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DLL4 Blockade Inhibits Tumor Growth and Reduces Tumor-Initiating Cell Frequency

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SUMMARY

Previous studies have shown that blocking DLL4 signaling reduced tumor growth by disrupting productive angiogenesis. We developed selective anti-human and anti-mouse DLL4 antibodies to dissect the mechanisms involved by analyzing the contributions of selectively targeting DLL4 in the tumor or in the host vasculature and stroma in xenograft models derived from primary human tumors. We found that each antibody inhibited tumor growth and that the combination of the two antibodies was more effective than either alone. Treatment with anti-human DLL4 inhibited the expression of Notch target genes and reduced proliferation of tumor cells. Furthermore, we found that specifically inhibiting human DLL4 in the tumor, either alone or in combination with the chemotherapeutic agent irinotecan, reduced cancer stem cell frequency, as shown by flow cytometric and in vivo tumorigenicity studies.

INTRODUCTION

Accumulating evidence has suggested that tumors are frequently composed of heterogeneous cell types and that tumor initiation and growth are driven by a subset of cells, termed cancer stem cells (CSCs) or tumor-initiating cells (Reya et al., 2001; Ailles and Weissman, 2007). In this model, tumors can be viewed as having a hierarchical organization with a tumorigenic cell population that can self-renew, and thereby proliferate indefinitely, at the top of the hierarchy. CSCs can also give rise to more differentiated progeny that comprise the bulk of the tumor, have reduced proliferative capacity, and are therefore less tumorigenic (Clarke et al., 2006). Although CSCs share certain properties with normal stem and/or progenitor cells, CSCs have accumulated oncogenic mutations and lost normal constraints on growth control. Evidence for tumor heterogeneity and CSCs was first provided in acute myeloid leukemia (AML) (Lapidot et al., 1994) and more recently extended to several human solid tumors, for example, breast, brain, prostate, colon, and pancreatic cancers (Al-Hajj et al., 2003; Singh et al., 2004; Patrawala et al., 2006; Galli et al., 2004; O'Brien et al., 2007; Ricci-Vitani et al., 2007; Dalerba et al., 2007; Li et al., 2007). Furthermore, several research reports have indicated that CSCs can be preferentially resistant to many current therapies, including various chemotherapeutic agents and radiation treatment (Dean et al., 2005; Costello et al., 2000; Matsui et al., 2004; Bao et al., 2006; Phillips et al., 2006; Dylla et al., 2008). Thus, therapeutic strategies that effectively target CSCs could have a major impact on cancer patient survival.

Delta-like 4 ligand (DLL4) is an important component of the Notch pathway and contributes to stem cell self-renewal and vascular development. Deletion of a single allele of DLL4 results in embryonic lethality due to defects in development of the vasculature (Duarte et al., 2004; Gale et al., 2004; Krebs et al., 2004). DLL4 overexpression is found in tumor vasculature and in tumor cells to activate Notch signaling (Patel et al., 2005; Yan et al., 2001). Previous studies have indicated that inhibition of DLL4 resulted in broad spectrum antitumor activity in cancer cell line-based xenograft models (Noguera-Troise et al., 2006; Ridgway et al., 2006; Scehnet et al., 2007). The antitumor effect was shown to be the result of dysregulated angiogenesis characterized by increased sprouting in endothelial tip cells leading to nonfunctional vasculature in the tumor. Thus, inhibiting DLL4 disrupts productive angiogenesis in a manner distinct from traditional antiangiogenic therapies by causing hyperproliferation of tumor vessels that leads to a reduction in tumor growth (Swanson and Harris, 2007; Thurston et al., 2007). In addition to its role regulating endothelial cells and tumor vasculature, the Notch pathway is known to play a key role in stem cell growth and differentiation in many lineages and tumor types (Wilson and Radtke, 2006). We developed neutralizing antibodies against human DLL4 to evaluate the effects of selectively targeting DLL4 expressed on tumor cells on tumor growth and CSC frequency. We also developed antibodies to murine DLL4 to further analyze the effects blocking signaling from DLL4 expressed in tumor stromal and vascular cells in xenograft models. The studies presented here focus primarily on early-passage colon tumor xenograft models. We found that blocking DLL4 selectively in the tumor resulted in a reduction in tumor growth and tumorigenic cell frequency independent of an angiogenic mechanism.

RESULTS

Identification and Characterization of Anti-Human DLL4

We first screened monoclonal anti-DLL4 antibodies from a murine hybridoma library for their ability to inhibit DLL4-induced

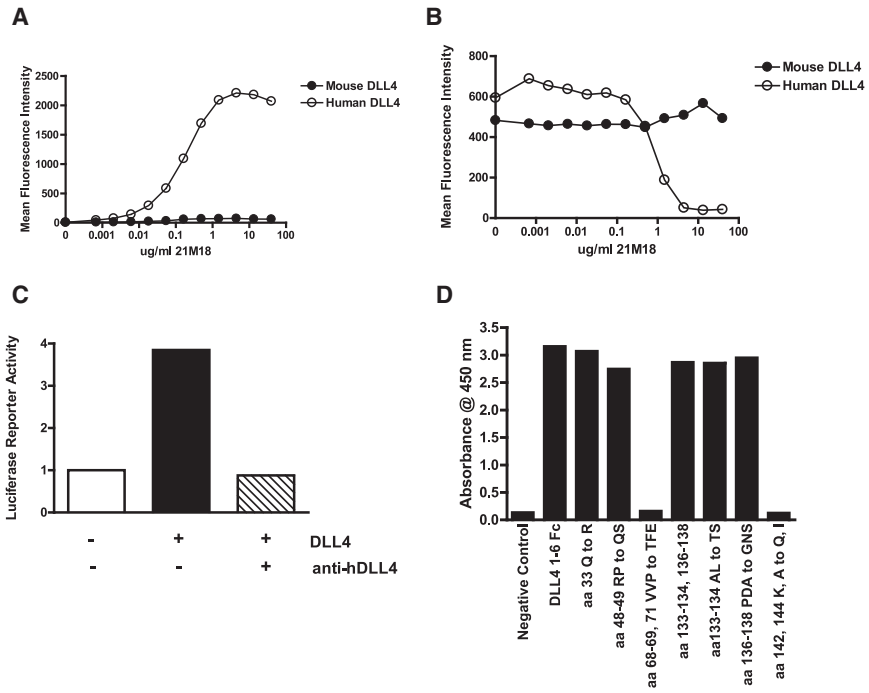

Figure 1. In Vitro Characterization of Anti-DLL4 Monoclonal Antibodies

(A and B) Activity of 21M18 in binding to human or mouse DLL4 (A) and blocking human or mouse DLL4 binding to Notch1-expressing cells (B). Each data point represents the average of two replicates. 21M18 was found to bind and inhibit human DLL4, but not mouse DLL4.

(C) Anti-hDLL4 inhibits hDLL4-induced Notch signaling using a luciferase reporter assay.

