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Dual Targeting of Tumor Angiogenesis and Chemotherapy by Endostatin- Cytosine Deaminase-Uracil PhosphoribosylTransferase

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Dual Targeting of Tumor Angiogenesis and Chemotherapy by Endostatin-Cytosine Deaminase-Uracil Phosphoribosyl Transferase

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Abstract

Antiangiogenesis is a promising anti-tumor strategy through inhibition tumor vascular formation to suppress tumor growth. Targeting specific VEGF/R has been shown therapeutic benefits in many cancer types and become a first approved antiangiogenic modalities by Food and Drug Administration (FDA) in United States. However, interruption of homeostasis in normal tissues that is likely due to the inhibition of VEGF/R signaling pathway induces unfavorable side effects. Moreover, cytostatic nature of antiangiogenic drugs frequently causes less tumor cell specific killing activity, and cancer cells escaped from cell death induced by these drugs even gain more malignant phenotypes, resulting in tumor invasion and metastasis. To overcome these issues, we developed a novel anti-tumor therapeutic EndoCD fusion protein which linked endostatin (Endo) to cytosine deaminase-uracil

phosphoribosyl transferase (CD). Endo targets unique tumor endothelial cells to provide tumor-specific antiangiogenesis activity and also carries CD to the local tumor area, where it serves nontoxic prodrug 5-fluorocytosine (5-FC) enzymatic conversion reaction to anti-metabolite chemotherapy drug 5-fluorouracil (5-FU). We demonstrated that 5-FU concentration was highly increased in tumor sites, resulting in high level of endothelial cells and tumor cells cytotoxic efficacy. Furthermore, EndoCD/5-FC therapy decreased tumor growth and colorectal liver metastasis incident compared with bevacizumab/5-FU treatment in human breast and colorectal liver metastasis orthotropic animal models. In cardiotoxicity safety profile, EndoCD/5-FC is a contrast to bevacizumab/5-FU; lower risk of cardiotoxicity induction or heart function failure was found in EndoCD/5-FC treatment than bevacizumab/5-FU does in mice. EndoCD/5-FC showed more potent therapeutic efficacy with high safety profile and provided stronger tumor invasion or metastasis inhibition than antiangiogenic drugs. Together, EndoCD fusion protein with 5-FC showed dual tumor targeting activities including antiangiogenesis and tumor local chemotherapy, and it could serve as an alternative option for antiangiogenic therapy.

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CHAPTER 1: INTRODUCTION

1.1 Tumor angiogenesis

Tumors initially grow as avascular nodules by absorbing nutrient and removing waste through simple diffusion. However, the growing beyond the size of approximately 1 mm diameter, the tumors require a delicate network of blood vessels to supply the nutrient and oxygen and remove waste products (Folkman, 1971). The neovasculation process in tumors is so called “tumor angiogenesis” or “ angiogenesis switch” (Bergers and Benjamin, 2003). Classically, the transition of vascularization results from the angiogenesis switch driven by hypoxia. Tumors can produce several angiogenic activators to attract and activate endothelial cells, which is a critical step to mediate angiogenesis. Activation of endothelial cells initiates the cell proliferation, which in turn induces sprouting from exiting vessels, migration, and adhesion of endothelial cells to from a lumen. New formation of vessels under angiogenesis process continues to provide the necessary nutrients for cancer cells to grow and survive (Bergers and Benjamin, 2003). Moreover, recent literatures show that glioblastma cancer stem cell by itself can differentiate to endothelium phenotype, and the neo-formed vessels contribute to tumor progression and metastasis (Bautch, 2010; Ricci-Vitiani et al., 2010; Wang et al., 2010b). Tumor angiogenesis may therefore occur through two distinct mechanisms, which by attracting endothelial cells to from vessels (classical angiogenesis process), or by differentiating from cancer stem cells themselves.

1.2 Antiangiogenesis therapeutic strategy

Classical angiogenesis process involves the interaction between angiogenesis factors as an inducer and endothelial cells as a responder. This angiogenesis process could be indirectly inhibited by neutralizing ligands (for example, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF)); blocking receptors tyrosine kinase activity (for example, vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors (PDGFR)); or directly suppressed endothelial cell proliferation and migration. Therefore, these can be classified two kinds of method to inhibit angiogenesis process. One is “direct antiangiogenesis” and another one is “indirect antiangiogenesis” (Kerbel and Folkman, 2002). The inhibitors that serve direct antiangiogenesis include endostatin (O'Reilly et al., 1997), angiostatin (O'Reilly et al., 1994), tumstatin (Sudhakar et al., 2003), and others (Cao, 2001). Most of them are endogenous proteins to be directly targeted to endothelial cells and restrain endothelial cell proliferation and migration. Indirect antiangiogenic inhibitors include VEGF (Kim et al., 1993) or PDGF monoclonal antibodies; or receptor tyrosine kinase inhibitor (TKI) (Ivy et al., 2009), which inhibit neovascularization by either neutralizing angiogenesis-inducing ligands or preventing receptors involved in angiogenesis pathways. Inhibition of tumor growth through antiangiogenesis therapeutic strategy may present certainly advantage of safety and low incident of drug resistant, and antiangiogenesis have potential to inhibit tumor invasion, and metastasis (Folkman, 2006).

1.3 Antiangiogenic drugs

There are many antiangiogenic targeting molecules tested in the clinical trials and pre-clinical studies. However, one of well recognized angiogenesis factor is vascular endothelial growth factor (VEGF), which has been demonstrated to play a crucial role in regulating tumor angiogenesis (Petrova et al., 1999) and normal vascular development (Fong et al., 1995; Shalaby et al., 1995). VEGF is secreted by starving cancer cells and bind to the receptors in endothelial cell to elicit several endothelial cells response including microvascular permeability (Dvorak et al., 1995), secretion of matrix-degrading enzymes, endothelial cell proliferation, migration, and survival (Terman and Stoletov, 2001). Therefore, antiangiogenesis by inhibiting of VEGF/VEGFR signal pathway was considered a good strategy for anti-tumor treatment. United states Food and Drugs Administration (FDA), up-to-date, has approved several antiangiogenic drugs which are shown promising anti-tumor results in the cancer patients in the clinic (Folkman, 2007). Bevacizumab (Bec or Avastin) is a monoclonal antibody that neutralizes VEGF to prevent new vascular formation. Bevacizumab is a first approved antiangiogenic drug in 2004 for combinational treatment with chemotherapy for metastatic colorectal cancer (Cohen et al., 2007b; Ratner, 2004) . Continuingly, bevacizumab was approved for treatment of patients with non-small cell lung cancer (Cohen et al., 2007a), metastatic breast cancer (Spalding, 2008), galioblastoma (Cohen et al., 2009), and renal cell cancer (Summers et al., 2010). However, currently FDA recommends removing bevacizumab from the treatment for metastatic breast cancer patients because clinical outcome doesn’t show significant tumor inhibition and better patent survival

(Burstein, 2010). Sorafenib and sunitinib are small molecular inhibitors that can block not only VEGFR tyrosine kinase activity but also PDGFR activity as well (Gotink and Verheul, 2010). In July 2011, FDA announces that bevacizumab is alternative option for some patients who treat in combination with chemotherapy. Sorafenib was approved by FDA for treatment of patients with renal cancer in 2005 (Eto and Naito, 2006) and hepatocellular carcinoma in 2007 (Flaherty, 2007); Sunitinib was first antiangiogenic drugs approved for two different cancer types at same time, renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stoma tumor (GIST) in 2007 (Rock et al., 2007). So far, three FDA approved antiangiogenic drugs (Bevacizumab, Sorafenib and Sunitinib) are all belong indirectly strategy to inhibit vascular growth.

1.4 Antiangiogenic drugs clinical hindrances

Ideal, anti-tumor drugs should have superior therapeutic window, i.e. high therapeutic efficacy and high safety. Chemotherapy can provide good anti-tumor activity (Morgan et al., 2004) but low safety because it lacks cancer cell specific targeting, resulting in frequently severe side effects (Orditura et al., 2004). On the other hand, antiangiogenic strategy is a quite different anti-tumor strategy from chemotherapy by blocking oxygen and nutrients supply to the tumors to suppress tumor growth. Because of this unique therapeutic strategy, it proposed a couple of advantages. First, it would be less possibility to induce drug resistance because it targets genetically stable endothelial cell instead of targeting genetically unstable

tumor cells. Second, it would have less off-targeting issues because tumor associated endothelial cells are uniquely proliferating which are different from quiescent normal endothelial cells (Augustin et al., 1994; Denekamp, 1984). *In vitro* preclinical data and *in vivo* animal models indeed provided the experimental results to support these predictions that antiangiogenic therapy is effective therapeutic strategy with low incident of drug resistance and without virtual toxicity (Boehm et al., 1997). Compared to side effects induced by traditional chemotherapy, the toxicity could be ignored in antiangiogenic treatment. However, accumulating clinical evidence has changed these principles and shown that antiangiogenic agents still induce drug resistance (Schmidt, 2009) and side effects (Hasani and Leighl, 2011).

1.4.1 Drug resistance

When cancer patients are treated with antiangiogenic agents, several mechanisms will respond to the inhibition of tumor vascular formation to avoid it. Those emerging mechanisms can generalize two models of antiangiogenic drug resistance, in specially targeting VEGF/VEGFR pathway: one, adaptive resistance; and the other, intrinsic (pre-existing) non-responsiveness resistance.

For adaptive resistance, tumor cells initially respond to anti-VEGF/VEGFR therapy and then adapt to treatment by inducing other angiogenic mechanisms to lead tumor relapse and progression. The induction of tumor vascular formation can be regulated by redundantly several angiogiogenesis mechanisms which contain at least four different mechanisms: activation other pro-angiogenic factors from tumor

cells, tumor-associated fibroblast, or stem cells (Fischer et al., 2007); bone marrow-derived progenitor cells recruitment; increasing vessels protection by pericyte coverage; and enhancement of tumor cell invasion for oxygen and nutrients requirement (Ebos et al., 2009; Paez-Ribes et al., 2009).

For intrinsic resistance, tumor vascular formation may regulate by multiple redundant angiogenesis factors which does not respond to antiangiogenesis monotherapy (Kerbel, 2009). Combination therapy to reduce drug resistance and further enhance therapeutic efficacy has been proposed; however, adverse effects cause patients in shorter progression-free survival (Tol et al., 2009).

1.4.2 Side effects

Anti-VEGF/VEGFR antibodies can block or neutralize angiogenesis induced by VEGF/VEGFR stimulation, and their less tumor specific targeting activity frequently lead off-target effects. In addition to tumor growth and survival, VEGF signaling pathway play an important role in normal physiological process to maintain homeostasis (Verheul and Pinedo, 2007). Example of side effects induced by anti-VEGF/VEGFR drug treatment includes hypertension, proteinuria, and impaired wound healing. In addition to management side effects, antiangiogenic treatment also induces potential life-threatening complications, gastrointestinal perforation responds in short-term treatment and cardiac function failure under long-term treatment (Force et al., 2007; Kramer and Lipp, 2007).

1.5 The concepts of antiangiogenic therapy potential prevent drug resistance

Multiple clinical trials have demonstrated that antiangiogenic therapy has clinical benefits, and some of antiangiogenic agents were approved by FDA. However, the emergence of drug resistant tumors in clinic has largely been unexpected compared with antiangiogenesis original principles. The potential mechanisms of drug resistance have been predicted by researchers and clinicians. They suggest some of possible treatment methods to ameliorate or avoid drug resistance.

1.5.1 Chemotherapy strategy contain antitangiogenic effect

The dose of chemotherapeutic agents is determined based on well established concept of maximum tolerant dose (MTD) in order to provide the best antitumor efficacy. However, 'the more frequent is better' or 'less is more' is a controversial issue. Higher dose is expected more anti-tumor effects but less survival benefits due to adverse effects in patents (Nieto, 2003; Roche et al., 2003). On the other hand, low dose of chemotherapy, which is also known as metronomic therapy, has been found to be able to reduce adverse effects but show antiangiogenic effects. In such low dose of chemotherapy, the dose sufficient to inhibit endothelial cells proliferation to from new vascular in tumor microenvironment but lower than the dose required killing the tumor cells is used (Citron et al., 2003; Tuma, 2003).

1.5.2 Combination therapy of antiangiogenic and chemotherapy agents

The anti-tumor efficacy of chemotherapeutic agents also depends on blood stream. Thus, one potential rationale for the combination of antiangiogenic agent and chemotherapy is that antiangiogenic therapy can normalize vascular flow, resulting in increased oxygenation and delivery of chemotherpetic agents (Brown and Giaccia, 1998). The other potential reason is that VEGF can serve as an anti-apoptotic molecule that protects endothelial cells as well as cancer cells from apoptosis induced by standard treatment. Therefore, it is reasonable to combine chemotherapy with antiangiogenesis to enhance therapeutic efficacy of both the cytostatic and cytotoxic effect (Sweeney et al., 2001).

1.5.3 Combination therapy of multiple antiangiogenic agents

In tumor progression process, VEGF is not the only angiogenic factor secreted by tumor cells. It has been already known that several angiogenic factors can redundantly regulate tumor angiogenesis. When patients are treated by anti-VEGF antibody, the hypoxia will be induced in tumor microenvironment. Not only tumor cells but also tumor-associated fibroblasts and microphages are stimulated by hypoxia, and then secret other angiogenic factors than VEGF to rescue hypoxia condition (Ivy et al., 2009). Therefore, it is not sufficient to inhibit tumor angiogenesis by monotherapy. Beside of VEGF, there are up to six different angiogenic factors and several intracellular factors have been recognized to modulate angiogenesis in different stages of breast cancer cell development (Relf et al., 1997). Thus,

treatment with multiple antiangiogenic agents may reduce the emergence of drug resistance risk in clinic.

1.5.4 Combination therapy of antiangiogenic agents and biological molecular targeting agents

Tumors with epidermal growth factor receptor (EGFR) overexpression/ mutation or human epidermal growth factor receptor-2 (HER-2) overexpression can be specifically selected for the treatments with the targeting agents that inhibit these receptor tyrosine kinases such as monoclonal antibodies or small molecule tyrosine kinase inhibitors. Because VEGF expression can be regulated by the EGFR family tyrosine kinases in tumors, these targeting agents also reach antiangiogenesis effects (Bruns et al., 2000; Clarke et al., 2001; Maity et al., 2000). However, clinical evidence shows that some tumor cells eventually become resistant to anti-EGFR antibody treatment, resulting in tumor recurrence because of increased VEGF expression (Viloria-Petit et al., 2001). Therefore, the combination therapy of antiangiogenic agents and biological molecular targeting therapy may be the alternative strategy to overcome targeted therapy resistance in some types of tumors.

1.5.5 Antiangiogenic agents itself as multiple targeting therapy

As mention previously, several angiogenic factors can contribute to tumor progression in different stages. Currently, next-generation antiangiogenic agents that target multiple molecules have been developed. For example, Sunitinib or sorafenib as multiple tyrosine kinase inhibitors which can inhibit PDGFR and VEGFR activity. These agents block multiple molecular targets, resulting in increased antitumor activity and decreased drug resistant potential at the same time (Teicher, 2010).

1.6 Broad-spectrum angiogenesis inhibitors

Over 100 years of cancer research, many critical signaling pathways involved in tumor initiation/progression has been identified. Now, it has been believed that several important signaling pathways can interplay with each other to redundantly regulate tumor progression. Therefore, even though one important oncogenic molecule is blocked by anti-tumor therapy, the other similar function molecular will express to rescue tumor development. Therefore, development of broad-spectrum angiogenic inhibitors is a new challenge for antiangiogenic agents.

