

# Chemokine Decoy Receptor D6 Plays a Negative Role in Human Breast Cancer

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## Abstract

**Chemokine binding protein D6 is a promiscuous decoy receptor that can inhibit inflammation *in vivo*; however, the role it plays in cancer is not well known yet. In this study, we showed for the first time that human breast cancer differentially expressed D6 and the expression could be regulated by some cytokines. More importantly, overexpression of D6 in human breast cancer cells inhibits proliferation and invasion *in vitro* and tumorigenesis and lung metastasis *in vivo*. This inhibition is associated with decreased chemokines (e.g., CCL2 and CCL5), vessel density, and tumor-associated macrophage infiltration. Furthermore, D6 expression is inversely correlated to lymph node metastasis as well as clinical stages, but positively correlated to disease-free survival rate in cancer patients. Therefore, D6 plays a negative role in the growth and metastasis of breast cancer. (Mol Cancer Res 2008;6(8):1276–88)**

## Introduction

Chemokines and chemokine receptors belong to a superfamily of small molecules that control the migration of leukocytes as well as many other cell types. This superfamily is classified into at least four groups, C, CC, CXC, and CX<sub>3</sub>C, based on the relative position of the first two cysteine residues on the NH<sub>2</sub> terminus (1, 2). In general, chemokines can also be divided into two functional subgroups: the inflammatory chemokines and the homeostatic chemokines. The inflammatory chemokines promote leukocyte infiltration to inflammatory sites and their expression is inducible. On the other hand, the homeostatic chemokines are constitutively expressed and involved in the

hematopoiesis and lymphoid organ development (3). Chemokine receptors are G-protein-coupled receptors expressed on the surface of various cells. Many studies have shown that chemokines and chemokine receptors are involved in the process of tumorigenesis and malignant progression (4-7). Cancer cells, stromal cells, and infiltrated immune cells could secrete chemokines. Cancer cells themselves can also express chemokine receptors and respond to these chemokines. This forms a complex chemokine network that influences tumor cell growth, survival, migration, and angiogenesis, as well as immune cell infiltration (8).

A typical receptor can bind its ligand with high affinity and specificity and elicit a cellular response. However, a group of special receptors that do not signal in response to binding of their ligands has been described in the chemokine system, including the Duffy antigen receptor for chemokines (DARC; ref. 9), D6 (10, 11), ChemoCentryx chemokine receptor (CCX-CKR; ref. 12), and so on. These molecules share the ability to bind chemokines with high affinity in the absence of any demonstrable signaling function, and therefore are now indicated as nonsignaling chemokine-binding proteins, decoy receptors, silent receptors, interceptors (internalizing receptors), chemokine sinks, sponges, or scavengers (13, 14), although they sometimes may act as chemokine reservoirs or transporters. Recent studies have suggested that DARC is a negative regulator of prostate cancer (15-17), lung cancer (18), and breast cancer (19) by sequestering chemokines. As for another decoy receptor, D6, research has shown that it is expressed on malignant vascular tumors (20), T-cell large granular lymphocyte leukemia cells (21), and choriocarcinoma cell lines (22). In addition, increased skin tumor burden induced by phorbol ester was observed in D6-deficient mice. Conversely, D6 transgenesis in keratinocytes can suppress skin tumor formation (23, 24). However, the expression spectra and possible role of D6 on other tumors including breast cancer are unclear.

In the present study, we investigated the expression of D6 in human breast cancers; we transfected pcDNA3.0/D6 into MDA-MB-231 and MDA-MB-435 human breast cancer cell lines, established D6-overexpressing clones, and then investigated the effects on tumorigenesis and metastasis. We showed for the first time that human breast cancer expresses D6 and the expression could be regulated by cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). D6 inhibits the proliferation and invasion of breast cancer cells *in vitro* as well as tumorigenesis and metastasis *in vivo*. This inhibition is associated with decreased intratumor chemokines, vessel density, and tumor-associated macrophage (TAM) infiltration.

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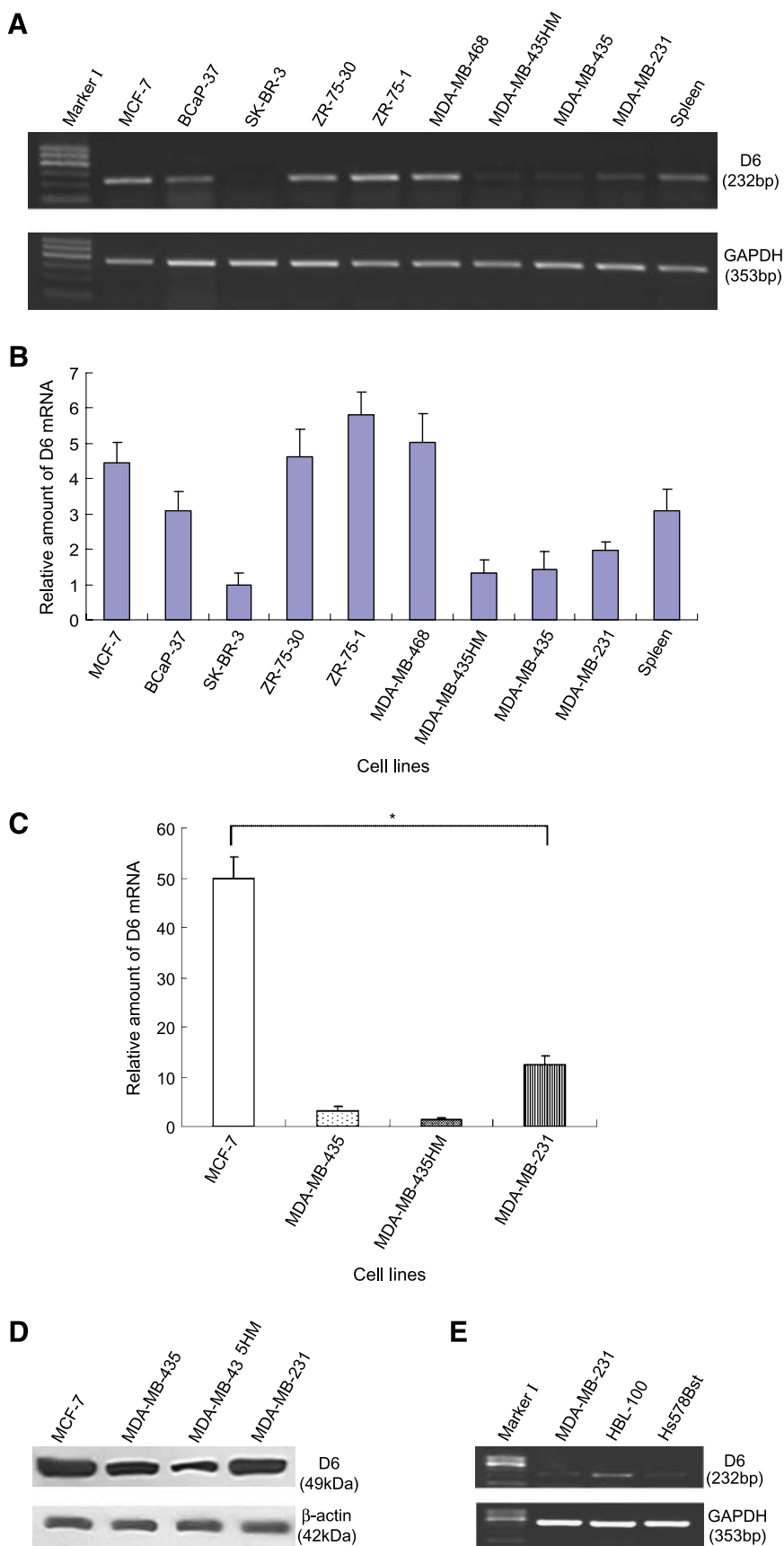
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**Note:** F-Y. Wu and Z-L. Ou contributed equally to this work.