(D) Amino acids from mouse DLL4 were substituted into the human DLL4 protein and tested for binding anti-hDLL4 in order to map its epitope. Two separate regions in the primary sequence were required for binding. One region is aa 68–71 in domain 1, and the second region is aa 142–144 in domain 2.

Notch pathway activation using a Notch-responsive luciferase reporter assay in HeLa cells. The antibody 21M18 showed strong inhibition in this assay and was further characterized. 21M18 binds to human DLL4 (hDLL4) but does not cross-react to murine DLL4 (Figure 1A), and correspondingly blocks human, but not murine, DLL4 binding to Notch1 receptor in a FACS-binding assay (Figure 1B). Anti-hDLL4 is completely specific for binding to DLL4 and does not bind detectably to any other Notch ligand, DLL1, DLL3, JAG1, or JAG2 (data not shown). Anti-hDLL4 is able to completely inhibit induction of a Notch-responsive reporter gene activated by hDLL4 stimulation (Figure 1C). We have determined the binding dissociation constant (K_d) to be 0.6 nM by Biacore assay using an Fc-hDLL4 protein containing the entire extracellular domain of hDLL4. To identify antibodies that recognize specific regions of the DLL4 extracellular domain, epitope mapping using various murine/human chimeras of DLL4 containing domains 1–6 of the N terminus was preformed. Our initial evaluation indicated that domains 1 and 2 (Parks et al., 2006) were important for binding, as replacement of either domain with the homologous murine sequence resulted in loss of binding to 21M18 (see Figure S1 available online). Although the Delta/Serrate/lag-2 (DSL) domain (Tax et al., 1994), also known as domain 3, was not sufficient for anti-hDLL4 binding, the presence of this domain was required for binding of anti-hDLL4 21M18 to hDLL4. Further analysis of amino acids in domains 1 and 2 by mutagenesis showed that amino acids VVP at positions 68, 69, and 71, and K and A at positions 142 and 144 were critical for anti-hDLL4 binding (Figure 1D). Thus, anti-hDLL4 21M18 recognizes a conformational epitope comprised of distinct regions in the primary sequence of hDLL4.

In Vivo Antitumor Activity of Anti-hDLL4

To determine the antitumor effect of anti-hDLL4 in vivo, we utilized human xenograft tumor models in NOD-SCID mice for our antibody screening and efficacy studies. The experiments presented in this report focus primarily on colon tumor xenografts. Tumors were directly transplanted into mice from patient samples and were minimally passaged in vivo without any selection for growth in cell culture. Importantly, these tumors retain much of the cellular heterogeneity of primary human tumors and contain CSCs as well as more differentiated nontumorigenic cells (Dalerba et al., 2007). The initial xenograft studies of anti-hDLL4 entailed implantation of tumor cells followed by initiation of treatments at day 2 (preventative dosing regimen). Using this experimental paradigm, we found that in OMP-C8, a human colon tumor xenograft derived from a liver metastasis, anti-hDLL4 produced a decrease in tumor growth (Figure 2A). We then explored the administration of anti-hDLL4 in combination with several chemotherapeutic agents commonly used for colon cancer. Anti-hDLL4 showed additive antitumor activity with 5-FU treatment (data not shown), and we found that OMP-C8 colon tumors were particularly sensitive to combination of anti-hDLL4 and irinotecan. For example, irinotecan at a dose of 7.5 mg/kg decreased OMP-C8 colon tumor volume by 80%, and, importantly, the combination of irinotecan plus anti-hDLL4 produced increased growth inhibition compared to the single agents alone (Figure 2A). In anti-hDLL4-treated OMP-C8 tumors, changes in the expression of Notch target genes *HES1* and *ATOH1* (Figure 2B) were consistent with the pattern of known antagonists of Notch signaling such as gamma secretase inhibitors (van Es et al., 2005). Further analysis showed that the effect of anti-hDLL4 on tumor growth was associated with a decrease in the frequency of proliferating cells, as indicated by a reduction of Ki67 expression in treated tumors, detected by immunohistochemistry (Figure 2C). Since very limited tumor growth was evident in the animals treated with the combination of anti-hDLL4 and irinotecan, we then investigated if tumor growth would resume if treatments were discontinued. We observed that tumors continued to grow progressively in the mice previously treated with irinotecan alone, whereas no tumors emerged and maintained growth in the animals previously treated with the combination of anti-hDLL4 and irinotecan (Figure 2D). A similar pattern was observed

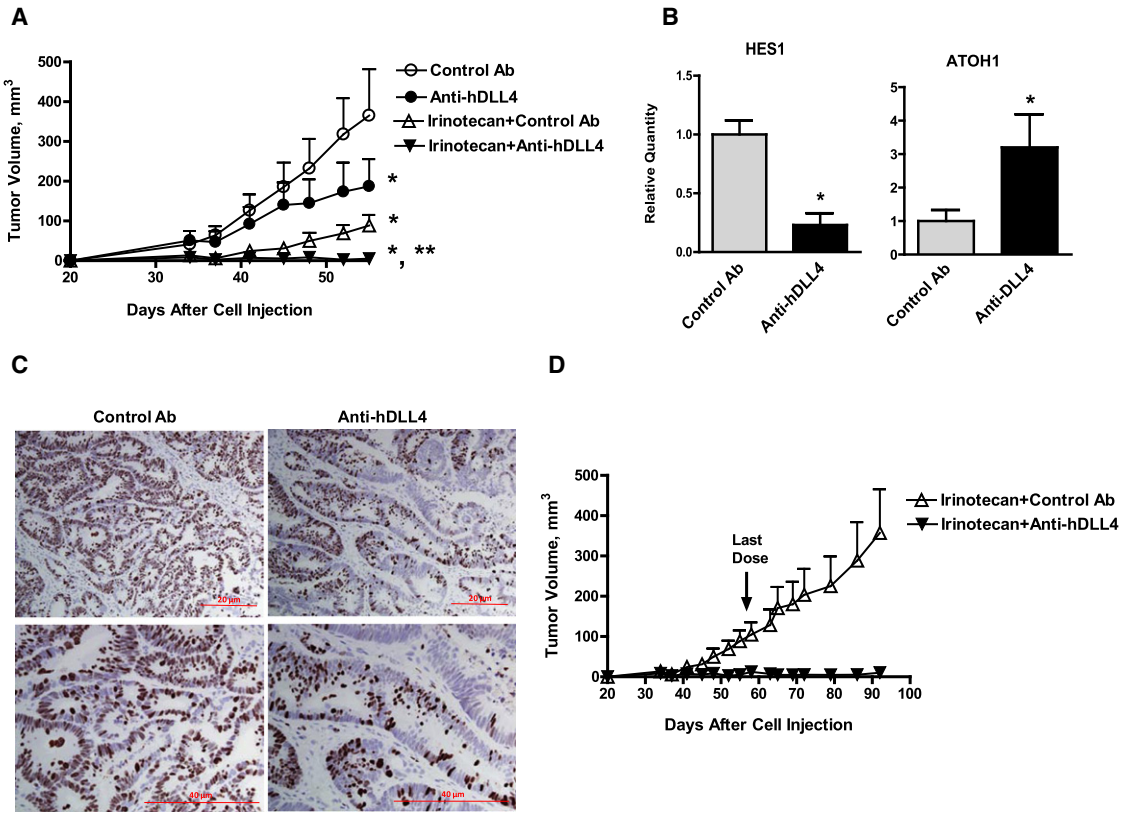

Figure 2. In Vivo Efficacy of Anti-hDLL4 Antibody 21M18

(A) Effect of control antibody (open circles), anti-hDLL4 (closed circles), irinotecan (dosed at 7.5 mg/kg once per week; open triangles), and the combination of anti-hDLL4 plus the same dose of irinotecan (closed triangles) on the growth of OMP-C8 colon xenograft tumors in NOD/SCID mice. There were ten mice used per treatment group; *$p < 0.05$ versus control Ab group; **$p < 0.05$ versus irinotecan plus control Ab group.