1.6.1 Endostatin

Endostatin is an endogenous angiogenesis inhibitor which is divided from C-terminal of collagen XVIII to become a 20 kDa fragment molecule. O'reilly and collogues discovered endostatin in 1997 (O'Reilly et al., 1997) and determined it can inhibit endothelial cell proliferation, migration by binding to $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$

integrin receptors (Sudhakar et al., 2003). Endostatin contains the broadest antiangiogenic spectrum activity though downregulation several angiogenesis pathway (Abdollahi et al., 2004) but induces less toxicity in mice (Zhang et al., 2010). Under phase I clinic trail, endostatin showed virtually no toxicity and no drug resistance respond from patients who were received endostatin treatment everyday over 3 years. However, no significant clinical outcome in multiple endostatin clinical trials was observed (Herbst et al., 2002; Yang et al., 2006) due to poor anti-tumor efficacy and short half-life (Fu et al., 2009; Kulke et al., 2006). In China, Wang and collogues succeed to overcome short half-life issue of endostatin. Endostatin was approved by the State Food and Drug Administration (SFDA) to use in non-small cell lung cancer in China (Wang et al., 2005). Although endostatin is not the only one factor containing broad antiangiogenic spectrum activity, this endogenous antiangiogenic protein has been tested in clinical trials more than any other proteins in recently decade.

1.6.2 Endostatin fusion protein

In order to overcome the weakness of endostatin, researchers have attempted to modify this protein to increase either protein stability and/or anti-tumor efficacy. It has been shown that Fc domain of IgG is linked to N-terminal of endostatin to prolong endostatin protein stability and anti-tumor efficacy in comparison with original endostatin (Lee et al., 2008). An additional metal-chelating sequence (MGGSHHHHH) was integrated at the N-terminus of endostatin to provide

additional zinc binding site, and the zinc-binding significantly reduced thermal induced protein degradation (Jiang et al., 2009). This modified endostatin, which is named as endostar, is approved for the treatment of non-small cell lung cancer patients in China. Moreover, endostatin has been fused with HER2 monoclonal antibody, angiostatin, or antagonist integrin receptor RGD peptide to increase anti-tumor efficacy and antiangiogenic activity in multiple cancer types including colon cancer, ovarian, and pancreatic cancer. (Belur et al., 2011; Jing et al., 2011; Shin et al.; Tysome et al., 2009; Tysome et al., 2011).

1.7 Enzyme-prodrug therapy

Enzyme-prodrug therapy is one of anti-tumor therapeutic strategies which need to metabolize or transform an inactive prodrug to an effective drug. Example of Enzyme-prodrug therapy are focused on inhibition of cell proliferation that preferable kill proliferation cell by blocking cell DNA/RNA synthesis and replication level. Enzyme-prodrug therapies can provide large amounts of tumor cells killing activity in short treatment cycles (Frei et al., 1988). However, there are some limitations for prodrugs in clinical application, including less tumor cell specific targeting activity, normal tissue off-targeting toxicity, and insufficient drug concentration in tumor sites by systemic treatment (Denny and Wilson, 1998; Evrard et al., 1999; Springer and Niculescu-Duvaz, 2000). When the cytotoxic drugs suppress tumor cell growth, they also kill the normal cell as well, particularly in the proliferating tissues such as bone marrow. Therefore, development of tumor specific targeting strategies for prodrug therapy becomes a big challenge in cancer research. After decades of cancer

research, researchers have identified several ways to activate prodrugs specifically in tumor sites, such as gene-directed enzyme prodrug therapy (GDEPT) (Dachs et al., 2009), virus-directed enzyme prodrug therapy (Grove et al., 1999), and antibody-directed enzyme prodrug therapy (Bagshawe, 2009).

Cytosine deaminase is a yeast enzyme which can catalyze enzymatic conversion of 5-flucytosine (5-FC) prodrug into chemodrug 5-fluorouracil (5-FU) (Pandha et al., 1999). Under this metabolism process, the cytosine deaminase linked with uracil phosphoribosyl transferase (we will refer to this fusion gene as CD) has been found to be able to enhance the enzymatic conversion compared to cytosine deaminase alone (Chung-Faye et al., 2001). This fusion strategy has been well established to enhance therapeutic effects in cancer cells (Erbs et al., 2000; Ramnaraine et al., 2003). However, systemic treatment with prodrugs induces off-target effects link to side effects, and general disadvantage of prodrug system is still tumor targeting difficulty as described above.

1.8 Working model Hypotheses

As we mentioned before, indirect antiangiogenic drugs are more likely to induce drug resistance than direct ones because their targets are genetically unstable cancer cells. Recently, it has also been found that glioblastoma cancer stem-like cells could differentiate to endothelial cells which continue to provide nutrient to cancer cells and maintain cancer cells growth and survival. Alternatively, the strategy for antiangiogenesis should specifically target tumor endothelial cells rather

than cancer cells, and then it would provide greater clinical benefits than targeting tumor cells (Bautch, 2010). Therefore, it is promising to choose direct antiangiogenic agents which specifically target genetically more stable endothelial cells (Kerbel and Folkman, 2002; Kerbel, 1991). However, the direct antiangiogenic agents are mostly endogenous molecules and have some disadvantages including low protein stability and low anti-tumor activity. By linking with therapeutic molecules, their weak anti-tumor activity and protein stability could be improved. In our study, we engineered endostatin used with CD in order to complement individual weakness and further provide a good therapeutic window including higher anti-tumor activity as well as low side effects and emergence of drug resistance. The major concept of this study is that EndoCD fusion protein has dual-targeting function. Not only does it have the capabilities of limiting endothelial cell growth (cytostaticity, by endostatin) but also killing cancer cells (cytotoxicity, by conversion of 5FC to 5FU at the tumor site). Endostatin is able to specifically target the fusion protein to tumor endothelial cells. CD is brought to tumor sites by its fusion with Endostatin, and therefore, 5-FC is converted to cytotoxic 5-FU only at the tumor sites. Therefore, EndoCD/5-FC provides dual targeting actvites including tumor antiangiogenesis and chemotherapy.

CHAPTER 2: MATERIAL AND METHOD

2.1 Reagents.

5-fluorouracil was purchased from InvivoGen (San Diego, CA), 5-fluorocytosine from Sigma (St. Louis, MO), and bevacizumab from the Department of Pharmacy at MD Anderson Cancer Center.

2.2 Cell Lines.

MDA-MB-231 and murine 4T1 breast adenocarcinoma cell lines were maintained in Dulbecco's modified Eagle's (DMEM)/F12 medium supplemented with 10% fetal bovine serum (FBS). HUVECs were cultured in endothelial cell medium-2 (Cambrex, East Rutherford, NJ). This colon cancer cell line was generated by our laboratory after several cycles of preselection from an orthotopic colon model that produced 100% liver metastasis and was maintained by G418 selection.

2.3 Recombinant Protein Purification.

The coding sequence of the human endostatin (Endo) was amplified from pPICZaA/hE (EntreMed) by polymerase chain reaction and cloned into the pET28 bacterial expression vector (Novagen) to generate pET28Endo. The yeast cytosine deaminase-uracil phosphoribosyl transferase (CD) was sub-cloned from pORF5-Fcy::Fur into pET28 (pET28CD). To generate pET28EndoCD, the fragment containing CD coding sequence was ligated to the 3' end of Endo to allow expression of the fusion protein as a single polypeptide. Recombinant proteins (Endo, CD, and EndoCD) were expressed from pET28Endo, pET28CD, and

pET28EndoCD and purified from a liter of IPTG-induced bacterial culture based on the procedures previously described (Huang et al., 2001). The pellet was resuspended in Buffer A (0.1 M Tris-HCl, pH8.0 and 5 mM EDTA, 0.1% sodium deoxycholate) and incubated at 4°C with the addition of lysozyme to a final concentration of 50μg/ml. The cells were sonicated and centrifuged at 8,000 X g for 10 min. The pellet was washed twice with Buffer A and resuspended in Buffer B (0.05 M Tris-HCl, pH 8.0, 1% sodium lauroyl sarcosine (SLS), and 1 mM DTT) 4°C overnight. After centrifugation, cleared supernatant was dialyzed sequentially against the following solution at 4°C: Buffer C (0.05 M Tris-HCl, pH 8.0, and 1 mM DTT), Buffer D (0.05 M Tris-HCl, pH 8.0), and Buffer E (0.05 M Tris-HCl, pH 8.0, 0.01 mM oxidized glutathione, and 1 mM reduced glutathione). A final dialysis step against 0.05 M Tris-HCl, pH 8.0 was performed to remove glutathione. The recombinant proteins were determined to be endotoxin free, and protein concentration was quantitated by using Bio-Rad dye method as described in the commercial protocol. The proteins were stored in aliquots with 0.05 M Tris-HCl, pH 8.0 buffer in the -80°C. The molecular weight of Endo, CD, and EndoCD is 20 kDa, 40 kDa, and 60 kDa, respectively. Therefore, an equimolar ratio (1:2:3) of the proteins was used for all experiments.

2.4 Cell Viability Assay.

5 X 10^4 cancer cells MDA-MB-231 were passed in a 96-well plate overnight. Endo, CD and Endo-CD recombinant proteins with 100 μg/ml 5-FC were put into each well. After 48 hr, MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium

bromide) solution at 50 μl per well (2 mg/ml; Sigma) was added into the cell culture and incubated for 2 hours, followed by addition of 100 μl of dimethyl sulfoxide (Sigma) to each well. Absorbance at 570 nm was measured immediately using a multi-well scanner (Labsystems, Helsinki, Finland).

2.5 Endothelial Tube Assay.

Matrigel (BD Biosciences, San Jose, CA) was added to each well of a 96-well plate and allowed to polymerize. A suspension of 5×10^3 HUVEC cells was passed into a Matrigel-coated well. The cells were treated with Endo, CD or EndoCD, and the treatment concentration was determined based on their respective molecular ratios. The cells were incubated for 4-6 hr at 37°C and viewed under a microscope. Five fields were viewed, and tubes were counted and averaged. All assays were performed in triplicate.

2.6 Migration Assay.

The inhibitory effect of endostatin on VEGF-induced chemotaxis was tested by using an 8-μm Boyden chamber (Costar, Acton, MA) assay. HUVECs ($1 \text{ X } 10^4$) were seeded in the upper chamber wells with 2% fetal bovine serum in the EBM medium (Cambrex, East Rutherford, NJ) and mixed together with Endo, EndoCD, or CD, and the treatment concentration was determined based on their respective molecular ratio. EGM2 medium (Cambrex) containing several growth factors were placed in the lower chamber as a chemo-attractant. The chamber was incubated at 37°C for 24 hr. After the non-migrated cells were discarded and the upper wells were washed

with PBS, the filters were scraped with a Q-tip, and the cells were fixed in 4% formaldehyde in PBS and stained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye. Three fields were viewed under a fluorescence microscope, and the cells were counted and averaged. All assays were performed in triplicate.

2.7 Animal Models.

All animals were maintained in the animal facility and experiments were carried out under the institutional guidelines of The University of Texas MD Anderson Cancer Center. For the syngeneic model, BALB/c mice were inoculated (mammary fat pad) with 1 X 10^5 4T1 murine breast adenocarcinoma cells. After the tumor volume reached 3-5 mm in diameter, equamolar amount of proteins (Endo, CD and EndoCD) were injected via tail vein (Endo 2.5 mg/kg) every other day. One hour after protein treatment, all groups received 5-FC (500 mg/kg) by intraperitoneal injection.

For the orthotopic xenograft model, nude mice were inoculated with 3 X 10^6 MDA-MB-231 human breast cancer cells in the mammary fat pad or 3 X 10^6 620-L-1 human colon cancer cells in the cecal wall. After tumors were established 7 days post injection, EndoCD (60 mg/kg, twice per week) or bevacizumab (Avastin, 10 mg/kg, once every two weeks) (Kabbinavar et al., 2003) was intravenously injected, and 5-FU (15 mg/kg, once per week) (Kabbinavar et al., 2003) or 5-FC (500 mg/kg; given 1 hr after EndoCD treatment) was administered by intraperitoneal injection. For practical clinical reasons, the treatment protocols for bevacizumab and 5-FU were essentially derived from previously established clinical doses and schedules

(Kabbinavar et al., 2003). Mice which received 10 mg/kg bevacizumab (once every two weeks, the clinical dose and schedule used in treating breast and colon cancer) or 60 mg/kg EndoCD (twice per week; protein dosage was based on endostatin clinical dosage 20 mg/kg and schedule was based on protein stability) via tail vein injection. Tumor volume was monitored by measuring luciferase signals using IVIS (In Vivo Imaging System; Xenogen, Alameda, CA). In a reduced-treatment experiment, the number of treatments given was decreased from 10 to 5. All protein treatments were given intravenously while chemical drugs were administered by intraperitoneal injection.

2.8 Immunofluorescence Staining.

Frozen sections (4-μm) were fixed in cold 100% acetone for 5 min and then air-dried. After immersion in 1X PBS for 15 min, the slides were incubated with rat monoclonal anti-CD31 antibody (BD Biosciences, San Jose, CA) at room temperature for 1 hr, rinsed with 1X PBS and then incubated with goat anti-rat immunoglobulin G conjugated to Texas Red (1:200; Jackson ImmunoResearch Laboratory, West Grove, PA) in the dark at ambient temperature for 60 min. CD31-positive blood vessels were counted in 10–30 fields at 200X magnification in a blinded fashion.

2.9 In vivo Apoptotic (TUNEL) Assay.

For *in vivo* apoptotic assay, tumors were fixed in 10% formalin and embedded in paraffin blocks. Tissue sections were incubated with proteinase K (20 mg/ml in

10mM Tris-HCl, pH 7.4–8.0, for 15 min at 37^{o}C), permeabilized in 0.1% Triton-X-100 in 0.1% sodium citrate, and then labeled with the TUNEL (deoxynucleotide transferase-mediated dUTP-biotin nick end labeling) reaction mixture (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, biotinylated nucleotide mix and TdT enzyme were added and incubated at 37^{o}C for 1 hr. The slides were washed in PBS, blocked in hydrogen peroxide, incubated in streptavidin horseradish peroxidase, developed in 3, 30-diaminobenzidine, and then counterstained with hematoxylin. The apoptotic cells (brown staining) were counted under a microscope. In each sample, 5 fields were randomly counted for the apoptotic cells.

2.10 In vivo BrdU Incorporation Assay.

MDA-MB-231 breast cancer cells were first injected into the mammary fat pad of nude mice, and when tumors reached 10 mm in diameter, mice were then treated once only with purified Endo (20 mg/kg), CD (40 mg/kg), EndoCD (60 mg/kg) proteins plus 500 mg/kg 5-FC, a clinically sufficient dose of 5-FU (15 mg/kg; 1X 5-FU), or 10 times the clinically sufficient dose (150 mg/kg; 10X 5-FU). The choice of 20 mg/kg Endo was based on a previous preclinical study(O'Reilly et al., 1997) and is also within the dosage range tested in the Phase I clinical trial(Herbst et al., 2002) (15-600 mg/m^2 in human is equivalent to 4.8-194.4 mg/kg in mouse (Freireich et al., 1966)). BrdU was intraperitoneally injected at 1 mg/kg 18 hr before tumors were harvested. Tumor sections were stained by BrdU antibody as previously described

(Mizoguchi et al., 2009), and the percentage of BrdU-positive cells was calculated by Automated Cellular Imaging System (ACIS III, Dako).