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**FIGURE 1.** The expression of D6 in breast cancer and normal mammary epithelial cells. **A** and **B.** Expression of D6 mRNA in nine human breast cancer cell lines and normal human spleen by RT-PCR (**A**) and the calculated relative expression levels in these cells (**B**). Bars, SE. **C** and **D.** Real-time PCR analysis of D6 mRNA (**C**) and Western blot analysis of D6 protein (**D**) in four cell lines (\*,  $P < 0.05$ ). **E.** Expression of D6 mRNA in normal mammary epithelial cell lines HBL-100 and Hs578Bst and breast cancer cell line MDA-MB-231.

**Table 1. Characteristics of the Four Breast Cancer Cell Lines**

	MCF-7	MDA-MB-435	MDA-MB-435HM	MDA-MB-231
Tumorigenesis	+	+++	++++	++
Metastasis	-	++	+++	+

NOTE: +, potential for tumorigenesis and metastasis.

In patients with breast cancer, D6 is negatively correlated to lymph node metastasis and clinical disease stage, but is positively correlated to overall survival rate.

## Results

### Constitutive Expression of D6 in Human Breast Cancer

To investigate the expression of D6 mRNA in human breast cancers, nine human breast cancer cell lines, four human breast cancer tissues, and two normal mammary epithelial cell lines have been detected by reverse transcription-PCR. We found that D6 mRNA is differentially expressed in these samples (Fig. 1). By analyzing the expression in the breast cancer cell lines, we found that different cell lines have different D6 mRNA expression levels, and it correlated with the tumorigenesis and invasiveness in which low-invasive cells such as MCF-7, ZR-75-30, ZR-75-1, and MDA-MB-468 express high levels of D6 mRNA, whereas high-invasive cells such as MDA-MB-435, MDA-MB-435HM, and MDA-MB-231 have low D6 expression (Fig. 1A). To further confirm the difference in D6 expression in the cells, we detected D6 mRNA expression in MCF-7, MDA-MB-435, MDA-MB-435HM, and MDA-MB-231 breast cancer cells by quantitative real-time PCR (Table 1). It was clearly shown that the expression of D6 mRNA in the low-invasive cell line MCF-7 is ~35 times higher than that in the high-invasive cell line MDA-MB-435HM, novel highly metastatic cells that are derived from MDA-MB-435 cells (25, 26). MDA-MB-435 has higher D6 mRNA expression than MDA-MB-435HM (Fig. 1C). The difference in D6 expression in these four cell lines was further confirmed at protein levels (Fig. 1D). Breast cancer tissues from four patients with different characteristics have different expression levels (data not shown). Normal mammary epithelial cells have higher D6 mRNA levels than cancer cells (Fig. 1E). All of these findings suggest that D6 may be a negative regulator of cell malignancy.

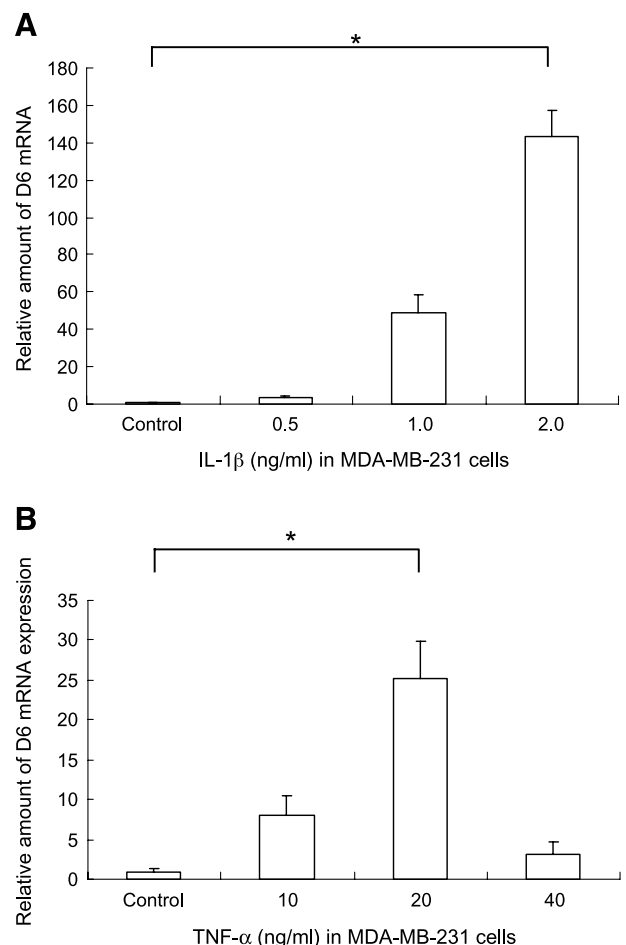
### D6 mRNA Expression Is Regulated by IL-1 $\beta$ and TNF- $\alpha$