(B) Effect of anti-hDLL4 on Notch target gene expression in OMP-C8 tumors as determined by quantitative PCR; *$p < 0.05$ versus control Ab group.

(C) anti-hDLL4 reduces cell proliferation in OMP-C8 tumors, determined by immunohistochemical staining for Ki67, a marker of cell proliferation. The top two photomicrographs were taken at 20× magnification and the bottom two at 40×.

(D) Continuation of the experiment shown in (A) following termination of the irinotecan and anti-hDLL4 treatments. The tumors in the group previously treated with irinotecan alone (open triangles) continued to grow, while the tumors in the group previously treated with anti-hDLL4 plus irinotecan (closed triangles) did not grow during the course of the experiment. Data are expressed as mean + SEM.

in OMP-C9 and OMP-C17 (Figure S2). Thus the combination of anti-hDLL4 and irinotecan resulted in increased antitumor activity in several colon tumor xenografts, relative to irinotecan alone. Consistent with our observations using the preventative dosing regimen, we found that 21M18 was efficacious alone and in combination with irinotecan in treating established OMP-C8 colon tumors (Figure S3).

Effect of Anti-hDLL4 on Tumor Recurrence

We developed a dosing regimen that would allow us to test the effect of anti-hDLL4 on tumor recurrence following chemotherapeutic treatment. In this tumor recurrence model, we treated mice bearing colon tumor xenografts of 200–500 mm^3 with 45 mg/kg irinotecan twice a week for 4–5 weeks. As seen in Figure 3A, this high dose of irinotecan induced OMP-C8 tumor regression; however, tumors reappeared about 25 days after treatment with irinotecan was terminated and grew progressively. Inclusion of anti-hDLL4 during irinotecan treatment did not affect the rate of irinotecan-mediated OMP-C8 tumor regression but significantly delayed tumor recurrence following termination of the chemotherapy (Figure 3A). Similar results were observed in an additional colon tumor model, OMP-C17 (Figure S4).

Effect of Anti-hDLL4 on Tumorigenic Cells

It has been proposed that CSCs are preferentially resistant to many standard therapies and that CSCs mediate tumor recurrence following such treatments (Dean et al., 2005; Bao et al., 2006). To determine whether the observed synergistic growth inhibition and delay in tumor recurrence by the combination of anti-hDLL4 and irinotecan was a result of these agents reducing tumor-initiating cell frequency, the CSC population was quantified in regressing tumors in three ways: flow cytometry, in vitro colony formation, and in vivo tumor growth. Tumors were harvested after 3 weeks of treatment at the point when they were reduced by 50% of the initial volume during irinotecan-induced tumor regression (as in Figure 3A). Flow cytometric analysis showed that cells expressing cell markers associated with tumorigenic colon cancer cells, $ESA^+/CD44^+/CD166^+$ (Dalerba et al., 2007), were increased from 28% in the control group to

43% following irinotecan treatment (Figure 3B, FACS plots are shown in Figure S5). In contrast, treatment with anti-hDLL4 resulted in a 50% decrease in the $ESA^+/CD44^+/CD166^+$ population compared to the control group. Notably, the combination of anti-hDLL4 and irinotecan further decreased the percentage of this triple-positive cell population in treated tumors (Figure 3B).

Tumor cells from the four treatment groups were processed to generate single-cell suspensions and assayed for their ability form colonies in vitro. This type of assay, a single-cell deposition-limiting dilution assay (SCD-LDA), has been used in the hematopoietic stem cell field to quantify normal and leukemic stem cells (Breems et al., 1994). We adapted this assay for studying epithelial tumor cells and have established culture conditions that support the growth over several weeks of tumorigenic, but not nontumorigenic, cells (Dylla et al., 2008). Irinotecan treatment was found to increase the percentage of cells capable of forming colonies, while the colony formation by tumor cells harvested from mice previously treated with the combination of anti-hDLL4 plus irinotecan was lower than both the control and the irinotecan alone groups (Figure 3C).

A functional test to determine CSC frequency is to transplant tumor cells from treated mice to another set of mice and quantify the number of cells required to generate new tumors by limiting dilution assay (LDA) (Wang et al., 1997). Equal cell numbers from each of the four treatment groups were injected into mice at four different cell doses, and tumor growth was assessed after 86 days (Figure S6). The frequency of tumor growth observed at the various cell numbers allows the determination of CSC frequency. As seen in Figure 3D, control mAb-treated tumors had a CSC frequency of approximately 1/160. Irinotecan-treated tumors had an approximately 2-fold higher CSC frequency compared to control. In contrast, treatment with anti-hDLL4 alone decreased the percentage of CSCs compared to control by approximately 2-fold. Strikingly, the combination of anti-DLL4 and irinotecan produced a further decrease in CSC frequency, approximately 5-fold lower than the control group and 9-fold lower than tumors treated with irinotecan alone (Figure 3D).

Additionally, we addressed the question of how treatment with anti-hDLL4 and irinotecan affected tumorigenicity by serially transplanting sorted cells derived from tumors after in vivo treatment. We purified tumor cells expressing colon CSC markers ($ESA^+/CD44^+/CD166^+$) (Dalerba et al., 2007) and injected 100 cells per mouse from either control Ab, irinotecan plus control Ab, or irinotecan plus anti-hDLL4-treated tumors. Cells from the first two groups grew readily and formed large tumors, whereas cells from tumors previously treated with the combination of chemotherapy and anti-hDLL4 exhibited reduced tumor growth frequency and rate (Figures 3E and 3F). Thus, the effect of anti-hDLL4 on reducing tumorigenicity is apparent not only when analyzing the whole tumor cell population but also when enriching for cells expressing CSC markers.

