INTERLEUKIN-1 BETA PROMOTES EPITHELIAL-MESENCHYMAL TRANSITION AND A STEM CELL PHENOTYPE OF COLON CANCER CELLS VIA ZEB1/2

by

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Abstract

Interleukin-1 beta (IL-1β) is an important mediator of inflammatory response, and the elevated expression of IL-1β is correlated with tumor growth and metastasis. Epithelial-mesenchymal transition (EMT) is a reversible transition between epithelial phenotype and mesenchymal phenotype. Usually, EMT can be identified by its unique morphology change and expression of EMT markers. In our study, we have found after treated HCT-116, a colon cancer cell line, and human primary colon cancer cells with IL-1β, cells began to display mesenchymal phenotype with highly down-regulated E-cadherin expression and up-regulated ZEB factors expression. For colon cancer cells, sphere formation assay in serum free medium (SFM) with the presence of growth factors is used to identify cancer stem cell population. We have shown that IL-1β can induce colon cancer stem cell proliferation and express stem cell markers (Bmi1, Nanog, and Nestin). In addition, besides the stem cell markers, we also found ZEB factors were highly up-regulated in spheroid cells as well. We silenced Zeb1 expression and investigated the effect of IL-1β on shZeb1 HCT-116 cells. The results indicated Zeb1 knockdown not only inhibited IL-1β-induced EMT but also reduced proliferation of spheroid cells and inhibited Bmi1 expression. Therefore, ZEB factors must play an important role in both EMT process and cancer stem cell development. From our data, we conclude that IL-1β promotes epithelial-mesenchymal transition and a stem cell phenotype in colon cancer via ZEB factors.

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Dedication

I would like to dedicate my parents, Jinxiang Li and Guang Gao, thank you so much for cheering me up all the time and the trust. Also, I would like to dedicate Chen Peng, my husband, for supporting me on my work and deep love.

Chapter 1 - Introduction

1.1 Colon cancer

Colon cancer, which is also called as colorectal cancer, is the cancer that originates from large intestine or rectum. According to American Cancer Society, it is one of the leading causes of cancer-related death every year in the United States (Compton, 2004). Colon cancer is the cancer of epithelial origin, and it starts in the large intestine or rectum as a benign polyp at the inner lining of intestine, then, the benign polyp slowly develops into cancer. Colon cancer is classified into five different stages, basing on the progression of cancer and the extent of cancer that has spread (O'Connel, Maggart et al., 2004). Stage 0 is the very early stage of colon cancer, in which the tumor remains on the innermost layer of intestine. Stage I is when the tumor is confined to the bowel wall, and locates on the inner layer of colon. After it develops into stage II, the tumor has grown and spread through the muscular layer of colon. At stage III, the tumor has been spread outside the colon to one or more lymph nodes. A stage IV colon cancer is defined as spreading of cancer cells to distant organs. Colon cancer is treatable if early diagnosis and treatments are applied, because the 5-year survival rate of an early stage colon cancer is relatively high compared with lung, liver, and stomach cancer.

Standard treatment for an early stage colon cancer patient involves surgical resection of the tumor and a certain amount of tissue surround the tumor. Chemotherapy may also be applied as a precaution against cancer recurrence. For stage IV colon cancer, radiation therapy could also be taken into account. Conventional cancer treatments are prone to tumor regression, however, they are often ineffective at preventing tumor recurrence. recent more evidence has shown that over 20% of patients with treated colon cancer see recurring signs within five years.

1.2 Colon cancer stem cell

Many studies have shown that there is a distinct and relatively rare population of "tumor-initiating" cells with stemness properties in a variety of tumor types (Pardal, Clarke et al., 2003). The best proof for the concept of cancer stem cells (CSCs) comes from the study of acute myeloid leukemia, in which a rare $CD34^{+}CD38^{-}$ population functioned as a source to reproduce acute myeloid leukemia in xenograft transplant system (Lapidot, Sirard et al., 1994; Bonnet and

Dick, 1997). Although these CSCs merely represent a small percentage of the overall tumor population, they are believed to be the only cells that can originate and induce tumor growth. These so-called CSCs have been successfully isolated from breast (Al-Hajj, Wicha et al., 2003), lung (Eramo et al., 2008), liver (Ma, Chan et al., 2007), and colon cancer (O'Brien and Pollet et al., 2006), and are defined by several surface markers such as $CD44^{+}/CD24^{-}$ (Fillmore and Kuperwasser, 2007), $ALDH^{+}$ (Jiang et al., 2009), $CD90^{+}/CD45^{-}$ (Yang and Ho et al., 2008), and $ESA^{+High}/CD44^{+}$ (Dalerba et al., 2007) respectively. The origin of CSCs are thought to be normal stem cells (SCs), progenitor cells, or bone-marrow-derived cells and they share remarkable similarities and properties like dividing and renewing themselves for a long period of time. CSCs are not destroyed by chemotherapeutics, which target at rapidly growing and differentiated tumor cells. These self-renewing CSCs are believed to mediate tumor recurrence after traditional cancer therapy (Todaro, Francipane et al. 2010).

Evidence has shown that human colon cancer stem cells (CCSCs) can form non-adherent floating spheres in a serum-free medium (SFM) (Kreso and O'Brien, 2008). The sphere formation assay is very important for identifying SCs in vitro. If cells maintain the property of SCs, they form spheres in SFM. It is believed that those spheres contain mostly SCs. In other words, the sphere assay is used to maximally enrich SCs in vitro. The sphere assay has been validated by use of xenotransplantation that is considered as a gold standard to evaluate CSC self-renewal and tumor initiation.

HCT-116, a human colon cancer cell line used for our study, grows as spheres in SFM (Figure 1.1), suggesting the cell line contains stem cell population. Addition of serum to SFM results in the differentiation of colon cancer cells to grow as adherent monolayer (Figure 1.2). However, for another cell line that we use, the human primary colon cancer cells (PCCCs), they fail to form spheres when culturing in SFM, suggesting CSCs in PCCCs were not active in this condition (Figure 1.3). However, when PCCCs are cultured in serum-containing medium (SCM), they can proliferate as multilayer cells (clusters), a typical morphology of cancer cells (Figure 1.4), suggesting PCCC are cancer cells.

1.2.1 Colon cancer stem cell characteristic: self-renew

Self-renewal ability is one of the most important properties of normal SCs and CSCs. In the hematopoietic system, a SC can give rise to two different types of daughter cells (Morrison,

Prowse et al. 1996; Reya, Morrison et al. 2001; Morrison and Kimble 2006). One daughter cell retains the special characteristic of self-renewal ability as its mother cell, whereas the other becomes a more specialized cell (Lin and Schagat, 1997). In addition, SCs can be classified into three different populations according to their capacity to differentiate—totipotent SCs that are able to give rise to new individuals on their own, developing significant self-renewal ability, pluripotent SCs that are capable of generating almost all tissues of the body, like embryonic SCs, and multipotent SCs that cannot renew themselves but are proficient to differentiate into a variety cell types on a certain location. SCs have the ability to retain the undifferentiated state by self-renewal and to differentiate into different cell types. Correspondingly, the small population of CSCs in tumor has the self-renewal ability to generate phenotypically diverse tumor cell population during tumorigenesis as well. Overexpression of WNT family genes, which work as crucial regulators of normal cell development, contributes to mammary-stem-cell pool development and cancer susceptibility (Liu, McDermott et al., 2004). In another study, cells that can undergo pluripotent differentiation and display SC properties have been successfully isolated from human central-nervous-system tumors (Singh et al., 2004; Hemmati et al., 2003; Singh, Clarke et al., 2004). These cells with stemness property express CD133, a cell surface antigen that is known as a hematopoietic SCs marker and later proved as a marker that can be expressed by SCs in other normal tissues (Yu and Zhang et al, 2004; Wu et al, 2006). Since the self-renewing CSCs are able to generate tumor at beginning or regenerate tumor after traditionally cancer therapies, it is imperative to identify the regulatory mechanisms and signaling pathways involved in CSC self-renewal (Dylla, Bevialia et al., 2008). The CSC therapy, which target on CSCs to suppress re-growth of tumor and elimination of cancer rather than differentiated tumors, provides a new sight in future cancer therapy (Serakinci, Guldberg et al., 2004).

Human CCSCs have shown the ability to form spheroid cells in SFM in the presence of certain growth factors, providing a way to establish a stem cell model in vitro (Kreso and O'Brien, 2008). In our study, self-renewal assay, an assay in which cells were cultured in a very low density in SFM, was applied to study HCT-116 and human PCCC, and identify their self-renewal ability.

1.2.2 Colon cancer stem cell characteristic: drug-resistance

Based on the CSC concept, CSCs are believed to mediate cancer recurrence after conventional therapies by their unique quiescence and DNA repair properties (Dean, Fojo et al., 2005). ATP-binding cassette drug transporters have been shown to protect CSCs from chemotherapeutic drugs. Recently, more and more evidence has shown that in vivo xenograft models of CSCs display chemoresistance (O'Brien, Kreso et al., 2009). For example, the initial evidence of CCSCs against chemotherapy was demonstrated by Todaro et al who conducted experiment to analyze viability of $CD133^+$ and $CD133^-$ cells after treatment with oxaliplatin and/or 5-fluorouracil. $CD133^+$ cells displayed much higher viability rate than $CD133^-$ cells in both in vitro and in vivo drug treatment, and impaired the effects of chemotherapy on a large scale (Todaro, Perez et al., 2008). Another study investigated the role of the chemoresistance of CCSCs by treating NOD/SCID mouse model with cyclophosphamide and/or innotecan. It showed that after treatment with chemotherapy, the residual tumors were enriched cells with CSC phenotype, and these cells could increase tumorigenic cell frequency by in vivo assay. The evidence suggests CSCs display strong chemoresistance and may participate in tumor recurrence after chemotherapy.

Carboplatin is the chemotherapy drug we used for investigating the drug resistant ability of HCT-116 spheroid cells. It is a widely used drug that against a variety of cancers. The cytotoxic platinum complex enables it to react with nucleophilic sites of DNA, leading to crosslink of intrastrandand-interstrand and DNA-protein (Kelland, 2007).

1.2.3 Colon cancer stem cell characteristic: expression of stem cell markers

Normal SCs and CSCs share some remarkable characteristics by expressing SC markers. The RNA-binding protein Musashi (Msi-1) was the first recognized as a human colon SC marker and it is believed to maintain the undifferentiated state of SCs through posttranscriptional control of downstream genes (Imai, Tokunaga et al., 2001; Battelli, Nikopoulos et al., 2006). CD29 was also isolated as a surface marker from the proliferative zone of human colonic crypt, which includes SCs and progenitor cells (Fujimoto, Beauchamo et al., 2002). These SC markers are highly expressed in colon cancer (Todaro, Perez et al., 2008; Vermeulen, Todaro et al., 2008). More importantly, recent studies also reveal the importance of Bmi1, a polycomb group protein, is a significant CSC marker that has been known for maintaining SCs, malignant transformation,

and biologic progression of several human carcinomas (Molofsky, Pardal et al., 2003). It has been known that the elevated expression of Bmi1 is notably associated with a poor 5-year survival rate in colon cancer patients, suggesting Bmi1 is an indispensable predictor of colorectal cancer prognosis (Du, Li et al., 2010). In addition, the overexpression of Bmi1 is related with tumorigenesis and CSC phenotype (Li et al, 2009; Dovey et al, 2008). Nestin, the marker for SCs from for central nervous system, is currently used as a marker for angiogenesis of colon cancer (Sheila et al., 2003). Nanog, is a transcription factor that is involved in self-renewal of undifferentiated embryonic stem cells, plays a prognostic role in colon cancer and promotes cell proliferation, invasion, and also participates in EMT process during cancer progression (Meng, Zheng et al., 2010).

1.3 Inflammation and cancer

More and more studies have proved that inflammation is closely related with cancer initiation and promotion. The concept of tumor microenvironment (TME) was first proposed by Stephan Paget but laid dormant for many years until 1970s. The TME mostly consists mostly of immunocytes, which play an essential role in tumorigenesis and especially in tumor progression (Witz, 2009). Moreover, the pathogenesis of cancer largely contributes to the interaction of cancer cells with its microenvironmental components that can determine whether the cancer cell will progress towards metastasis or will stay latent. Cytokines released by the tumor associated immunocytes within TME mediate the link between inflammation and cancer progression (Kaler et al., 2009). It has been known that macrophages-derived IL-1β within the TME promotes Wnt signaling in colon cancer cells and further enhances the proliferation, exerting the protumorigenic activity.

1.3.1 Interleukin-1 beta (IL-1β)

Interleukin-1 beta (IL-1β) is a very important pro-inflammatory cytokine that involves in a variety of cellular activities, including cell proliferation, differentiation, and apoptosis (Fleisher et al., 1995; Wang et al., 2007). Produced by monocytes, macrophages, and epithelial cells, IL-1β is also essential for host defense mechanisms and repair (Vonk et al., 2006). In addition, previous studies show that the concentration for IL-1β is highly elevated at the tumor site (Apte et al., 2006), where the enforced expression of IL-1β are correlated with tumor progression and advanced metastatic disease in cancer patients (Pantschenko et al., 2003). In the tumor

microenvironment, IL-1β released by tumor-associated macrophages can always promote angiogenesis, tumor growth and metastasis (Elaraj et al., 2006). In colon cancer, IL-1β produced in the TME protects colon cancer cells from TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis (Kaler et al., 2010). Therefore, inactivation of IL-1β by neutralizing IL-1β antibody or silencing IL-1β in macrophages may inhibit tumor invasion and progression.

1.4 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) is a reversible transition in which epithelial cells undergo transformation to a mesenchymal phenotype and give rise to fibroblasts and myofibroblasts (Willis and Borok, 2007). It is required for tissue morphogenesis during normal embryonic development and can also occur as pathological events during adult life in cancer. Epithelial and mesenchymal cells can be identified by their unique visual appearance and multicellular structure. A typical epithelium is a sheet of cells with regularly spaced cell-cell junctions and adhesions between neighboring epithelial cells. When triggered by the down-regulated E-cadherin expression, epithelial cells begin to lose cell-cell adhesion and show an increased migratory ability. Mesenchymal cells, which have an elongated shape, exhibit neither regimented structure nor tight intracellular adhesion compared with epithelial cells, and provide invasive properties of cells. In addition, the migration of mesenchymal cells is more dynamic than epithelial cells which move as a sheet en block (Thiery, 2002). In the normal embryonic development, the EMT process enables epithelial cells to loss polarity and gain motile characteristics of mesenchymal cell, which simultaneously correspond to the metastatic cancer cells during malignant progression (Yang and Weinberg, 2008). Interestingly, these properties of EMT have also been endorsed to normal SCs and CSCs.