2.11 LC/MS/MS.

The HILIC and mass spectrometry condition was modified based on the previous studies (Kosovec et al., 2008; Pisano et al., 2005). First, tumor-bearing mice were administered with 500 mg/kg 5-FC, 10X-5-FU, or 60 mg/kg EndoCD plus 500 mg/kg 5-FC. Tumor samples were harvested after 5-FU or EndoCD/5-FC treatment for 2 hours. Depending upon the weight of the tissues to be processed, a 100 mg/mL tissue suspension in methanol containing 100 ng/mL of 5-Bromouracil (5-BrU, Sigma) was prepared as an internal standard; calculate the tissue volume as 1 µL per mg of tissue weight. Tissue weight >200 mg prepare 100 mg/mL suspension; tissue weight <200 mg prepare 50 mg/mL suspension. (For example: 250 mg tumor tissue = 250 µL volume; to prepare a 100 mg/mL suspension in methanol add 2250 µL of methanol + 100 ng/mL 5-BrU, the final volume upon homogenization will be 2500 µL at 100 mg/mL concentration). Tumor was homogenized by Mistral Ultrasonic tissue homogenizer and samples centrifuged for 5 min at maximum speed at 4˚C to pellet any solid material. The cleared supernatant was then transferred into a sample vial for analysis by LC/MS/MS.

2.12 Small Animal MRI.

Cardiac MRI was carried out as previously described (Wang et al., 2010a). Mice were anesthetized with isoflurane in a circulatory heating stage throughout the

procedure. The dose was adjusted to maintain a respiratory rate between 20 and 50 breaths per minute. Magnetic resonance imaging of the heart was conducted with a Bruker 7.0T scanner located in Small Animal Imaging Facility at the University of Texas MD Anderson Cancer Center. Image streams of serial short axis sections covering the whole heart (1.0 mm in thickness) were obtained with IG-Flash-cine sequences. End-systolic volume (ESV) and end-diastolic volume (EDV) of each section were manually segmented, and left ventricular ejection fraction was calculated by the following formula: LVEF = (ΣEDV-ΣESV)/ΣEDV.

2.13 Hydroxyproline assay.

This assay was modified from a previously described protocol (Kliment et al., 2009; Woessner, 1961). Hearts harvested from treated and untreated mice (13-18 mg) were hydrolyzed in 6N HCl at 50°C overnight in a glass tube and neutralized with NaOH and vacuum dried at 40°C. The pellet was resuspended in 1 ml of 5 mM HCl. A 1:10 dilution of each samples in a total volume of 200 µl was mixed with 100 µl of chloramine T solution (2 ml H_2O, 0.14 g chloramine-T, 8ml hydroxyproline assay buffer (11.4 g sodium acetate anhydrous, 7.5 g trisodium citrate dihydrate, and 77 ml isopropanol; final volume was 200 ml with H_2O, pH 6.0, and 1.25 ml of Erlich's reagent containing 6 g p-dimethylaminobenzaldehyde, 18 ml 60% perchlorate, and 78 ml isopropanol). The samples were incubated at 55°C for 20 min and read at OD_{570} nm. T*rans*-4-hydroxy-L-proline was used as standard curve to determine hydroxyproline concentration. The control group was set as basal level 1.

2.14 Masson's trichrome staining.

Masson's trichrome staining solution was purchased from Sigma. Tissue section was deparaffinized, rehydrated and put in Bouin's Solution at room temperature overnight. The samples were washed in running tap water for 5 min to remove the yellow color from the section, stained in Weigert's Iron Hematoxylin Solution for 5 min, washed again in running tap water for 5 min, and then stained in Biebrich Scarlet-Acid Fuchsin for 5 min. The slides were then placed in phosphomolybdic/ phosphotungstic acid solution for 10 min, transferred to Aniline blue for 5 min, placed in 1% acetic acid solution for 3 min, and then rinsed in distilled water. Finally, the section was washed with 1% acetic acid for 1 minute and rinsed in distilled water.

CHAPTER 3: ENDOCD FUSION PROTEIN PURIFICATION

In our previously study, we used gene therapy based strategy to determine anti-tumor activity and antiangiogenic function of EndoCD (Ou-Yang et al., 2006). Although the technology of gene therapy is conceptually encouraging, it requires improvements in delivery methods to be efficiently used in clinical settings and to enhance its therapeutic effectiveness. The major concerns of gene therapy include delivery difficulties, low transfection efficiency, and unpredictable dose response. However, the technology for using protein therapy (e.g., antibodies) has been well established over the past decade. It has advanced to the extent that targeting delivery and dose responsiveness are all well controlled. Thus, the FDA has approved multiple antibodies for cancer therapy, and protein therapy has now become one of the important strategies in cancer treatment. Therefore, we expect that EndoCD fusion protein therapy will overcome deficiency of gene therapy and provide a new strategy for targeting angiogenesis and targeting chemotherapy to increase anti-tumor activity and reduce side effects.

3.1 Construction of EndoCD protein expression

To obtain EndoCD fusion protein, we first constructed human endostatin (Endo) or yeast cytosine deaminase-uracil phophoribosyl transferase (CD) protein expression plasmid (Figure 1A). Each Open Reading Frame (ORF) was subcloned into pET28 protein expression vector. We also subcloned both Endo and CD into a single pET28 vector to link Endo DNA sequence with CD DNA sequence (Figure

1B). Then, we purified each protein as described in Materials and Methods. For further experiments, we need to adjust each protein concentration to become equimolar. The approximately 1:2:3 ratios was based on 20 kDa of Endo, 40 kDa of CD, and 60 kDa of EndoCD protein molecular weight and would perform in the all experiments (Fig. 2).

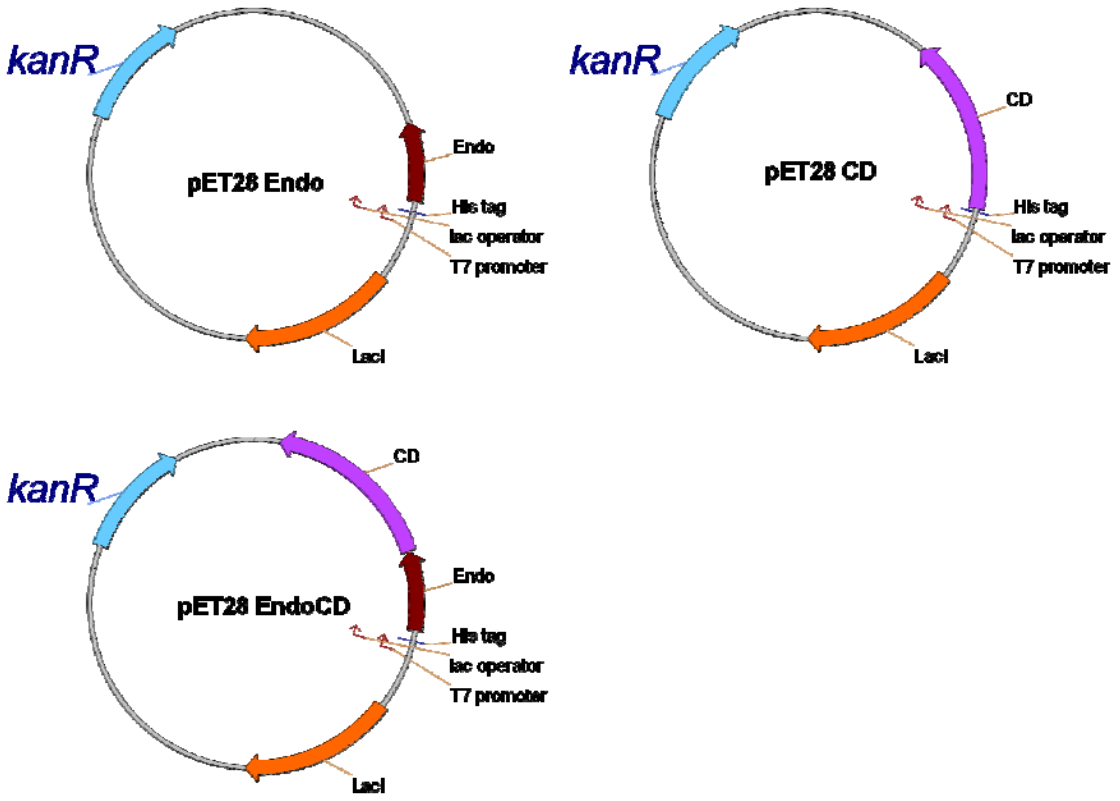
Figure 1. Construction of Endo, CD, and EndoCD protein expression vector.

(A) Vector map of the Endo, CD, and EndoCD fusion protein. EndoCD was clone into the pET28 vector (Novagen) and expressed in *Escherichia coli* (BL21) with an N-terminal histidine tag.

(B) Amino acid sequence of EndoCD fusion protein. Endostatin (red), CD (blue).

Figure. 1

A.



B.

MHSHRDFQPVLHLVALNSPLSGGMRGIRGADFQCFQQARAVGLAGTFRAFLSSRLQDLYSIVRRA
DRAAVPIVNLKDELLFPSWEALFSGSEGPLKPGARIFSFDGKDVLRHPTWPQKSVWHGSDPNGRR
LTESYCETWRTEAPSATGQASSLLGGRLLGQSAASCHHAYIVLCIENSFMTASKEFVTGGMASKW
DQKGMDIAYEEAALGYKEGGVPIGGCLINNKDGSVLGRGHNMRFQKGSATLHGEISTLENCGRLE
GKVYKDTTLYTTLSPCDMCTGAIIMYGIPRCVVGENVNFKSKGEKYLQTRGHEVVVVDDERCKKIM
KQFIDERPQDWFEDIGEMNPLFFLASPFLYLTYLIYYPNKGSFVSKPRNLQKMSSEPFKNVYLLPQT
NQLLGLYTIIRNKNTTRPDFIFYSDRIIRLLVEEGLNHLPVQKQIVETDTNENFEGVSFMGKICGVSIVR
AGESMEQGLRDCCRSVRIGKILIQRDEETALPKLFYEKLPEDISERYVFLLDPMLATGGSAIMATEVL
IKRGVKPERIYFLNLICSKEGIEKYHAAFPEVRIVTGALDRGLDENKYLVPGLGDFGDRYYCV#

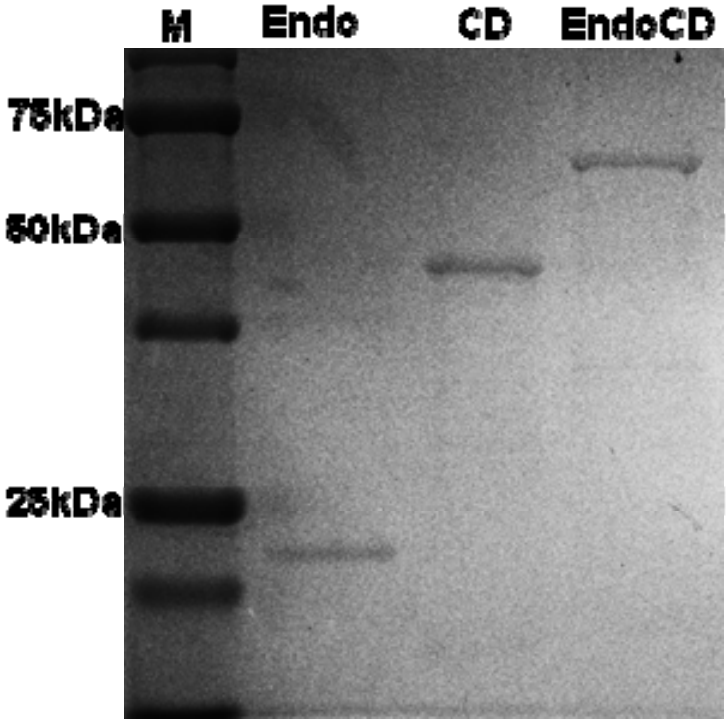
Figure 2. Endo, CD, EndoCD protein purification

(A) SDS-PAGE analysis of the purified Endo, CD, and EndoCD protein.

Molecular weights of Endo, CD, and EndoCD are 20, 40, and 60 kDa, respectively.

Figure 2.

A.



CHAPTER 4: INVESTIGATION OF ENDOCD FUSION PROTEIN BIOLOGICAL FUNCTION

4.1 To study EndoCD antiangiogenic function and cell killing activity in vitro

To characterize the biological activities of EndoCD, we purified the His-tagged recombinant proteins by using bacteria protein expression system. We then tested the antiangiogenesis activity of purified Endo and EndoCD by tube formation and migration assays using human umbilical vein endothelial cells (HUVEC). As shown in Figure 3, inhibition of angiogenesis by EndoCD was similar to that of Endo as both significantly decreased tube formation (Figure 3, upper panels) and the number of migrated cells (Figure 3, lower panels) compared with control (mock) or CD treatment. Next, we examined CD activity by measuring the enzymatic conversion of the prodrug 5-FC to cytotoxic 5-FU activity by using a cell viability assay in different cancer cell lines including 4T1 mouse mammary carcinoma; panO2 mouse pancreatic adenocarcinoma; MDA-MB-231, MDA-MB-468, and MCF7 human breast cancer cells; BE3, BIC-1, and SKG-4 human esophageal cancer cells lines (Figure 4A-C). As shown in Figure 4, EndoCD evidently suppressed cell viability nearly as effectively as CD alone, suggesting the fusion protein maintains the 5-FC prodrug converting enzyme activity.

Figure 3. Antiangiogenic activites of EndoCD protein *in vitro*.

Human Umbilical Vein Endothelial Cell *(*HUVEC*)* was treated with 2.5 μM Endo, CD, or EndoCD. Upper panels, inhibition of HUVEC tube formation by EndoCD was similar to that of Endo. Tube formation was counted in three randomly selected areas. Lower panels, HUVEC migration was blocked by EndoCD under VEGF-attracted condition.

Figure 3.

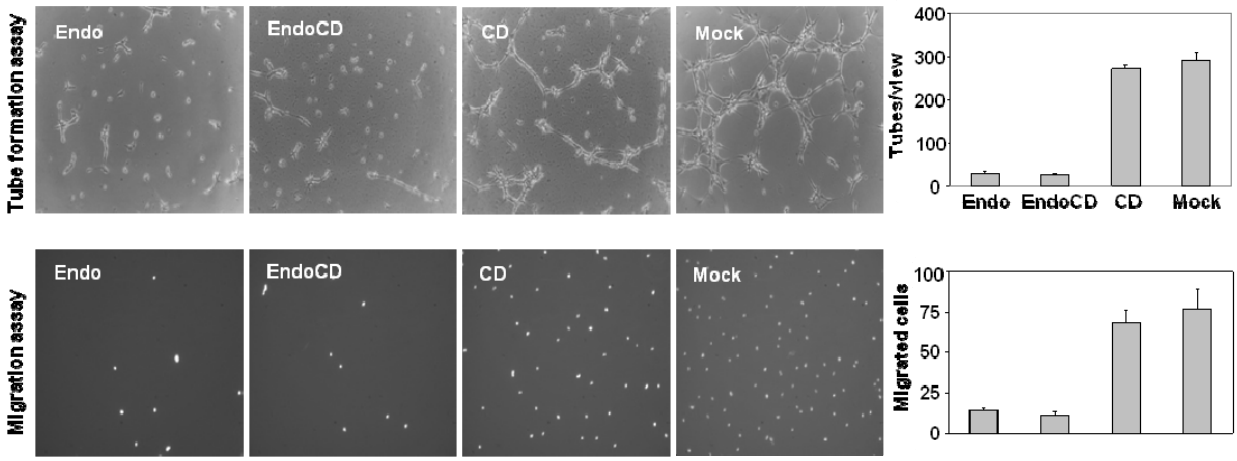

Tube formation assay
Endo
EndoCD
CD
Mock
Tubes/view
400
300
200
100
0
Endo
EndoCD
CD
Mock
Migration assay
Endo
EndoCD
CD
Mock
Migrated cells
100
75
50
25
0

Figure 4. Cancer cell killing activities of EndoCD/5-FC protein *in vitro.*

Cancer cells lines that were treated with 100 μg/ml of 5-FC and various concentrations of Endo, CD, or EndoCD. The cell viability of 5-FC alone group was set as 100%. ■, Endo/5-FC; △, CD/5-FC; ○, EndoCD/5-FC.