Effect of Anti-hDLL4 on Gene Expression in CSCs

To investigate the mechanism of action of anti-hDLL4 on reducing cancer stem cell frequency, we then determined the effect of anti-hDLL4, irinotecan and the combination on gene expression in sorted cells expressing colon CSC markers ($ESA^+/CD44^+/CD166^+$) (Dalerba et al., 2007) as well as in nontumorigenic cells ($ESA^+/CD44^-/CD166^-$) from OMP-C8 tumors. DLL4 was expressed at the RNA level in both cell populations, and we found that anti-hDLL4 treatment resulted in repression of *HES1* and activation of *ATOH1* expression in both cell populations (Figures 4A and 4B) similar to the effect seen in the whole tumor (Figure 2B). Thus, anti-hDLL4 appears to have a direct effect in modulating Notch signaling in the CSC-enriched subpopulation within the tumor. We also found that anti-hDLL4 modulated the expression of other genes that shed light on its mechanism of action. For example, we found that anti-hDLL4 induced the expression of chromogranin A (CHGA), a marker of endocrine differentiation in colon cells (Hendy et al., 1995). Genes that regulate apoptosis were altered by anti-hDLL4. For example, while irinotecan treatment induced the expression of antiapoptotic genes such as *HSPA6* and *BIRC3*, anti-DLL4 reversed this effect in the combination group. The genes mentioned above were regulated equivalently in both $CD44^+/CD166^+$ cells and $CD44^-/CD166^-$ cells. In contrast, certain genes were found to be differentially regulated in the CSC-enriched cells versus the nontumorigenic cells, including the proapoptotic gene *PDCD4*, the Wnt inhibitors *DACT1* and *AXIN2*, and genes involved in oxidative stress, *FOXO1* and *TXNIP*.

Targeting Murine and Human DLL4 in Xenografts

Our antibody, 21M18, recognizes human but not rodent DLL4. Therefore, with this antibody we are not able to assess the impact of blocking DLL4-Notch signaling in the tumor stroma and vasculature as previously described (Noguera-Troise et al., 2006; Ridgway et al., 2006). To dissect the mechanisms involved in anti-DLL4 inhibition of tumor growth, we developed an anti-mouse DLL4 antibody, designated 21R30, which is capable of blocking binding and signaling of murine but not human DLL4 (Figure S7). We confirmed that this anti-mouse DLL4 antibody has antitumor activity through deregulating tumor angiogenesis. Similar to previous studies, tumors treated with anti-mouse DLL4 showed increased tumor vasculature as seen by anti-CD31 staining (Figure 5A). In contrast, there was no evidence for endothelial cell hyperproliferation after anti-hDLL4 treatment. The combination of antibodies also showed an effect on increasing endothelial cell staining similar to anti-mDLL4 (Figure 5A). We then tested the anti-mDLL4 and anti-hDLL4 antibodies individually and in combination for their effect on C8 tumor growth and found that each antibody reduced tumor growth by approximately 50%, and the combination of the two antibodies produced additive antitumor efficacy (Figure 5B). We next tested the effect of chemotherapeutic treatment in the context of inhibiting DLL4 in both the stroma and the tumor, and found irinotecan combined with anti-hDLL4 and anti-mDLL4 resulted in additive antitumor activity and regression of established C8 tumors (Figure 5C).

Activity of Anti-DLL4 in Breast Tumors

Previous studies in this paper have focused on colon tumors. We have also observed that blocking DLL4 in both the tumor and in the stroma/vasculature has antitumor efficacy in a wide range of human tumor xenografts from various tumor types including colon, breast, lung, and pancreas. An example in a breast tumor xenograft is shown in Figure 6. Established UM-PE13 breast tumors were treated with high-dose taxol (paclitaxel) to reduce tumor volume together with either a control Ab or the

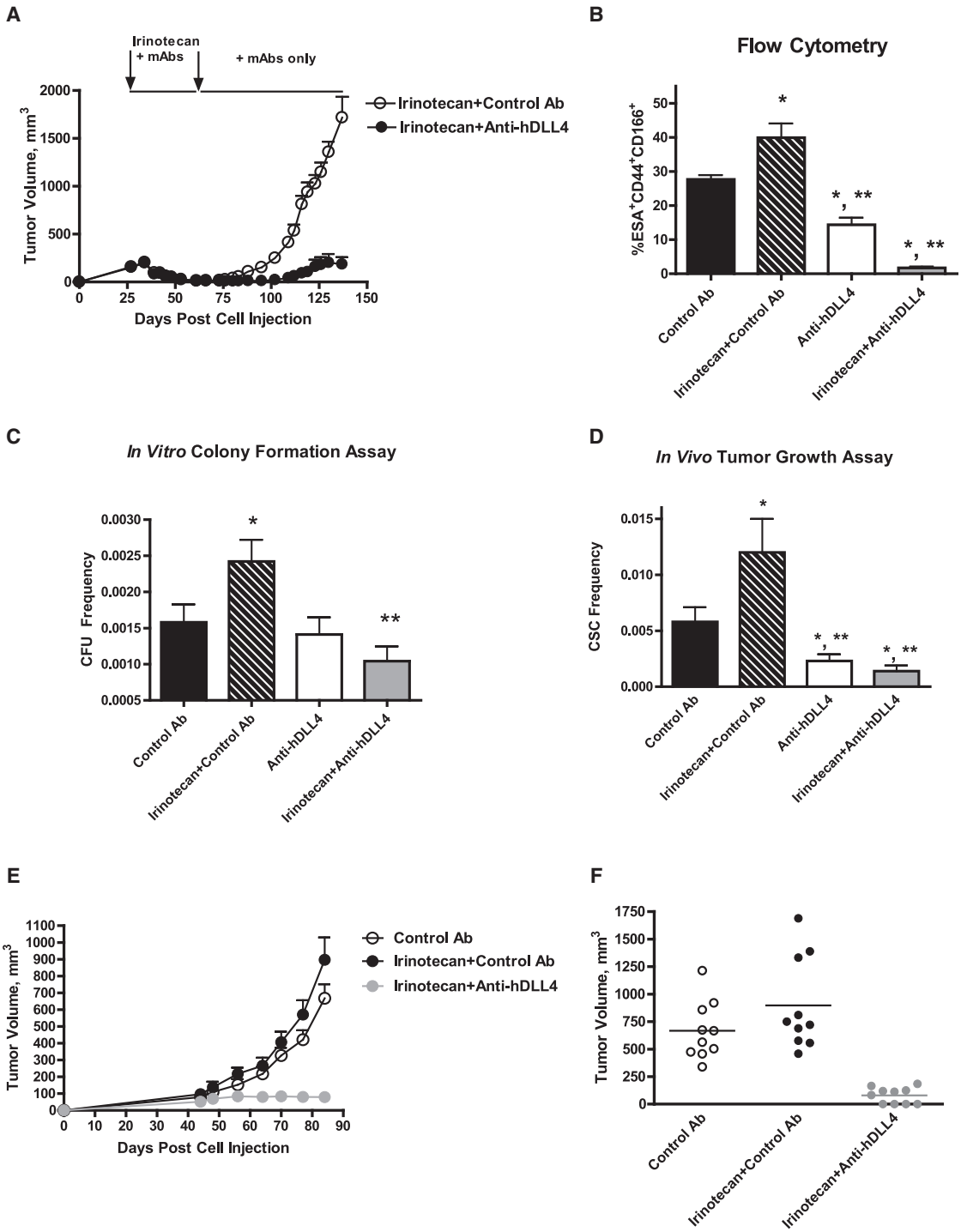

Figure 3. Effect of Anti-hDLL4 on Colon Tumor Recurrence

(A) Treatments were initiated when OMP-C8 colon tumors were approximately 200 mm^3 (left arrow). Irinotecan was dosed at 45 mg/kg twice per week, either together with control Ab (open circles; n = 12) or together with anti-hDLL4 (closed circles; n = 11). Irinotecan treatments were stopped at day 60 (right arrow), and the antibody treatments continued. The rates of tumor growth in the two groups are shown. Anti-hDLL4 reduced the rate of tumor recurrence.