1.4.1 EMT-associated transforming growth factor-beta (TGF-β) modulation

The process of EMT can be regulated by a variety of cytokines and growth factors. Secreted inducers of EMT include transforming growth factor-beta (TGF-β), basic fibroblast growth factor (FGF2), epidermal growth factor (EGF), insulin-like growth factor-2 (IGF-2), and hepatocyte growth factor (HGF) (Thiery, 2002). TGF-β is a multifunctional polypeptide which signals through receptor serine/threonine kinase complexes, is one of the most well studied EMT inducer (Miettinen et al., 1994). The TGF-β signaling pathway has been proved associated with induction and maintenance of EMT (Strutz et al., 2002), indicating the role of TGF-β as an

essential mediator of tumor progression (Border and Noble, 1994; Derynck et al., 2001; Bottinger and Bitzer, 2002; Roberts and Wakefield, 2003). In response to TGF-β-ligand binding, the tight complexes formed by TGF-β receptors contribute to the phosphorylation of Smad protein family, Smad2 and Smad3 (Massague, 2000). The phosphorylated Smads then translocate to nucleus with cytoplasmic Smad4 and accumulate in nucleus to control transcription of target genes (Derynck and Zhang, 2003). In addition, Smad3-dependent transcriptional regulation is believed to control the TGF-β target gene expression, as TGF-β fails to induce EMT in primary tubular epithelial cells derived from kidneys of Smad3 knockout mice. However, hepatocytes isolated from liver-specific Snad2 knockout mice spontaneously transition to mesenchymal cells without any stimulation of TGF-β, indicating Smad2 function to maintain cells in epithelial phenotype (Piek, Ju,. 2001; Goumans et al., 2003, Zavadil et al., 2004). Recent studies also revealed the controversial role of TGF-β as a growth inhibitor and promoter during cancer development (Derynck, Akhurst et al., 2001). During early stages of cancer progression, TGF-β signals as a tumor suppressor pathway, and exhibits strong growth inhibitory and apoptotic effects resulting in tumor suppression. Whereas during later stages of tumorigenesis, TGF-β plays a pro-metastatic role that drives EMT and promotes CSC maintenance in breast cancer, since the metastatic breast CSCs with $CD44^{high}/CD24^{low}$ population express the TGF-β pathway signature (Shipitsin et al., 2007).

1.4.2 EMT-related epithelial and mesenchymal cell markers

The EMT process can be characterized by definite gene profile changes. Epithelial and mesenchymal cells can be distinguished by the expression of a number of canonical markers that are specifically used to identify whether the cells are epithelial or mesenchymal cells (Nawshad et al., 2005). For instance, E-cadherin, one of the most important epithelial markers, is an adherent and tight junction protein (Zeisberg and Neilson, 2009). It belongs to the family of transmembrane glycoproteins and can maintain tissue integrity. A high level of E-cadherin expression is related with suppression of invasion and growth of many epithelial cancers. During the process of EMT, the expression of E-cadherin is highly diminished (Vleminckx, Vakaet et al., 1991; Perl, Wilgenbus et al., 1998). The Zinc-finger enhancer binding (ZEB) protein like Zeb1 or Zeb2 is a crucial EMT activator in many human cancers (Vandewalle, Van Roy et al., 2008). According to previous study, the enforced expression of ZEB factors results in a rapid EMT.

Both Zeb1 and Zeb2 are transcriptional regulators and can suppress the expression of E-cadherin (Aigner et al., 2007; Graham et al., 2008). For mesenchymal cells, extracellular matrix (ECM) component fibronectin and intermediate filament protein vimentin are the two widely used markers. In more recent studies, the theory of ZEB/miR-200 feedback loop has been established to illustrate the cellular plasticity of EMT, CSCs, and cancer progression (Burk, Schubert et al., 2008; Wellner, Schubert et al., 2009; S. Brabletz and T. Brabletz, 2010). The microRNA 200 family members contribute to EMT by targeting at ZEB proteins and are down-regulated by TGF-β.

1.4.3 EMT-associated cancer progression

The mechanisms that regulate EMT are now being discovered and the implication between EMT and cancer prognosis has been uncovered as well. During cancer progression, EMT typically contributes to tumor invasiveness, intravasations and extravasations of metastatic cells (Thiery, 2002). Normal epithelium lining by the basement membrane give rise to adenoma when an abnormal growth occurs. Epigenetic changes and genetic alterations will further transform the adenoma into a carcinoma in situ. Further alternation of normal epithelium contributes high stage carcinoma in situ that transition the epithelial phenotype into mesenchymal phenotype by abolishing E-cadherin, and at the main time the basement membrane becomes fragmented. The highly-migrated mesenchymal cells can intravasate into lymph or blood vessels through capillaries over the tumor and travel throughout the body. Extravasated at a secondary site, the solitary carcinoma cells will either remain solitary or form a new carcinoma through mesenchymal-epithelial transition (MET). In summary, EMT process may be a prerequisite for cancer cell metastasis.

1.4.4 EMT-induced formation of CSCs

When tissue injury or tumorigenesis occurs, the differentiated cells are triggered to gain SC properties through EMT induction by Wnt, Notch and Hedgehog signaling pathways, which drive both normal SCs and CSCs self-renewal and maintenance (Huber et al., 2005; Malanchi et al., 2008; Peacock and Watkins, 2008). In addition, metastatic cancer cells, which undergo EMT process, exhibit a CSC phenotype. For example, a $CD44^{high}$, $CD24^{low}$ CSC-like population was found in disseminated breast cancer cells (Al-Hajj et al, 2003). Importantly, CD44 was reported as a supporter of the EMT-associated Wnt signaling pathway in CSC maintenance (Wielenga et

al., 1999). In colon cancer, the overexpression of CD44 can be found at both the mRNA and protein levels (Wielenga et al., 1993). By using CD44 deficient mice, which are more susceptible to apoptosis compared with $CD44^{+}/^{+}$ mice, a recent study proved the protective role of CD44 against apoptosis by apoptotic stimulus of irradiation, (Lakshman et al., 2005). A study conducted by Mani et al. has connected the EMT process to the emergence of CSC. They showed that when mammary epithelial cells undergo EMT, either induced by TGF-β or forced expression of E-cadherin transcriptional repressors, can give rise to a $CD44^{high}$, $CD24^{low}$ CSC-like population (Mani et al., 2008). Simultaneously, SCs isolated from normal breast tissue or CSCs from breast cancers express classical EMT markers. Therefore EMT not only contributes to cellular motility, but also the induction of CSC phenotype and prevents cells from apoptosis and senescence. It has been known that Zinc-finger enhancer binding (ZEB) factors (Zeb1 and Zeb2) are crucial EMT activators and they are the essential targets of microRNA-200 family members (Burk et al., 2008). In return, all the microRNA-200 family members are transcriptional targets of ZEB factors as well (Bracken et al., 2008). ZEB factors and microRNA-200 family members not only have opposite functions, but also reciprocally control the expression of each other. In addition, ZEB factors will suppress the expression of E-cadherin by down-regulate microRNA-203 and microRNA-183, which together with microRNA-200 to block expression of SC factors, connecting the induction of EMT and maintenance of stemness together (Wellner et al., 2009). Since microRNAs are known to control central cellular process, the EMT process may regulate by microRNAs as well. Thus the balanced expression of ZEB factors and microRNA-200 by ZebMicRNA-200 feedback loop is a "molecular motor" of cellular plasticity in development of disease, especially for the progression of cancer to metastasis from the view of CSCs (S. Brabletz and T. Brabletz, 2010).

1.5 Overall hypothesis

Since it has been proven that IL-1β is highly up-regulated in colon cancer, and is correlated with colon cancer growth and metastasis, and EMT can generate cells with the property of CSCs, we hypothesize that IL-1β may promote EMT and a colon cancer stem cell phenotype.

Figure 1.1

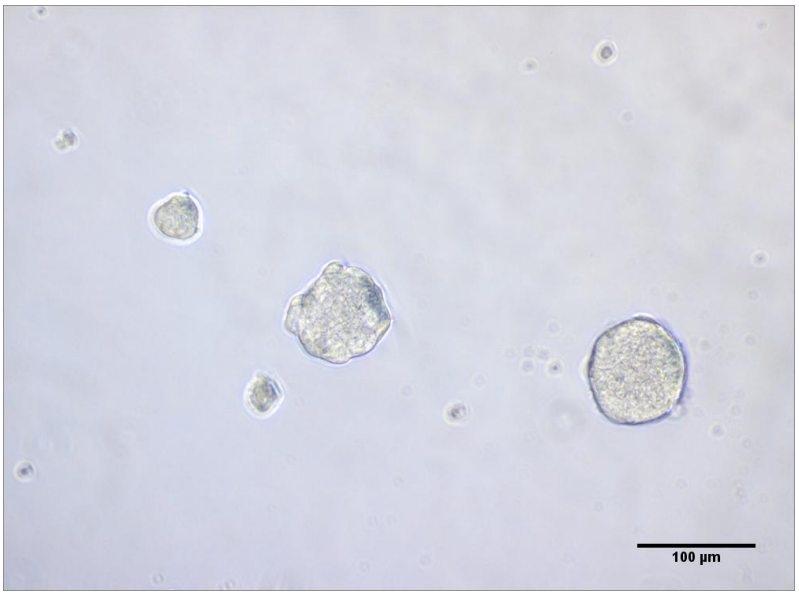


Figure 1.1 Sphere formation of HCT-116 in serum-free medium (SFM). After cultured in SFM, HCT-116 cells, a human colon cancer cell line, form free-floating spheres, suggesting that the cell line itself maintains stemness property. Picture was taken on day 7. Scale bar = 100 µm.

Figure 1.2

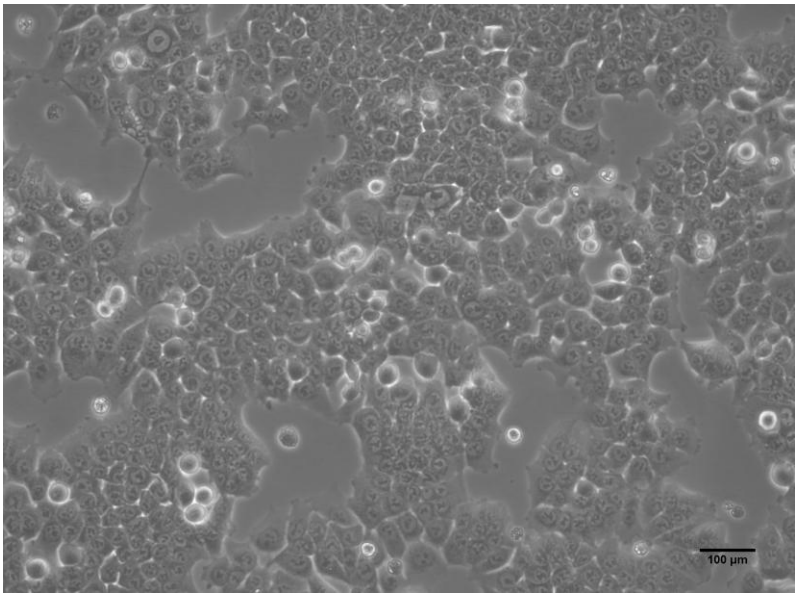


Figure 1.2 HCT-116 formed monolayer in serum-contain medium (SCM). In SCM, HCT-116 cells form monolayer instead of spheroid cells and exhibits typically epithelial property. Scale bar = 100 µm.

Figure 1.3

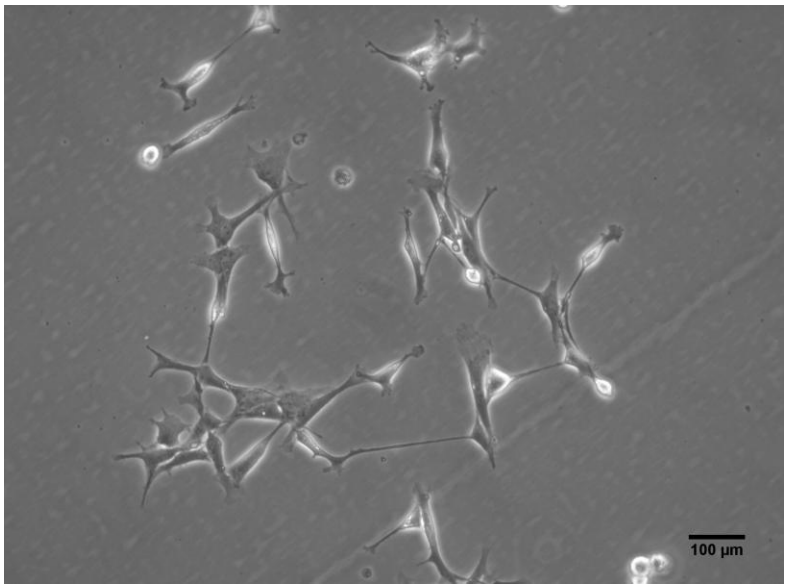


Figure 1.3 Human primary colon cancer cells form monolayer in serum-free medium (SFM). In contrast of HCT-116 cells that form spheres in SFM, the human primary colon cancer cells form an attached monolayer instead. Picture was taken on day 8. Scale bar = 100 µm.

Figure 1.4

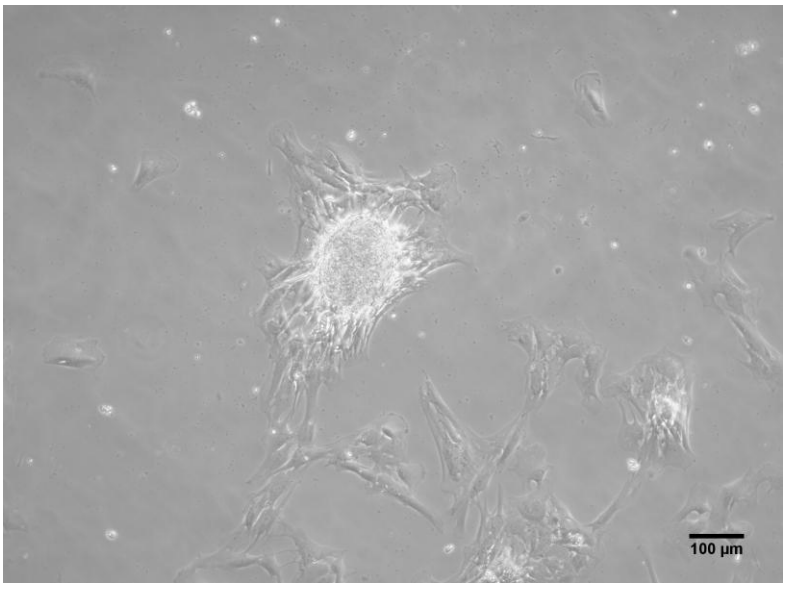


Figure 1.4 Human primary colon cancer cells form both monolayer cells and multilayer cells in serum-containing medium (SCM). The human primary colon cancer cells form both monolayer cells and multilayer cells, which is one of the typical characteristic of cancer cells, after cultured in SCM, suggesting the primary colon cells are cancer cells. Picture was taken on the 31st day after culturing in SCM. Scale bar = 100 µm.