(A) Mouse 4T1 mammary carcinoma and panO2 pancreatic adenocarcinoma;

(B) Human MDA-MB-231, MDA-MB-468, and MCF7 breast cancer cell lines;

(C) Human BE3, BIC-1, and SKG-4 esophageal cancer cell lines

Figure 4A.

4T1 **panO$_2$**

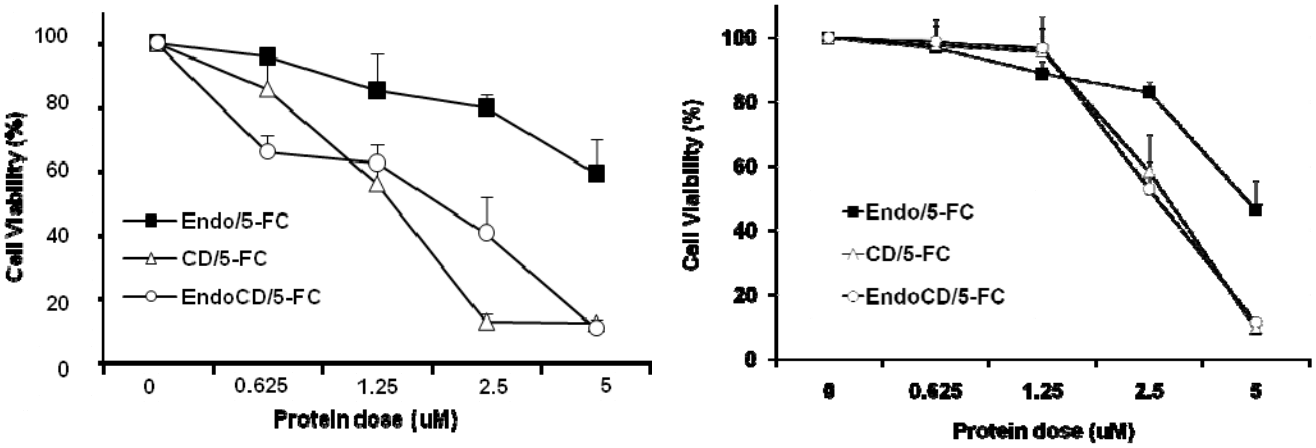
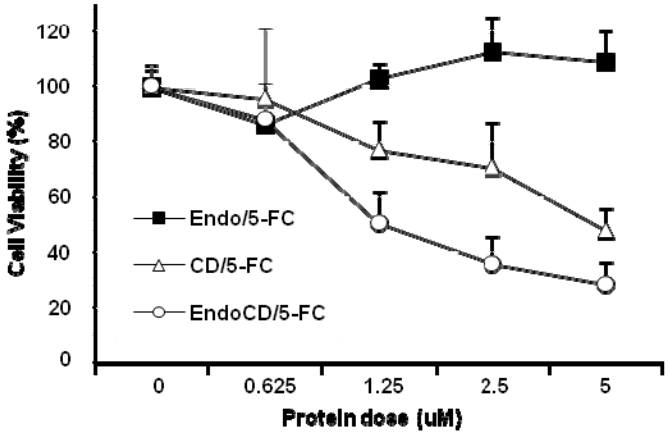
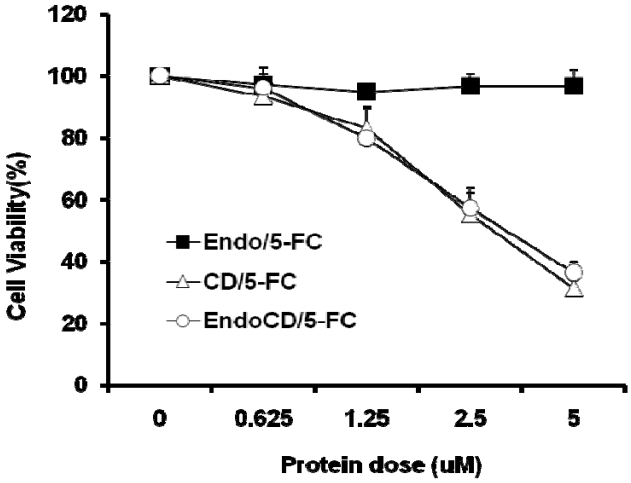


Figure 4B.

MBA-MB-231



MBA-MB-468



MCF7

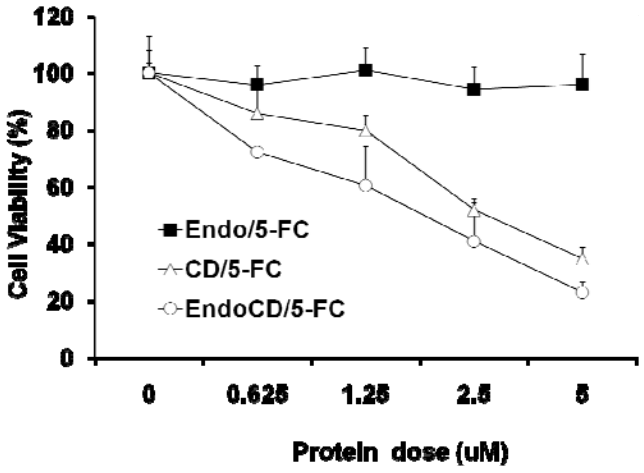
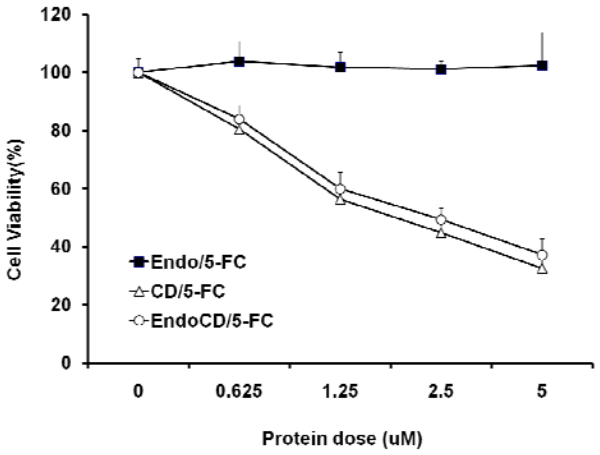
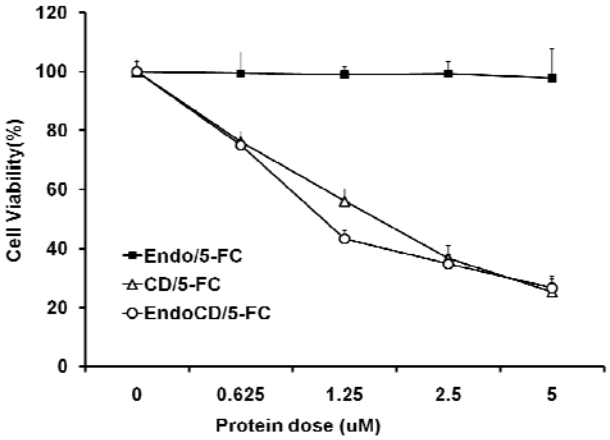


Figure 4C.

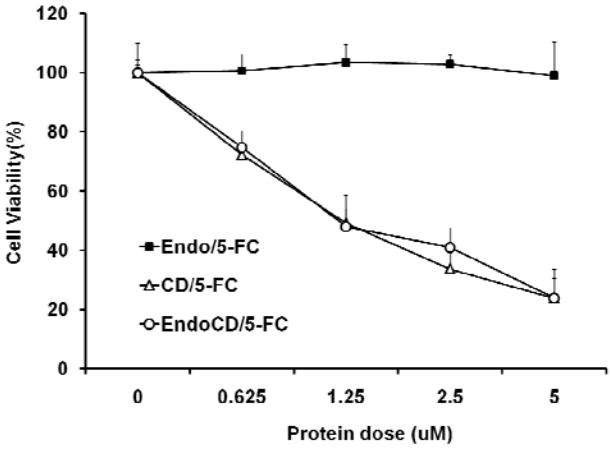
BE3



BIC1



SKGT-4



4.2 To investigate EndoCD/5-FC biological activities in vivo

4.2.1 To investigate the anti-tumor efficacy of EndoCD/5-FC *in vivo*

We tested EndoCD/5-FC antitumor activity *in vivo* in a syngeneic mammary tumor model in which 4T1 breast cancer cells were injected into the mammary fat pad of BALB/c mice. An equamolar of each protein (2.5 mg/kg of Endo, 5 mg/kg of CD or 7.5 mg/kg of EndoCD) was administered into the tail vein of mice every other day (marked by arrows) for a total of 10 treatments, with 500 mg/kg 5-FC (Chung-Faye et al., 2001) injection given 1 hr after protein treatment. The EndoCD/5FC-treated mice showed more potent tumor suppression (Figure 5A) and prolonged the overall mean survival rate (Figure 5B) compared with Endo/5-FC- or CD/5-FC-treated ones. These results indicate that the EndoCD fusion protein inhibits tumor growth more effectively than the two proteins alone.

4.2.2 To investigate the biological function of EndoCD/5-FC-induced anti-tumor and antiangiogenic activities *in vivo*

The tumor samples were harvested from the protein-treated mice to analyze angiogenesis suppression as well as cancer cell death caused by the protein therapy. Tumor tissues were subjected to immunofluorescence staining with CD31 (a marker for endothelial cells) antibody and terminal deoxynucleotidyl transferase dUTP nick end labeling *(*TUNEL*)* assay to characterize program cell death. The results showed that EndoCD/5-FC decrease tumor vascular density and cause endothelial and cancer cell apoptosis more significantly than Endo/5-FC and CD/5-FC alone. We further merged signals from blood vessels and apoptosis by

double staining in all protein-treated tumor samples. One important finding, which was specially observed in the EndoCD/5-FC treatment tumor samples, is the majority of apoptotic signals (green color) surrounded and existed in endothelial cells (red color) (TUNEL/CD31 panel and inset, Figure 6). These phenomena indicate that apoptosis was ongoing in tumor endothelial cells treated with EndoCD/5-FC. Furthermore, apoptosis signals around the endothelial cells suggest that tumor cell also underwent program cell death process in EndoCD/5-FC treated tumor samples but not in other protein treatment samples. These results suggest that the specific cytotoxic activity observed in EndoCD/5-FC treated mice may come from increased 5-FU local concentration.

On the other hand, we also investigate the effects of EndoCD/5-FC protein on cancer cell proliferation. We established orthotopic human breast cancer animal model and treated them with the fusion protein with the 5-FC or 5-FU under clinical condition. Then, we further determined the effects of fusion proteins on cell proliferation by *in vivo* BrdU incorporation analysis. The results indicate that the cells in EndoCD/5-FC-treated tumor samples exhibited less cell proliferation activity than the cells from tumor samples treated with other protein/prodrug combinations (Figure 7A). This cell proliferation suppression activity of EndoCD/5-FC is even 10 times more potent than 5-FU treatment (Figure 7B), which encouraged us to measure 5-FU concentration at local tumor area.

4.2.3 To quantify 5-FU concentration in tumor

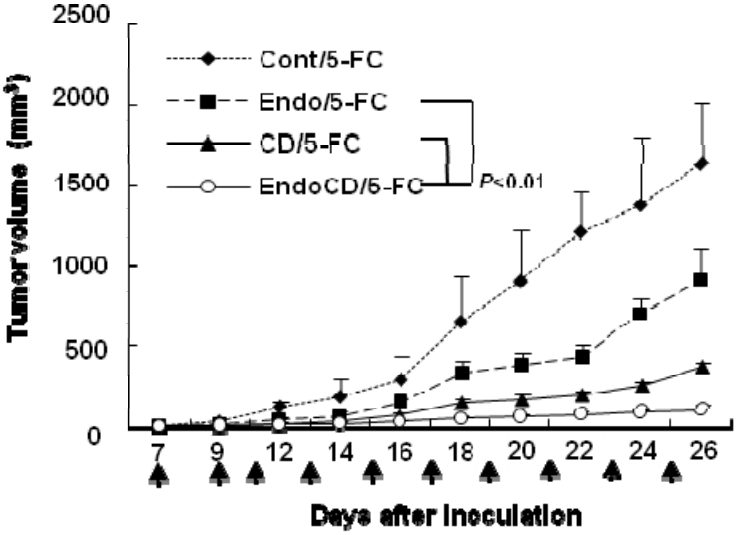
In order to further verify whether induction of cancer cell apoptosis and inhibition of cancer cell proliferation are indeed caused by high concentration of 5-FU, we further determined 5-FU local concentration by liquid chromatography-mass spectrometry (LC/MS/MS) in tumors. According to LC/MS/MS analysis, 5-FU concentration from EndoCD/5-FC-treated tumors was about 7 times higher than that detected from the 10X 5-FU-treated tumors (Figure 8). Together with those *in vivo* function assays, we conclude that EndoCD/5-FC possesses ability to decrease density of tumor blood vessels and accumulate 5-FU concentration in tumor to induce apoptosis in both tumor and tumor endothelial cells.

Figure 5. EndoCD/5-FC anti-tumor activity in breast cancer orthotopic animal model

(A) BALB/c mice were injected with 4T1 cells into mammary fat pad. Equimolar of each protein was injected intravenously and all mice were given 5-FC by intraperitoneal injection 1 hr after protein treatment. Arrow marker represents protein treatment times. EndoCD/5-FC had the best therapeutic efficacy in suppressing tumor growth and prolonged the overall mean survival of mice (B).

Figure 5.

A. 4T1



B

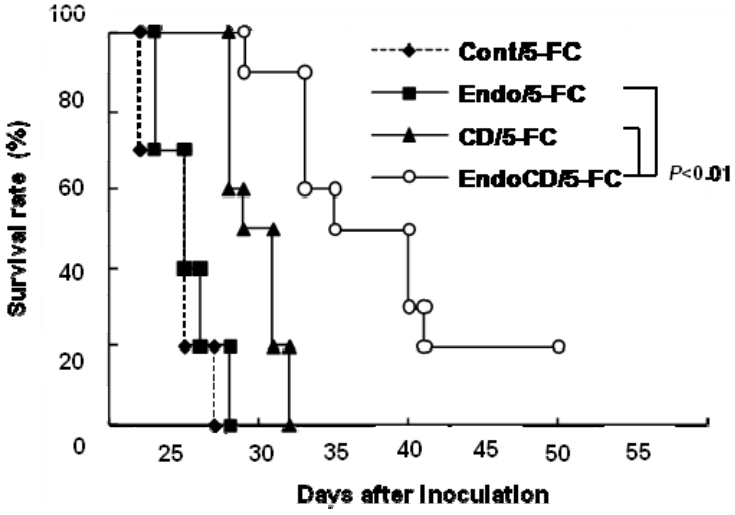


Figure 6. Biological activity by EndoCD/5-FC in tumor microenvironment.

