	Peroxisome
Ppp1r3g	Protein phosphatase 1, r3G	4.30	Down	n/a
Ccrn4l	NOCTURNIN	4.24	Down	Cell Cycle Control by BTG Proteins
Cx3cr1	Chemokine (C-X3-C) receptor 1	4.09	Up	Chemokine signaling pathway

Liver: 9 Days (Pre-metastatic) vs. Mets (Metastatic)

ID	Gene Name	Ratio	Direction	Pathway
Reg3b	Regenerating islet-derived 3b	311.01	Up	n/a
Hsd3b5	Hydroxy-delta-5-steroid dehydrogenase, 3b5	266.94	Up	Steroid hormone biosynthesis
Lcn2	SV-40 induced 24p3	140.45	Up	n/a
Sult1e1	Sulfotransferase family 1E1	136.50	Up	Steroid hormone biosynthesis
Hsd3b4	Hydroxy-delta-5-steroid dehydrogenase, 3 beta4	133.02	Up	Steroid hormone biosynthesis
Fmo3	Flavin containing monooxygenase 3	107.31	Up	Drug metabolism - cytochrome P450
Slco1a1	Organic anion transporting polypeptide 1	95.94	Up	Bile secretion
Igdcc4	DDM36E	85.74	Down	n/a
Bmper	BMP-binding endothelial regulator	82.67	Down	n/a
Ngp	Neutrophilic granule protein	79.59	Up	Ribosome biogenesis in eukaryotes

Liver: Sham vs. Mets (Metastatic)

ID	Gene Name	Ratio	Direction	Pathway
Lcn2	SV-40 induced 24p3 mRNA	388.24	Up	n/a
Hsd3b5	Hydroxy-delta-5-steroid dehydrogenase, 3b5	212.34	Down	Steroid hormone biosynthesis
Reg3b	Regenerating islet-derived 3b	201.98	Up	n/a
Ngp	Neutrophilic granule protein	167.94	Up	Ribosome biogenesis in eukaryotes
Stfa2l1	Stefin A2 like 1	141.38	Up	n/a
Igdcc4	DDM36E	124.04	Up	n/a
Hsd3b4	Hydroxy-delta-5-steroid dehydrogenase, 3b4	111.77	Down	Steroid hormone biosynthesis
Camp	Cathelicidin antimicrobial peptide	91.55	Up	Tuberculosis
Bmper	BMP-binding endothelial regulator	90.69	Up	n/a
Sult1e1	Sulfotransferase family 1E, member 1	90.16	Up	Steroid hormone biosynthesis

These genes potentially play a role in metastasis-related interactions between cancer and host cells that promote the progression of metastasis in the liver. Since cytokines are commonly the molecular signals that mediate these interactions, analysis of cytokines and cytokine receptor pathways can lead to the identification of metastasis-promoting host-tumor interactions. Therefore, the genes involved in cytokine-cytokine receptor interaction were focused on in this study.

As known, the changes in the liver microenvironment during the progression of metastasis are a dynamic process. To begin to understand the role of the differentially regulated cytokine-related genes in the process of liver metastasis, the results from pairwise A, B and C with respect to the time of induction or repression of each identified cytokine-related genes were combined to determine their sequential expression pattern as liver metastasis progressed. This analysis will allow us to infer their potential roles, based on the assumption that tumor progression dictates genetic alterations in the liver in response to stimuli emanating from the primary tumor through endocrine and paracrine mechanisms, self-regulation of the liver under pathological pressure, and varying liver components such as cell death, proliferation and inflammatory cell infiltration.

Based on the features of the microarray analysis, eight possible sequential patterns of gene expression were defined and enumerated (Figure 3.5). Then all the cytokine-related genes whose expression was significantly changed by at least two-fold in the liver tissues were identified and assigned into one of the eight types of sequential expression patterns, according to their appearances in the pairwise comparisons in A, B, and C (Table 3.7).

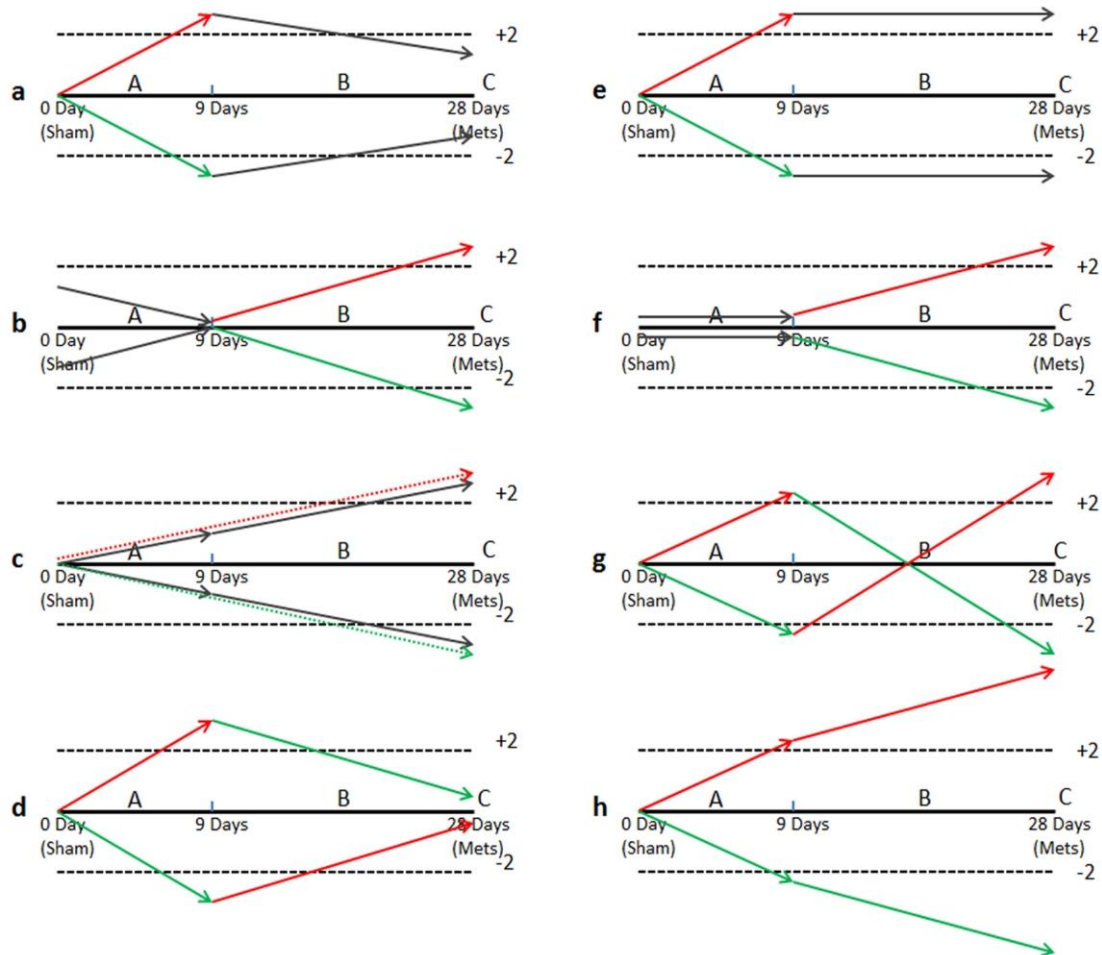


Figure 3.5 Sequential expression patterns of cytokine-related genes in liver during metastasis. A, B, and C represents the three pairwise comparison of liver tissues at three time points during the progression of metastasis (0 day, 9 days and 28 days). Green arrow means down regulation of gene expression, red arrow means increasing gene expression, black arrow means no significant change in expression, 2 means statistically significant two-fold change in expression.

Assuming that the cancer cells continued to influence gene expression in the liver as the tumor continued to grow and metastasis progressed, the molecular signals that mediate the cross-talk between the primary tumor and the liver will be more likely amplified in the liver. Thus, the genes encoding these signals that were up-regulated in the liver were particularly focused.