Chapter 2 - Materials & Methods

2.1 Cell line

The HCT-116 cell line is involved in colorectal carcinoma, and has the morphology as epithelial cells. The complete growth medium for HCT-116 is McCoy's 5A Medium Modified (Invitrogen) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO) in a humidified incubator at 37 ℃ with 5% CO_2.

The primary colon tumor tissues were obtained from Surgical Associates in Manhattan, Kansas. The sample was from an 84-year old Caucasian female who was diagnosed with colorectal cancer. The complete growth medium for primary colon cancer cells is McCoy's 5A Medium Modified (Invitrogen) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (GIBCO) in a humidified incubator at 37 ℃ with 5% CO_2.

2.2 EMT Morphology study

HCT-116 cells and PCCCs all grew as monolayers and attached to T25 flasks in 10% serum-contain medium (SCM). When cells became ~80% confluent, the culture medium was removed and the cells were rinsed by 1 x PBS. Trypsin-EDTA (0.25%, Cellgro, Inc) was added to cells and incubated for 5 minutes at 37 ℃ to dissociate cells from the flask. After the majority of cells had detached from flask, 5 mL SCM was added to flask to inhibit the function of Trypsin-EDTA. Cell suspension was centrifuged at 1500 rpm, 24 ℃ for five minutes. After supernatant was removed and cell pellet was re-suspended with 1 x PBS to exclude any remained serum around cells, cell suspension was centrifuged again as described previously at 1500 rpm, 24 ℃ for five minutes. Then, supernatant was removed and cells were re-suspended by 1% SCM with 1% FBS. Cells were seeded at a density of 5×10^4 cells/well in 6-well plates with or without 4 ng/mL Interleukin-1 beta (IL-1β, R&D) and plates were placed in incubator at 37 ℃ and 5% CO_2. HCT-116 cells were cultured for 10 days and PCCCs were cultured for 19 days before harvesting cells for RNA extraction. IL-1β (4ng/mL) was added every other day from the first day of culture, and the culture medium was changed every four days. Images were taken for IL-1β-induced EMT cells after 10 days or 19 days of culture for HCT-116 or PCCC, respectively.

2.3 Wound healing assay

HCT-116 cells ($5x10^4$/well) were cultured in SCM with 10% FBS in 6-well plates until in they were confluent. Then cells were starved with SCM with 0.5% FBS overnight. After 12 hours, cells were re-cultured in SCM with 1% FBS. This time point was defined as time 0. At time 0, two lines were scratched in each well and cells were treated with IL-1β (4ng/mL) or without IL-1β. Images were taken at different time points from 0h, 12h, 24h, 36h, to 48h. Wound width was measured at every time point and exact the same site of each line. For each line, two images were taken, and the mean of the wound width was defined as the wound width of that line. For cell proliferation assay, 1000 HCT-116 cells per well were seeded in 1% SCM in 96-well plate with or without IL-1β (4ng/mL) for 48h. Cell number was counted every 12 hours from the time point of seeding.

2.4 Sphere assay

HCT-116 cells and PCCCs were cultured in 10% SCM in T25 flask as described in EMT morphology study. When cells became ~80% confluent, the culture medium was removed and the cells was rinsed by 1 x PBS. Trypsin-EDTA (0.25%, Cellgro, Inc) was added to cells and incubated for 5 minutes at 37℃ to dissociate cells from the flask. After the majority of cells had detached from flask, 5 mL SCM was added to flask to inhibit the function of Trypsin-EDTA. Cell suspension was centrifuged at 1500 rpm, 24℃ for five minutes. After supernatant was removed and cell pellet was re-suspended with 1 x PBS to exclude any remained serum around cells, cell suspension was centrifuged again as described previously at 1500 rpm, 24℃ for five minutes. Then, supernatant was removed and cells were re-suspended by serum free medium. The serum free medium comprised of neurobasal-A medium (GIBCO/Invitrogen) with supplements 1 x B27 (Invitrogen), 20ng/ml of epidermal growth factor (EGF, R&D systems), 20ng/ml of basic fibroblast growth factor (bFGF, R&D systems), 1 x GlutaMAX-I supplement (Invitrogen), 50ng/ml heparin (Sigma-Aldrich) and 1% penicillin-streptomycin. HCT-116 cells were seeded at a very low density of 100 cells/well in 96-well plates while primary colon cancer cells were seeded at a density of 500 cells/well with or without IL-1β, and then plates were placed in incubator at 37℃ and 5% CO2 for seven days. IL-1β (4ng/mL) was added every other day from the first day of culture, and the culture medium was changed every four days. After seven days of culture, images were taken and the sphere number was counted.

2.5 Cell proliferation assay

HCT-116 cells and PCCCs were cultured in SFM as described in self-renewal assay. On the seventh day of culture, the cell proliferation ratio was measured by cell proliferation kit (MTT, Roche Applied Science) as followed: 10μl/well of MTT labeling reagent was added, mixed and incubated in a humidified incubator at 37℃ for four hours, then solubilization solution was added, mixed and incubated in the incubator overnight. After 12 hours, the OD value was evaluated with a spectrophotometer at 550 nm with a reference wavelength of 690 nm. For cell counting proliferation assay, spheroid cells were first dissociated with 0.025% Trypsin-EDTA for 10 minutes in 37℃ incubator, then mixed 1:1 with Trypan blue(GIBCO). Cell number was counted under microscope.

2.6 Drug resistant assay

HCT-116 cells were cultured as self-renewal assay described above. 1000 cells/well of 96-well plate were seeded with 100 μl SFM for five days with or without IL-1β (4 ng/ml). IL-1β was added every other day and the medium was changed on the fourth day. On the fifth day of culture, cells were treated with different concentration (0 μM, 250 μM, 500 μM, or 1000 μM) of Carboplatin for 48 hours. After 48 hours, cells were stained with Trypan blue (Amresco Inc.) and cell number was counted under the microscope. The viability was determined by the percentage of live cells over the sum of live and dead cells.

2.7 RNA extraction

1 x 10^4 cells/well of HCT-116 cells were cultured in 6-well plate in 1% SCM and SFM with or without IL-1β stimulation for seven days as indicated above for the sphere assay, then was followed by RNA extraction by TRI reagent (Sigma). For primary colon cancer cells, 1 x 10^4 cells in each well of 6-well plate were cultured in 1% SCM and SFM for twenty-two days with or without IL-1β stimulation before the RNA extraction. Cells were first rinsed by ice-cold 1 x PBS then directly lysed by 1mL of TRI Reagent in the well. Cell lysates were prepared by vigorously pipetting up and down several times. Chloroform (Fisher) was added at 0.2ml/1ml of TRI Reagent, and samples were vortexed vigorously for 15 seconds before incubating at room temperature for two minutes. Samples were centrifuged at 14,000 rpm for 15 minutes at 4℃. After centrifugation, the upper aqueous phase was transferred without disturbing the interphase

into a fresh tube. The RNA was then precipitated by mixing with 0.5 ml isopropyl alcohol (Sigma) per 1ml of TRI Reagent. Samples were incubated at 4 ℃ for 30 minutes and centrifuged at 14,000 rpm for 15 minutes. The supernatant was removed completely and the RNA pellet was washed with 1 ml 75% ethanol per ml of TRI Reagent. Then, RNA was centrifuged at 10,000 rpm for 5 minutes at 4 ℃. After removing supernatant, RNA pellet was air-dried for 10 minutes before dissolving in DEPC-treated water. To avoid contamination of genomic DNA, RNA samples were digested by a DNAse kit (Ambion, AM1906) as followed: 0.1 volume 10 x DNase I buffer and 1 µl rDNase I were added to the RNA. After incubating the enzymes and RNA in 37 ℃ for 20 minutes, 0.1 volume of DNase inactivation reagent was added to the RNA and the reaction was incubated at room temperature for 2 minutes. Samples were centrifugation at 10,000 g for 1.5 minutes. The supernatant that contained the RNA was collected.

2.8 Gene expression

Relative mRNA expression was performed by two-step qRT-PCR. cDNA was reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad 170-8891). A total volume of 20 µl, consisting of 500 ng RNA, 4 µl 5 x iScript reaction mix, 1 µl iScript reverse transcriptase and the balance as water were mixed per reaction.

qPCR were performed by using SsoFast Eva Green Supermix kit (Bio-Rad 172-5201). The reaction mix consisted of 10 µl Eva Green mix buffer, 0.5 µl forward primer, 0.5 µl reverse primer, and 0.5 µl template, which was obtained from the reverse transcription step as described above. The condition of the qPCR steps was as followed: enzyme was activated by 30 seconds at 95 ℃, denatured at 95 ℃, then annealing and extension at 62 ℃ for 30 seconds, and last, melting at 78 ℃ for 20 minutes and increase 0.5 ℃ in 10 seconds per step from 65 ℃ to 85 ℃. The amplification of cDNA was performed with the primers.

2.9 Protein extraction

Protein from HCT-116 was extracted after culturing 1 x 10^4 cells in 1% SCM and SFM with or without IL-1β stimulation for seven days in 6-well plate. Protein from primary colon cancer cells was extracted after culturing 1 x 10^4 cells in 1% SCM and SFM for 22 days with or without IL-1β stimulation. Cells were rinsed with ice-cold PBS once and lysed directly in the well by adding 500 µl RIPA buffer. Plate was incubated at room temperature for 1 minute before

the cell lysate was pipetted up and down vigorously for at least ten times by syringe. After pipetting, cell lysate was vortexed for 30 second. Then, protein was placed into -80 ℃ for storage.

2.10 Western blot and quantification

Buffer for SDS-PAGE, membrane transfer and other process was prepared before Western blot, recipes were as followed: 1 x SDS running buffer contained 16 g Glycine, 3.36 g Tris-Base, and 1.1 g SDS, water was added up to 1 L. 1 x Transfer buffer contained 3.79 g Tris-Base, 18 g Glycine, and 250 mL methanol, water was added up to 1 L. 1 x TBS buffer contained 2.42 g Tris-Base, 8 g NaCl, water was added up to 1 L, then 0.1% Tween 20 was added into TBS.

In the stage of SDS-PAGE, protein concentration was determined by using NanoDrop. Then extract was mixed 1:1 with 2 x SDS sample buffer with beta-mercaptoethanol and heat to 100 ℃ for 5 min. Mixture was briefly centrifuged before ran on the SDS-PAGE. Running condition was 75V for 3 hours. For the process of membrane transfer, gel was blotted onto a pre-weted nitrocellulose membrane by transfer buffer. The stack was in following order: case (clear side), sponge, Whatman paper, membrane, gel, Whatman paper, sponge, and case (black side). Blotting condition was 90V for 1 hour, and ice-pack was used to cool down the apparatus. After that, membrane was blocked for 2 hours in 10 mL 1 x TBST dissolved with 5% non-fat dry milk in a small Tupperware dish on a shaker. Then, membrane was incubated with primary antibody (anti-Bmi1: mouse monoclonal, dilution factor: 1:500; anti-Nestin: mouse monoclonal, dilution factor: 1: 500; anti-Zeb1: rabbit polyclonal, dilution factor: 1:500; anto-Zeb2: mouse monoclonal, dilution factor: 1:500; anti-actin: rabbit monoclonal, dilution factor: 1:1000; anti-E-cadherin: rabbit monoclonal; dilution factor: 1:1000) diluted in 2.5 ml 1 x TBST and 2.5 mL 5% BSA in 50 mL conical tube and rotated on the nutator at 4 ℃ overnight. After blocking stage, membrane was washed 3 x for 5-10 min in 15 mL 1 x TBST at RT in a small Tupperware on a shaker. Then, secondary antibody (anti-rabbit-HRP, dilution factor: 1:2000; anti-mouse-HRP, dilution factor: 1:2000) was added to the membrane for 1 hour at RT in 2.5 mL 1 x TBST + 5% BSA in a 15 mL conical tube on a rotator. Membrane was washed 3 x for 10 min each in 15 mL 1 x TBST. After washing, substrate was added onto the membrane, and membrane was wrapped in a plastic and exposed to film.

The graph digitizing software UN-SCAN-IT was used for the quantification of Western Blot membrane. After opening an image (TIFF, JPEG, BMP, GIF, etc.) with the software, it can

convert the image into the underlying (x, y) values automatically. Background was first set, and each band was circled with the exactly same area. The average pixel was shown by comparing the total pixel with segment size. Then relative abundance was obtained by comparing the average pixel of aimed band and actin.

2.11 Primary colon cancer cell isolation

Tumor tissue was first placed into a 6-well plate, and then cut with scissors into tiny pieces for at least 5 minutes. Then, 3 mL (100 U/ml) Collagenase A was added to the tissue and mixed well. The plate was placed into 37℃ incubator, 1 hour for further digestion. During the one hour, cell suspension was mixed every 15 minutes. 20 μL 0.5M EDTA was added to the well to stop digestion. Then, all the tissue and cell suspension in 6-well plate was transferred into a homogenizer for quick mixing. The liquid phase was collected into a 50 mL conical tube. 1 x DPBS was used to wash homogenizer for two times. Cell suspension was then filtered to remove remaining debris. Cells were then spun down and resuspended into 10% serum-containing medium.

2.12 shZeb1 transduction

1 x 10^4 HCT-116 cells were placed into 1.5 mL SCM in 6-well plate 24 hours prior to shZeb1 lentiviral infection. When cells became 50% confluent, medium was removed and replaced by fresh SCM with 5μg/mL Polybrene (Santa Cruz). ShZeb1 and scramble shRNA lentiviral particles (Santa Cruz) were thawed at room temperature and mixed gently before use. Cells were infected by adding lentiviral particles to culture. Then plate was swirled to mix thoroughly and incubated overnight. Medium was replaced and cells were incubated for 1-2 days before selecting stable clones expressing shRNAs. Cells were split 1:5 according to the cell number and were continued to incubate for 48 hours in SCM. Stable clones were selected via Puromycin dihydrochloride selection (10μg/mL). Puromycin-containing medium was replaced every 3-4 days for 1 month to obtain stable transduced cells. Stable multiple clones were then expanded in SCM.