To investigate the possibility that cytokines, which are produced by tumor cells, adjacent cells, or inflammatory cells, may regulate D6 expression, we conducted experiments using IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  to determine how cytokines affect D6 expression in MDA-MB-231 cells. D6 mRNA expression was analyzed by real-time PCR. As shown in Fig. 2A, IL-1 $\beta$  could promote the expression of D6 mRNA in a dose-dependent manner. TNF- $\alpha$  could also enhance the expression of D6 mRNA, with the strongest stimulation at the concentration of 20 ng/mL (Fig. 2B). Quantitation analysis showed an increase of 8-, 25-, and 3-fold with TNF- $\alpha$  at 10,

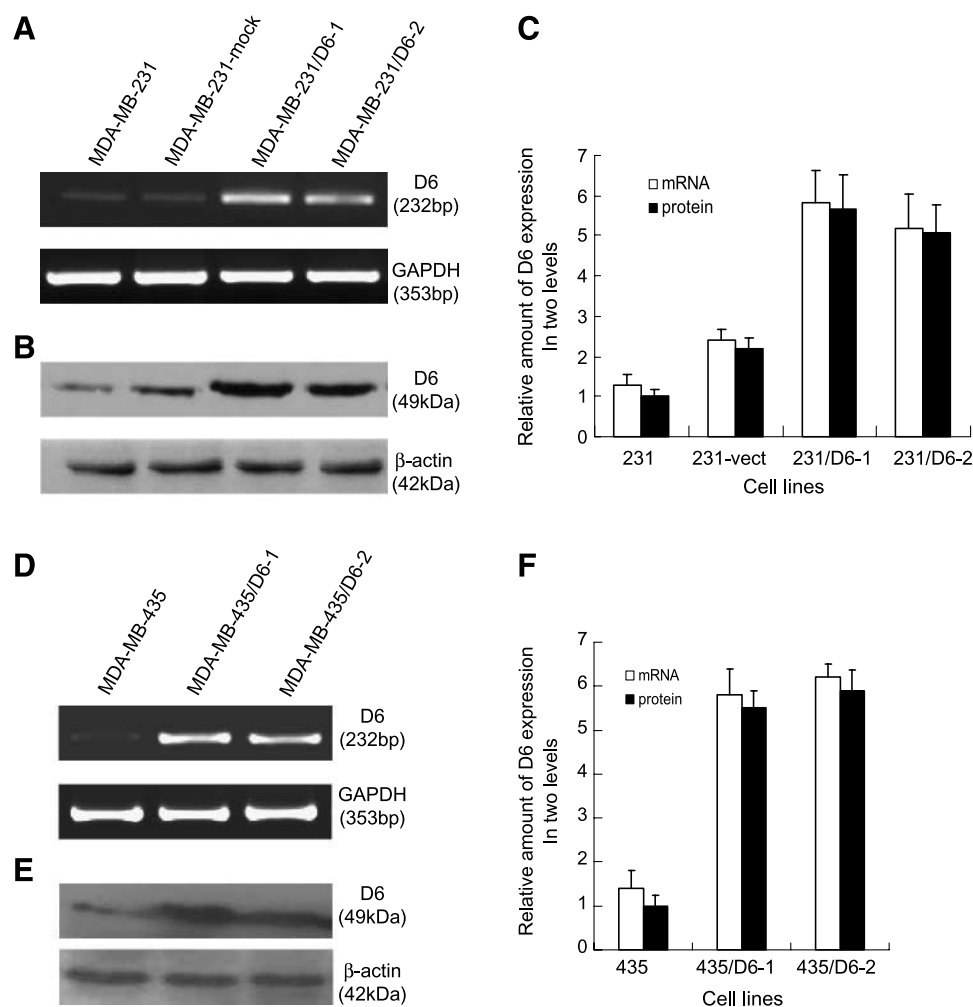
20, and 40 ng/mL, respectively. In contrast, IFN- $\gamma$  does not have significant influence on D6 mRNA expression at the concentrations of 10, 20, and 40 ng/mL (data not shown).

### Stable Transfection of D6 cDNA into MDA-MB-231 and MDA-MB-435 Cells

We speculate that D6 may be a negative regulator of cell tumorigenesis and metastatic potential. To further investigate this possibility, we transfected D6 expression vectors pcDNA3.0/D6 into MDA-MB-231 and MDA-MB-435 cells and generated stable transfectants. D6 stably transfected clones from MDA-MB-231 and MDA-MB-435 cells were named MDA-MB-231/D6-1, MDA-MB-231/D6-2, MDA-MB-435/D6-1, and MDA-MB-435/D6-2. D6 expression in these clones was detected by reverse transcription-PCR and Western blot. The levels of D6 mRNA and protein expression in D6-transfected clones were significantly increased compared with parental cells in both cell lines (Fig. 3). The clones expressing high levels of D6 were used in further experiments.



**FIGURE 2.** Real-time PCR analysis of D6 mRNA expression in MDA-MB-231 cells induced by IL-1 $\beta$  and TNF- $\alpha$ . **A.** IL-1 $\beta$  could promote the expression of D6 mRNA in a dose-dependent manner (\*,  $P < 0.05$ ). **B.** TNF- $\alpha$  could also enhance the expression of D6 mRNA, with the strongest stimulation at the concentration of 20 ng/mL (\*,  $P < 0.05$ ).