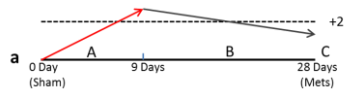
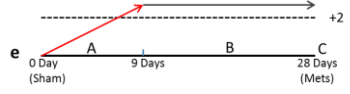
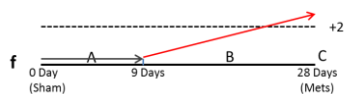
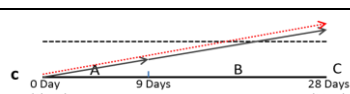
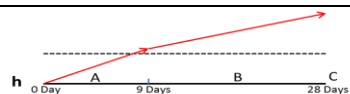


Table 3.7 Genes encoding cytokines follow sequential expression patterns. Up and Down means up-regulated or down-regulated during progression from normal to pre-metastatic to metastatic stage.

Pattern	Up	Down
a	Ccl2	N/A
b	N/A	Cxcl10, Pgf, Bmp4, Flt3l
c	Pbp, Il24, Ltb, Tnf, Pdgfb, Cxcl16, Tnfsf13b, Bmp5, Vegfc, Tgfb1	Vegfa
d	N/A	N/A
e	Bmp2, Csf1	N/A
f	Cxcl14, Cxcl13, Ccl6, Ccl8, Il6, Il1b, Il7, Il12a, Tnfsf12, Ccl24, Pf4, Ccl17, Kitl, Ccl12, Il1a	Cxcl9, Bmp8a, Vegfb, Tnfsf8, Ccl27a, Ccl25, Amh, Bmp6, Bmp7, Ifna9
g	N/A	N/A
h	Cxcl1, Hgf, Tnfsf15, Pdgfc	N/A

Based on the characteristics of trends shown in Table 3.7, the up-regulated genes were further classified into 3 categories and assign their role into a particular stage of metastatic progression (Table 3.8).

Table 3.8 Three categories of liver signal molecules during metastasis.

Category	Cytokine Gene	Pattern	Feature
Pre-Metastasis	Ccl2	a	
	Bmp2, Csf1	e	
Pre-Metastasis to Metastasis	Cxcl14, Cxcl13, Ccl6, Ccl8, Il6, Il1b, Il7, Il12a, Tnfsf12, Ccl24, Pf4, Ccl17, Kitl, Ccl12, Il1a	f	
Metastasis	Pbp, Il24, Ltb, Tnf, Pdgfb, Cxcl16, Tnfsf13b, Bmp5, Vegfc, Tgfb1	c	
	Cxcl1, Hgf, Tnfsf15, Pdgfc	h	

As shown in Table 3.8, genes from type a and e are up-regulated in the pre-metastasis stage, but either decrease or remain at similar level in the transition from pre-metastatic to metastatic stage. Genes from type f sequence were classified as metastasis factors because their expression levels only increase at the metastasis stage when the tumor has metastasized to the liver. On the other hand, genes from type c and h are likely involved throughout the metastasis progression because their expression levels increase in the pre-metastasis stage and continue to increase as the tumor cells arrive and establish themselves in the liver. The difference between type c and type h is that type c genes increase gradually as the metastasis is established and could be thought of as accompanying events, while type h genes are more potently expressed and could be critical events in metastasis. Based on this classification, the pre-metastasis signaling molecules Ccl2, Bmp2, Csf1 and the highly expressed pre-metastasis to metastasis signaling molecules Cxcl1, Hgf, Tnfsf15, and Pdgfc can be viewed having special roles in the pre-metastatic phase and may be involved in establishing the pre-metastatic niche. These genes could potentially be important biological markers for prediction or early diagnosis, or targets for preventing or interfering with liver metastasis in colon cancer.

In summary, applying the sequential expression pattern analysis to the differentially expressed genes encoding secretory signal molecules such as cytokines, chemokines, and growth factors indicate that their mRNA levels accumulate at different rates in the liver where they can exert functions such as stimulation and chemotaxis that can shape a unique environment at each stage of liver metastasis and dictate its interactions with the primary tumor. These interactions are most likely carried out by endocrine mechanisms in the pre-metastasis phase and then by both endocrine and

paracrine mechanisms in the metastasis phase as cancer and stromal cells are recruited to the liver, which could explain the higher expression levels in the metastasis phase as compared to the pre-metastasis phase.

In addition to the cytokine-related genes, other genes which that are classically associated with establishment of the pre-metastatic niche and or have been shown to promote metastasis were found in the liver tissue microarray analysis. These include S100a8, S100a9, Saa3, Mmp9, and Egfr. These genes follow similar sequential expression patterns as the cytokine-related genes including Cxcl1, Hgf and Lcn2 (the top 1 changed genes in liver). Their expression levels increase in the liver during the process of metastasis (Figure 3.6), suggesting that they are mediators of metastasis-promoting host-tumor interactions in the liver.

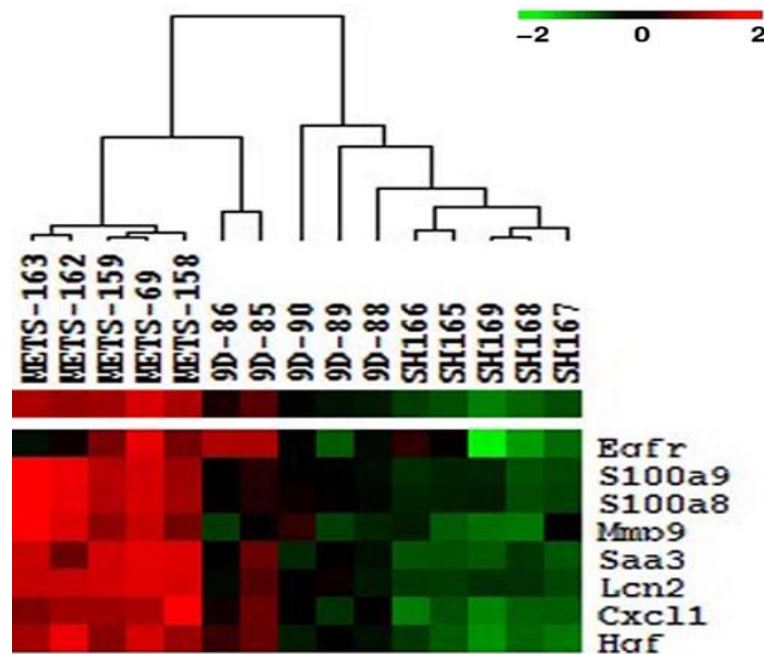


Figure 3.6 Unsupervised hierarchical cluster analysis of changes in gene expression in liver from Sham, pre-metastatic liver (9 Days), and liver with metastasis (Mets). Red = up-regulated, Green = down-regulated, and Black = unchanged.

### 3.5 SUMMARY AND DISCUSSION

In this chapter, RNA microarray analyses using an Agilent whole mouse genome 44x44K array was used to profile the gene expression signatures of colon carcinoma cells with varying potentials for metastasis and liver tissue from tumor bearing mice at various stages of metastasis with the ultimate goal of elucidating the complex crosstalk between the primary tumor and target organ that is required for metastasis. By KEGG pathway analysis of the results from the pairwise comparison gene expression in CT26 and CT26-FL3 cell lines, the highly liver metastatic CT26-FL3 cell line was found to have an enhanced ability for inducing immune responses and releasing cytokines. This implies that CT26-FL3 might be a better manipulator of host-tumor interactions than the CT26 parental cell line, which could contribute its highly liver metastasis tendency. Based on these observations, the sequential expression patterns of the differentially expressed genes cytokine-related genes obtained by pairwise comparisons of liver samples from mice bearing tumors from CT26-FL3 cells that were collected at 0, 9, and 28 days (4weeks) after cecum implantation were analyzed. A number of these genes have been proven to play important roles in metastasis including Hgf and Cxcl1 that were found to be elevated in all stages of liver metastasis examined. HGF can promote the growth, dissociation and migration of cancer cells, as well as promote basement membrane breakdown, angiogenesis, anti-apoptosis, etc. which are important for metastasis (Mizuno and Nakamura 2013). CXCL1 promotes tumor invasion and metastasis (Cheng, Wang et al. 2011), as well as manipulate the tumor microenvironment and host stromal cells to direct a signaling network that promotes metastasis (Acharyya, Oskarsson et al. 2012). Ccl2, which is elevated in the pre-metastasis stage, has been shown to mediate the cross-

talk between cancer cells and stromal fibroblasts (Tsuyada, Chow et al. 2012). In all, by the analyses of their sequential expression patterns, the genes that might direct various stages of metastatic progression were identified. Here the focus was the genes in the cytokine/cytokine-receptor pathways that encode cellular messengers that mediate the crosstalk between the primary tumor and the target metastatic organ. Other pathways remain to be analyzed. In all, the results from the microarray analyses could lead to the identification of genes or combination of genes that may be used for early diagnosis of metastasis, or targets of therapeutic intervention to alleviate morbidity and mortality from this disease.