(B) Established tumors were treated for 2 weeks with either control Ab, anti-hDLL4, irinotecan (45 mg/kg), or anti-hDLL4 plus irinotecan and analyzed by flow cytometry for the expression of the CSC markers CD44, CD166, and ESA. Five tumors from each treatment were analyzed. *$p < 0.05$ versus control Ab group; **$p < 0.05$ versus irinotecan plus control Ab group.

(C) Tumors previously treated as described in (B) were analyzed for their ability to form colonies when cultured in vitro.

(D) Tumors treated as described in (B) were analyzed for CSC frequency by serial in vivo transplant, LDA. There were ten mice per group and four cell doses analyzed. The appearance of tumors on day 86 was used to calculate the tumor-initiating cell frequency. Anti-hDLL4 treatment (open bars) reduced CSC

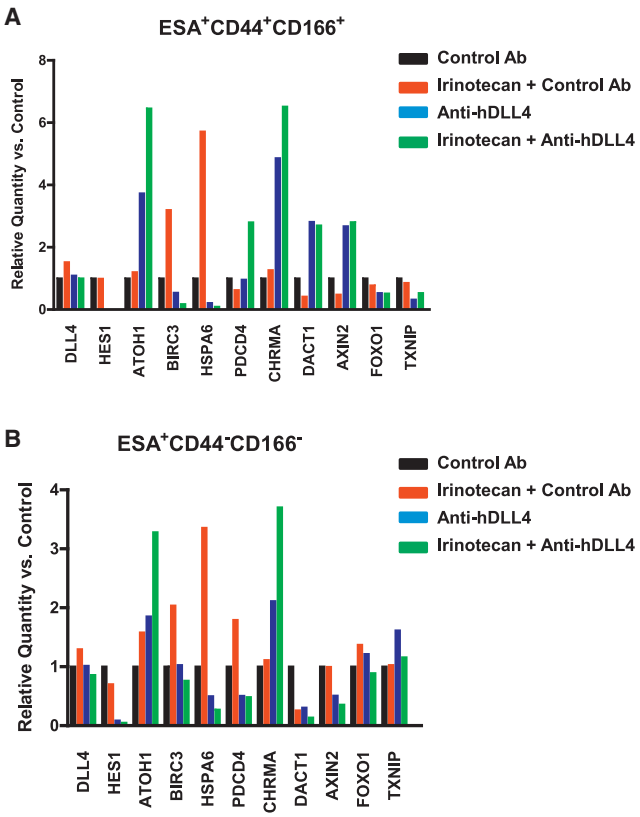

Figure 4. Effect of Anti-hDLL4 on Gene Expression in Sorted Enriched CSC and Non-CSC Populations

OMP-C8 tumors treated with either control Ab (black bars), irinotecan (red), or anti-hDLL4 (blue), or the combination of irinotecan and anti-hDLL4 (green). After 2 weeks of treatment, tumors were processed, and tumor cells were sorted by flow cytometry into CD44+CD166+ and CD44-CD166− populations. Levels of gene expression determined by quantitative PCR in the double-positive cells (A) and double-negative cells (B) are shown. Values are relative to the control Ab-treated group.

combination of anti-hDLL4 plus anti-mDLL4. Blocking DLL4 with this pair of antibodies had a dramatic effect in delaying tumor regrowth after cessation of the taxol treatment (Figure 6A). We observed no significant regrowth of tumors for approximately 80 days after stopping chemotherapeutic treatments. UM-PE13 tumors treated with either control Ab, taxol plus control Ab, anti-hDLL4 plus anti-mDLL4, or the combination of taxol and the anti-DLL4 antibodies were analyzed by serial transplant, LDA to quantify tumor-initiating cell frequency. Taxol treatment was found to increase CSC frequency approximately 3-fold, while inhibiting DLL4 decreased CSC frequency, either alone or, more significantly, in combination with taxol (Figure 6B).

DISCUSSION

Our findings demonstrate that blocking DLL4 signaling inhibits tumor growth through multiple mechanisms, including a reduction in CSC frequency. In addition to the previously described effect on deregulating angiogenesis by targeting DLL4 in the vasculature, we show that selectively inhibiting DLL4 signaling in human tumor cells with anti-hDLL4 21M18 leads to a decrease in colon tumor growth, a delay in tumor recurrence after chemotherapeutic treatment, and a decrease in the percentage of tumorigenic cells. While inhibition of DLL4-Notch signaling is a new and promising strategy for cancer treatment, the utility of disrupting angiogenesis via this mechanism (Thurston et al., 2007) has yet to be proven effective in the clinic. DLL4 blockade has been shown to increase proliferation of endothelial cells; however, the resulting vessels are not perfused by intravascular tracers, such as lectin, suggesting that these vessels lack a functional lumen. An interesting question yet to be resolved concerns the long-term fate of this abnormal neovasculature and whether it could ever be normalized into functional tumor vasculature. Further experiments and future clinical testing are required to address this important issue.

Understanding the molecular mechanisms involved in the effect of anti-hDLL4 on tumor-initiating cell frequency is an important question. It is possible that DLL4-Notch signaling is required for the self-renewal ability of the CSCs and maintains the tumorigenic cells in a more undifferentiated cell state. Consistent with this possibility is the repression of *HES1* observed in tumor cells after anti-hDLL4 treatment. *HES1* expression has been shown to be important in maintaining cells in an undifferentiated, pluripotent state (Sang et al., 2008). Conversely, upregulation of *ATOH1* is indicative of differentiation of colon cells to mucin-secreting goblet cells, and ATOH1 has recently been shown to have the properties of a tumor-suppressor gene in colorectal cancer (Bossyut et al., 2009). Anti-hDLL4 treatment also induced expression of CHGA, a known marker for endocrine cell differentiation in various organs including colon (Hendy et al., 1995). Significantly, we observed regulation of Notch target genes, *HES1* and *ATOH1*, after anti-hDLL4 treatment in RNA prepared from whole tumors (Figure 2B) and in FACS-sorted tumorigenic and nontumorigenic cells (Figures 4A and 4B). Thus, anti-hDLL4 can directly regulate Notch signaling in the enriched tumorigenic CSC subset of cells within the tumor. In addition to promoting differentiation of stem/progenitor cells, repression of DLL4-Notch signaling in CSCs may also promote cell death or inhibit proliferation of the tumorigenic cells, particularly in combination with chemotherapeutic agents.