**FIGURE 3.** Expression of D6 in the cell clones with gene modification. **A** and **B.** Expression of D6 in MDA-MB-231 cells after transfection was detected by RT-PCR (**A**) and Western blot (**B**). **C.** Relative mRNA expression and protein levels in MDA-MB-231/D6 clones. **D** and **E.** Expression of D6 in MDA-MB-435 cells after transfection was detected by RT-PCR (**D**) and Western blot (**E**). **F.** Relative mRNA and protein levels in MDA-MB-435/D6 clones. Values are expressed to relative controls, which was given an arbitrary value of 1. Bars, SE.

#### *D6 Inhibits the Proliferation and Invasion Abilities while Enhancing the Clearance of Chemokines at the Protein Level in In vitro Studies*

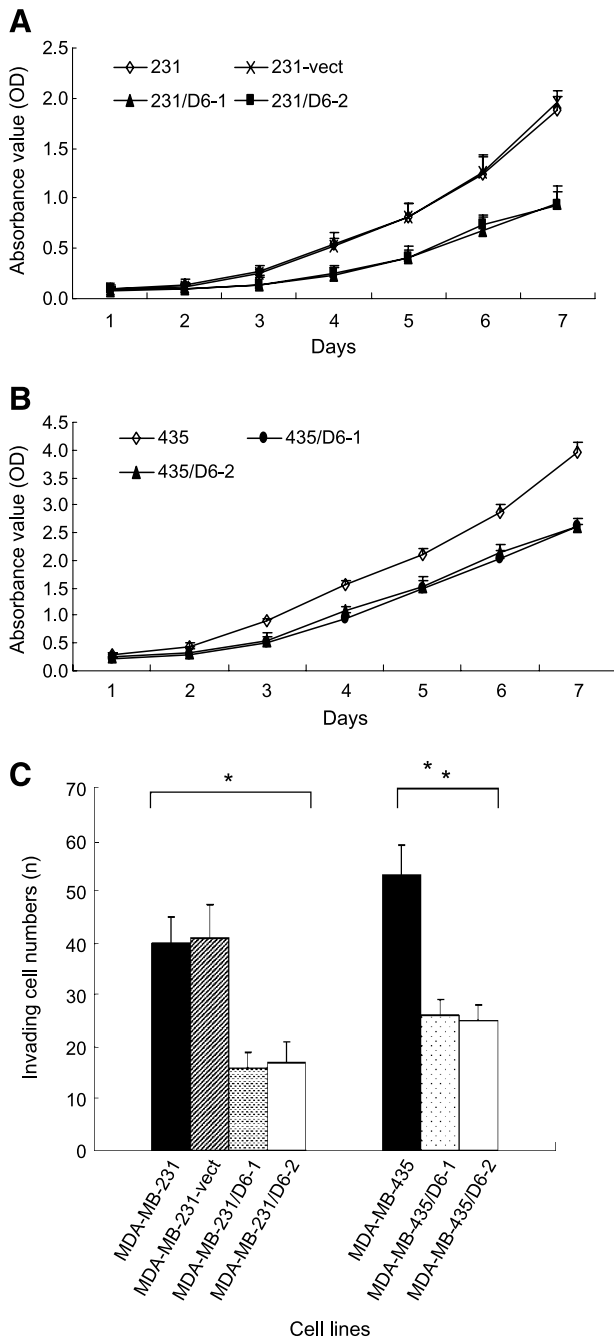
To investigate whether D6 overexpression could modulate the proliferation and invasion of MDA-MB-231 and MDA-MB-435 cells *in vitro*, we assessed the proliferation and invasion assay in D6-transfected and parental cells. As shown in Fig. 4A to C, D6-transfected clones MDA-MB-231/D6 and MDA-MB-435/D6 have significantly slower proliferation and lower invasion potential when compared with mock-transfected and wild-type cells. Because D6 could bind and scavenge chemokines, we detected the amount of its ligands in the conditioned cell supernatant by ELISA. As a result, most of the ligands decreased in the D6-overexpressing clones, with CCL2, CCL4, CCL13, CCL17, and CCL22 decreased significantly (Fig. 5), whereas no significant changes in mRNA expression could be seen in these chemokines (Fig. 6A and B). In addition, no obvious changes in the cell cycle distribution could be seen in D6-overexpressing cell clones, and no significant effect could be seen on matrix metalloproteinase-1, matrix metalloproteinase-2, matrix metalloproteinase-9, vascular endothelial cell growth factor, basic fibroblast growth factor, and cyclin E mRNA expression (data not shown).

#### *D6 Inhibits Tumor Growth, Metastasis, Angiogenesis, and TAM Infiltration by Clearing the Chemokines in In vivo Studies*

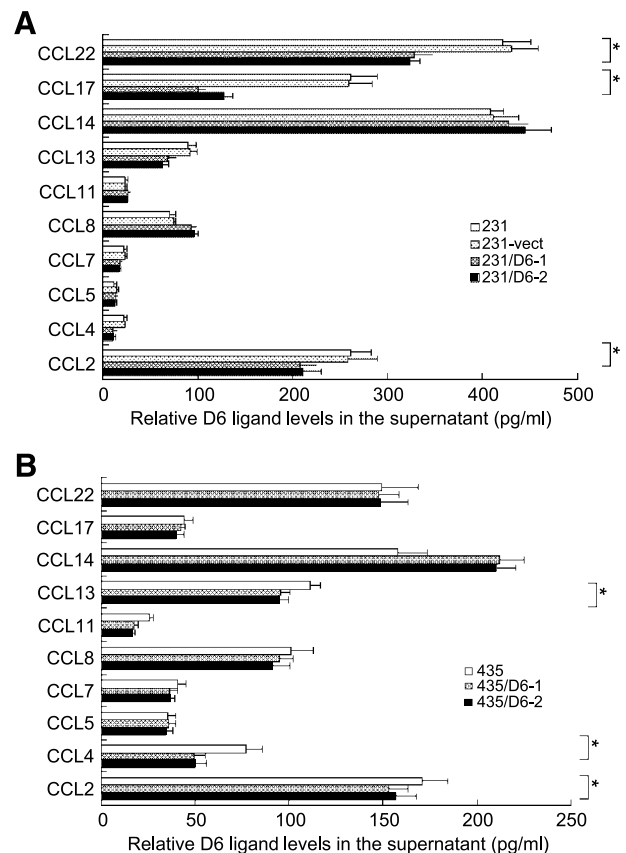
The effect of D6 on tumor growth and metastasis was further assayed using an orthotopic xenograft tumor model in athymic mice. Results reveal that D6-transfected MDA-MB-231 and MDA-MB-435 cells grew much slower than mock-transfected and wild-type cells in nude mice (Fig. 7A and B;  $P < 0.05$ ). To study pulmonary metastasis, lungs were examined physically at autopsy and then subjected to microscopic examination for morphologic evidence of tumor cells by light microscopy on H&E-stained paraffin sections. The incidence of lung metastasis was decreased from 55% to 12% in mice bearing MDA-MB-435/D6 clone xenografts compared with parental cell xenografts (Fig. 8A and B). In view that MDA-MB-231 cells have very low potential to form lung metastasis, we did not find any statistically significant difference in lung metastasis between the two groups (data not shown).