Finally, some genes that were found to be highly altered in the metastatic tumor cells or in the target organ were located, such as *Il33* in cancer cells and *Lcn2* in liver tissue microenvironment. These genes have tremendous fold change and belong to top 10 changed genes, which imply a potential role in colon cancer liver metastasis. Studies examining the role of these genes in the seed and the soil are currently being undertaken.

## CHAPTER 4

### IDENTIFY GENES THAT MEDIATE METASTATIC-PRONE HOST-TUMOR INTERACTIONS IN COLON CANCER

In the previous chapter, a microarray technique was used to profile gene expression signatures of colon cancer cells and the target liver microenvironment to identify genes that promote liver metastasis of colon cancer. Mainly based on genes related to cytokines and cytokine receptors interactions, the characteristics of host-tumor interactions during liver metastasis were delineated in the mouse model. These dynamic host-tumor interactions induced by CT26-FL3 cells could be one of the most important factors that underlie the higher incidence of liver metastasis in tumor bearing mice as, compared to mice bearing tumors from the parental CT26 cells. Therefore, the goal of the studies in this chapter was to identify candidate genes which mediate host-tumor interactions to promote liver metastasis in colon cancer.

#### 4.1 DETECTING TARGET GENE FROM CANCER CELLS.

Although the host-tumor interactions are intricate and dynamic, the initiating molecular signals must come first from the cancer cells and induce reciprocal signaling responses from the host environment or target organ. Therefore, the candidate genes that mediate host-tumor interactions to promote liver metastasis should be found among the differentially expressed genes when comparing CT26 and CT26-FL3 cells. In table 3.4,

interleukin 33 (Il33) with a fold change of 34.41 belongs to the top 10 most significantly changed genes in CT26-FL3 as compared to CT26 cells. Il33 is a pivotal gene in the cytosolic DNA-sensing pathway. In immunology, this pathway is involved in host response to infection with viruses and other pathogens and can detect microbial RNA and DNA and subsequently activate downstream signaling pathways for the induction of interferons and proinflammatory cytokines (Cao 2009). Because the highly expressed cytokines and enhanced capability to induce immune responses were found in CT26-FL3 cells, Il33 became the top gene of interest.

#### 4.1.1 IL33 IS AN ALARMIN.

IL33 is a relatively newly discovered interleukin, that was first found in 2003, and is expressed in the nucleus of non-hematopoietic cells such as fibroblasts and epithelial and endothelial cells of various tissues (Moussion, Ortega et al. 2008). Because of its role in anti-viral responses and pro-inflammatory effects, IL33 was classified as an alarmin (Haraldsen, Balogh et al. 2009; Zhao and Hu 2010). Alarmins are a group of molecules that are the endogenous equivalent of pathogen-associated molecular patterns and they function to alert the host immune system of cell and tissue trauma (Coffelt and Scandurro 2008). They are rapidly secreted from stimulated leukocytes and epithelia, passively released from necrotic cells but not apoptotic cells, can activate receptor-mediated responses, and bridge cellular and adaptive immunity (Oppenheim and Yang 2005). As potent mediators of inflammation, alarmins play a fundamental role in the pathogenesis of a wide range of sterile or infection-induced immune and inflammatory disorders (Chan, Roth et al. 2012). Some alarmin molecules such as defensins, LL-37, high-mobility group

box 1 (HMGB1) protein, and S100 protein family have been recently addressed regarding their role in tumorigenesis and cancer progression (Salama, Malone et al. 2008).

Until now, the role of IL33 in cancer has not been widely studied; its functions were mainly discovered in immune diseases, like infection, asthma and allergy. IL33 is a member of the IL-1 superfamily of cytokines and its expression is up-regulated following pro-inflammatory stimulation. It can function both as a traditional cytokine and as a nuclear factor regulating gene transcription (Miller 2011). IL-33 mediates its biological effects by interacting with the receptors ST2 and IL-1 receptor accessory protein (IL1RAP), activating NF- $\kappa$ B and MAP kinase signaling pathways (Liew, Pitman et al. 2010) and stimulating the production of pro-inflammatory mediators, and induce IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 production (Moulin, Donze et al. 2007). On the other side, IL-33 strongly induces Th2 cytokine production from T helper 2 (Th2) cells and can promote the pathogenesis of Th2-related disease such as asthma (Miller 2011). Therefore, IL-33 appears to be an immune mediator in tissue damage or stress.

#### 4.1.2 ACTIVATION OF IL33-ASSOCIATED PATHWAYS IN COLON CANCER CELLS.

Based on the known functions of IL33, there were some indirect correlations suggesting that IL33 might play a role in mediating metastasis-promoting host-tumor interactions in the mouse model of colon cancer. First, KEGG pathways analyses of the pairwise comparison of CT26 and CT26-FL3 cell lines showed that the main affected pathways include cytokine-cytokine receptor interactions, Jak-STAT signaling pathway, toll-like receptor signaling pathway, cytosolic DNA-sensing pathway, and African



trypanosomiasis (Table 3.3). Similar to the cytosolic DNA-sensing pathway wherein IL33 is directly involved, activation of the toll-like receptor signaling pathway and the African trypanosomiasis pathway were also related to immune responses induced by pathogen infection. These pathways are involved in the MAPK signaling pathway which activates NF- $\kappa$ B to release numerous cytokines like TNF alpha, IFN, and IL6 (Pecaric-Petkovic, Didichenko et al. 2009). For example, IL6 found overexpressed in colon cancer is a major mediator of inflammation and an activator of signal transducer and activator of transcription 3 (STAT3) to block apoptosis in cells during the inflammatory process (Hodge, Hurt et al. 2005). These raise the possibility that IL33 can induce the MAPK signaling pathway to mediate the crosstalk with toll-like receptor signaling and African trypanosomiasis pathways. To verify this, total protein extract from CT26 and CT26-FL3 cell were analyzed by Western blotting to determine the expression levels of key molecules associated with these pathways. The results showed elevated expression of p-ERK, p-P38, and p-STAT3 in CT26-FL3 as compared to CT26 cells, indicating that the MAPK signaling pathway is activated in CT26-FL3 cells (Figure 4.1).

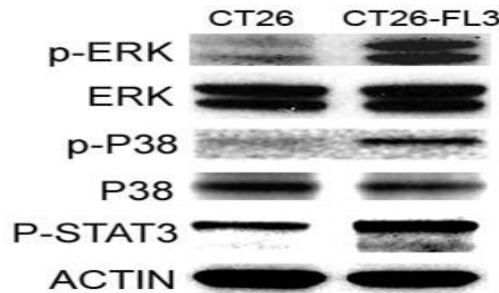


Figure 4.1 Activation of MAPK and STAT3 signaling in CT26-FL3 cells. Western blot analysis showed that CT26-FL3 cells had increased levels of proteins in the MAPK and STAT3 signaling pathways as compared to CT26 cells.