Immunofluorescence staining of tumor tissues from mice treated with the indicated proteins and 5-FC combination (from Figure 5). Blood vessel was stained with vascular marker CD31 antibody (red) and apoptosis signal was detected by TUNEL assay staining (green). EndoCD/5-FC induced tumor vascular density reduction and endothelial and tumor cell apoptosis. Represent imaging is shown in left and quantification of each signal is shown in right.

Figure 6.

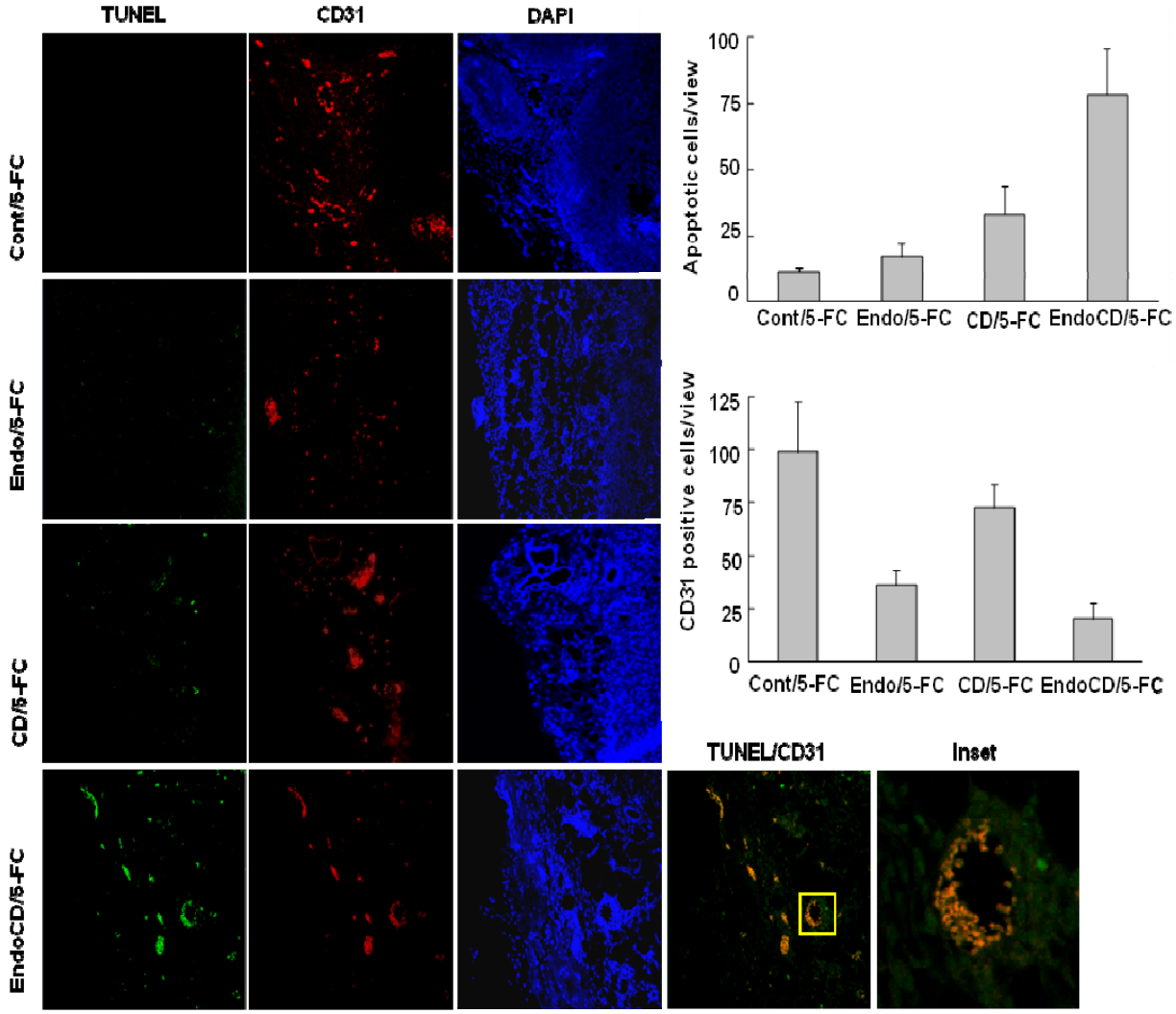

TUNEL
CD31
DAPI
Cont/5-FC
Endo/5-FC
CD/5-FC
EndoCD/5-FC
Apoptotic cells/view
100
75
50
25
0
Cont/5-FC
Endo/5-FC
CD/5-FC
EndoCD/5-FC
CD31 positive cells/view
125
100
75
50
25
0
Cont/5-FC
Endo/5-FC
CD/5-FC
EndoCD/5-FC
TUNEL/CD31
Inset

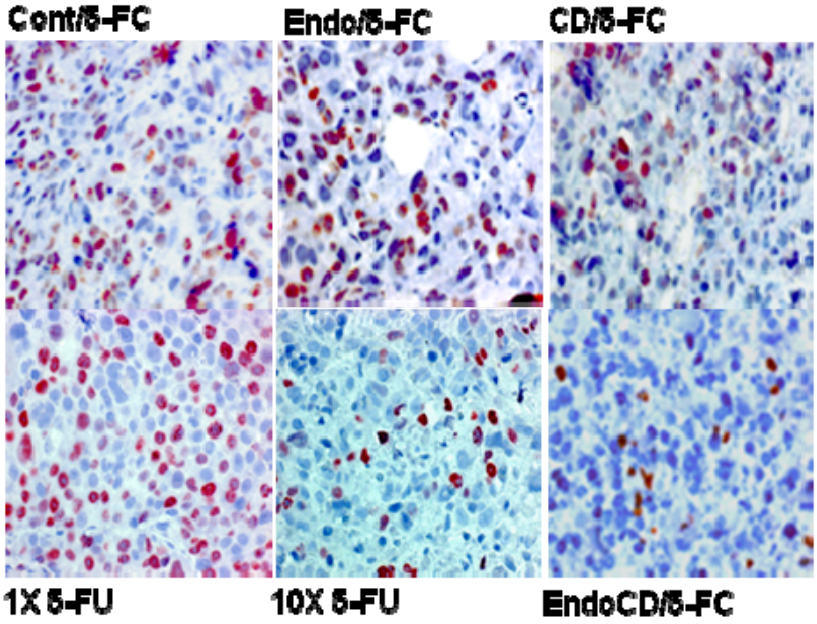
Figure 7. Inhibition tumor cell proliferation by EndoCD/5-FC

(A) Tumor samples were labeled with BrdU (brown) antibody.

(B) Quantification of BrdU signals. EndoCD/5-FC has more potent to inhibit cancer cell proliferation than with 10X 5-FU.

Figure 7.

A.


Cont/5-FC
Endo/5-FC
CD/5-FC
1X 5-FU
10X 5-FU
EndoCD/5-FC

B.

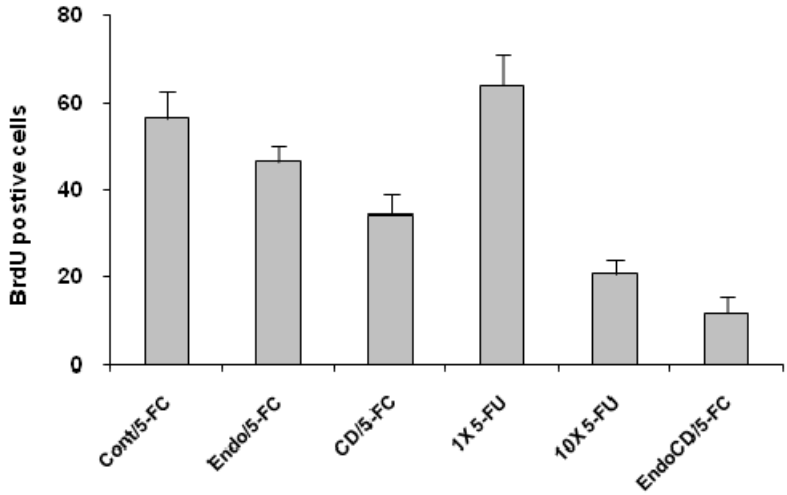
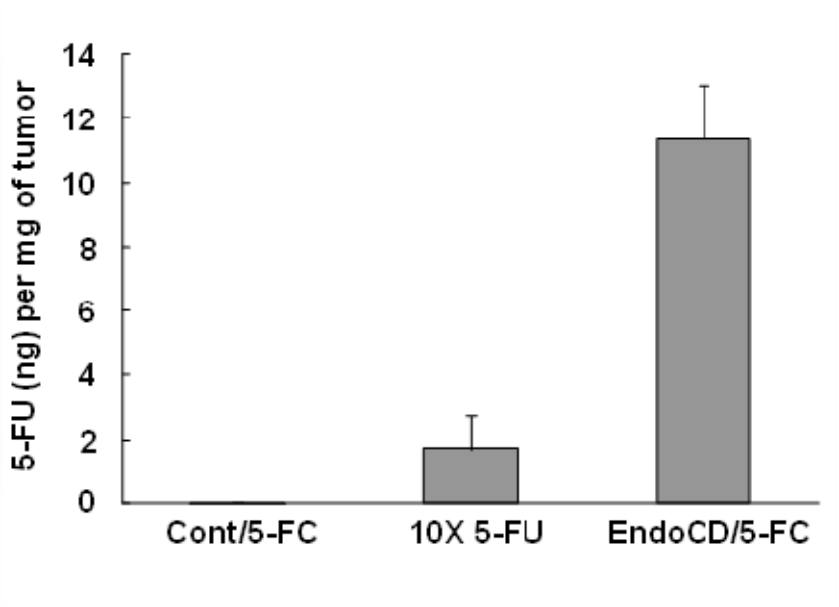

BrdU positive cells
80
60
40
20
0
Cont/5-FC
Endo/5-FC
CD/5-FC
1X 5-FU
10X 5-FU
EndoCD/5-FC

Figure 8. Increased 5-FU concentration by EndoCD/5-FC in tumor microenvironment.

5-FU concentration was detected by LC/MS/MS in tumor. Mice injected 5-FC, 10X-5-FU via intraperitoneal injection, or EndoCD/5-FC via intravenous injection.

Figure 8.


5-FU (ng) per mg of tumor
14
12
10
8
6
4
2
0
Cont/5-FC
10X 5-FU
EndoCD/5-FC

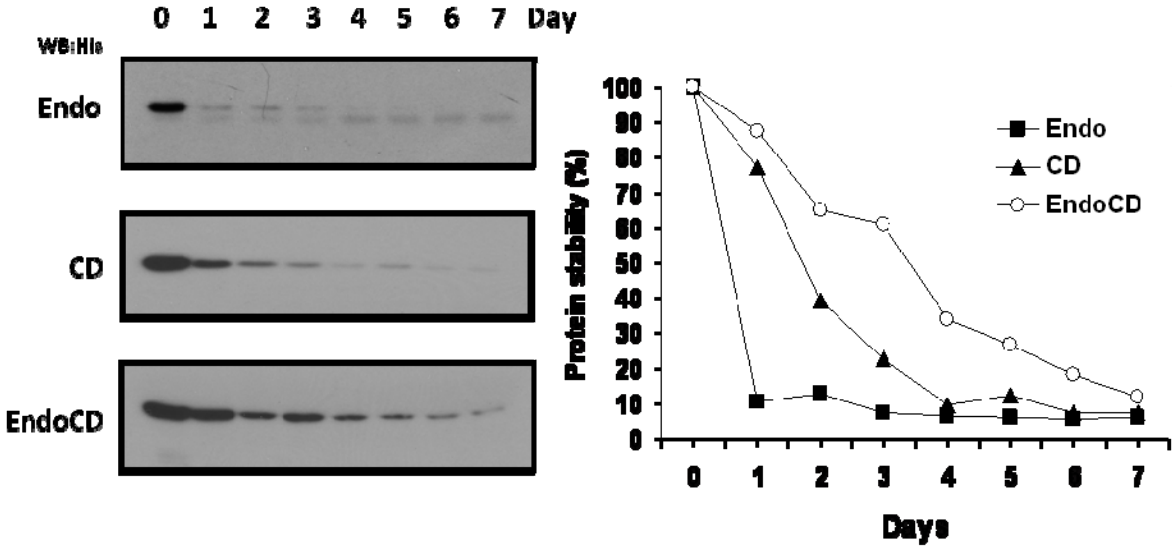
CHAPTER 5: INVESTIGATION OF ENDOCD PROTEIN STABILITY

To analyze the protein stability, we mixed an equimolar amount of each protein (Endo, CD, and EndoCD) with mice serum and incubated at 37 degree for the number of days. The samples were then subjected to western blotting and hybridized by anti-his-tag antibody. Quantification result shows that the half-life of Endo protein is less than one day, which consistent with reports from previous clinical (Eder et al., 2002). However, the EndoCD fusion protein has much longer half-life, which is about three days in the presence of mice serum (Figure 9).

Figure 9. The protein stability of EndoCD fusion protein.

The stability of the EndoCD fusion protein is longer than Endo. (a) 12.5 μM of each Endo, CD, or EndoCD were incubated in mice serum, and at the indicated time points, protein samples were harvested and analyzed by immunoblotting. (b) Protein bands were quantified and normalized to the day 0. ■, Endo; ▲, CD; ○, EndoCD.

Figure 9.