Puromycin selection: 1,000 HCT-116 cells were cultured in SCM in 96-well plate until they were almost confluent. Puromycin was added at various concentrations, ranging from 2 to 10μg/mL to cells and incubated for two days. 10μg/mL was picked up as the right concentration to the non-transduced cells.

2.13 qRT-PCR analysis

Relative mRNA expressions were analyzed by $2^{-\Delta\Delta CT}$ method (Winer, Jung et al. 1999). β-actin was used as internal control gene. The C_T values were provided from real time instrumentation (AB system). The data was analyzed using the equation: amount of target = $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = (C_T.\text{ target} - C_T.\text{ Actin})\text{ treated} - (C_T.\text{ target} - C_T.\text{ Actin})\text{ control}$. The fold change in the target gene, normalized to β-actin and relative to the expression of control, was calculated for each sample.

2.14 Statistic analysis

Statistical analyses were compared by a paired, two-tailed student's t test using excel program. A p value of less than 0.05 was considered significant. Analysis of variance was used for comparison of multiple groups.

Table 2.1 Sequences of primers were used in qRT-PCR

	Forward primer sequence	Reverse primer sequence

β-actin	5’-CCTGGGCATGGAGTCCTGTGG-3’	5’- CTGTGTTGGCGTACAGGTCTT-3’
E-cadherin	5’-GTCCTGGGCAGACTGAATTT-3’	5’-GACCAAGAAATGGATCTGTGG-3’
Zeb1	5’-AAGAATTCACAGTGGAGAGAAGCCA-3’	5’-CGTTTCTTGCAGTTTGGGCATT-3’
Zeb2	5’-CGCTTGACATCACTGAAGGA-3’	5 ‘-CTTGCCACACTCTGTGCATT-3’
Bmi1	5’-TCATCCTTCTGCTGATGCTG-3’	5’-GAACTTGCCGGAACTGAGAAC-3’
Nestin	5’-GAAACAGCCATAGAGGGCAAA-3’	5’-TGGTTTTCCAGAGTCTTCAGTGA-3’
Nanog	5’-TGATTTGTGGGCCTGAAGAAAA-3’	5’-GAGGCATCTCAGCAGAAGACA-3’

Chapter 3 - Result and Discussion

3.1 IL-1β-induced epithelial-mesenchymal transition (EMT) in HCT-116 and primary colon cancer cells (PCCC)

At first, we studied the morphological changes of HCT-116 and PCCC in SCM with 1% FBS in the presence or absence of IL-1β. We hypothesized that IL-1β could induce the EMT

process by switching the epithelial phenotype to a mesenchymal phenotype based on the fact that IL-1β is correlated with tumor progression and advanced metastatic disease (Pantschenko et al., 2003) and EMT may be required for cancer cell metastasis (Thiery, 2002). There are two different treatments: one is control (CTL) without any cytokine added, and the other is IL-1β treatment. In the absence of IL-1β, HCT-116 cells formed monolayer with very tight cell-cell junction and cells were densely packed together to form islets, a typically epithelial phenotype. However, after treated with IL-1β, cells became elongated and segregated from each other, indicating that IL-1β had induced the process of EMT (Figure 3.1 A-D). The morphology changes in HCT-116 and PCCC cells were similar to each other, which indicated that IL-1β induced EMT process in both colon cancer cell line and primary colon cancer cells. In addition, islet numbers were counted for HCT-116 cells. In the absence of IL-1β, the average islet number was approximately 14 per microscopic field whereas in IL-1β treated group, no islets were observed as the mesenchymal cells were all segregated from each other (Figure 3.1 E).

3.2 IL-1β-induced HCT-116 EMT cells have a higher migration and proliferation rate

As a result of EMT, cells lose tight cell-cell connection and gain much stronger migration ability. Here, the wound healing assay was applied to investigate the directional migration of HCT-116 treated with or without IL-1β. Wound width was measured from 0h right before IL-1β stimulation until 48h (Figure 3.2 A-D). After addition of IL-1β, the wound width showed a significantly decrease beginning at 24h and 80% wound was almost healed at 48h, which indicated IL-1β can induce a higher migration rate of HCT-116. However, to exclude the possibility that the healing of the wound may be due to a higher proliferation rate induced by IL-1β rather than higher migration ability, proliferation assay was then applied to study the difference of cell number between control and IL-1β treatment. It turned out that between 0h to 36h, there was no difference in cell number in control and IL-1β-treatment groups. However, by 36h, cells after treated with IL-1β showed a significant increase in cell number. Together, migration assay and proliferation assay indicated that during 0h to 36h, there was basically no difference in cell proliferation between control and IL-1β treated group. Thus the decrease of the wound width was due to a higher migration rate cells since the cell number failed to show any

difference between control and IL-1β treated group. The decrease in the wound width between 36h to 48h was attributed to both higher migration rate and proliferation rate (Figure 3.2 E-F).

3.3 IL-1β-induced HCT-116 and primary colon cancer EMT cells express EMT marker genes and proteins

After studying the morphological changes, we then analyzed the molecular biological evidence of IL-1β-induced EMT by gene and protein expression. Since the EMT process can be characterized by several defined gene profile changes, we determined expression of E-cadherin and Zeb factors as important EMT markers (Zeisberg and Neilson, 2009; Aigner et al., 2007; Graham et al., 2008) in cells treated with or without IL-1β using qRT-PCR and immunoblot assays. HCT-116 cells treated with IL-1β showed a significant up-regulated Zeb1 expression and down-regulated E-cadherin expression. In PCCC, IL-1β induced expression of Zeb2 instead of Zeb1, and suppressed the expression of E-cadherin (Figure 3.3 A-B). Zeb1 and Zeb2 shared the same conservative DNA binding domains. They both function as crucial EMT activators and induce the loss of E-cadherin expression. The protein expression determined by Western blot analysis was consistent with the gene expression, which confirmed the down-regulated E-cadherin and up-regulated Zeb1 expression in HCT-116 (Figure 3.3 C-D). In addition, the quantification of western blot images showed 15% increase of Zeb1 and 35% decrease of E-cadherin expression (Figure 3.3 E-F).

3.4 IL-1β-promoted sphere formation of HCT-116 in SFM

Based on the fact that SCs can form spheres in SFM and elevated IL-1β expression is correlated with colon cancer growth and metastasis (Kreso and O'Brien, 2008; Pantschenko et al, 2003), we hypothesized that after HCT-116 and primary colon cancer cells were treated with IL-1β, SCs would be maximally enriched and spheres would be formed in SFM. Therefore we cultured HCT-116 in SFM with or without IL-1β. The sphere assay, which is widely utilized as a method to determine and enrich stem cells, showed that after 7 days of culture, spheres in IL-1β treated group showed a significant increase in size and number than those in control. From these images, we can see that in control without any IL-1β, HCT-116 can form spheres itself, which suggested that this type of cells already contained some stem cells, or contained some cells that

had the property as stem cells. However after cells were treated with IL-1β, the morphological change was more obvious, the size and number of spheres both increased (Figure 3.4 A-B).

3.5 IL-1β-promoted sphere formation of HCT-116 in SFM at a very low cell density

The morphology study of sphere assay suggested that IL-1β could promote the sphere formation of HCT-116. Therefore, self-renewal assay was performed to investigate the self-renewal ability, which is one of the most important characteristics of CSCs (Pardal, Clarke et al., 2003). In self-renewal assay, a very low density of cells, 1 cell per ul, was cultured in 96-well plates containing 100 ul SFM per each well for 7 days with or without IL-1β stimulation. After 7 days of IL-1β treatment, the size and number of spheres all showed a significant increase compared with control. In addition, when spheres were separated into three different groups according to their size, IL-1β was observed to have the ability to stimulate HCT-116 to form more spheres that had a size bigger than 90um (diameter). This result suggested that IL-1β can promote the self-renewal ability of HCT-116 (Figure 3.5 A-C).

3.6 IL-1β-promoted HCT-116 sphere cells proliferate at a higher rate

To further determine the effect of IL-1β on CSCs proliferation, proliferation assays were applied to HCT-116 right after self-renewal assay. After HCT-116 were cultured in SFM with or without IL-1β for seven days, proliferation was measured by MTT assay and cell counting. The proliferation ratio by OD values showed that cells treated with IL-1β had a significant increase in proliferation compared with cells in the absence of IL-1β. In addition, cell counting result showed an increase in cell number in IL-1β treated group, consistent with proliferation ratio determined by MTT assay. This indicated that HCT-116 proliferated at a high rate after IL-1β stimulation (Figure 3.6 A-B).

3.7 IL-1β-induced sphere formation of primary colon cancer cells in SFM at a very low cell density

Beside the colon cancer cell line, we determined twhether IL-1β could promote or induce the sphere formation and self-renewal ability of PCCCs in SFM as well. Therefore, self-renewal assay was applied to PCCCs. Five cells per ul were cultured in SFM with or without IL-1β for 8 days. The result showed that PCCC cells all formed monolayer that attached to the plate in SFM

without IL-1β, different from HCT-116 in SFM. Whereas in IL-1β treated group, spheres were formed (Figure 3.7 A-D). These results suggested that PCCCs contained no CSCs or some inactivated CSCs. However, after treated with IL-1β, cells began to form spheres, the characteristic of CSCs (Kreso and O'Brien, 2008).

3.8 IL-1β-induced primary colon cancer sphere cells proliferate at a high rate

Proliferation assay was applied to primary colon cancer sphere cells after they were cultured in SFM for 8 days with or without IL-1β. Cells were dissociated first and then cell number was counted. The number of cell in IL-1β treatment showed a significant increase compared with the cell number in CTL, which suggested that IL-1β could induce proliferation of primary colon cancer sphere cells at a high rate (Figure 3.8).

3.9 IL-1β-treated HCT-116 sphere cells have enhanced drug resistance ability

CSCs have enhanced drug resistant ability. Therefore, we determined whether cells treated with IL-1β could have the increased drug. Carboplatin, a widely used chemotherapy drug against a variety of cancers, was the drug used for drug resistant assay for HCT-116 with or without IL-1β treatment. 1,000 HCT-116 cells were cultured in SFM in the presence or absence of IL-1β for 5 days, and then, cells were incubated with various concentration of carboplatin (0 μM, 250 μM, 500 μM, and 1000 μM) for 48 h. After 48 h of incubation, IL-1β treated cells began to show a higher drug resistant ability compared with control. When the drug concentration reached 1000uM, the viability of control cells decreased to 15%, whereas in IL-1β treated group, cells still remained 50% alive (Figure 3.9). These data suggested IL-1β-treated HCT-116 sphere cells had an enhanced drug resistant ability, which was also another important character of CSCs (Dean, Fojo et al. 2005; Kang and Kang 2007).

3.10 IL-1β-induced HCT-116 and primary colon cancer sphere cells increased stem cell gene and protein expression

Since results from both the self-renewal assay and the drug-resistant assay of HCT-116 cells and PCCCs indicated that cells treated with IL-1β gained CSC properties, qRT-PCR was performed to detemine the SC gene expression profile. For HCT-116, the SC gene markers we examined were Bmi1, Nestin, and Nanog. Bmi1 is a crucial predictor of colorectal cancer prognosis and the overexpression of Bmi1 is related with tumorigenesis and CSC phonotype (Du,

Li et al., 2010; Li et al, 2009; Dovey et al, 2008). Nestin is a marker for angiogenesis of colon cancer (Sheila et al., 2003) and Nanog is involved in SC self-renewal. Expression of Bmi1, Nestin, and Nanog all were increased in cells treated with IL-1β compared with that in control cells. In PCCCs, Bmi1 expression was up-regulated as well. Besides the SC marker genes, it was surprising to find that gene expression of Zeb1 and Zeb2 were up-regulated in IL-1β-induced HCT-116 and PCCC sphere cells respectively, which were also up-regulated in IL-1β-induced EMT cells (Figure 3.10 A-B). In addition, Western blot analysis was conducted after protein was extracted from 1 x 10^4 HCT-116 to confirm protein expression. The protein expression was consistent with gene expression (Figure 3.10 C-F).

3.11 Zeb1 knockdown inhibited IL-1β-induced EMT in HCT-116

From the qRT-PCR gene expression analysis of IL-1β-induced EMT cells and spheroid cells, we found the common genes that were up-regulated in both HCT-116 and PCCCs. They were ZEB family gene and Bmi1, which suggested these two genes may play very significant role in CSC self-renewal and EMT process. Since ZEB factors and Bmi1 are linked together by ZEB/miRNA-200 feedback loop, it was important to understand the role of Zeb1 in cancer prognosis. Zeb1 was knocked down using shRNAs to determine its role in IL-1β-induced EMT and self-renewal in HCT-116 cells. The morphology study was performed at first. Cells expressing scramble or shZeb1 formed monolayer culture and displayed very tight cell-cell connection. However, after treated with IL-1β, cells expressing scramble became elongated and cells were segregated from each other, while shZeb1 cells appeared resistant to the effect of IL-1β on cells, and maintained epithelial morphology (Figure 3.11 A-D). In addition, the Western blot analysis confirmed that in scramble shRNA cells, the Zeb1 expression was increased and E-cadherin expression was decreased after IL-1β stimulation; whereas shZeb1 cells failed to show an increase of Zeb1 expression and decrease of E-cadherin expression after IL-1β treatment, which indicated Zeb1 knockdown can inhibit IL-1β-induced EMT in HCT-116 (Figure 3.11 E).

3.12 Zeb1 knockdown reduced proliferation of sphere cells and inhibited IL-1β-induced Bmi1 expression in SFM

ZEB factors and Bmi1 were linked together by ZEB/miRNA-200 feedback loop, and we had shown that shZeb1 can block the IL-1β-induced EMT process, therefore self-renewal assay

and western blot were performed to study whether shZeb1 knockdown would affect CSC self-renewal. 1 cell per 10 μl was cultured in SFM with or without IL-1β for 7 days to measure the proliferation rate. The shZeb1 cells showed a significant decrease in proliferation compared with scramble cells, while after treated with IL-1β, shZeb1 cells showed increased proliferation, reaching to similar levels with scramble cells without IL-1β. However, compared to scramble cells treated with IL-1β, shZeb1 cells treated with IL-1β, showed a significant decrease in proliferation (Figure 3.12 A). The Western blot result showed decreased expression of Zeb1 and Bmi1 as well. These results indicated that Zeb1 regulates Bmi1 expression and mediates IL-1β-induced Bmi1 expression in HCT-116 cells as well as cell proliferation in SFM (Figure 3.12 B).