#### 4.1.3 IL33 EXPRESSION IN COLON CANCER

To begin to understand the role of IL33 in colon cancer liver metastasis, the expression levels of IL33 were validated in the colon cancer mouse model. The mRNA levels of IL33 and its receptor St2 were measured by real-time PCR. IL33 mRNA level in CT26-FL3 was found approximately 40-fold higher as compared to that in CT26 cells, which was consistent with the microarray data. On the other hand, the mRNA level for the St2 receptor in CT26-FL3 was only two-fold higher over that in CT26 cells (Figure 4.2a). Also the intracellular protein levels of IL33 from total cells extracts were determined by Western blotting. The results showed that intracellular levels of IL33 in CT26-FL3 cells were elevated only by approximately 2- to 3-fold, in spite of the 40-fold increase in mRNA levels (Figure 4.2b). This suggests that most of the IL33 is probably secreted from the cancer cells into the surrounding microenvironment where it could exert its effect on cells in the tumor stroma or target organ. Therefore, the protein levels of IL33 in blood serum from mice bearing tumors from CT26 or CT26-FL3 cells, or from sham control mice were determined by western blotting, using albumin as a control for equal loading in each lane. The results showed that sera from mice bearing CT26-FL3 derived tumors had a higher level of IL33 as compared to sera from mice bearing tumors from CT26 cells (Figure 4.2c) while sera from sham injected control mice had basal levels of IL33 (Figure 4.2c). Immunohistochemical staining for IL33 in primary tumor sections from the cecum further showed that IL33 levels were higher in tumors derived from CT26-FL3 as compared to those from CT26 (Figure 4.2d). Collectively, these results indicated that the highly metastatic cell line CT26-FL3 can secrete higher levels of

IL33 into circulation where it can potentially influence the host tumor microenvironment in a paracrine fashion.

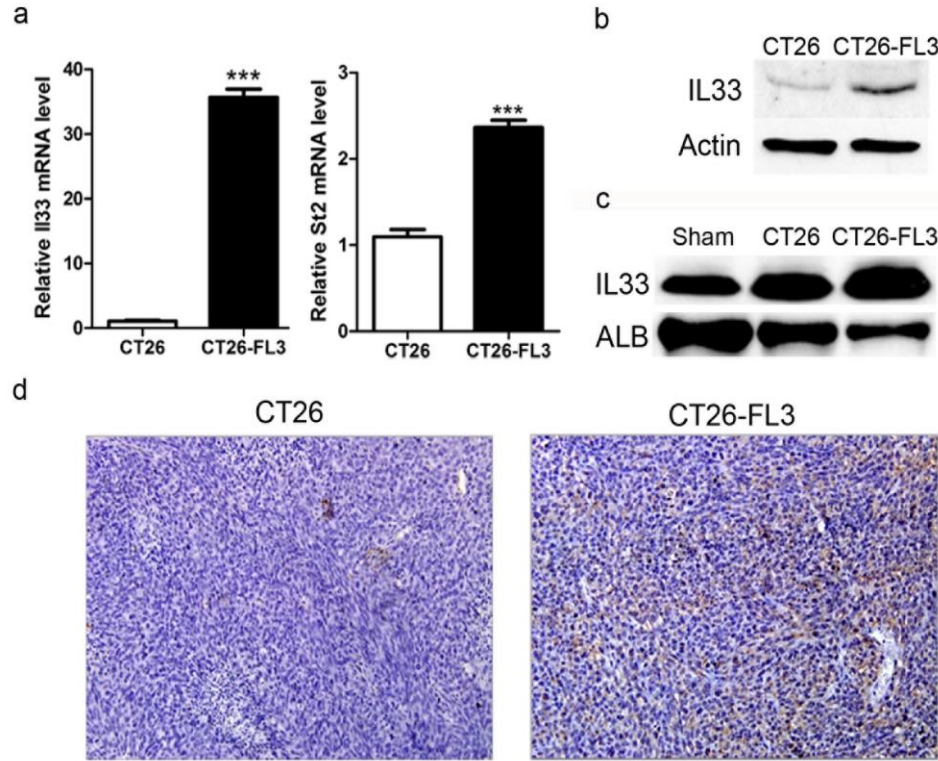


Figure 4.2 Increased expression of IL33 in highly metastatic CT26-FL3 cells and in tumors derived from these cells. a. mRNA levels of IL33 and its receptor St2 in CT26 and CT26-FL3 were measured by qRT/PCR. The mRNA expression levels were normalized against  $\beta$ -actin mRNA. b. Total protein extracts from CT26 and CT26-FL3 cells were analyzed by Western blotting to detect IL33 protein levels. c. Sera taken from mice bearing tumors from CT26 or CT26-FL3 cells or sham injected mice at four weeks after cecal implantation were analyzed by Western blotting to detect serum levels of IL33. d. Immunohistochemical analysis of sections from primary cecal tumors derived from CT26 and CT26-FL3 using antibodies against IL33.

To determine the stage in tumor development at which IL33 expression becomes elevated, its mRNA level was measured in early stage non-invasive, non-metastatic intestinal adenomas in the  $Apc^{Min/+}$  mouse, a genetic model of intestinal tumorigenesis.

The  $Apc^{Min/+}$  mouse is derived from the C57BL/6J background and has a mutation in the tumor suppressor *Apc* (Adenomatous polyposis coli) gene. These mice spontaneously develop multiple adenomas in the small intestine with a few in the colon. Tumors and non-tumor regions of the small intestine were collected from  $Apc^{Min/+}$  mice and from normal intestinal tissues from wild type C57BL/6J mice.

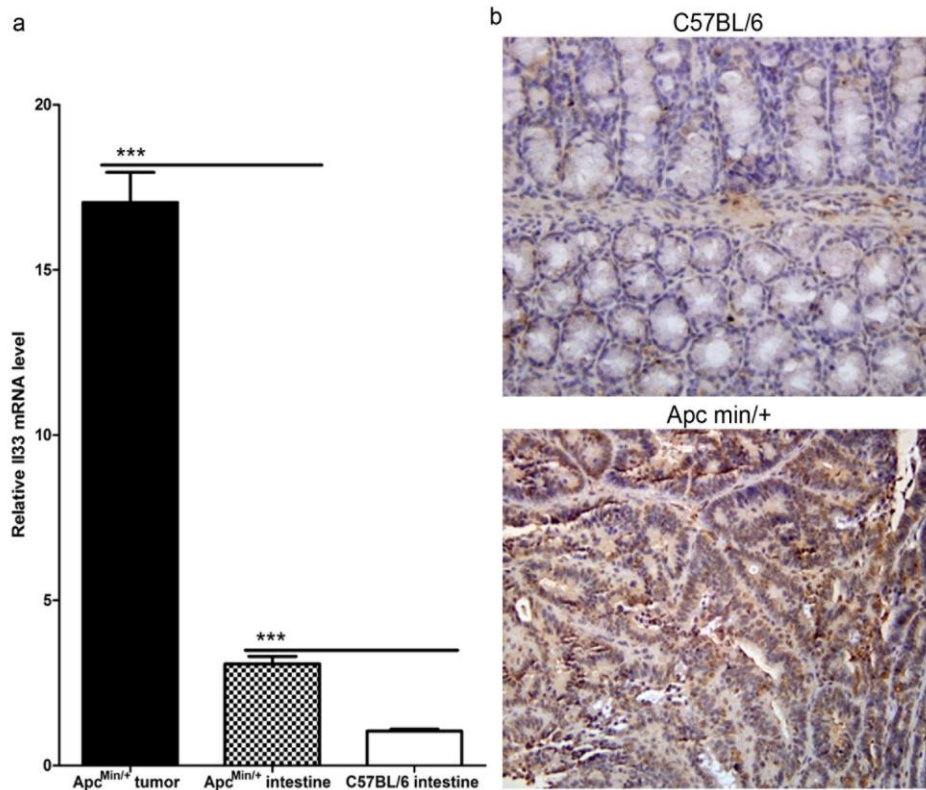


Figure 4.3 Increased expression of IL33 in tumor tissue from  $Apc^{Min/+}$  mice. a. mRNA levels of IL33 in tumor and intestine from  $Apc^{Min/+}$  mice, and intestine from C57BL/6J mice were measured by qRT/PCR. The mRNA expression levels were normalized against  $\beta$ -actin mRNA. b. Immunohistochemical analysis of sections from tumor tissue of  $Apc^{Min/+}$  and intestinal tissue from C57BL/6J mice using antibodies against IL33.

mRNA was isolated from these tissues and IL33 mRNA levels were determined by real-time PCR (Figure 4.3a). IL33 protein levels were assessed by immunohistochemical staining of adenomas from  $Apc^{Min/+}$  mice and intestinal tissues from wild type mice

(Figure 4.3b). The results showed that even in early stage adenomas, before the tumor becomes invasive, IL33 mRNA levels were already elevated by approximately 7-fold over non-tumor intestinal sections from Apc<sup>Min/+</sup> mice or 17-fold over normal intestinal sections from non-tumor bearing C57BL/6 wild type mice. Immunohistochemical analysis further showed that IL33 was highly expressed in tumor tissue from Apc<sup>Min/+</sup> mice as compared to normal intestinal sections from C57BL/6 mice.