We observed that irinotecan treatment induces a stress response in tumor cells and an increase in the expression of certain antiapoptotic genes such as *HSPA6* (also known as inducible HSP70) and *BIRC3* (also known as cIAP2). Anti-hDLL4

frequency relative to control-treated tumor (black bars). Irinotecan treatment (cross-hatched bar) increased CSC frequency, and the combination of anti-hDLL4 plus irinotecan group (gray bar) resulted in tumors with a lower CSC frequency than both the control and irinotecan alone groups. *$p < 0.05$ versus control Ab group; **$p < 0.05$ versus irinotecan plus control Ab group.

(E) Serial transplant of tumor cells from treated animals from the experiment shown in (A). One hundred CD44+CD166+ cells were injected into ten animals each, and tumor growth was monitored over 84 days.

(F) Individual tumor measurements on day 84 from each of the ten animals shown in the groups in the serial transplant experiment (E) are shown. Data are expressed as mean + SEM.

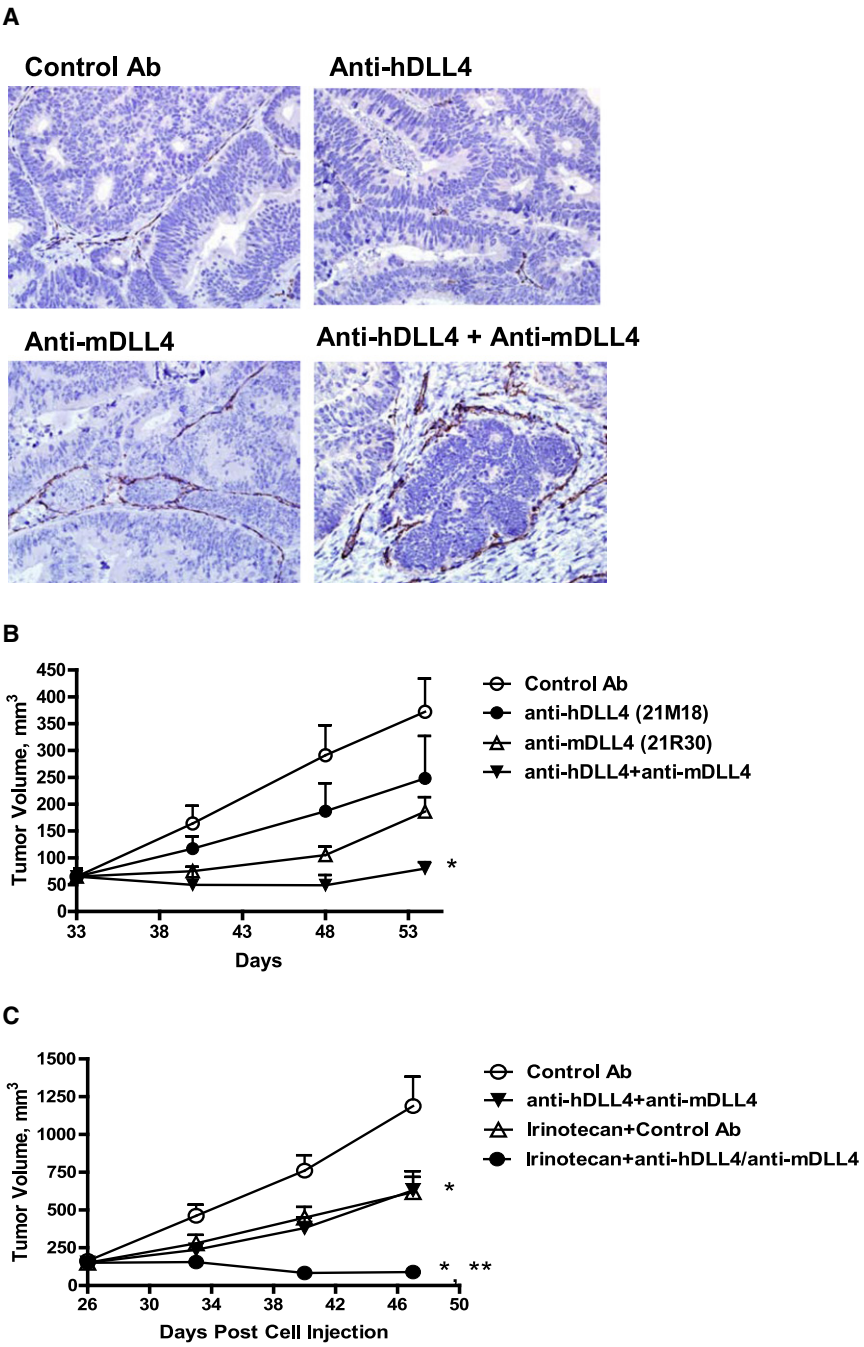

Figure 5. Effect of Anti-hDLL4 in Combination with Anti-Mouse DLL4

(A) Effect of anti-DLL4 antibodies on tumor vasculature as shown by anti-CD31 staining. Tumors treated with anti-mouse DLL4, 21R30 show increased anti-CD31 staining, while tumors treated with anti-hDLL4 look similar to control.

(B) Established OMP-C8 tumors were treated with either control Ab (open circles), anti-hDLL4 (filled circles), anti-mDLL4 (open triangles), or both antibodies (filled triangles). There were ten animals in each treatment group; *p < 0.05 versus control Ab group.

(C) Established OMP-C8 tumors were treated with either control Ab (open circles), anti-hDLL4 plus anti-mDLL4 (filled triangles), irinotecan (dosed at 7.5 mg/kg once per week) plus control Ab (open triangles), or the combination of irinotecan plus anti-hDLL4 and anti-mDLL4 (filled circles). *p < 0.05 versus control Ab group; **p < 0.05 versus irinotecan plus control Ab group. Data are expressed as mean + SEM.

treatment inhibits induction of these genes, potentially sensitizing tumor cells to cell death in response to the chemotherapeutic treatment.

All the genes discussed above were regulated in a similar manner to the tumorigenic $CD44^+/CD166^+$ and nontumorigenic $CD44^-/CD166^-$ cell populations. Interestingly, certain genes were found to be differentially regulated in the two cell populations. For example, the proapoptotic tumor suppressor gene *PDCD4* (Wang et al., 2008) was found to be induced by the combination of anti-DLL4 and irinotecan in the enriched tumorigenic cell population, but not the $CD44^-/CD166^-$ cells. We also observed selective activation of the Wnt pathway inhibitors *AXIN2* and *DACT1* in the tumorigenic cells. AXIN2 is a well-characterized inhibitor of canonical Wnt signaling through β-catenin (Jho et al., 2002), and *DACT1* (also known Dapper 1) encodes a Dishevelled-interacting protein that also blocks Wnt signaling (Zhang et al., 2006). The Wnt-β-catenin pathway is known to be important is maintaining normal and CSCs in the colon (van Es et al., 2005). These data provide evidence for crosstalk between the Notch and Wnt pathways in CSCs, which has been observed in other settings (for example, Phng et al., 2009).