3.13 Discussion

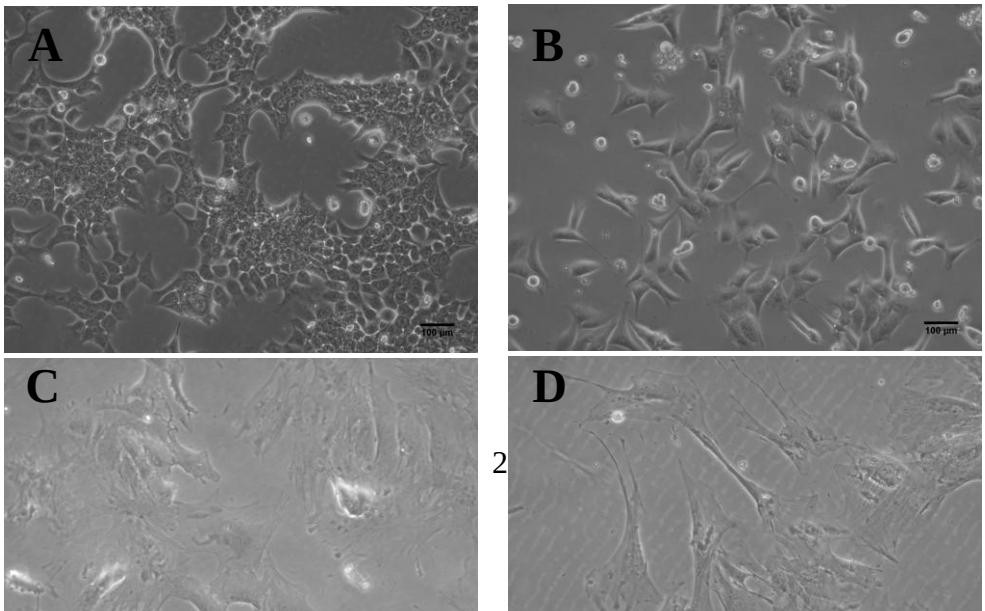
The elevated expression of CSC markers has been observed in colon cancer (Fujimoto, Beauchamo et al., 2002). The small population of CSCs may be in charge of tumor recurrence after traditional treatments. There is no doubt that targeting on CCSCs would provide a new approach of colon cancer therapy.

IL-1β, which plays a key role in the tumor microenvironment, has connected inflammation and cancer progression together by promoting Wnt signaling pathway and increasing cell proliferation in colon cancer cells (Pantschenko et al., 2003; Kaler et al., 2009). From our study, we found that IL-1β had significant effects on cancer progression and CCSCs development. Our data showed that HCT-116 EMT cells induced by IL-1β display the following characteristics: significant epithelial to mesenchymal phenotype transition, strong migration ability, and EMT marker expression. In addition, the spheroid cells induced by IL-1β exhibit SCs properties: enhanced self-renewal ability, strong anti-cancer drug resistance, and SC marker expression. Overall, our data indicate that IL-1β induce EMT and CSC development in colon cancer cells.

Zeb1 is a very important regulator involved in EMT and CSC self-renewal. According to our data, we have observed that IL-1β can promote epithelial-mesenchymal transition and a stem cell phenotype in colon cancer cells through ZEB factors. In IL-1β-induced colon cancer EMT cells, expression of Zeb1 or Zeb2 was up-regulated and the expression of E-cadherin was suppressed, indicating IL-1β triggered the EMT process. In IL-1β-induced sphere cells, expression of Bmi1 and Zeb1 or Zeb2 was increased. It has been reported that Zeb1/Zeb2 works

through MicroRNA-200 family to regulate the expression of Bmi1 (Burk, Schubert et al., 2008; Wellner, Schubert et al., 2009; S. Brabletz and T. Brabletz, 2010). Therefore, our results suggest that Zeb1/Zeb2 regulates IL-1β-induced Bmi1 expression in colon cancer cells. The important role of Zeb1 in regulation of IL-1β-induced EMT and self-renewal in HCT-116 cells was further confirmed using shRNAs to knockdown of Zeb1 in HCT-116 cells. Zeb1 knockdown affected measured properties associated with EMT and CSCs in HCT-116 cells, namely morphological change between epithelial and mesenchymal phenotype, sphere formation, and characteristic marker expression. Our finding indicates that Zeb1 regulates IL-1β-induced EMT and CSC self-renewal ability in HCT-116.

Figure 3.1



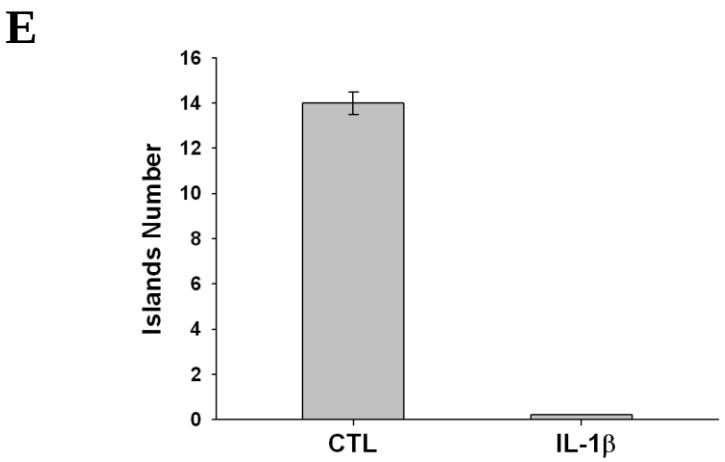
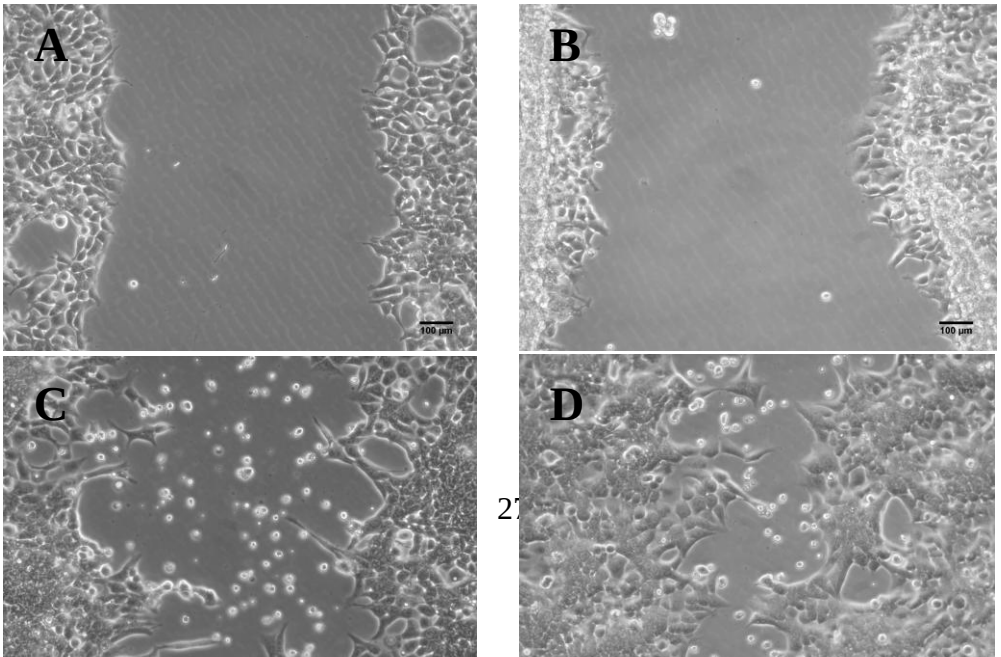


Figure 3.1 IL-1β-induced EMT cells lost cell-cell adhesion and increased cell mobility. 1 x 10^4 HCT-116 and 1 x 10^5 PCCC were cultured in 1% SCM in 6-well plate with the presence or absence of IL-1β for 10 days and 19 days respectively. Morphological change was observed. **A.** HCT-116 control, cells formed monolayer and densely packed together to form closely connected network. **B.** After treated HCT-116 cells with IL-1β, cells became elongated and segregated from each other. **C.** PCCC in control without IL-1β and **D.** with IL-1β displayed similar morphology compared with HCT-116. Cells were tightly adhered before IL-1β stimulation. **E.** Islet numbers counted per field of 6-well plate of HCT-116 confirmed with the morphology study, cells were segregated after IL-1β treatment. 50 x objective.

Figure 3.2



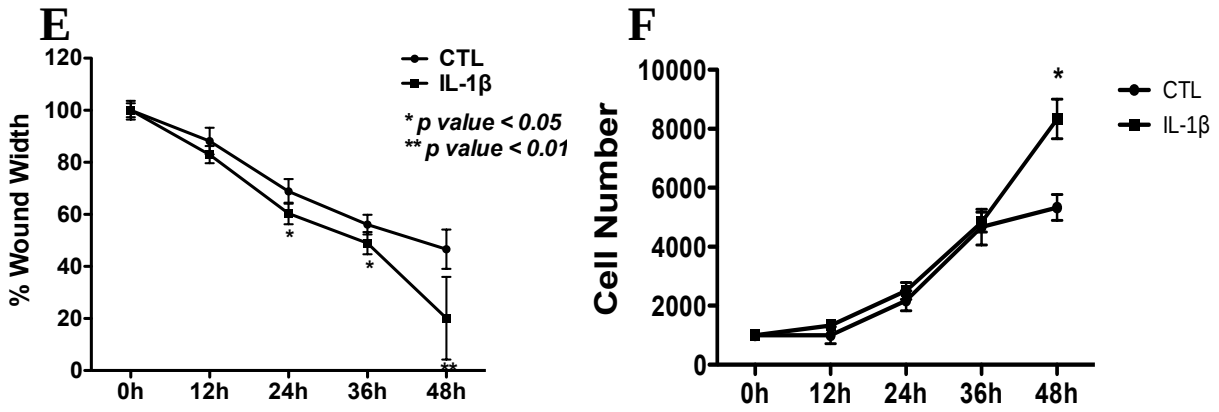
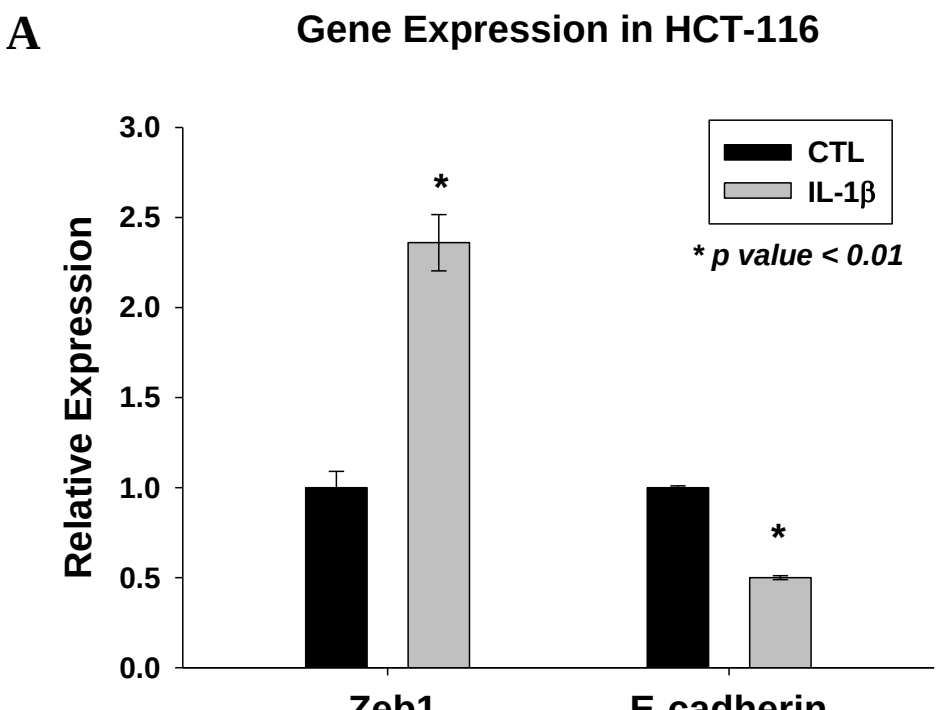


Figure 3.2 IL-1β-induced HCT-116 EMT cells had a higher migration and proliferation rate in vitro. Monolayer was wounded and wound width was measured every 12 h in control and IL-1β treated group. For **A.** HCT-116 control and **B.** HCT-116 IL-1β, the wound width at 0 h right before adding IL-1β was almost the same. **D.** HCT-116 cells treated with IL-1β. After 48 h of IL-1β treatment, the wound was almost healed for 80% compared with **C.** HCT-116 CTL, which healed only 50%. **E.** IL-1β treated cells had increased migration. The increased healing rate for IL-1β treated cells started to show significant difference from 24 h. **F.** IL-1β increased cell proliferation after 36 h. There was no significant difference during 0 h to 36 h in cell proliferation between cells treated with and without IL-1β. Scale bar = 100μm. * $p < 0.05$, ** $p < 0.01$. Figures are representative for two independent experiments.