To determine if the IL33 is similarly induced in human colorectal cancer, tissues representing different stages of cancer progression from colon cancer patients were acquired from tissue bank at the Center for Colon Cancer Research (CCCR) of the University of South Carolina. Analyses of these cells would validate the correlation of IL33 expression observed in the mouse model to that of colon cancer in the clinical setting. Tissue sections from non-tumor regions and from stage 1, 2, 3, and 4 from colon cancer patients were analyzed by immunohistochemical staining to assess the levels of IL33 and ST2 proteins. Based on the staining intensity, the results indicated that the expression levels of both IL33 and ST2 are associated with colon cancer stages; they increase during the progression of colon cancer in patients (Figure 4.4a). To semi-quantify the expression levels of IL33 and ST2, total RNA was isolated from tissues and measured by real-time PCR. Although variations in tissue samples from same stage existed, the trend of IL33 and ST2 expression was consistent with results from immunohistochemical staining (Figure 4.4b). The low levels of IL33 and ST2 observed in samples from stage 4 cancer may have resulted from the massive necrosis of cancer cells found inside the tumor lesion that typically occurs at the late stage of cancer. It is unclear why the mRNA expression of ST2 is higher in non-tumor region of the patient samples. It

is possible that elevated levels of IL33 in the patient could induce the expression of St2; further studies and a larger number of tissue samples will need to be examined.

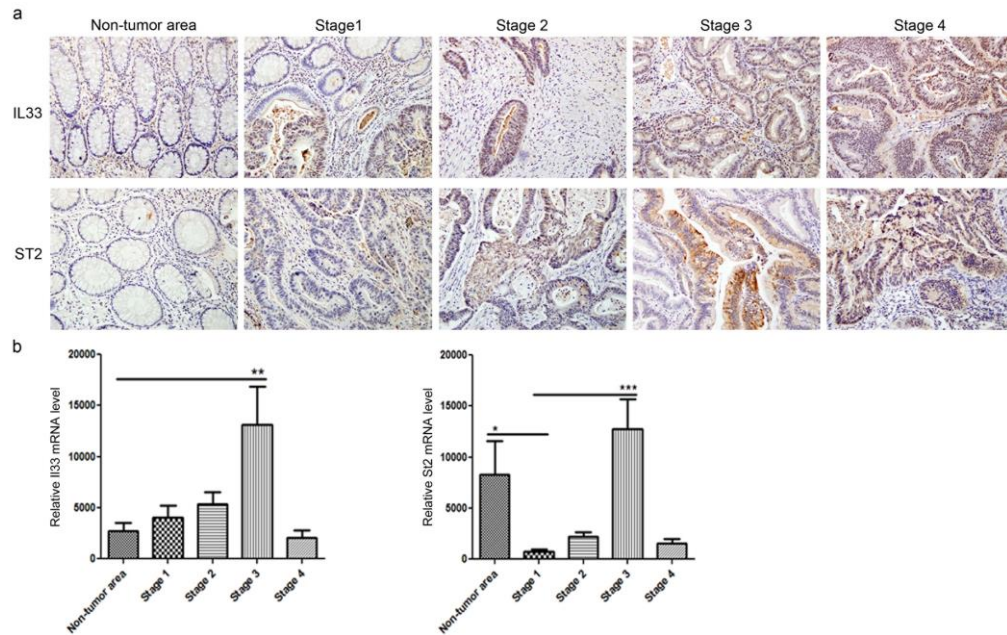


Figure 4.4 Expression levels of IL3 and ST2 are associated with advancing stages of colon cancer in patents samples. a. Immunohistochemical analysis of sections from tissue from colon cancer patients at stage 1, 2, 3, and 4. Non-tumor region were collected from intestine non-tumor area patients at stage 1, 2 and 3. b. mRNA levels of Il33 and St2 tissue from colon cancer patients at stage 1, 2, 3, and 4 were measured by qRT/PCR. The mRNA expression levels were normalized against  $\beta$ -actin mRNA.

In summary the results from qRT/PCR and immunohistochemical staining indicate that both in mouse and human colon cancer tissues, IL33 mRNA and protein levels are elevated as early as the adenoma stage, suggesting an important and early role in the etiology of the disease.

#### 4.1.4 THE ROLE OF IL33 IN COLON CANCER LIVER METASTASIS

To explore the role of IL33 in colon cancer progression and liver metastasis, the mouse IL33 cDNA was cloned into the expression plasmid pcDNA3.1 (Figure 4.5a). In

order to utilize the Il33 knockout mouse which is in the C57BL/6 genetic background, the C57BL/6-derived mouse colon carcinoma cell line MC38 was transfected with either pcDNA3.1 empty vector or pcDNA3.1-mIl33 and stable transfectants were generated by selection in zeocin. mRNA and protein levels of mouse Il33 were assessed by qRT/PCR and western blotting, respectively, to verify its expression in the stable transfectants. As shown in Figure 4.5 b, Il33 mRNA levels in two single clones isolated from the stable transfectants, IL33-1 and IL33-2 were approximately 60-fold higher than that in untransfected MC38 cells, and in cells that were transfected with the empty vector. As previously observed in CT26 and CT26-FL3 cells, protein levels in IL33-1 and IL33-2 were approximately two-fold higher than the untransfected and vector only transfected cells in spite of the high mRNA levels.

To determine the effect of increase Il33 expression on tumor growth and metastasis,  $2 \times 10^5$  MC38-vector or MC38-mIl33 cells were harvested and injected into spleen of eight-week old C57BL/6 mice (ten mice each). After 3 weeks, mice were sacrificed and tissues isolated for histological analysis. Results showed that 100% of the injected mice developed a tumor in the spleen; however, the tumor sizes in mice injected with MC38-mIl33 cells were significantly larger than that in mice injected with MC38-vector cells. The weights of spleen bearing tumors from MC38-mIl33 cells were about 5 folds heavier as compared to those from MC38-vector cells (Figure 4.5 f), suggesting that the MC38 cells overexpressing Il33 exhibited an increased proliferation *in vivo* as compared to the vector controls. Moreover, in 100% of mice (10 out of 10) injected with MC38-mIl33 cells, multiple visible nodular metastatic tumors were observed in the liver.