Certain genes regulated by oxidative stress were also found to be regulated by anti-DLL4, including *FOXO1* and *TXNIP*. The FoxO family of transcription factors has been previously found to be required for maintenance of hematopoietic stem cells and protects these cells from oxidative stress (Tothova et al., 2007). Resistance to oxidative stress has recently been shown to be a property of CSCs, and *FOXO1* and *TXNIP* were found to upregulated in tumorigenic cells (Diehn et al., 2009). Reduction of expression of these genes after anti-DLL4 treatment provides further insight into the mechanism of action in the tumorigenic cell subset.

Inhibition of DLL4-Notch signaling in tumor cells also appears to affect proliferation of the nontumorigenic, or bulk tumor, cells as demonstrated by the effect of anti-hDLL4 on the number of Ki67-positive cells. The CSC frequency of C8 tumor xenografts was determined to be less than 1% by LDA (Figure 3D), while anti-hDLL4 reduced the number of proliferating cells in tumors by a far larger extent, apparently greater than 50% (Figure 2C). This is consistent with our finding that anti-DLL4 treatment

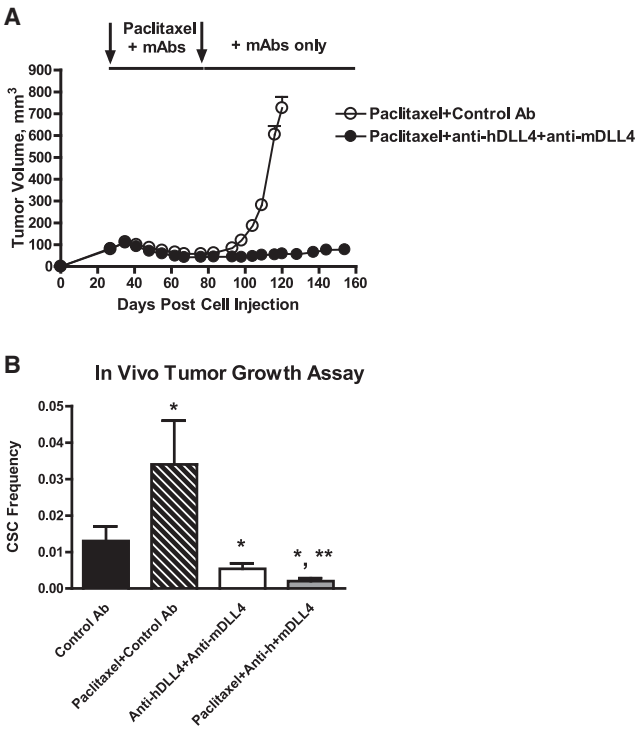

Figure 6. Effect of Anti-hDLL4 and Anti-mDLL4 in Combination with Paclitaxel on Breast Tumor Recurrence and Cancer Stem Cell Frequency

(A) Established UM-PE13 tumors were treated with either control Ab plus paclitaxel (open circles) or a mixture of anti-hDLL4 and anti-mDLL4 plus paclitaxel (filled circles). Paclitaxel was dosed at 15 mg/kg twice per week. Paclitaxel treatments were stopped at day 75, and the antibody treatments were continued. Tumor growth recurred in the control group after the cessation of paclitaxel treatments, but not in the anti-DLL4 group.

(B) Tumors were harvested from the experiment shown in (A) along with tumors treated with control Ab alone or anti-hDLL4 plus anti-mDLL4 and analyzed for CSC frequency by serial transplant, in vivo limiting dilution analysis. There were ten mice per group, and three cell doses were analyzed for each group (50, 150, and 500 cells). The appearance of tumors on day 75 was used to calculate the tumor-initiating cell frequency. Anti-hDLL4 + anti-mDLL4 treatment (open bar) reduced CSC frequency relative to the control Ab-treated tumors (black bar). Paclitaxel treatment (cross-hatched bar) increased CSC frequency, and the combination of anti-hDLL4 plus irinotecan group (gray bar) resulted in tumors with a lower CSC frequency than both the control and irinotecan alone groups. *$p < 0.05$ versus control Ab group; **$p < 0.05$ versus irinotecan plus control Ab group. Data are expressed as mean + SEM.

blocked Notch signaling in the $CD44^{-}/CD166^{-}$ bulk tumor cells as well as the $CD44^{+}/CD166^{+}$ tumorigenic cells.

This study presents an experimental approach and highlights the utility of using human tumor xenografts to study the effect of drug treatments. An advantage of these models over conventional cell line-based models is that the tumor-derived xenografts retain much of the cellular heterogeneity of original tumor. Importantly, use of the human tumor xenografts allows the quantification of a drug's effect on tumor-initiating cell frequency by carrying out in vivo serial transplantation of cells from treated tumors. Although technically demanding and time consuming, the in vivo LDA is a very powerful, functional assay that makes no assumptions about the frequency, flow cytometry marker profile, or heterogeneity of the tumor-initiating cell population within a tumor and therefore is widely applicable to the preclinical study of many different tumor types and therapeutic agents. A key challenge in the field is to find surrogate markers that reliably correlate with tumor-initiating cell frequency across a broad range of tumors. This advance would enable the quantification of CSCs in clinical tumors samples—for example, before and after treatment, an important application for which the LDA is not a practical option.

We provide evidence that it is possible to increase or decrease tumorigenic cell frequency in treated tumors. For example, treatment with the chemotherapeutic agents irinotecan or taxol, while effective at reducing tumor volume, increased the frequency of tumorigenic cells. These data provided further experimental support for a widely held hypothesis in the field proposing that CSCs are preferentially resistant to many current therapies (Dean et al., 2005; Wicha et al., 2006) and are consistent with other studies (Bao et al., 2006; Dylla et al., 2008). Our findings have important potential implications in cancer treatment and drug discovery. Agents that reduce cancer cell frequency hold the promise of improving cancer treatment by delaying or preventing tumor recurrence and reducing the metastatic spread of disease. In particular, blocking DLL4 function is an attractive strategy for novel therapeutics, since this approach attacks the tumor through multiple mechanisms including inhibiting productive angiogenesis, inhibiting proliferation of bulk tumor cells, and reducing CSC frequency.

EXPERIMENTAL PROCEDURES

Antibodies

Anti-human DLL4 antibodies including 21M18 were generated by immunizing mice with purified human DLL4 (an Fc-DLL4 fusion protein containing the entire extracellular domain of DLL4), derived from a baculovirus expression system, followed by hybridoma generation and characterization. For production of 21M18, the hybridoma cells were injected in mice intraperitoneally, and antibody was purified from the ascites fluid. Anti-mouse DLL4 antibodies, including 21R30, were generated by panning a phage display library obtained from Morphosys (Rothe et al., 2008). DNA fragments encoding the fAb generated from the phage display libraries were subcloned into a full-length IgG expression vector, and the antibody was expressed in CHO cells and purified. The negative control Ab was 1B711, a murine monoclonal directed against dinitrophenol, (also known as anti-hapten) and obtained from the American Type Tissue Collection (ATCC).