0 1 2 3 4 5 6 7 Day
WB:His
Endo
CD
EndoCD
Protein stability (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7
Days
Endo
CD
EndoCD

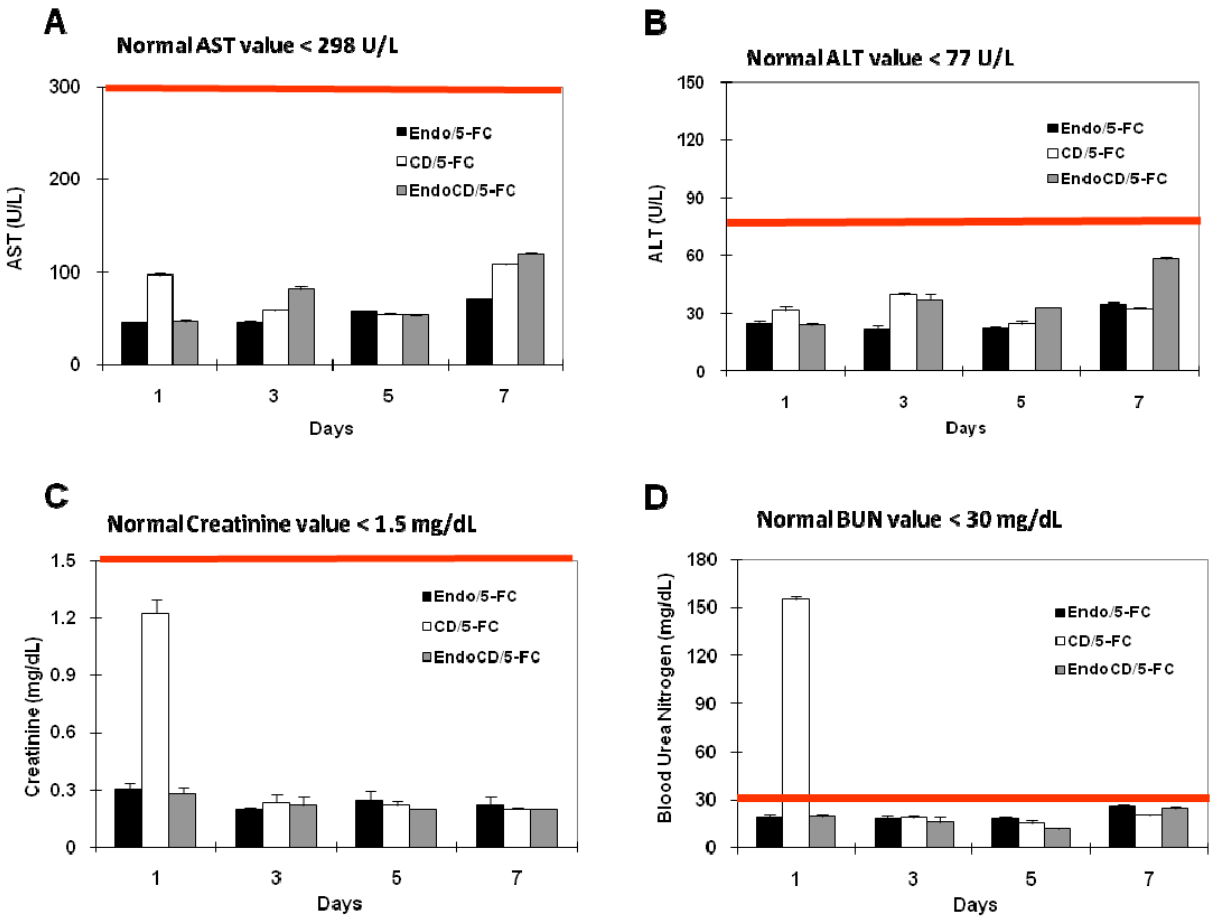
CHAPTER 6: INVESTIGATION OF ENDOCD/5FC TOXICITY

To studies the acute toxicity of EndoCD fusion protein, mice were given 60 mg/kg EndoCD by intravenous injection and then 500 mg/kg 5-FC intraperitoneally injected 1 hr after protein treatment. Mice blood were collected from orbital sinus every other day for one week, and liver functional markers aspartate aminotransferase (AST) and alanine aminotransferase (ALT), as well as kidney function markers blood urea nitrogen (BUN) and creatinine in mice blood were detected. The analysis results indicate that all organ functional markers of liver and kidney (AST, ALT, BUN and creatinine) in EndoCD/5-FC-treated mice were in the normal range (Khatri et al., 2006) (Figure 10). Moreover, there were no sick signs found in EndoCD/5-FC treatment group. For example, no mice died more than two months, nor exhibited less appetite, less activity, and hair loss (data no shown). Together, the results indicate that EndoCD/5-FC would not have any acute toxicity nor induce anylife-threaten side effects in mice.

Figure 10. Acute Toxicity Assay of Endo, CD, and EndoCD fusion protein.

EndoCD/5-FC has virtually no toxicity in mice. 20 mg/kg of Endo, 40 mg/kg of CD, or 60 mg/kg of EndoCD was given to mice by intravenous injection, and all mice were injected with 500 mg/kg of 5-FC intraperitoneally 1 hr after the protein injection. Mice blood were then collected from orbital sinus every other day for one week, and liver and kidney function markers in the blood were determined. AST (A) and ALT (B) represent liver function; creatinine (C) and BUN (D) represent kidney function. The red line indicates the normal value. AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen.

Figure 10.


A
Normal AST value < 298 U/L
AST (U/L)
300
200
100
0
1
3
5
7
Days
Endo/5-FC
CD/5-FC
EndoCD/5-FC
B
Normal ALT value < 77 U/L
ALT (U/L)
150
120
90
60
30
0
1
3
5
7
Days
Endo/5-FC
CD/5-FC
EndoCD/5-FC
C
Normal Creatinine value < 1.5 mg/dL
Creatinine (mg/dL)
1.5
1.2
0.9
0.6
0.3
0
1
3
5
7
Days
Endo/5-FC
CD/5-FC
EndoCD/5-FC
D
Normal BUN value < 30 mg/dL
Blood Urea Nitrogen (mg/dL)
180
150
120
90
60
30
0
1
3
5
7
Days
Endo/5-FC
CD/5-FC
EndoCD/5-FC

CHAPTER 7: ANTI-TUMOR ACTIVITY COMPARISON BETWEEN ENDOCD/5-FC AND BEC/5-FU

To demonstrate EndoCD/5-FC would be a novel anti-tumor drug in clinic, we should compare therapeutic efficacy of EndoCD/5-FC and current clinical drugs. Based on biological function, Endo provide antiangiogenesis activity while cancer cell killing effects mostly come from 5-FU that is converted from 5-FC by CD. The mechanism of anti-tumor function of EndoCD/5-FC is similar with bec/5-FU which is used in several cancer types including metastatic colorectal cancer, non-small cell lung cancer (NSCLC), and breast cancer.

To compare the therapeutic efficacy of EndoCD/5-FC and bec/5-FU, we performed two orthotopic tumor models including human breast cancer (MDA-MB-231) and human liver metastasis colorectal cancer (620-L-1). SW620 is highly liver metastasis colon cancer cell line which was generated by several times re-transplantation liver metastatic cancer cell in colon. 620-L-1 was developed by our laboratory and stably expresses luciferase protein for *in vivo* life image detection. Breast cancer cells and colon cancer cells were injected into mammary fat pad and cecal wall of colon, respectively. One week after tumor cell injection, EndoCD/5-FC or bec/5-FU was injected into the mice at equivalent clinical dose and treatment schedules. The results shows that EndoCD/5-FC provided significantly better anti-tumor activity than bevacizumab or 5-FU alone (Figure 11A and 12A) and also prolonged overall mean survival rate than bev/5-FU ($p=0.004$) in the colon cancer model (Figure 12B). However, EndoCD/5-FC showed a similar therapeutic efficacy to bev/5-FU under this treatment schedules (Figure 11A and 12A).

To compare the therapeutic efficacy of EndoCD/5FC and bev/5-FU, we reduced total drug treatments which from 10 times treatment reduce to 5 times treatment and results shows that EndoCD/5-FC had better tumor suppression activity than bev/5-FU in the MDA-MB-231 breast cancer mouse model (Figure 11B) and the metastatic colon cancer mouse model (Figure 12C) when the tumor sizes were majored on tumor inoculation Day 42 and Day 35, respectively.

As we mentioned in Chapter 1, anti-VEGF/VEGFR drug treatment could suppress tumor angiogenesis and tumor growth; however, it has recently been suggested that tumor cells escaped from cell death induced by these therapies may become refractory tumors with high invasive and metastatic properties (Loges et al., 2009). To further determine whether EndoCD/5-FC therapy also has this clinical weakness, we used 620-L-1 liver metastasis colorectal cancer cells as an analysis model. To monitor cancer cell growth and indicate metastatic tumors, 620-L-1 cancer cells were trasnfected to stably express luciferase protein, which can be tracked by IVIS-100 live image system. On 35 days after tumor inoculation, mice treated with EndoCD/5-FC did not show significant liver metastasis, while the significant liver metastasis was observed in the mice treated with bev/5-FU (Figure 13). Taken together (Figure 11, 12, and 13), these results suggest that EndoCD/5-FC has potent therapeutic activity to control tumor growth and survival as well as metastasis.

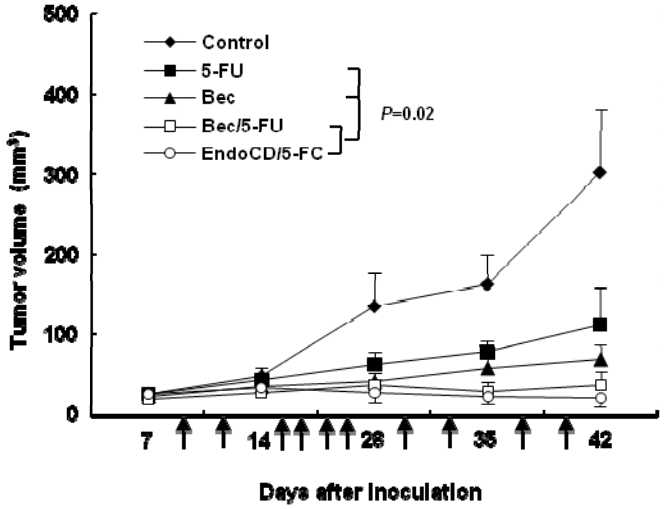
Figure 11. Anti-tumor activity comparison of EndoCD/5-FC and bev/5-FU in an orthotopic human breast cancer mouse model.

(A) Mice bearing 231 breast tumors were treated with the indicated drug combination, and growth of tumor volumes were monitored. EndoCD/5-FC showed significantly better anti-tumor activity than bevacizumab or 5-FU alone. However, EndoCD/5-FC showed a similar therapeutic efficacy to bev/5-FU under this treatment schedules. Arrows represent each protein treatment.

(B) Reduced total drug treatments and EndoCD/5-FC had tumor suppression activity better than bev/5-FU in MDA-MB-231 breast cancer mouse model on tumor inoculation Day 42. (For detail schedule of reduced treatments, please refer to material and method.)

Figure 11.

A. MDA-MB-231



B. MDA-MB-231

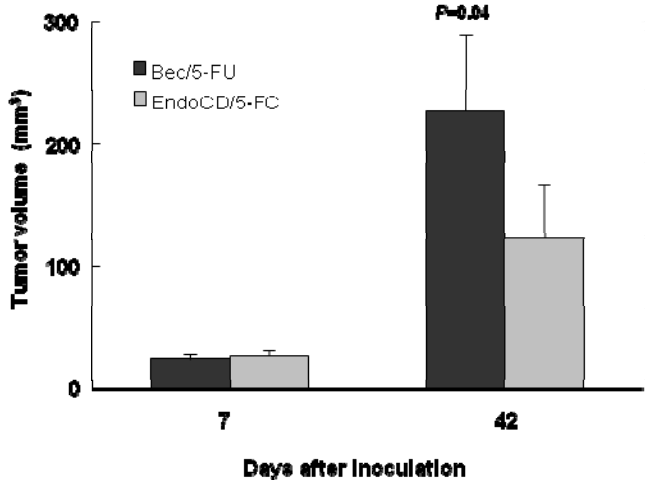


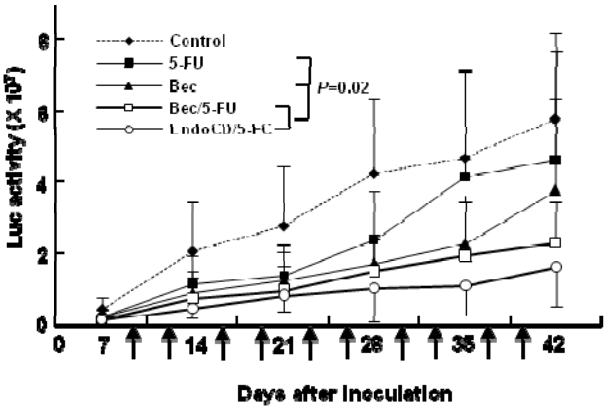
Figure 12. Anti-tumor activity comparison between EndoCD/5-FC and bev/5-FU in an orthotopic human liver metastasis colorectal cancer mouse model.

(A)EndoCD/5-FC shows significantly better anti-tumor activity than bevacizumab or 5-FU alone and also prolong mice overall mean survival rate than bev/5-FU ($p=0.004$) in colon cancer model (B). However, EndoCD/5-FC did not show a significantly better therapeutic efficacy than bev/5-FU under this treatment schedules.

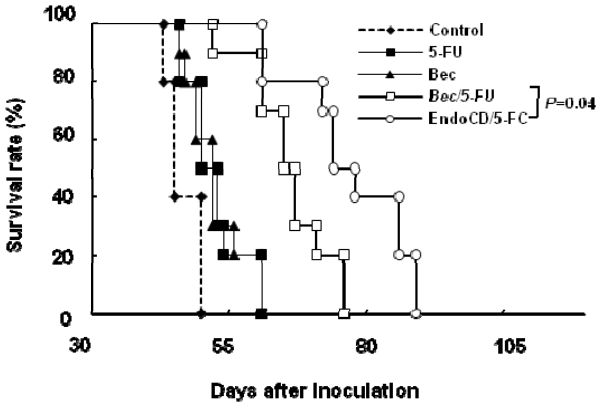
(C) Reduced total drug treatments and EndoCD/5-FC had tumor suppression activity better than bev/5-FU on tumor inoculation Day 35. Arrows represent each protein treatment. (For detail schedule of reduced treatments, please refer to material and method.)

Figure 12.

A. 620-L-1



B. 620-L-1



C.620-L-1

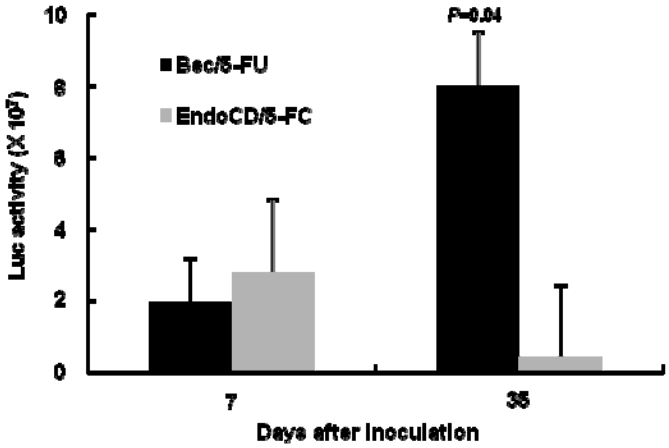
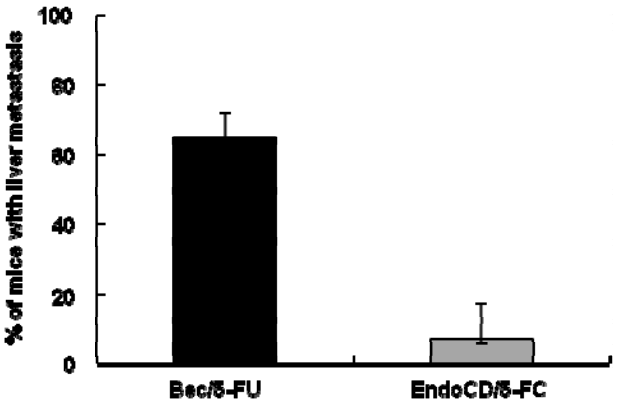


Figure 13. Liver metastasis comparison between EndoCD/5-FC and bev/5-FU in an orthotopic human liver metastasis colorectal cancer mouse model.

Mice bearing 620-L-1 colon cancer expressing luciferase were treated by EndoCD/5-FC or bev/5-FU. Tumor metastasis signal was tracked by IVIS-100 image on Day 35 after inoculation. The result shows EndoCD/5-FC treatment did not increase liver metastasis compared with bev/5-FU treatment group.

Figure 13.