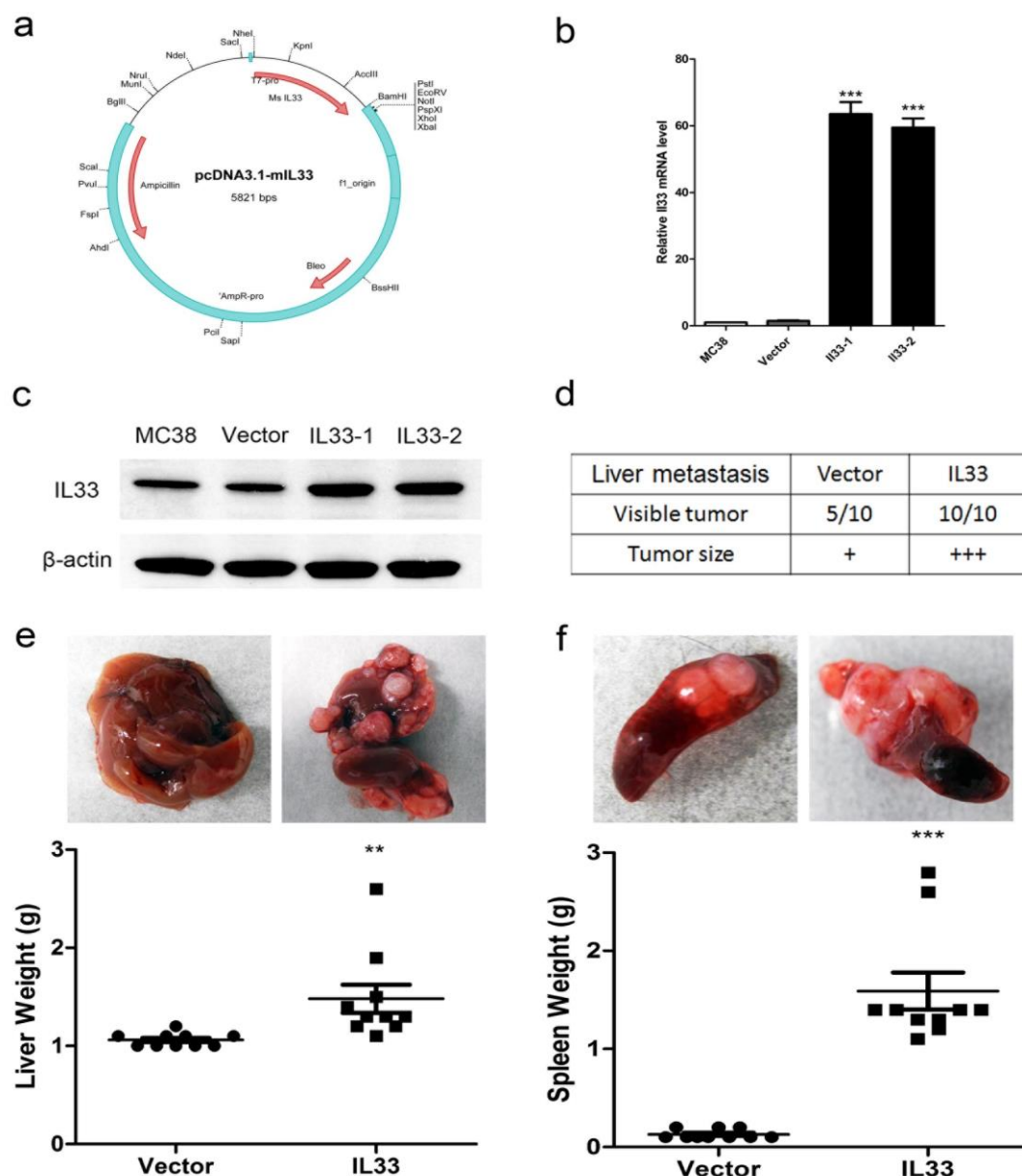


Figure 4.5 Overexpression of IL33 promotes tumor malignancy and liver metastasis of colon cancer in mice. a. pcDNA 3.1-mIL33 plasmid. b and c. expression levels of IL33 from MC38 stable transfected with pcDNA3.1 or pcDNA3.1-mIL33 were measured by qRT/PCR (b) and western blotting (c). d. Incidence of liver metastasis and tumor size of mice injected with MC38-vector or MC38-mIL33 cells in spleen. (+ indicate mild, ++ moderate, +++ severe.) e and f. The weight of liver and spleen of mice injected with MC38-vector or MC38-mIL33 cells in spleen.

On the other hand, in mice injected with MC38-vector cells, only 50% of mice (5 out of 10) developed metastatic tumors in the liver and the tumor size were considerably



smaller than that of mice injected with MC38-mIL33 cells (Figure 4.5 d, e). These results suggest that overexpression of IL33 in MC38 cells promotes tumor malignancy and enhanced liver metastasis in this experimental model of liver metastasis of colon cancer.

## 4.2 EXPLORING TARGET GENES FROM THE LIVER MICROENVIRONMENT.

Tumors induce reciprocal host-tumor interactions to establish metastasis. As the main target organ in colon cancer metastasis, the liver is the pivotal organ exhibiting these interactions and responding with genetic alteration that prepare a fertile environment for the arrival of metastatic cells, such as establishment of pre-metastatic niche. Therefore, liver can be a valuable resource for identifying genes that promote liver metastasis.

### 4.2.1 LCN2 IS THE MOST CHANGED GENE IN METASTASIS-BEARING LIVER IN THE COLON CANCER MOUSE MODEL.

Based on the results from microarray analyses of changes in gene expression in liver from CT26-FL3 bearing mice, Lipocalin 2 (Lcn2) with 388-fold change over non-tumor bearing mice, is the most altered gene in liver during metastasis (Table 3.6). LCN2 (also known as 24p3 or NGAL), is a 25-kDa secretory glycoprotein that was originally identified in mouse kidney cells and human neutrophil granules (Kjeldsen, Johnsen et al. 1993). It has been implicated in diversified functions such as apoptosis and innate immunity (Xu, Ahn et al. 2012). Under inflammatory stimuli, such as lipopolysaccharides and IL1 $\beta$ , lipocalin-2 can be induced to express and secrete in neutrophils. The proinflammatory transcription factor NF- $\kappa$ B has been shown to

transactivate Lcn2 expression by binding to a consensus motif in the promoter region of the Lcn2 gene, suggesting that this secretory protein might be involved in the inflammatory responses (Fujino, Tanaka et al. 2006). Moreover, LCN2 interacts with its receptor -24p3R to mediate iron trafficking. By increasing intracellular iron accumulation, it has been shown to induce apoptosis (Xu, Ahn et al. 2012). In cancer, complexes of LCN2 with matrix metalloproteinase matrix metalloproteinase-9 (MMP-9) were found in the urine obtained from breast cancer patients, suggesting a possible role for lipocalin-2 in the protection of MMP-9 against autolysis (Kubben, Sier et al. 2007). The specific role of LCN2 in liver metastasis is still unclear as conflicting data regarding its role in carcinogenesis has been reported. In 2009, LCN2 was reported to promote tumorigenesis and metastasis in breast cancer (Leng, Ding et al. 2009). However, LCN2 was also shown to suppress tumor invasion and liver metastasis of colon cancer cells (Lee, Lee et al. 2006). Most of these studies have focused on the expression of LCN2 in the cancer cells themselves, but very little is known regarding its role in the tumor or host microenvironment.

To determine the expression of Lcn2 in our mouse model of colon cancer, blood serum was collected from mice bearing CT26-FL3 cells and analyzed by western blotting using antibodies against LCN2 (Figure 4.6). The result showed that serum LCN2 progressively increased during growth of the tumor in cecum from 0 to 4 weeks after implantation, which suggest that Lcn2 is associated with colon cancer progression. Since LCN2 was thought to protect MMP9 from degradation, MMP9 level was also determined in serum samples. The enhanced MMP9 level was consistent with accumulation of LCN2 during progression to metastasis. In addition, IL-33 administration can increase the

expression of Lcn2 in osteoclasts (Schulze, Bickert et al. 2011). Since IL33 has been shown to be up-regulated in CT26-FL3, it is possible that Il33 might play a role in the induction of Lcn2 expression, and that both molecules might play an important role in host-tumor interactions that might enhance liver metastasis of colon cancer. Further studies to determine the role of Lcn2 in colon cancer liver metastasis are being pursued by lab colleague Daniel Hughes.

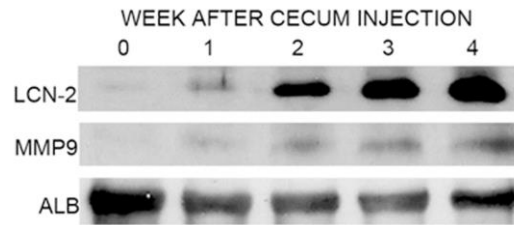


Figure 4.6 Elevated serum levels of LCN2 and MMP9 in mouse model after cecum implantation of CT26-FL3 cells, serum were taken from 5 mice at each time point.

#### 4.3 SUMMARY AND FUTURE DIRECTIONS

Based on the microarray results from last chapter, potential target genes that may mediate host-tumor interactions that promote liver metastasis of colon cancer were chosen from cancer cells and liver environment, respectively. Il33 is the target gene derived from the highly metastatic colon cancer cell line. In the colon cancer mouse model and patients, elevated Il33 expression were verified and found associated with stages of colon cancer progression. By overexpression Il33 in mouse colon cancer line MC38, Il33 were found to promote tumor malignancy and liver metastasis in splenic injection mouse model. Lcn2 is the top 1 significantly changed gene in metastasis-

bearing liver in mouse model. Its serum level was kept increasing during the tumor progression, which imply it might have unique role in colon cancer metastasis. Further study will be continued by Daniel Hughes.