Generation of Human and Murine DLL4 Stable Cell Lines

Human and murine DLL4 overexpressing cells were generated by transfecting HEK293 cells (ATCC) with FuGENE 6 (Roche) and either full-length human DLL4 (amino acids 1–685) or murine DLL4 (amino acids 1–686) in pcDNA 3.1 (Invitrogen) and selected with G418 for individual clones.

Characterization of Anti-DLL4 Antibodies

Luciferase reporter assays were carried out in HeLa cells (obtained from ATCC) that stably express human Notch2. Cells were cotransfected with pGL4 luc (Promega) driven by a synthetic 8× CBS promoter element, MAML, and pCMV Renilla (Promega) as a transfection control. Cells were incubated with 100 ng hDLL4 ligand (R&D Systems) coated on 96-well optical plates and assayed 18 hr later using the Dual-Glo luciferase assay reporter system (Promega). Antibody binding to cells was determined by incubation of cells on ice with indicated antibody concentrations and detected by goat anti-mouse PE (Caltag). Ligand/receptor blocking studies were done by first incubating the cells with the indicated antibodies, then adding hN1 10–15 Fc (a human Notch1 peptide corresponding to amino acids 375–601 (which correspond to EGF repeats 10–15) in-frame with the human Fc constant region),

which was detected by goat anti-human Fc PE (Jackson Immunoresearch) and analyzed by flow cytometry (FACS Calibur, Becton Dickinson).

Epitope Mapping

Mammalian expression plasmid vectors comprising a CMV promoter upstream of polynucleotides that encode fragments of the extracellular human DLL4 domain or murine/human chimeras as Fc fusion proteins were generated using standard recombinant DNA technology. Recombinant proteins were then expressed in cultured HEK293 cells by transient transfection, and conditioned medium was collected and used in ELISA assay to identify the region of human DLL4 required for interaction with 21M18.

Xenograft Models

The establishment and characterization of in vivo CSC-driven colon and breast xenograft models were described previously (Dalerba et al., 2007). Tumors were passaged in vivo from one generation of mice to the next without any intervening cell culture. Tumor cells were stored at −80°C. Experiments for testing antibodies were initiated from frozen cell stocks. Breast tumors from UM-PE13 were used at passage 5. Passage 2 was used for all OMP tumors (C8, C9, and C17). In general, there were ten animals used for each treatment group.

Immunohistochemistry

Tissue samples were either stored in formalin for immunohistochemical analysis or frozen in optimal cutting temperature (OCT) medium for immunofluorescence. For IHC, specimens were embedded in paraffin, and 5 micron tumor sections were stained with anti-CD31 antibody using standard protocols (Vectastain kit, Vector Labs).

Flow Cytometric Analysis

Single-cell suspensions obtained from control and treated tumors were stained with stem cell markers and analyzed by FACS (BD Biosciences) for their expression of epithelial specific antigen (ESA or EpCAM), CD44, CD166.

In Vitro Culture

Plates (96-well) were prepared to accept human tumor cells by preplating either Mitomycin-C-treated 3T3 fibroblasts or murine embryonic fibroblasts in DMEM +10% fetal calf serum 24 hr prior to experiment initiation. Immediately prior to depositing individual human tumor cells into these plates in limiting dilution, the media was replaced with serum-free Medium-D (3:1 low glucose DMEM:F-12 Media, B27 supplement, ITS-X, Pen/Strep [all from Invitrogen, Carlsbad, CA] and 0.5 μg/mL hydrocortisone [Stem Cell Technologies, Vancouver, BC]), supplemented with 20 ng/mL bFGF and EGF, 5 u/mL heparin and 1×10^6 u/mL LIF. After cell deposition, plates were gently spun at 500 rpm for 5 min at room temperature to promote cell attachment. When cultured in vitro for more than 7 days, media was changed weekly.

In Vitro Limiting Dilution Assay

To evaluate CSC frequency in vitro, cells were seeded by fluorescence-activated cell sorting (FACS) into 96-well plates prepared as described above at cell doses of 40, 120, 360, and 720 cells per well (24 wells per dose) and incubated at 37°C/5% CO_2/5% O_2 for 21 days. Wells with colony growth were then determined by visual inspection. Based on frequency of wells with no colony growth, CSC frequency was determined using *Poisson* distribution statistics and the L-Calc Version 1.1 software program (StemCell Technologies, Inc., Vancouver, Canada).

In Vivo Limiting Dilution Assay to Determine Cancer Stem Cell Frequency

For the experiment shown in Figure 3D, OMP-C8-bearing animals were treated with Control Ab, 1B711, anti-hDLL4, irinotecan, or the combination of anti-hDLL4 and irinotecan. Control and treated tumors were then harvested after 3 weeks, when the irinotecan-treated tumors had regressed to approximately 50% of their volumes before treatment. The harvested tumors from each treatment group were pooled and processed to dissociate into single cells. Tumor cells were then incubated with biotinylated mouse antibodies (α-mouse CD45-biotin 1:200 dilution and rat α-mouse H_2Kd-biotin 1:100 dilution, BioLegend, San Diego, CA) on ice for 30 min followed by addition of streptavadin-labeled magnetic beads (Invitrogen, Carlsbad, CA) to remove mouse cells. Some portion of cells (about 300,000 cells per sample) was processed for phenotyping analysis as described in the Flow Cytometry Analysis section, and for subsequent RNA purification, the remaining human cells in the suspension were collected, counted, and diluted to appropriate cell doses (50, 100, 300, and 900 cells), mixed in the mixture of 1:1 (v/v) FACS buffer (Hank's balanced salt solution [HBSS] supplemented with 2% heat-inactivated fetal bovine serum and 20 mM HEPES) and Matrigel, and injected subcutaneously in NOD/SCID mice (10 mice per cell dose per treatment group). Tumors were allowed to grow for 86 days. The individual tumor measurements are shown in Figure S6. Tumor-initiating cell frequency was calculated using the L-calc program (StemCell Technologies Inc.). The experiment shown in Figure 6B with UM-PE13 breast tumors was carried out in a similar way as described above. The cell doses used were 50, 150, and 500 cells from each of the experimental groups.

Data Analysis

Data are expressed as mean ± SEM. Differences in mean values between groups were analyzed by nonparametric t test. Multiple comparisons used one-way ANOVA test with post hoc t test comparison. Differences of $p < 0.05$ were considered significantly different. Software for statistical analysis was by GraphPad Prism4 (GraphPad Software Inc).

SUPPLEMENTAL DATA

Supplemental Data include seven figures and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00228-8.

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