To illuminate the changes in the xenografts, we detected the expression of D6 protein by immunohistochemistry to verify that higher D6 protein expression exists in the D6-transfected cell xenografts compared with control xenografts (Fig. 8C). We further examined overall microvessel density by anti-CD34

antibody staining. It was clearly shown that D6 decreased the vessel density by 50% in the MDA-MB-231/D6 clone and by 40% in the MDA-MB-435/D6 clone xenografts compared with control xenografts (Fig. 8D).



**FIGURE 4.** D6 inhibits the proliferation and invasion of cells *in vitro* and promotes the clearance of chemokines at the protein level. **A** and **B.** Growth curves for MDA-MB-231, MDA-MB-231/vect, MDA-MB-231/D6-1, and MDA-MB-231/D6-2 (**A**) and for MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 (**B**) in *in vitro* proliferation assay. Points, mean of three independent experiments; bars, SE. **C.** *In vitro* invasion assays of the invasive potential of MDA-MB-231, MDA-MB-231/vect, MDA-MB-231/D6-1, and MDA-MB-231/D6-2 and of MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 using Matrigel invasion Transwell chamber. \*,  $P < 0.05$ , versus control (Student's *t* test).



**FIGURE 5.** **A** and **B.** Representative median levels of human CC chemokine proteins as D6 ligands in MDA-MB-231, MDA-MB-231/vect, MDA-MB-231/D6-1, and MDA-MB-231/D6-2 (**A**) and in MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 (**B**) in the cell supernatant. Columns, mean of three independent experiments; bars, SE.

Because CCL2 and CCL5 concentration was correlated significantly with TAM accumulation (27), we further examined the TAM infiltration in the xenografts of MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 cells by anti-CD68 antibody staining. As shown in Fig. 8E, TAMs were greatly reduced in the D6-overexpressing xenografts when compared with control cell xenografts.

D6 is known as a promiscuous chemokine decoy receptor. It can specifically bind and scavenge almost all the inflammatory CC chemokines (28). Therefore, we decided to detect the amount of mouse CCL2, CCL4, CCL5, and CCL13 proteins in the xenografts and of CCL2 and CCL5 in mouse blood. The results indicate that a significant decrease of CCL2, CCL5, and CCL13 in xenografts (Fig. 9A) and of CCL2 and CCL5 in the blood (Fig. 9B) were seen compared with control cells.

#### D6 Is Associated with Lymph Node Metastasis and Survival Rate in Patients

First, 72 breast cancer cases and 32 normal breast cases were used in the real-time PCR study to monitor the gene expression of D6. The patients' characteristics are shown in Table 2. Clinical samples were collected from January 2004 to April 2007. The expression of D6 mRNA in these tissues was

detected and the relationship with clinical characteristics was analyzed. The results are shown in Table 3. A negative correlation was found between *D6* gene expression and lymph node metastasis, with the relative *D6* expression being  $0.327 \pm 0.013$  in the lymph node–positive patients and  $0.362 \pm 0.012$  in the lymph node–negative patients ( $P < 0.05$ ). *D6* expression is also inversely related with clinical stages. We did not find any correlation between *D6* expression and estrogen receptor, progesterone receptor, and human epidermal growth factor receptor-2 status. In addition, we selected 32 breast cancer cases by randomization and compared the *D6* expression with those in 32 normal breast tissues. The relative *D6* expression was  $0.306 \pm 0.015$  in cancer tissues and  $0.349 \pm 0.306$  in normal breast tissues. Although normal breast tissues have a slightly higher *D6* expression than cancer tissues, this difference failed to achieve statistical significance.

Second, other 97 invasive breast cancer cases were used in the immunohistochemistry study to monitor the protein level of *D6*. The patients' characteristics are shown in Table 4. In this group of cases, tumor samples were collected from September 2004 to November 2005. As a result, *D6* positive percentage was 69.6% in patients without lymph node metastasis and 39.2% in patients with lymph node metastasis ( $P < 0.05$ ; Fig. 10A), suggesting that *D6* protein is also significantly negatively correlated to lymph node metastasis. The typical diffuse