Figure 3.3


A
Gene Expression in HCT-116
Relative Expression
3.0
2.5
2.0
1.5
1.0
0.5
0.0
CTL
IL-1β
* p value < 0.01
*
*
Zeb1
E-cadherin

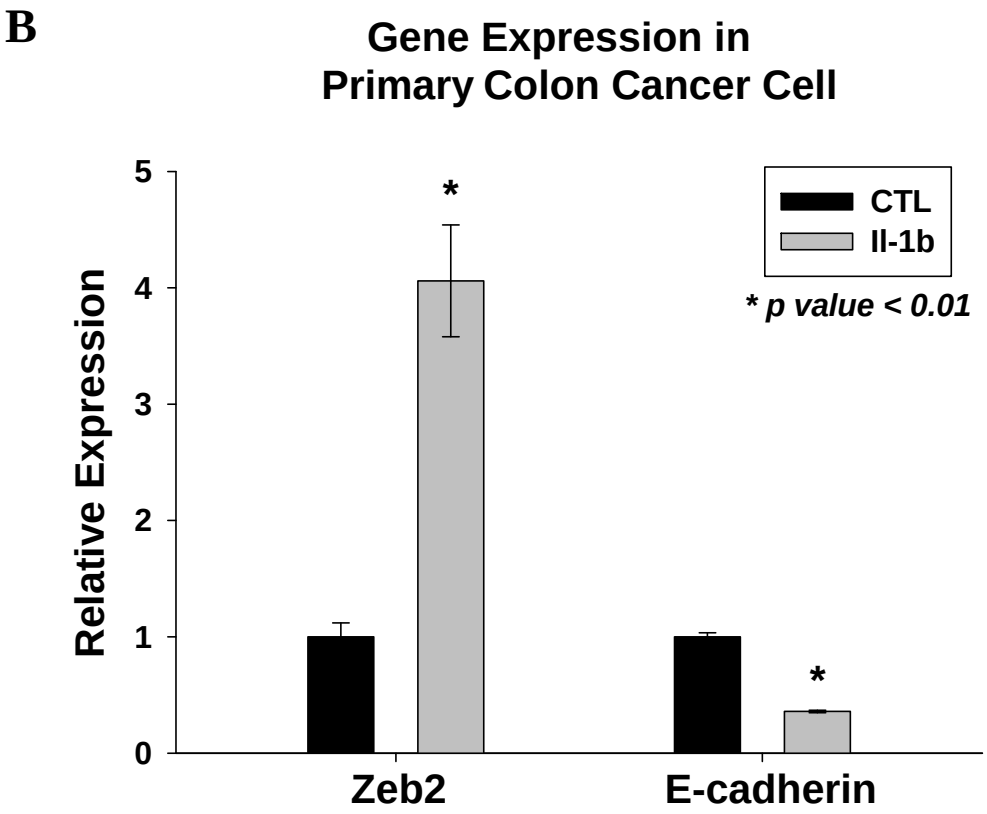
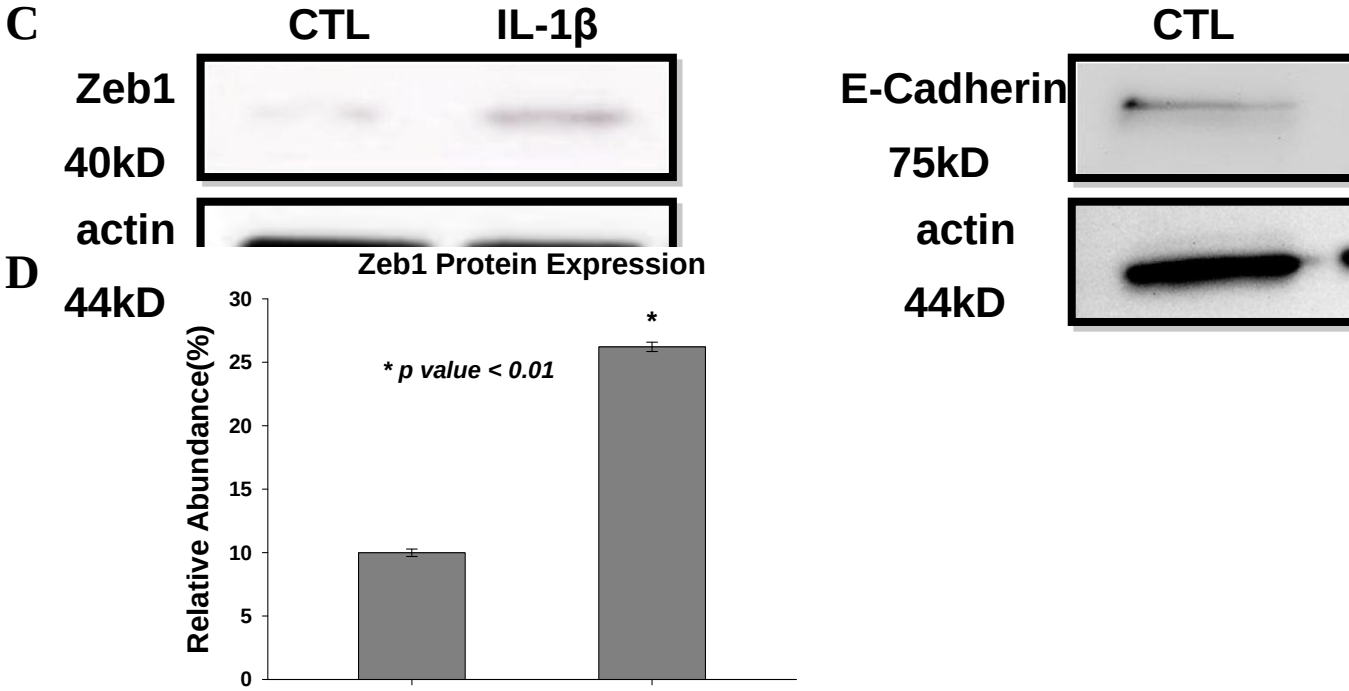

B
Gene Expression in
Primary Colon Cancer Cell
Relative Expression
5
4
3
2
1
0
*
*
CTL
Il-1b
* p value < 0.01
Zeb2
E-cadherin

Figure 3.3


C
CTL
IL-1β
Zeb1
40kD
actin
44kD
CTL
E-Cadherin
75kD
actin
44kD
D
Zeb1 Protein Expression
Relative Abundance(%)
30
25
20
15
10
5
0
*
* p value < 0.01

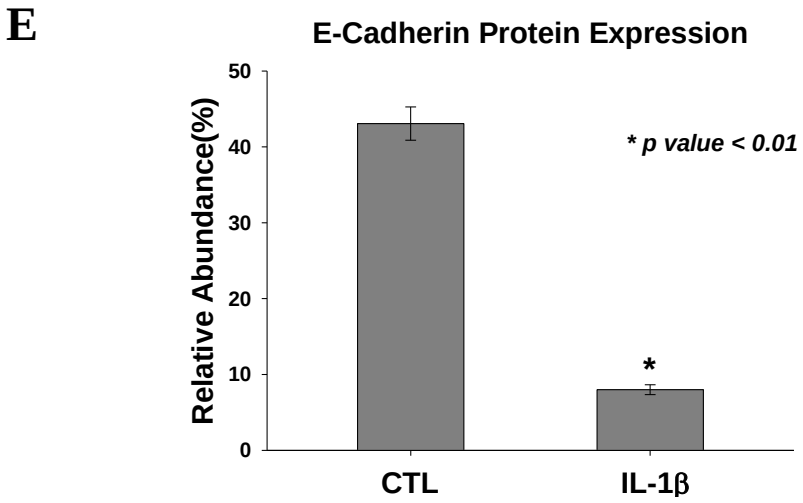
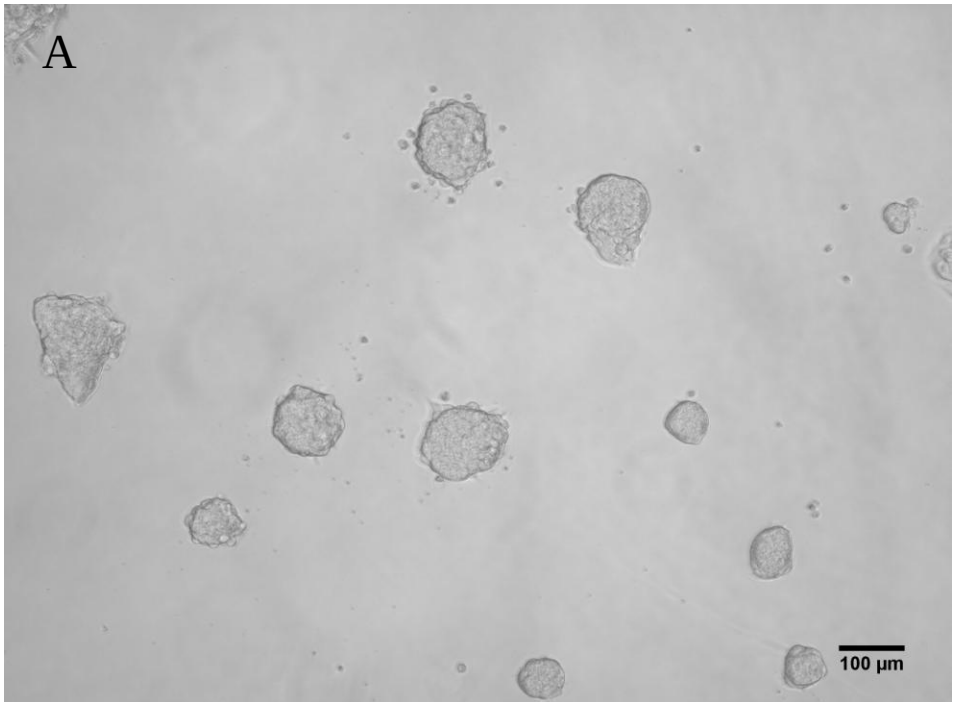


Figure 3.3 IL-1β-induced EMT cells express EMT marker genes and proteins. A-B. qRT-PCR analysis was performed to detect the mRNA level of EMT markers Zeb1 and E-cadherin in HCT-116 cells **(A)**, and Zeb2 and E-cadherin in PCCC **(B)** treated with or without IL-1β. With IL-1β treatment, more Zeb1/Zeb2 and less E-cadherin were expressed. **C.** The protein expression of Zeb1 and E-cadherin in HCT-116 was consistent with mRNA expression. The quantification in **D.** and **E.** showed the percentage of increase in Zeb1 protein and decrease in E-cadherin protein expression. Western blot result was consistent with qRT-PCR result. β-actin mRNA and protein levels were used as an internal control. * $p < 0.01$.

Figure 3.4



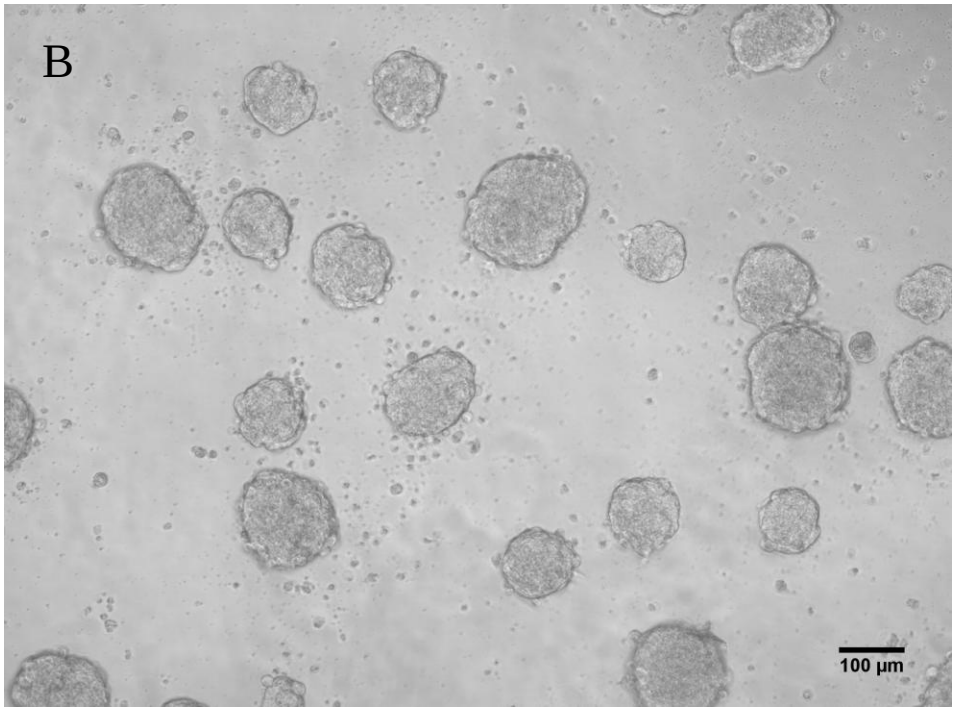
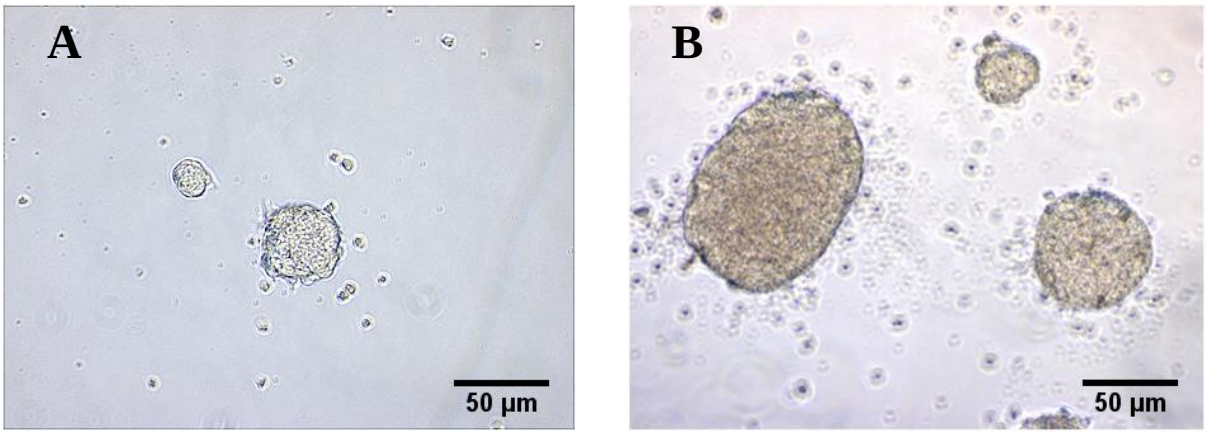


Figure 3.4 IL-1β promoted HCT-116 sphere formation. HCT-116 cells (1 x 10^5/well) were cultured in SFM in 6-well plates in the presense or absense of IL-1β for 7 days. **A.** In control, cells formed spheres without IL-1β. **B.** With IL-1β treatment, spheres became larger and increased in number. Scale bar = 100µm.