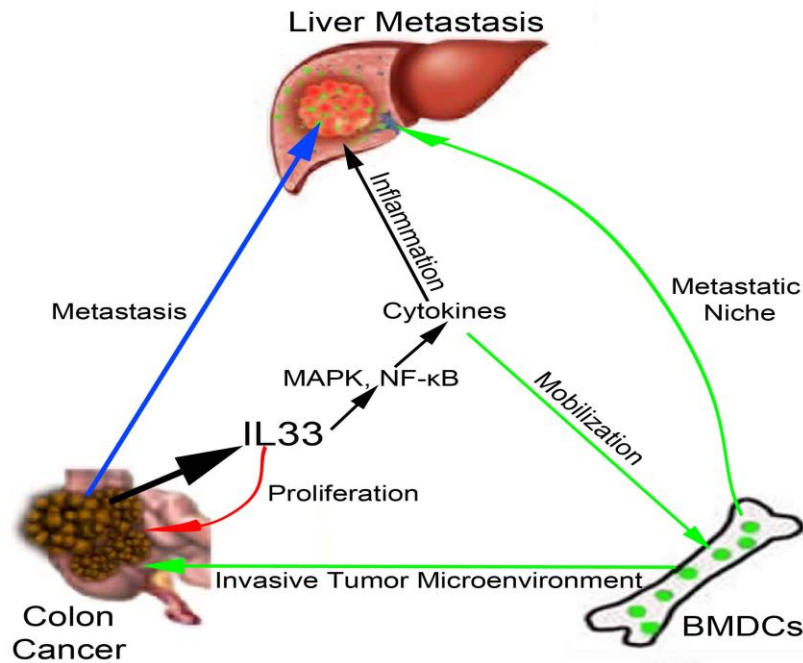


Figure 4.7 A working model on the role of IL33 in tumorigenesis and liver metastasis in colon cancer. Produced by cancer cells in colon, IL33 enhances tumor proliferation in the primary tumor by paracrine mechanisms. IL33 induces the release of various cytokines by activating MAPK and NF- $\kappa$ B pathways in the host environment which causes the inflammation in liver and enhance the recruitment of BMDCs to the primary tumor and target organ to promote liver metastasis of colon cancer.

Based on the findings in this study, a working model of IL33 tumorigenesis and liver metastasis in colon cancer were proposed as a foundation for next research (Figure 4.7). In future, the role of IL33 will be confirmed in other colon cancer models such as cecum implantation model. Because IL33 is a cytokine and also can be released by other normal cells in tumor bearing host, IL33 knockout mice will be used to locate the resource

of IL33 in tumorigenesis and liver metastasis in colon cancer. Then the capabilities such as invasion, angiogenesis, proliferation and tumor stroma remodeling influenced by IL33 overexpression will be detected to discover the mechanism of IL33 in promoting tumor malignancy and metastasis. At last, the experimental therapy will be test on mouse model by administering IL33 recombinant protein and/or IL33 antagonist, or soluble ST2 receptor.

## CHAPTER 5

### MATERIALS AND METHODS

#### Cell culture

The Balb/cByJ-derived mouse colon carcinoma cell line CT26 was purchased from American Type Culture Collection (ATCC), and cultivated in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and 1% Penicillin/Streptomycin (Pen/Strep, Mediatech, Manassas, VA) at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere.

Transfection of cell lines was performed using Lipofectamine 2000 (Invitrogen, Grand Island, NY) following manufacturer's instructions. CT26 cells were stably transfected with pGL4.13-mCherry-Hygro vector containing the mCherry red fluorescent protein (RFP) and the hygromycin resistance gene. Stable transfectants were selected in the presence of 500 µg/ml hygromycin (Hygrogold, Invivogen, San Diego, CA).

#### Mice

CByJ.B6-Tg (UBC-GFP) 30Scha/J mice expressing enhanced green fluorescent protein (eGFP) under the control of the ubiquitin promoter were used as donors for bone marrow transplantation (BMT). Balb/cByJByJ mice were used as BMT and orthotopic homograft recipients. Both strains were purchased from Jackson Laboratories (Bar

Harbor, ME) but were bred and maintained at the Mouse Experimentation Core Facility of the Center for Colon Cancer Research at the University of South Carolina (USC), Columbia, SC. All animal experiments were conducted according to the guidelines and approval of USC Institutional Animal Care and Use Committee.

#### Orthotopic homografting in Mice

For cecal implantations, sub-confluent cells were harvested and washed in phosphate buffered saline (PBS) just prior to implantation. Eight-week-old male Balb/cByJByJ mice were anesthetized by inhalation of 2% isoflurane in oxygen and placed in supine position. A midline incision was made to exteriorize the cecum. Using a 33-gauge micro-injector (Hamilton Company, Reno, NV),  $2 \times 10^6$  cells in 10-15  $\mu$ L were injected into the cecum subserosal. The injection site was sealed with a tissue adhesive (3M, St. Paul, MN) and sterilized with 70% alcohol to kill cancer cells that may have leaked out. The cecum was replaced in the peritoneal cavity, and the abdominal wall and skin closed with 6-0 polyglycolic acid sutures (CP Medical, Portland, OR). Sham control mice underwent similar surgery, but no cells were implanted into the cecum.

#### Establishment of tumor cell lines

Tumor specimens were excised from Balb/cByJ mice that were implanted with CT26 cells subcutaneously, in the cecum, or from liver metastases. They were dissected free of necrotic areas, connective tissue, and blood clots then rinsed 3 times with cold (4°C) DMEM containing 1% FBS and 2% Pen/Strep. Tissues were sliced into 1-3 mm<sup>3</sup> fragments and then subjected to sequential enzymatic digestion for 30 minutes each at

37°C in DMEM containing collagenase type I (200 units/ml), DNase (270 units/ml), or hyaluronidase type IV (35 NF units/ml) (Sigma, St. Louis, MO). The resulting cell suspension was maintained at 4°C, filtered through a 70 µm nylon cell strainer (BD Biosciences, Bedford, MA), washed in PBS, and then grown in culture in as described above.

## Histology

Tumor-bearing mice were humanely sacrificed and the entire intestine, primary cecal tumor, and liver were excised, fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.2. Tissue blocks were embedded in paraffin, 5 µm sections obtained and then stained with hematoxylin and eosin (H&E) (VWR, West Chester, PA) for visual examination. The stained slides were reviewed and screened for representative tumor regions by a pathologist.

## Immunohistochemistry

The paraformaldehyde-fixed, paraffin-embedded tissue sections were deparaffinized, rehydrated, then incubated in a microwave oven with 0.01M citrate buffer, pH 6.0 for 10 minutes for antigen retrieval. Endogenous peroxidases were blocked with 3% H<sub>2</sub>O<sub>2</sub> for 15 min. Nonspecific epitopes were blocked with normal horse serum (Jackson ImmunoResearch, West Grove, PA) for 1 hour. The sections were incubated overnight at 4°C with antibodies against one of the following proteins: proliferating cell nuclear antigen (PCNA, 1:300 dilution), matrix metalloproteinase 9 (MMP9), matrix metalloproteinase 2 (MMP2), vascular endothelial growth factor (VEGF), VEGF receptor



1 (VEGF-R1), S100A8 (all from Abcam, Cambridge, MA), lysyl oxidase (LOX), c-MYC, Cyclin D1 (CCND1) (all from Santa Cruz Biotechnology, Santa Cruz, CA), or S100A9 (R&D Systems, Minneapolis, MN) (all at 1:100 dilution). This was followed by incubation with the corresponding secondary antibody conjugated to horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA) for 1 hour at room temperature (RT). Antigen signals were detected using the 2-Solution Diaminobenzidine (DAB) Kit (Invitrogen, Frederick, MD), counterstained with hematoxylin, mounted in Acrymount (StatLab, McKinney, TX), and visualized under a light microscope.