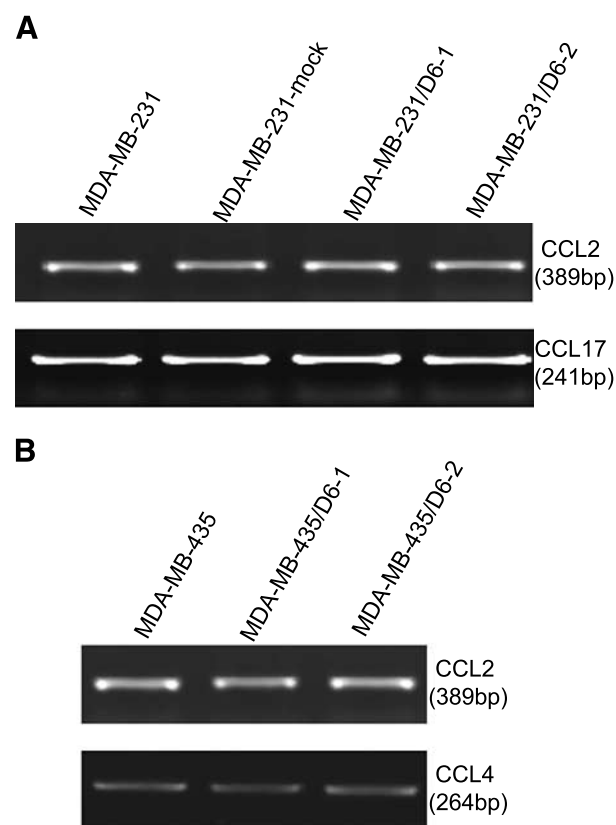
cytoplasmic staining of *D6* protein can be found in many tumor cells but not in the stromal cells (Fig. 10B). In addition, our study revealed that *D6* protein level is positively correlated to the disease-free survival of all patients, especially in patients with lymph node metastasis ( $P < 0.05$ ; Fig. 11A and B).

## Discussion

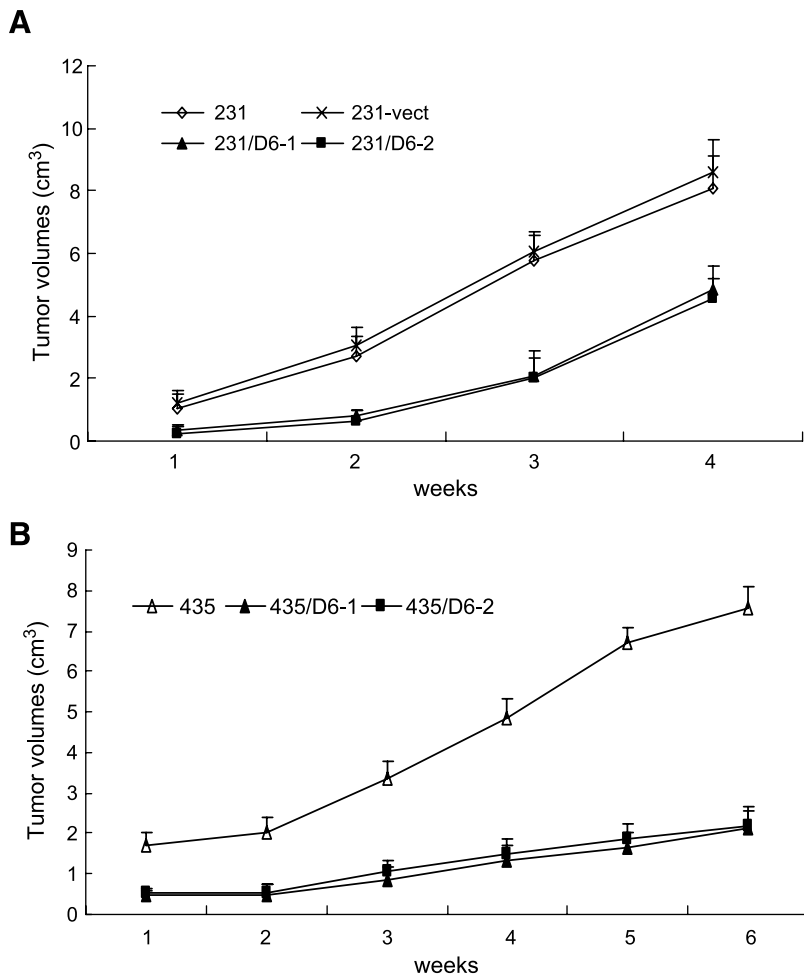
*D6*, also known as chemokine binding protein 2, chemokine binding receptor 9, CC chemokine receptor (CCR)-9, or CCR10, is an atypical nonsignaling chemokine receptor. It binds almost all inflammatory CC chemokines (CCL2, CCL3L1, CCL4, CCL5, CCL7, CCL8, CCL11–CCL14, and CCL22, and weakly to CCL17), but not homeostatic chemokines (28–30). Even among inflammatory CC chemokines, *D6* recognition is strictly restricted to the biologically active form (31). Conventional chemokine receptors can transduce intracellular signals after binding its ligands. However, a significant body of evidence shows that *D6* is not involved in signaling activities that are typically observed after chemokine receptor triggering, such as calcium fluxes and chemotaxis. *In vitro* biochemical investigations have revealed that *D6* seems to be specialized for the rapid and continuous internalization and degradation of its chemokine ligands (32, 33). *In vivo*, *D6* inhibits inflammation in skin induced by chemicals (34, 35), lungs challenged by allergens (36), and placenta irritated by lipopolysaccharides or antiphospholipid autoantibodies (22), which is prevented by blocking inflammatory chemokines, suggesting that *D6* is a potent CC chemokine scavenger and negative regulator of inflammatory responses (37).

In malignancies, *D6* has been detected in malignant vascular tumor cells, T-cell large granular lymphocyte leukemia cells, and choriocarcinoma cell lines. It could suppress the development of chemically induced skin tumors (23). The expression profile of *D6* and the role it plays in other tumors are unclear thus far. In our study, we found that human breast cancer cell lines and human breast cancer tissues differentially expressed *D6*, with a negative profile with cell tumorigenesis and invasiveness. Generally speaking, inflammatory cytokines and chemokines in the tumor microenvironment may affect the production and activity of each other in the process of tumor progression. We speculated that *D6* expression may be linked with cytokines, and we found that *D6* mRNA expression could be promoted by IL-1 $\beta$  and TNF- $\alpha$ , but not influenced by IFN- $\gamma$ . At a TNF- $\alpha$  concentration of 40 ng/mL, *D6* expression decreased and this may be linked to cell damage. These results provide an initial clue to understand the regulation mechanism of *D6* itself in tumors, although their detailed kinetics, interaction, and importance need to be further investigated.