Figure 3.5



C

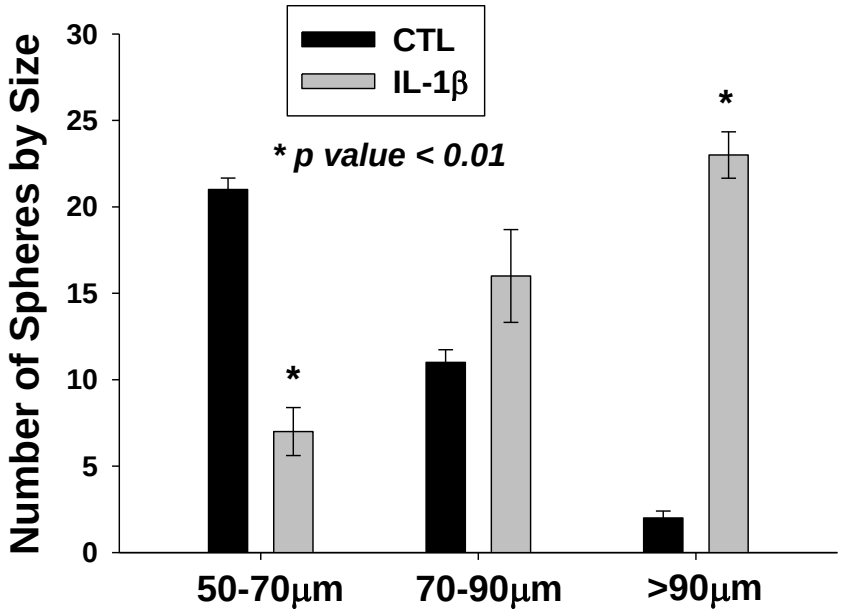
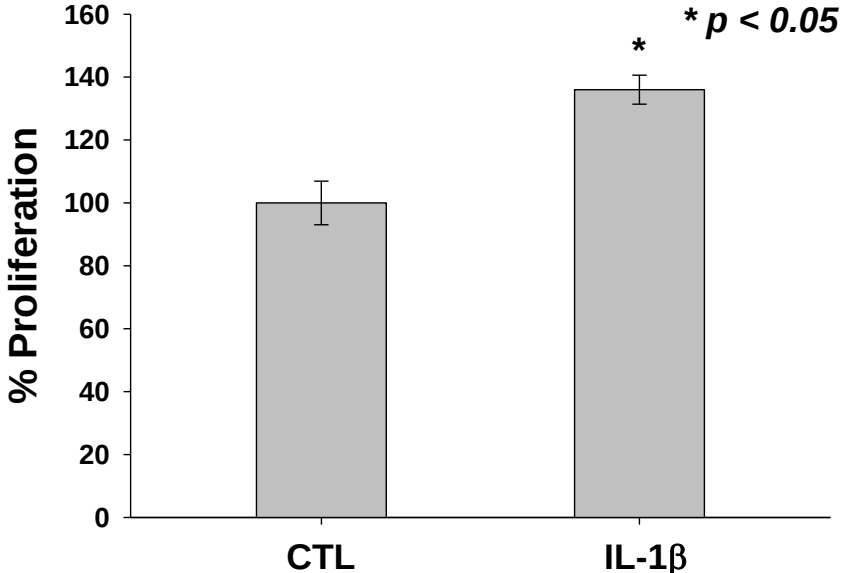


Figure 3.5 IL-1β-promoted the self-renewal ablity of HCT-116. To study the self-renewal ablity of HCT-116, cells were cultured in a very low cell density (100 cells per well of a 96-well plate) in SFM for 7 days. **A.** In control, spheres formed without IL-1β stimulation. **B.** Sphere formation after IL-1β treatment. **C.** Spheres were divided into 3 different groups according to their size. Scale bar = 50μm. * $p < 0.01$. Figures are representative for three independent experiments.

Figure 3.6

A



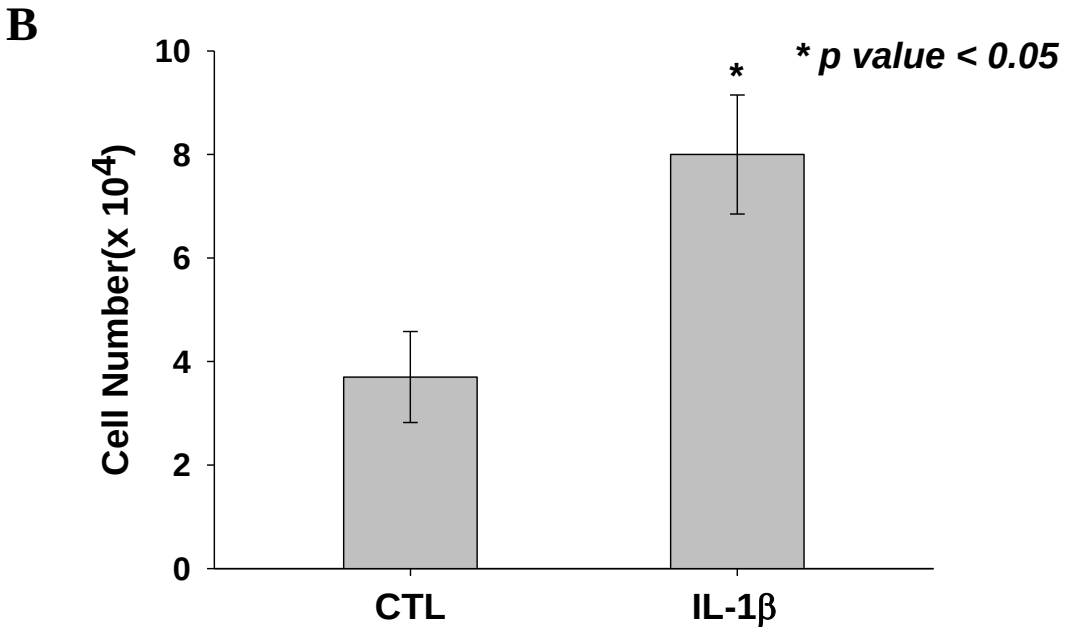
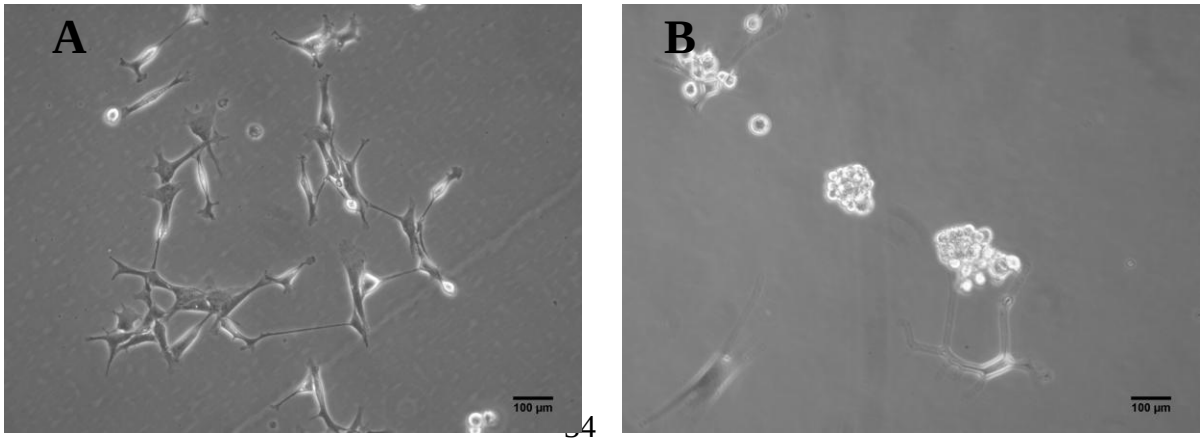


Figure 3.6 IL-1β-promoted proliferation of colon cancer stem cells of HCT-116. HCT-116 cells (100 cells/well) were cultured in SFM in 96-well plates for 7 days before the proliferation assay. **A.** and **B.** showed the proliferation assay results measured by MTT proliferation kit and cell counting, respectively. These two results corresponded with each other, which all showed significant increase in either proliferation ratio of cell number. * $p < 0.05$. Figures are representative for three independent experiments.

Figure 3.7



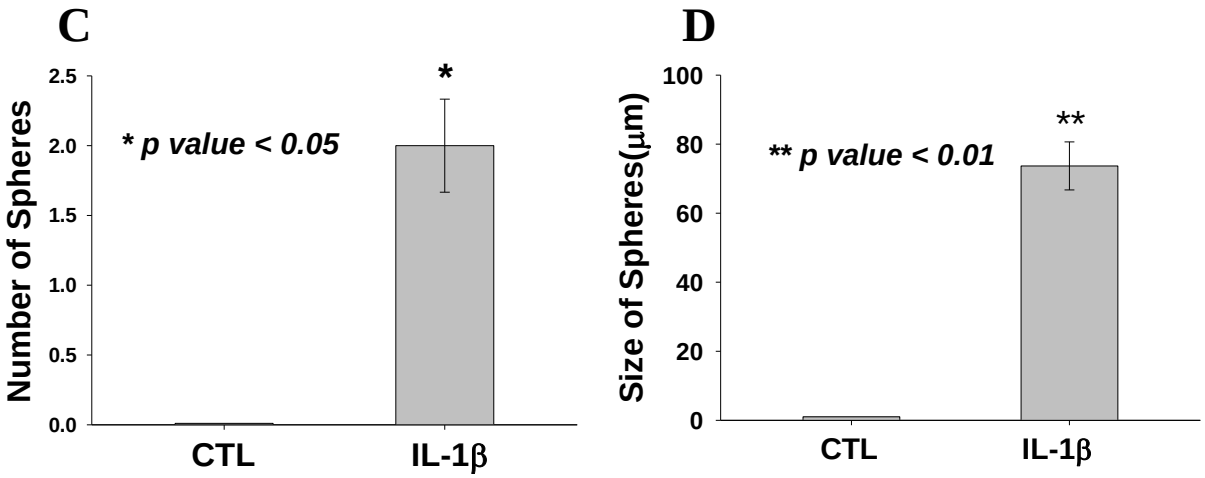


Figure 3.7 IL-1β-induced sphere formation and self-renewal ability of PCCCs. A. and **B.**, self-renewal assay of PCCCs. 500 cells/well of PCCC were cultured in SFM with the presence **(B)** or absence **(A)** of IL-1β for 8 days. **C.** the number of spheres per well of field in IL-1β treatment was around 2, and **D.** the size of spheres was around 76μm. Scale bar = 100μm. * $p < 0.05$. ** $p < 0.01$. Figures are representative for two independent experiments. The results from the two independent experiments are consistent with each other.

Figure 3.8

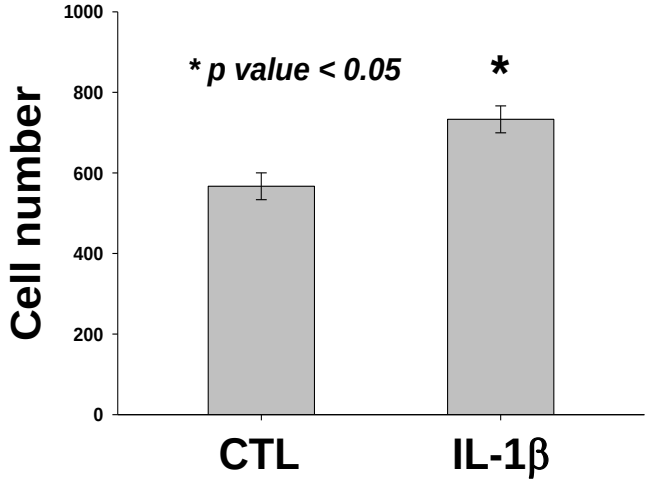


Figure 3.8 IL-1β-promoted proliferation of PCCCs at a very low cell density in SFM. Cell counting proliferation assay was performed immediately after self-renewal assay. The PCCCs proliferation measured by cell counting also showed a significant increase in IL-1β treated group, which was similar with HCT-116 as well. * $p < 0.05$. Figures are representative for two independent experiments.

Figure 3.9

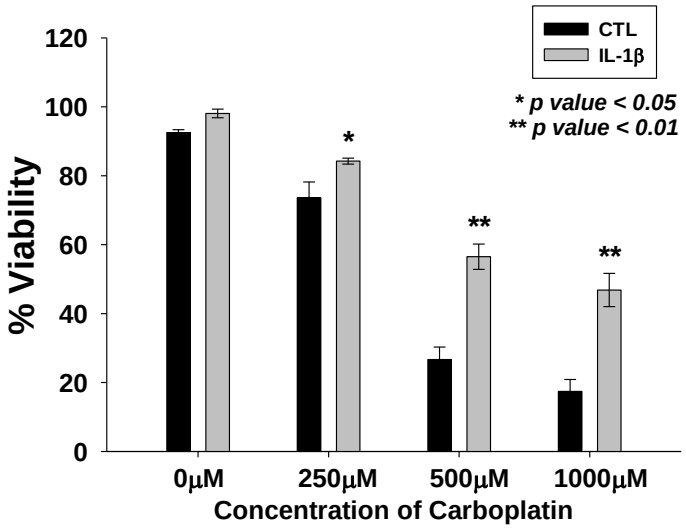
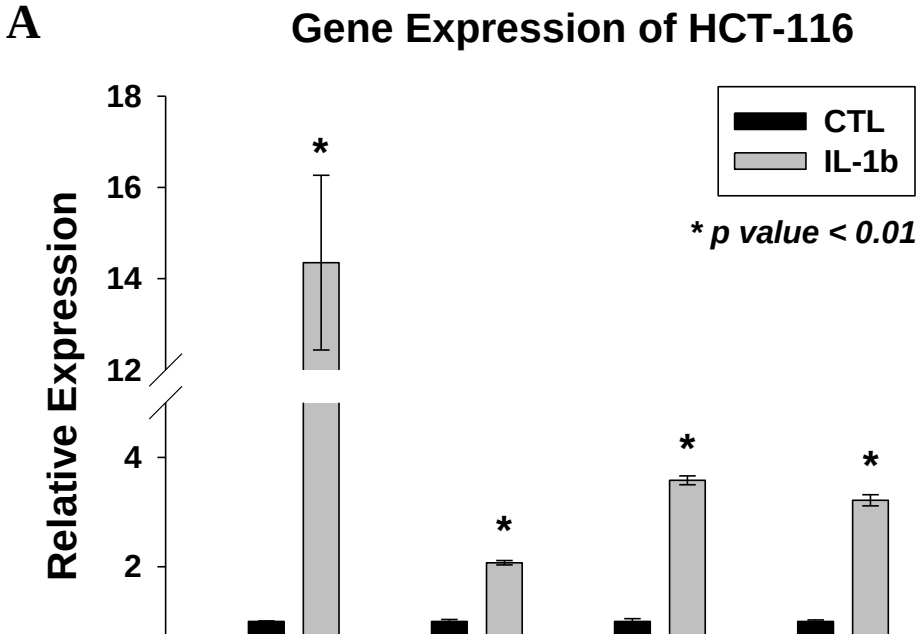
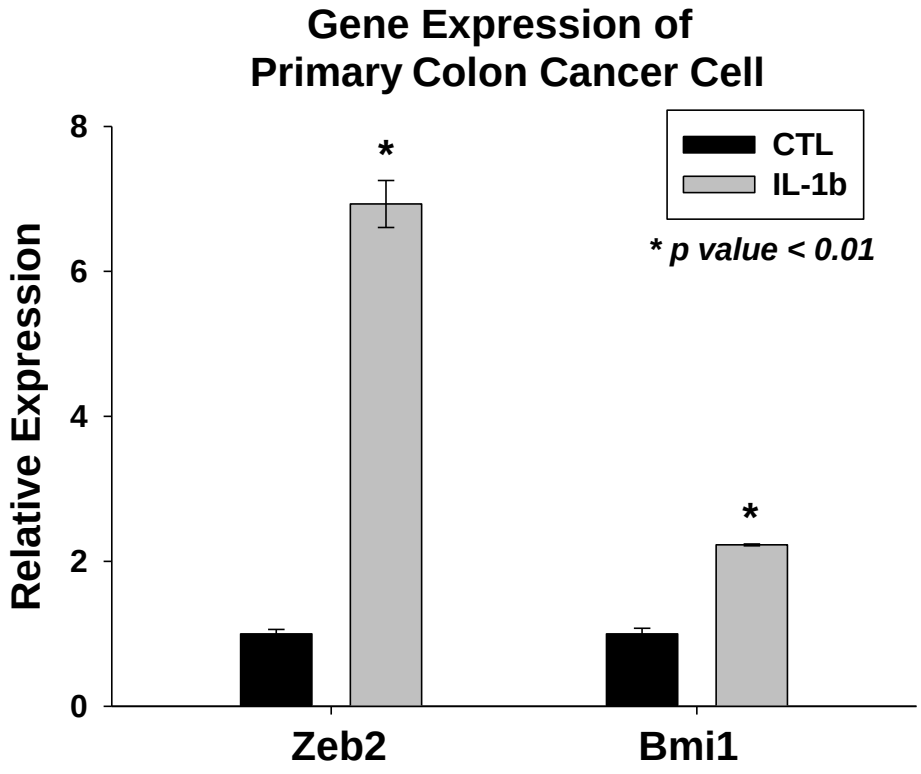


Figure 3.9 IL-1β-enhanced drug-resistant ability of HCT-116. 1,000 cells/well of HCT-116 were cultured in SFM in 96-well plate with or without IL-1β for 5 days. The viability of cells was much higher after treated cells with IL-1β. * $p < 0.05$. ** $p < 0.01$. Figures are representative for two independent experiments. The results for both experiments were consistant.