620-L-1



CHAPTER 8: COMPARISON OF CARDIOTOXICITY BETWEEN ENDOCD/5-FC AND BEC/5-FU

It has been known that bevacizumab can cause 1.7 to 3% left ventricular dysfunction incidence, and 5-FU is also well studied to induce ischemic complications in cancer patients (Yeh and Bickford, 2009). To determine the cardiotoxicity effects of those drugs including EndoCD/5-FC and bev/5-FU, we harvested serums from drugs-treated mice (from figures 11 and 12) to further detect troponin I, which is a biological marker for damage of cardiomyocyte. Troponin I serum level was dramatically increased in bev- and bev/5-FU-treated breast cancer mice model. On the other hand, mice treated with bev/5-FU are the only group showed high level of troponin I in colon cancer model (Figure 14). These results suggest bev/5-FU treatment may cause cadiomyocyte damage but EndoCD/5-FC may not.

To further examine whether EndoCD/5-FC protein treatment affects cardiac function, we used small animal MRI to analyze end-diastolic volume (EDV) and end-systolic volume (ESV) that allowed us to calculate the left ventricular ejection fraction (LVEF; LVEF = (ΣEDV-ΣESV)/ ΣEDV) (Wang et al., 2010a) of mice before (pretreatment basal level) and after treatment with bev/5FU or EndoCD/5FC. Representative EDV and ESV images are shown in upper panel of figure 15 and LEVF amounts are shown in lower panel of figure 15. LEVF was significantly decreased in bev/5FU-treated mice in post three-month treatment. On the other hand, LEVF was only slightly changed in EndoCD/5FC-treated mice even after six-

month treatment. Therefore, EndoCD/5-FC protein therapy may provide great advantage because of minimal cardiac impact.

To study the effect of EndoCD/5-FC and bev/5-FU on heart tissue, we analyzed the incidence of cardiac fibrosis which has abnormal collagen accumulation. Collagen amounts in heart tissues can be determined by indirectly detecting hydroxyproline or direct collagen trichrome staining. The heart tissues were collected from mice used in figure 15, and we found that higher hydroxyproline amount (Figure 16A) and collagen accumulation (blue color, Figure 16B) in heart from bec/5-FU-treated mice than hearts from the control mice and EndoCD/5-FC-treated mice.

One of critical VEGF biological function is maintain myocardial angiogenesis; and it has been demonstrated that ischemic cardiomyopathy would be induced by loss of VEGF in mice (Carmeliet et al., 1999). To exam the effects of EndoCD/5-FC and bev/5-FU on mice myocardial angiogenesis, we measured serum VEGF levels and also determined coronal vessels density by staining with vascular marker CD 31 antibody. Then, we found that circulating VEGF levels significantly reduced in mice treated bev/5-FU but not in one treated with EndoCD/5FC (Figure 17). Moreover, CD 31 signals, which indicate coronal vessel density, were also decreased in heart tissues of mice treated bev/5-FU but not in one treated with EndoCD/5FC (Figure 18). Together, these results indicate that bev/5-FU treatment would potentially induce cardiomyopathy and/or cardiac function failure compared to EndoCD/5-FC treatment in cancer patients.

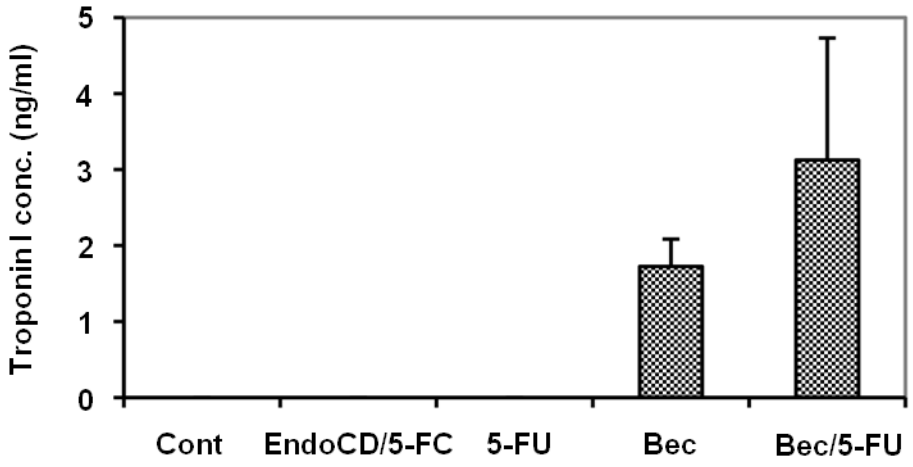
Figure 14. Cardiotoxicity Assay by detecting troponin I level.

Drug-treated mouse serum were collected from two orthotopic mice tumor model including human breast cancer model (MDA-MB-231) and human liver metastasis colorectal cancer model (620-L-1) to detect circulating troponin I level by ELISA. EndoCD/5-FC treatment group did not induce cardiotoxicity in both mice tumor therapy model. Bec, bevacizumab

(A) High troponin I level induced by bec or bec/5-FU treatment group in breast cancer model, indicating that Bec and bec/5-FU treatment could cause mice cardiacmyocyte damage.
(B) Cardiactoxicity was observed only in bec/5-FU treatment mice in human liver metastasis colorectal cancer model.

Figure 14.

A. MDA-MB-231



B. 620-L-1

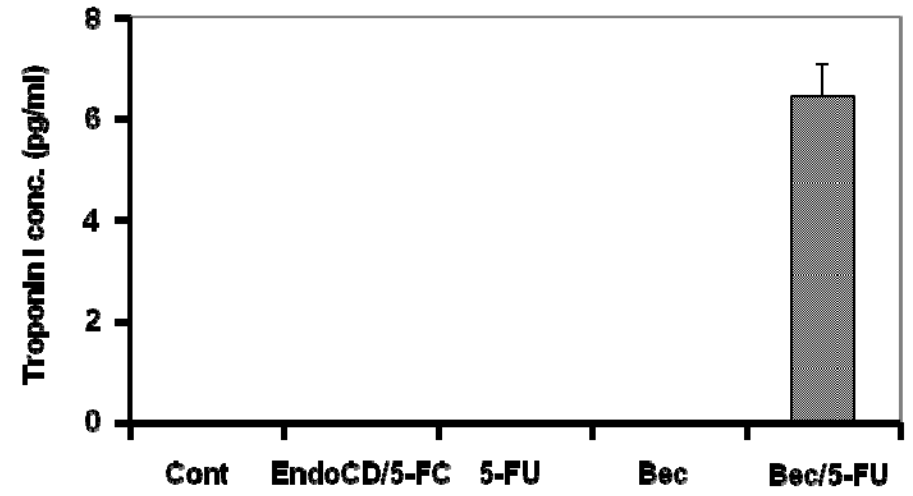


Figure 15. Cardiac function detection by magnetic resonance imaging (MRI) in drugs-treated mice

Upper panel is representative image of EDV (end of diastolic volume) and ESV (end of stoic volume).

Left ventricular ejection fraction (LVEF) was calculated by (ΣEDV-ΣESV)/ ΣEDV from each treatment mice and shown in lower panel. Before drugs treatment, LVEF value was set as mice normal value. LVEF value was significantly reduced in bev/5-FU treatment group and there was no significant change of LVEF value in EndoCD/5-FC treatment group after three- and six- month treatment. NS, no significance. (For detail schedule of reduced treatments, please refer to material and method.)

Figure 15.

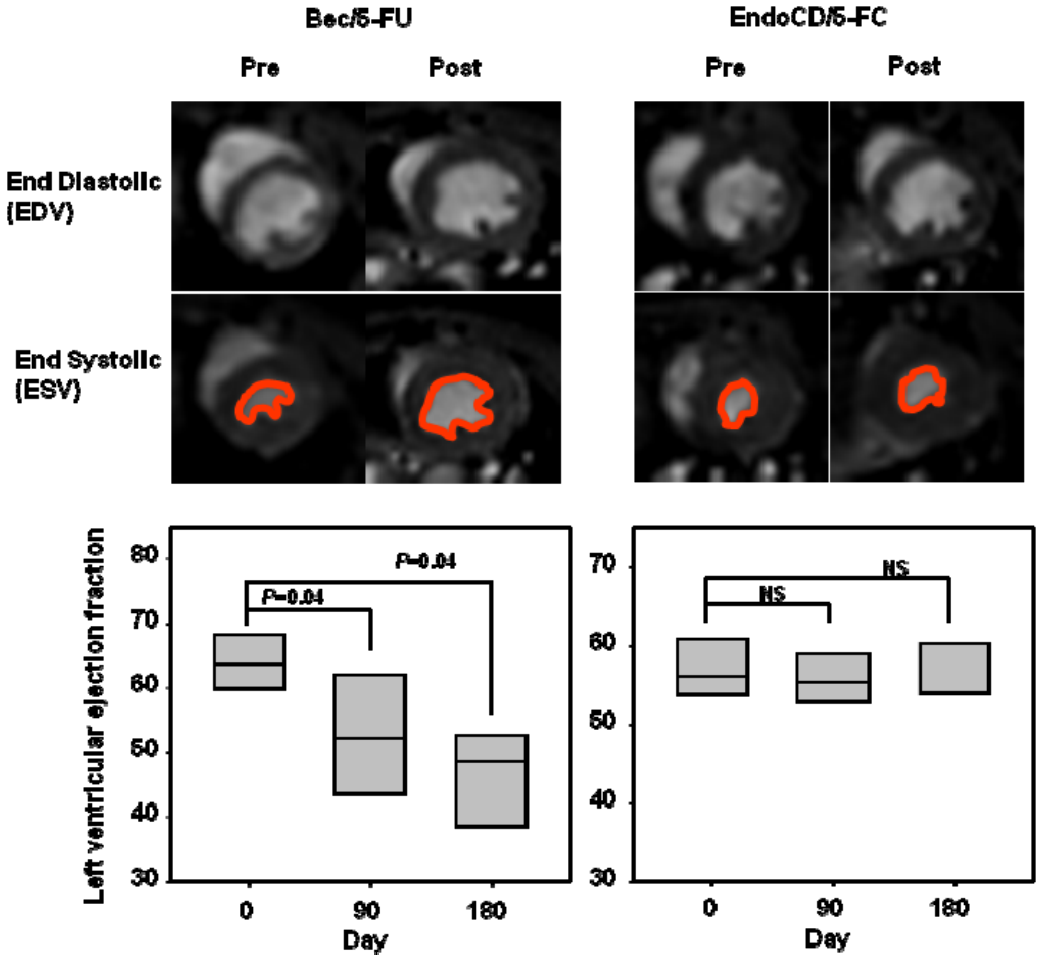

Bec/5-FU
EndoCD/5-FC
Pre
Post
Pre
Post
End Diastolic (EDV)
End Systolic (ESV)
Left ventricular ejection fraction
80
70
60
50
40
30
P=0.04
P=0.04
0
90
180
Day
70
60
50
40
30
NS
NS
0
90
180
Day

Figure 16. Cadiac fibrosis detection in EndoCD/5-FC and bec/5-FU treatment mice heart.

(A)Hydroxyproline assay.

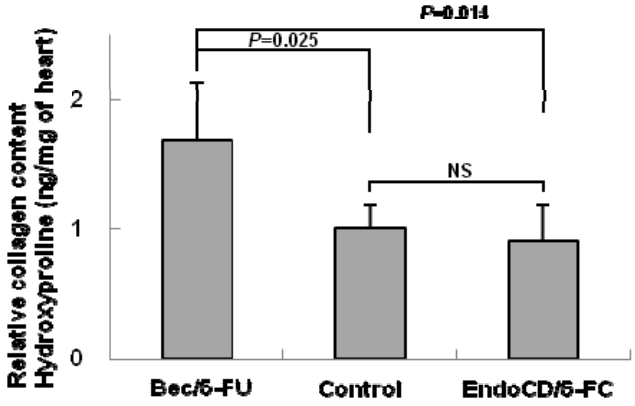
The hearts were harvested from mice treated with EndoCD/5-FC or bec/5-FU. We also collected the same age of mice heart as a normal control. The detection amount was normalized with the weight of heart tissue. The high proline hydroxylation was detected in the bev/5-FU treatment group, which is significantly higher than control mice. In contrast, there was no significant difference in proline hydroxylation between EndoCD/5-FC group and control mice. NS, no significance.

(B) Trichrome staining.

Direct method shows fibrosis phenomena in mice heart. The presence of fibrosis is shown in blue by trichrome staining of heart histological section. Similar results as (A) are shown.

Figure 16.

A.Hydroxyproline assay.



B. Trichrome staining

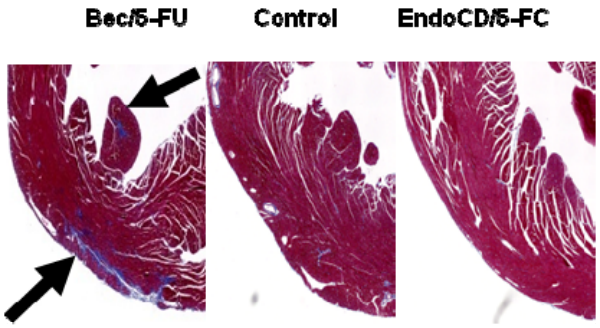


Figure 17. Circulating VEGF level comparison in EndoCD/5-FC and bec/5-FU treatment mice

Circulating VEGF level was lower in bev/5-FU-treated mice than control mice and there were no significantly changes in EndoCD/5-FC-treated mice compared with control mice. NS, no significance

Figure 17.

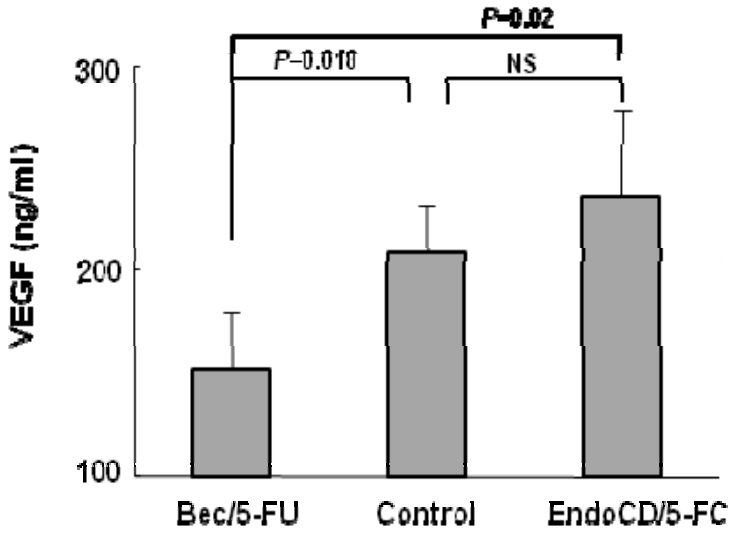
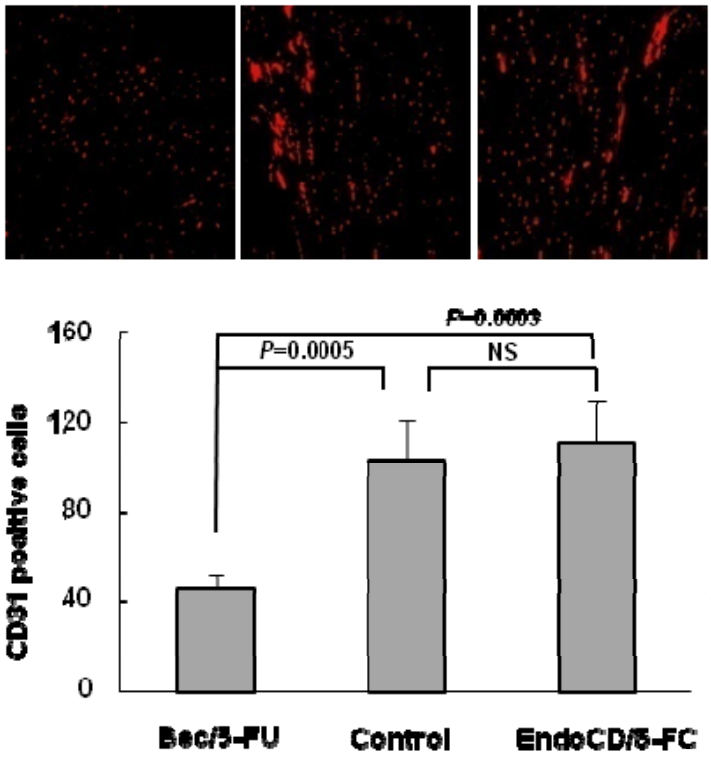

P=0.02
P=0.010
NS
300
200
100
VEGF (ng/ml)
Bec/5-FU
Control
EndoCD/5-FC

Figure 18. Coronal vessels density comparison in EndoCD/5-FC and bec/5-FU treatment mice

Upper panels are representative images of coronal vascular density hybridizing by CD31 antibody (red) in mice heart tissue.