Chemokines play multifaceted roles in tumor malignancy, inducing intratumor leukocyte infiltration and regulating immune functions, directing the migration of tumor cells to specific organs or tissues (the CXCL12–CXCR4 axis and CCL19/21–CCR7 axis), regulating angiogenic processes (mainly ELR<sup>+</sup> CXC and some CC chemokines), and acting directly on tumor cells to control their malignancy-related functions (38, 39). The chemokine network partly dictates the fate of the malignancy cascade. In view of the *D6* function as a promiscuous chemokine decoy receptor by clearing



**FIGURE 6.** **A** and **B.** Expression of CCL2 and CCL17 mRNA in MDA-MB-231, MDA-MB-231/vect, MDA-MB-231/D6-1, and MDA-MB-231/D6-2 (**A**) and of CCL2 and CCL4 mRNA expression in MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 (**B**).



**FIGURE 7.** D6 inhibits tumor growth and lung metastasis in *in vivo* studies. The inhibition is associated with decreased angiogenesis and TAM infiltration and enhanced clearance of chemokine CCL2, CCL4, CCL5, and CCL13 in the xenografts. **A** and **B**. Growth curves for MDA-MB-231, MDA-MB-231/vect, MDA-MB-231/D6-1, and MDA-MB-231/D6-2 (**A**) and for MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 (**B**) in *in vivo* proliferation assay. Columns, mean of three independent experiments; bars, SE.

inflammatory CC chemokines, we speculate that D6 may play a role in tumor progression through regulation of the chemokine balance. We successfully established D6-overexpressing cell clones MDA-MB-231/D6 and MDA-MB-435/D6 by stable transfection and found that D6 could inhibit proliferation and invasion in both cell lines. The detection of the chemokines in the media indicated that most of the D6 ligands decreased in the D6-overexpressing cell clones at the protein level, whereas no changes in these ligands at mRNA levels could be observed. This reveals that the inhibitory function of D6 is accomplished by the clearing of the target chemokine and the regulation is posttranslational. No significant influence of D6 on the cell cycle distribution and on the mRNA expression of matrix metalloproteinase, vascular endothelial cell growth factor, transforming growth factor- $\beta$ , and cyclin E was observed. *In vivo*, we found that D6 could significantly inhibit orthotopic xenograft growth and pulmonary metastasis. These inhibitions were associated with a remarkable decrease in the amount of D6 ligands CCL2, CCL5 (RANTES), and CCL13 in the xenografts and of CCL2 and CCL5 in the serum. The inflammatory chemokines can act directly on tumor cells through chemokine receptors that are expressed by these cells. The two chemokines, CCL2 and CCL5, are prominently associated with breast malignancy: Elevated levels of CCL2 and CCL5 expression in

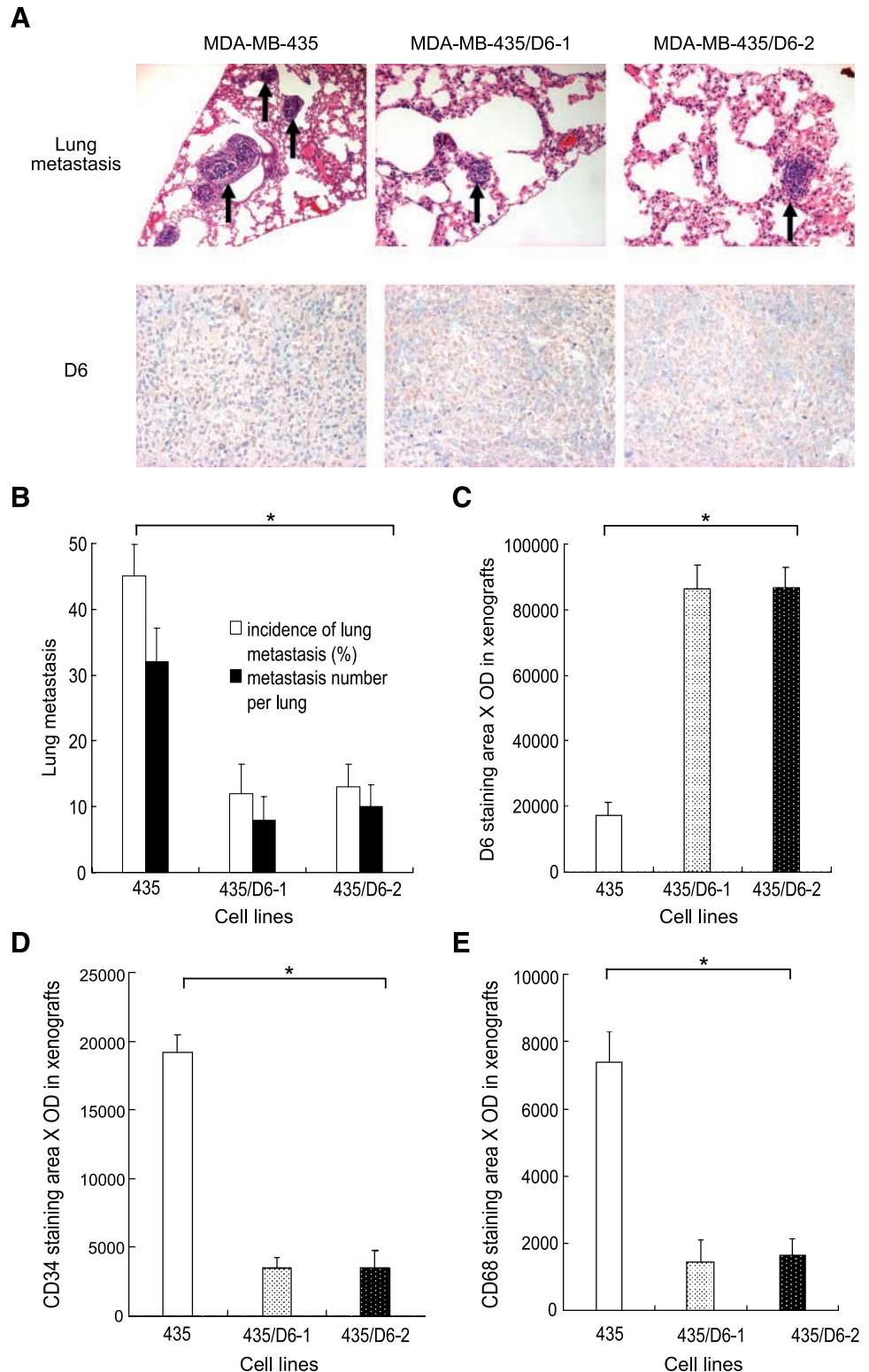
breast cancer patients were significantly correlated with early relapse, advanced tumor stage, and poor prognosis (40-43). Direct and causative roles in breast malignancy were assigned for CCL2 and CCL5 in several animal model systems (44-46), and CCL2 and CCL5 potently up-regulate the expression of promalignancy activities in breast cancer cells (47). In our study, D6 scavenges CCL2 and CCL5 in the tumor and may directly inhibit the promalignancy function of these chemokines.