#### Boyden Chamber cell invasion and wound healing assays

The ability of CT26 and CT26-FL3 cells to invade through Matrigel-coated filters was measured using transwell chambers (Costar, Cambridge, MA) with polycarbonate membranes (8.0- $\mu$ m pore size) coated with 100 $\mu$ l Matrigel (BD Biosciences, Bedford, MA) on the top side of the membrane. The upper surface of the matrix was challenged with 10,000 cells kept in serum-free medium containing 0.1% bovine serum albumin (BSA). The lower chamber contained medium supplemented with 10% FBS. After 16 hours, the cells were stained with 0.1% crystal violet solution. Cells and Matrigel on the upper surface of the membrane were removed carefully with a cotton swab. Cells that invaded through the matrix were visually counted at five randomly chosen field views. Each experiment was performed in triplicate wells and repeated three times.

For the wound healing assay, confluent monolayer cultures of CT26 and CT26-FL3 cells plated in 6-well plates were wounded with a sterile 200  $\mu$ l pipet tip and incubated with DMEM containing 1% FBS. Representative fields of wounded

monolayers containing wounds of the same width were photographed under an inverted microscope at 40× magnification after incubation for 1-4 days at 37°C in a humidified CO<sub>2</sub> atmosphere. The extent of wound repair was evaluated by measuring the area of the wound by computerized image analysis using the Image J image software (NIH, Bethesda, MD). Each experiment was performed in quadruple wells and repeated three times.

#### Cell proliferation assay and *In vivo* monitoring of tumor growth

To determine the growth rate of CT26 and CT26-FL3 *ex vivo* in culture, 10,000 cells in 2 ml of DMEM with 10% FBS were plated per well in 6-well plates. The number of cells was counted after incubation for 3 to 8 days at 37°C. Assays were performed in triplicate and repeated three times. To monitor tumor growth, cells ( $2 \times 10^6$  in 100  $\mu$ l) were injected subcutaneously into Balb/cByJ mice. Tumor size was measured with calipers and tumor volume (mm<sup>3</sup>) was calculated as  $\text{width}^2 \times \text{length} / 2$ . Measurements were taken from four mice per group and repeated three times.

#### Western Blotting

Sera from CT26- and CT26-FL3- tumor bearing mice were analyzed by immunoblotting. Antibodies against the following proteins were used as probes: MMP9, VEGF (both from Abcam, Cambridge, MA), osteopontin (OPN), serum amyloid A3 (SAA3), S100A8, S100A9 (all from Santa Cruz Biotechnology, Santa Cruz, CA). The blots were incubated with primary antibody (1:1000) overnight at 4°C, washed three times with PBS/0.01% Triton X-100, followed by HRP-conjugated secondary antibody

(Bio-Rad, Hercules, CA)(1:5000) for 1 hour at room temperature. The blots were visualized using an ECL enhanced chemiluminescence kit (GE Healthcare, Piscataway, NJ). As internal controls for equal protein loading, blots were stripped and probed with antibodies against albumin (Santa Cruz Biotechnology, Santa Cruz, CA).

#### RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT/PCR)

Total RNA was isolated from CT26 and CT26-FL3 cells using RNeasy RNA isolation kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA using a cDNA synthesis Kit (Bio-Rad, Hercules, CA). qRT/PCR was performed on an iCycler iQ5 PCR Thermal Cycler using SYBR green supermix (Bio-Rad, Hercules, CA). Validated gene specific primer sets for hepatocyte growth factor (HGF), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF- $\alpha$ ), interferon-gamma (IFN- $\gamma$ ), colony stimulating factors 2 and 3 (CSF2 and CSF3), CXCL1, CXCL4, CXCL11 and  $\beta$ -actin were obtained from RealTimePrimers (Elkins Park, PA).  $\beta$ -actin was used for normalization. Assays were run in five replicates.

#### Bone marrow isolation and transplantation

CByJ.B6-Tg(UBC-GFP)30Scha/J mice were anesthetized with isoflurane by inhalation and humanely sacrificed. Bone marrow (BM) cells were flushed from femur and tibia using a 21-gauge needle into PBS containing 2% FBS. Four-week-old recipient Balb/cByJByJ mice were lethally irradiated (950 rads administered at 200 rads/min) using a Varian Clinac linear accelerator.  $3-5 \times 10^6$  mono-nucleated cells were transplanted

into the recipient mice by tail vein injection. Transplanted mice were administered sterile water containing 0.018% Baytril antibiotic (Bayer, Shawnee, KS) for two weeks post-transplantation to prevent infection. To assess BM engraftment, peripheral blood was drawn from the retro-orbital sinus of recipient mice at 4 weeks post-transplant. Red blood cells were lysed with ammonium chloride lysis buffer (150mM NH<sub>4</sub>CL, 10mM Na<sub>2</sub>CO<sub>3</sub>, 0.1mM EDTA, pH 7.4). Leukocytes were then incubated with PE-Cy5 conjugated anti-CD45 antibody (BD Pharmingen, San Diego, CA), and analyzed in a Beckman Coulter Epics-XL Flow Cytometer and CXP analysis software.

#### Confocal microscopy

The liver was excised from sham control and CT-26 or CT26-FL3-bearing Balb/cByJ mice, and fixed in freshly prepared 4% paraformaldehyde in PBS, pH 7.2. Following fixation, the tissues were rinsed with PBS and vibratome sections were cut at 100  $\mu$ m thickness. Samples were stained with phalloidin conjugated to Alexa 633 (Invitrogen, Carlsbad, CA, 1:100 dilution) to visualize tissue morphology. Nuclei were stained with 1:10,000 dilution of 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, CA). Samples were imaged on a Zeiss LSM510 META confocal scanning laser microscope.

#### Microarray analysis

Total RNA was isolated using Qiagen's RNeasy Mini Kit according to manufacturer's protocol. RNA quantity was assessed using an Agilent 2100 Bioanalyzer and RNA Integrity Numbers (RIN) ranged from 8 to 10.0. Microarrays experiments were

performed using Agilent's platform. Total RNA was amplified and labeled using Agilent's Low Input Quick Amp Labeling Kit (Agilent, Wilmington, DE) according to the manufacturer instructions. Then labeled RNA was purified using Qiagen's RNeasy Mini Kit (Qiagen, Valencia, CA) and assessed dye incorporation and cRNA yield. Labeled cRNA samples were hybridized to Agilent Whole Mouse Gene Expression Microarrays 4x44K (Agilent, Wilmington, DE) using Agilent's Gene Expression Hybridization Kit (Agilent, Wilmington, DE) according to the manufacturer's instructions. After washes and drying, arrays were scanned for both the Cy3 and Cy5 channels at 5  $\mu$ m resolution using a ProScanArray Express HT scanner (Perkin Elmer Life and Analytical Sciences) and the ScanArray Express SP3 software. The scanned images were saved as TIFF files and fluorescence intensities were quantitated using ImaGene 8.0.1 software (BioDiscovery). Raw intensities for backgrounds and foregrounds (spots) were uploaded into limmaGUI where features were background corrected using the Normexp method with offset equal to 50. Subsequently, data was normalized within arrays using the locally weighted scatterplot smoothing (LOESS) algorithm and between the arrays performing scale normalization. Normalized data (M and A values) were exported from limmaGUI and normalized intensities for both Cy3 and Cy5 channel were calculated for all arrays by solving the equations for M and A, being  $M = \log_2(R/G)$  and  $A = 1/2 [\log_2(R) + \log_2(G)]$ . R = Cy5 channel intensity (Red), and G = Cy3 channel intensity (Green). In the next step, normalized intensities were uploaded into GeneSifter analysis software (Geospiza, Inc.) where sample groups were contrasted.

## Statistical Analysis

Data were expressed as the mean  $\pm$  standard deviation (SD). Statistical analysis was performed by the Students' t-test when only two value sets were compared, and one-way analysis of variance (ANOVA) followed by Dunnett's test when the data involved three or more groups.  $P < 0.05$ ,  $P < 0.01$  or  $P < 0.001$  was considered statistically significant and indicated by \*, \*\* or \*\*\*, respectively.

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