Lower panel shows that coronal vascular density was significantly decreased in bev/5-FU treatment mice compared to control mice but not in EndoCD/5-FC-treated group. NS, no significance

Figure 18.


P=0.0005
NS
160
120
80
40
0
CD31 positive cells
Bec/5-FU
Control
EndoCD/5-FC

CHAPTER 9: SUMMARY OF CHAPTER 3 TO 8 AND DISCUSSION

Antiangiogenesis, the novel anti-tumor concept by Judah Folkman, has become reality during over last three decades and been applied in clinic. Inhibition tumor growth by anti-VEGF/VEGFR monoclonal antibodies became a first approved antiangiogenic modality in clinic. Although anti-tumor efficacy was fully tested in preclinical studies, accumulating clinical reports have shown that these drugs have cytostatic function without curative potent and further induced tumor recurrence, tumor invasion and metastasis (Loges et al., 2009). Although targeting therapy provides predictable safety profile, systemic treatment interrupts normal organ homeostasis to induce side effects (Verheul and Pinedo, 2007). Therefore, development of the strategies to enhance therapeutic efficacy and targeting specificity under center principle of Judah Folkman will become a new challenge in next decade.

In this study, we set up this challenge as a goal. Namely, we have attempted to develop a novel antiangiogenic drug which provides high therapeutic efficacy and decreases incident of tumor recurrence and the risk of tumor invasion and metastasis. Moreover, to provide the high efficacy, we also tried to increase targeting specificity to prevent off-target side effect and increase safety. Then, we finally developed a novel fusion protein EndoCD. Endostatin is broad-spectrum antiangiogenesis protein which can specifically target tumor vascular system (Avraamides et al., 2008). In addition, the fusion protein can increase 5-FU concentration, which is converted from 5-FC by CD, in the tumor microenvironment. Thus, EndoCD/5-FC offers not only antiangiogenesis by tumor vascular targeting but

also tumor targeting chemotherapy. Dual targeting effect shown here will provide curative benefits to cancer patients.

Clinical antiangiogenic drugs belong to indirect antiangiogenesis agents that selectively target VEGF pathway. Although, VEGF pathway is important for tumor angiogenesis, it is also known essential for normal physiological maintenance (Verheul and Pinedo, 2007). Indirect antiangiogenesis agents also block VEGF function in normal organ, and it perhaps causes side effects. In the principle of EndoCD fusion protein, it is directly targeted to uniquely proliferating endothelial cell in tumor sites, and thus it would have decreased off-target potential (Kerbel, 1991). Moreover, by carrying chemotherapeutic drugs to tumor area, EndoCD enhances cytostatic effect at the same time. In order to prove the concept, we purified EndoCD fusion protein from bacteria expression system and determine antoangiogenic and cell killing activities *in vitro* and *in vivo*. EndoCD/5-FC induced endothelial cells and tumor cells apoptosis and inhibited tumor cell proliferation, and these anti-tumor activities further reduced tumor cell invasion and metastasis. We also demonstrated that EndoCD/5-FC fusion protein has dual targeting tumor antiangiogenic and tumor local chemotherapeutic activities. Together, EndoCD is expected to be able to decrease potential tumor recurrence and tumor metastasis.

In the present study, we also compared EndoCD/5-FC anti-tumor activity and safety with a clinical antiangiogenic drug, bevacizumab and 5-FU combination. EndoCD/5-FC showed better anti-tumor and metastasis inhibition activities than bec/5-FU. In the safety profile, EndoCD/5-FC provide virtually no toxicity, especially cardiotoxicity and cadiac function failure, while bec/5-FU exhibited these toxicities.

Therefore, our studies demonstrated that EndoCD/5-FC may resolve the weakness of antiangiogenic and chemotherapeutic drugs and reveal a novel potential of antiangiogenic modality in clinic.

CHAPTER 10 FUTURE DIRECTIONS

10.1 Improvement of currently protein expression system

In our current protein purification system, when EndoCD fusion protein was expressed in E. coli, the majority of protein become aggregated and found in inclusion body of bacteria. Therefore, we first purified the inclusion body to get high purity of EndoCD protein, and then EndoCD was denatured and refolded though dialysis procedures. During dialysis procedure, EndoCD structure may be refolded from linear structure to different stages of tertiary structure. The quality of protein in each purification batches may vary due to dialysis procedure.. Therefore, we need to improve or modify currently protein expression system for future clinical application.

10.1.1 To improve protein solubility in different protein expression.

To improve protein solubility, we will test different approaches in our currently expression system including different expression vectors, different induction temperature, and modification of the linker. In figure 19A modification strategy map, we can either change linker or use different tag to test in different induction temperature. In this expression vector, high solubility of EndoCD protein can be induced in room temperature (23 ºC) or 4 ºC degree (Figure 19B). We will further purify soluble protein in large scale E. coli expression system.

Figure 19. Solubility improvement of EndoCD protein in E. coli system

(A) The strategy map of protein expression map.

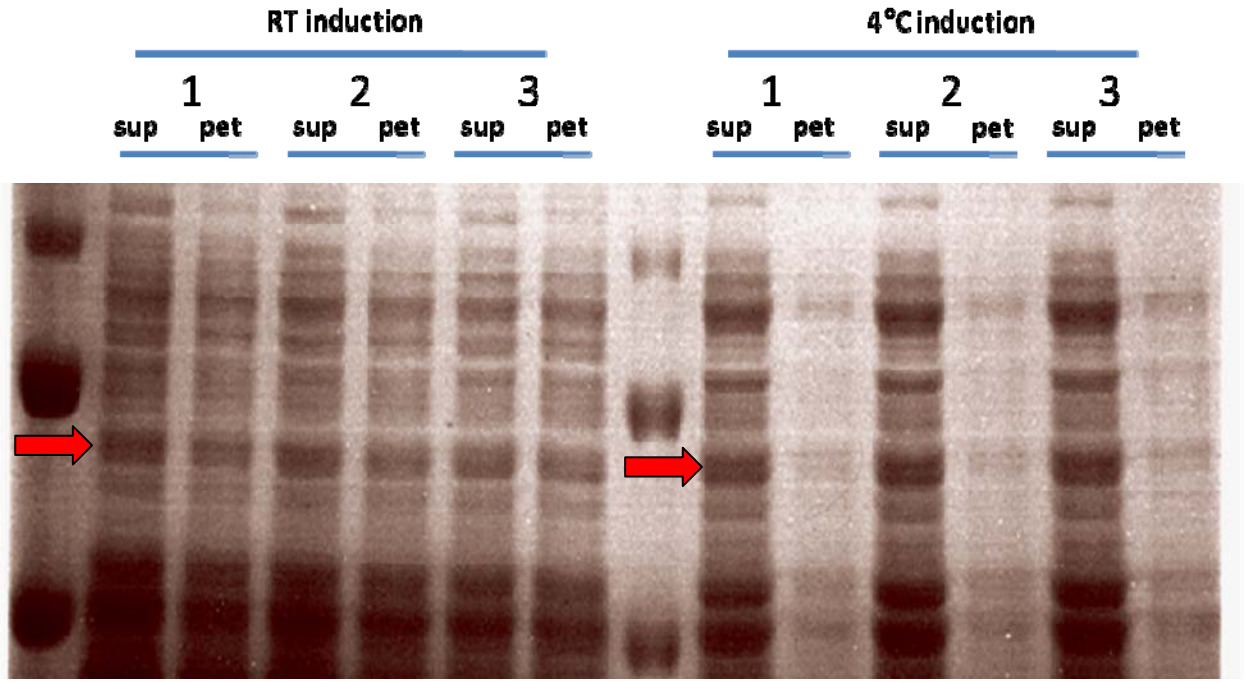
(B) EndoFlexCD protein (red color arrow) induction in different temperature. RT, room temperature; sup, soluble protein; pet, insoluble protein

Figure 19.

A.


GST
His
Endo
Linker
CD

B.


RT induction
4°C induction
1
2
3
1
2
3
sup
pet
sup
pet
sup
pet
sup
pet
sup
pet
sup
pet

10.2 To test the cancer stem cells (CSCs, TICs: tumor-initiating cells) killing activity of EndoCD/5-FC.

Clinical data now suggest that antiangiogenic therapy leads to the progression of tumors by increasing invasion and metastasis, likely due to activation of the cancer stem cell population (Ebos et al., 2009; Paez-Ribes et al., 2009). Cancer stem cells (CSC, or tumor-initiating cells, TICs) have been considered to contribute to cancer initiation, progression and chemotherapeutic resistance (Al-Hajj and Clarke, 2004; Reya et al., 2001; Rossi et al., 2008). It has also been shown that glioma initiating cells have a greater ability to promote vascular endothelial growth which may confer enhanced angiogenesis for tumor cell survival and proliferation (Ricci-Vitiani et al., 2010; Wang et al., 2010b). Currently, there are no effective ways to target CSCs. CSCs in different types of cancer have been gradually identified. For example, breast cancer stem cells (BCSCs, alternatively called breast tumor initiation cells, BTICs) can be isolated by sorting for CD44+CD24-/low cells (Al-Hajj et al., 2003) or Hoechst negative side population (SP) cells (Patrawala et al., 2005), and can also be enriched by spheroid culture and serial transplantations in immunodeficient mice (Ponti et al., 2005). These CSCs not only harbor the capability of self-renewal, but also are able to differentiate into multiple lineages of tumor cells growing in various types of distal organs. They have been shown to be resistant to the standard chemotherapy. Thus, we hypothesized that EndoCD/5-FC may selectively reduce breast cancer stem cells populations.

10.2.1 To determine EndoCD/5-FC CSCs killing activities

After EndoCD/5-FC protein treatment, we found that EndoCD/5-FC selectively reduced the MDA-MB-231 and MCF-7 $CD44^{+}/CD24^{-}$ population in a dose dependent manner (Figure 20A) as well as mammosphere formation (Figure 20B). These results suggest that EndoCD/5-FC may inhibit BCSC growth. We will expand the experiments to multiple breast cancer cell lines even primary tumor samples with different criteria of CSC phenomena *in vitro* and *in vivo*. The molecular mechanism for EndoCD/5FC-mediated BCSCs suppression should be interesting to further pursue. For instance, if Endo is critical for targeting BCSCs, integrin αvβ1 in BCSCs may be important for maintenance of BCSC. Once it becomes clear, we will logically pursue the appropriate direction. Alternatively, we can always use a nonbiased approach such as antibody arrays to identify which signal pathways might be activated/inactivated by EndoCD/5-FC treatment.

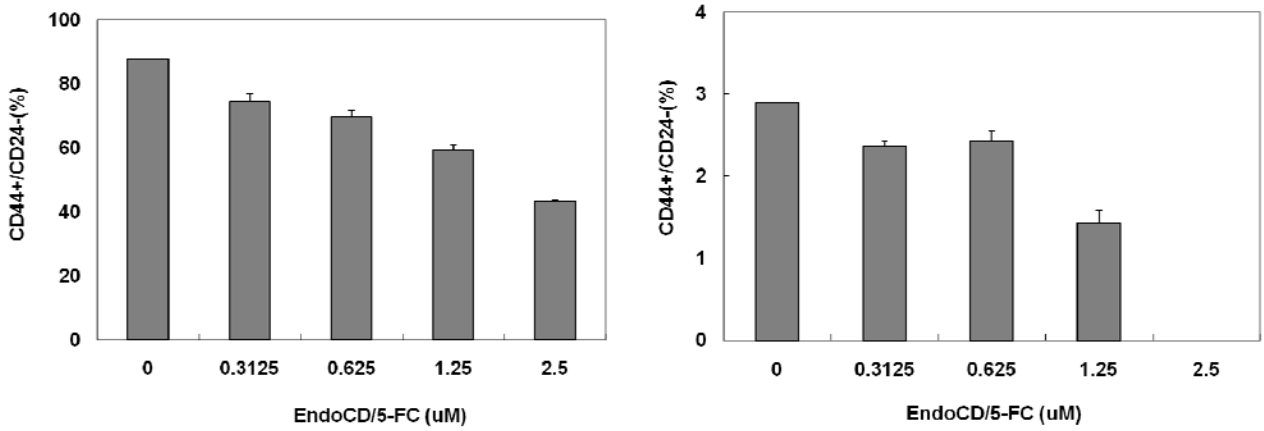
Figure 20. EndoCD/5-FC can selectively reduce breast cancer stem cell population.

(A) To detect the CSC (TICs) population, surface markers CD44+/CD24- serve as an index. After EndoCD/5-FC treatment in two human breast cancer cell lines (MDA-MB-231 and MCF-7) for 48 hours, $1X10^6$ cells were processed for staining with FITC-conjugated CD44 and PE-conjugated CD24 antibodies, respectively, and then analyzed or sorted with BD flow cytometer. EndoCD/5-FC decreased breast stem cell population by dose dependent manner.

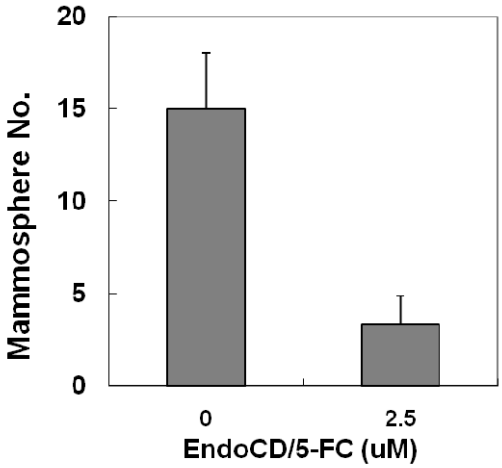
(B) CSC (TICs) population was also validated by culturing mammospheres using MammoCultTM medium containing 4 ug/ml Heparin and 0.48 μg/ml hydrocortisone. EndoCD/5-FC has ability to inhibit breast cancer mammopheres formation.

Figure 20.

A.MDA-MB-231 **MCF-7**



B.



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VITA

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