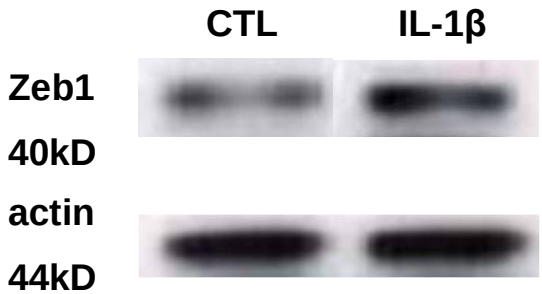
Figure 3.10

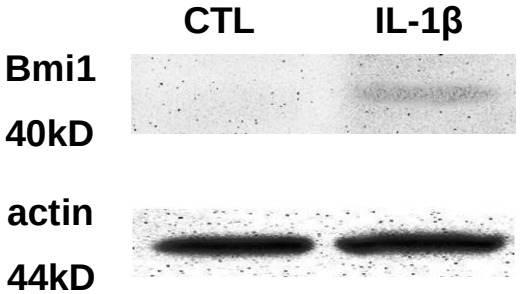


B

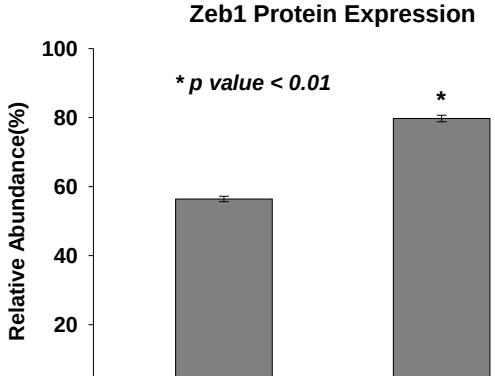

Gene Expression of
Primary Colon Cancer Cell
CTL
IL-1b
* p value < 0.01
Relative Expression
8
6
4
2
0
*
*
Zeb2
Bmi1

C

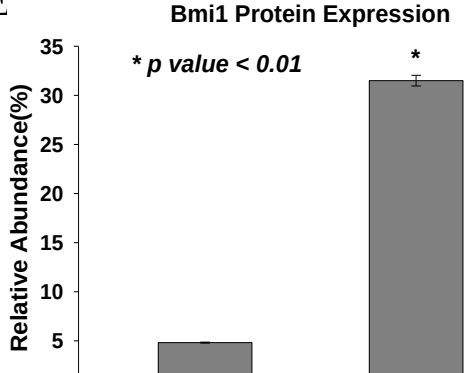

CTL
IL-1β
Zeb1
40kD
actin
44kD


CTL
IL-1β
Bmi1
40kD
actin
44kD

D


Zeb1 Protein Expression
* p value < 0.01
Relative Abundance(%)
100
80
60
40
20
*

E


Bmi1 Protein Expression
* p value < 0.01
Relative Abundance(%)
35
30
25
20
15
10
5
*

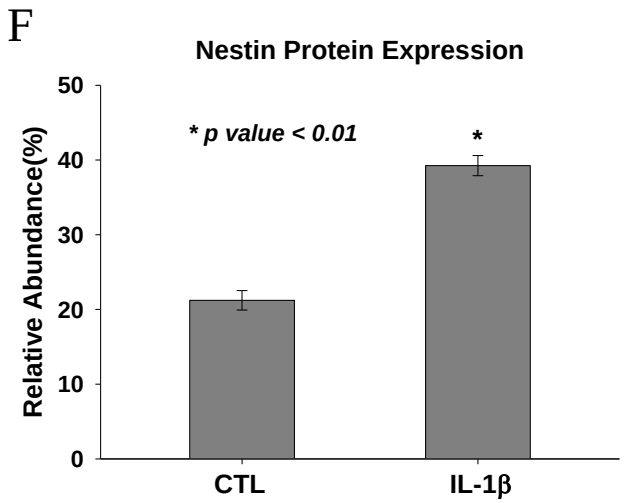
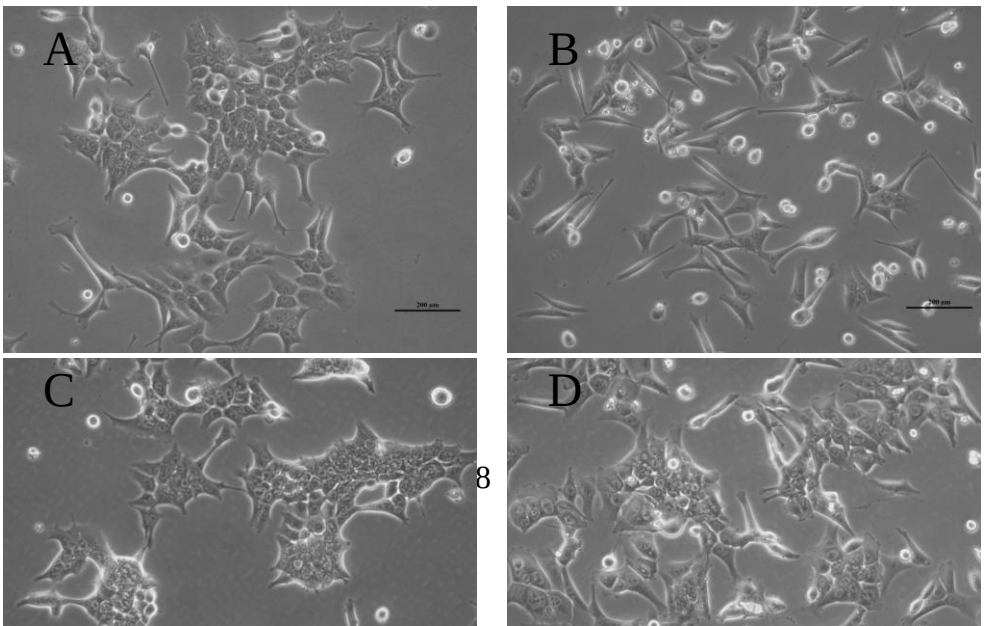


Figure 3.10 IL-1β-stimulated cells express stem cell markers. A. SC gene expression in HCT-116 cells. After IL-1β treatment, more Bmi1, Nestin, and Nanog were expressed in HCT-116 cells. Beside the stem cell genes, Zeb1 was elevated as well. **B.** SC gene expression in PCCC. Bmi1 and Zeb2 were both increased. **C.** The western blot result confirmed the gene expression. **D.**, **E.**, and **F.** showed the relative abundance of protein expression. β-actin mRNA and protein levels were used as an internal control. * $p < 0.01$. Western blot results were consistent with qRT-PCR gene expression

Figure 3.11



E

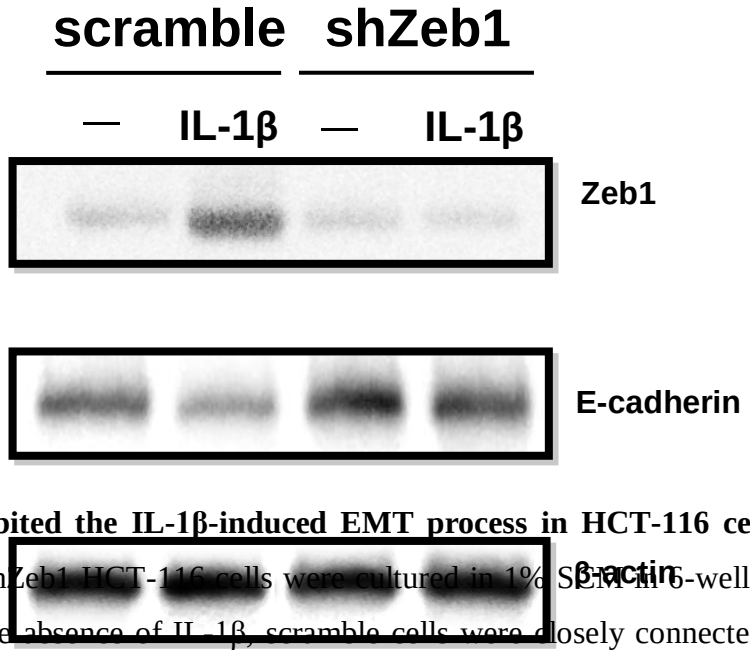
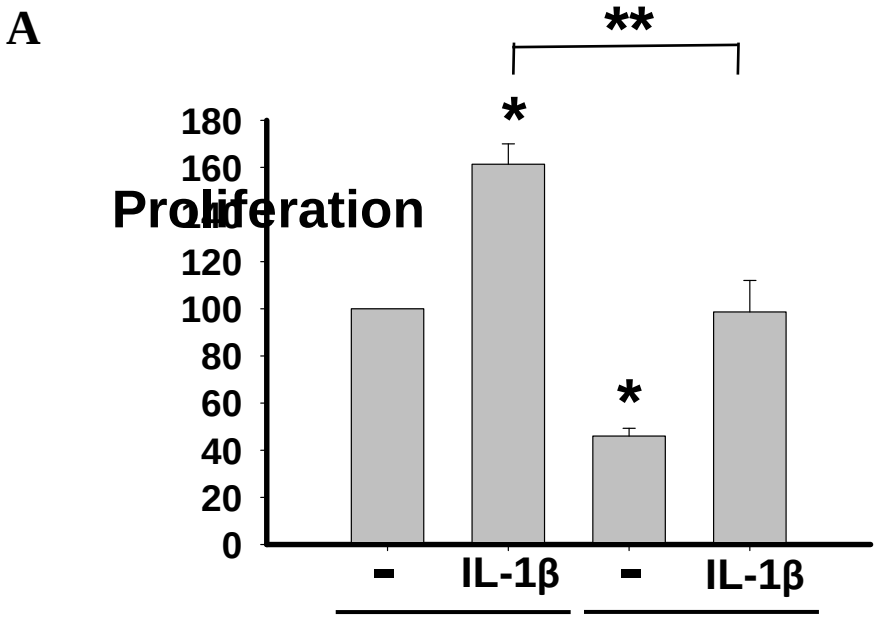


Figure 3.11 shZeb1-inhibited the IL-1β-induced EMT process in HCT-116 cells. 1 x 10^4 cells/well of shCTL and shZeb1 HCT-116 cells were cultured in 1% SFM in 6-well plate w/wo IL-1β for 7 days. **A.** In the absence of IL-1β, scramble cells were closely connected with each other. **B.** After IL-1β treatment, scramble cells gained an elongated mesenchymal phenotype. **C.** In the absence of IL-1β, shZeb1 cells were closely adhered together, whereas after IL-1β treatment **(D)**, cells were not all separated from each other, they began to show an adhered morphology. **E.** Western blot results were consistent with morphology study. β-actin protein levels were used as an internal control. Scale bar = 100 μm.

Figure 3.12



B

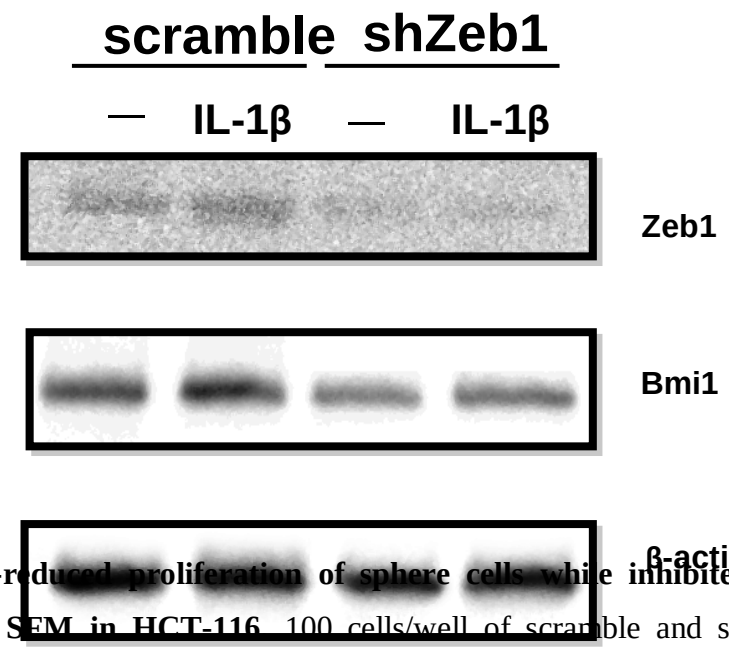


Figure 3.12 shZeb1-reduced proliferation of sphere cells while inhibited IL-1β-induced Bmi1 expression in SEM in HCT-116. 100 cells/well of scramble and shZeb1 cells were cultured in 96-well plate w/wo IL-1β for 7 days. **A.** Proliferation assay. In scramble control, IL-1β increased proliferation. In the absence of IL-1β, shZeb1 cells showed a significant decrease in cell number compared with scramble control. Although in IL-1β treatment, proliferation ratio showed an increase, however, it failed to increase as much as in scramble IL-1β treatment. Figures are representative for three independent experiments. **B.** Western blot results showed the knockdown of Zeb1 expression and decrease in Bmi1 expression. β-actin protein levels were used as an internal control. Scale bar = 100 μm.

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