It has been suggested that both CCL2 and CCL5 increase the angiogenesis and vascularity in breast cancer (47, 48). In the present study, we show that the overall vessel density decreased significantly along with lower amounts of CCL2 and CCL5 in the xenografts from D6-overexpressing clones compared with that from the mock-transfected and parental cells.

TAM infiltration in the tumor was found to promote tumor invasion and metastasis by secreting a variety of tumor-supporting factors: angiogenic factors (such as vascular endothelial cell growth factor), proteases degrading the extracellular matrix (such as matrix metalloproteinases), growth factors (such as epidermal growth factor), and more (49, 50). Obvious candidates responsible for high TAM presence in tumors are chemokines, especially CCL2 and CCL5 (27, 51, 52). Our observations show that TAM infiltration is greatly reduced in

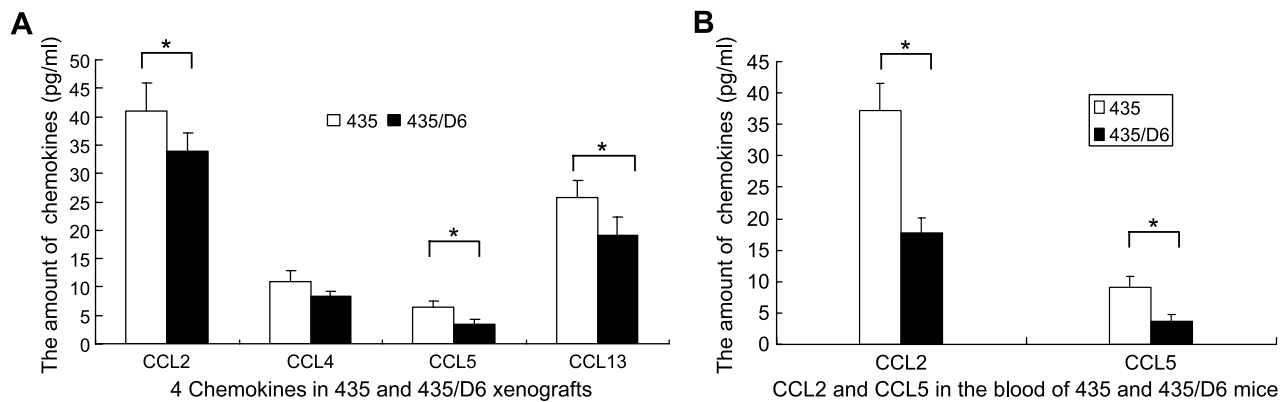
D6-overexpressing xenografts. This inhibition is correlated with the decreasing amount of CCL2, CCL5, and CCL13. It suggests that D6 can reduce TAM infiltration indirectly by clearing the chemokines in the tumor. Decreased TAM infiltration may also contribute to the inhibition of tumor growth and metastasis.

In the present study, we examined *D6* gene expression in 72 cases of human breast cancer samples and 32 cases of normal human breast tissues by real-time PCR procedure. We also examined the D6 protein level in other 97 cases of invasive breast cancer by immunohistochemical method. We found that



**FIGURE 8.** **A.** H&E staining of pulmonary metastasis tumors (arrows, metastasis) and immunohistochemical examination of D6 protein ( $\times 100$ ), CD34 (hotspots; arrows, blood vessels;  $\times 100$ ), and CD68 (hotspots;  $\times 100$ ) in xenografts of MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 tumor-bearing mice at 7 wk. **B.** Representative tumor metastasis rate and metastasis numbers per lung. **C** to **E.** Quantitative analysis of D6 (**C**), CD34 (**D**), and CD68 (**E**) staining in immunohistochemistry slides of 7-wk xenografts of MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-35/D6-2 cells. Columns, mean of three independent experiments; bars, SE, \*,  $P < 0.05$ .





**FIGURE 9.** **A.** Relative amount of mouse chemokine CCL2, CCL4, CCL5, and CCL13 in 7-wk xenografts of MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 cells. Columns, mean of three independent experiments; bars, SE. \*,  $P < 0.05$ . **B.** Relative amount of mouse chemokine CCL2 and CCL5 in 7-wk serum of MDA-MB-435 and MDA-MB-435/D6 cells. Columns, mean of three independent experiments; bars, SE. \*,  $P < 0.05$ .

both the mRNA expression and protein level of D6 are negatively related to lymph node metastasis in spite of these two completely different patient groups. In addition, D6 mRNA expression was negatively related to clinical disease stages. Furthermore, the immunohistochemical staining results revealed that D6 protein level is significantly positively related to disease-free survival rate both in lymph node metastasis and all patients.

However, no significant relationship was found between D6 expression and estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (ErbB2) status in patients with breast cancer. Furthermore, although the normal tissues have a little more D6 expression than cancer tissues, the difference is not statistically significant. This could be explained partly by the fact that D6 can only regulate some inflammatory chemokines, whereas it has no influence on the homeostatic chemokines. It is also possible that D6 is actually

indispensable in both pathologic and physiologic conditions. Except for D6, other chemokine decoy receptors such as DARC, CCX-CKR, and probably CXCR7 (RDC1) also participate in the regulation of the chemokine network, although their relationship is not well known.

Chemokines and chemokine receptors in the tumor micro-environment play a multifaceted role in malignancy progression. Some can promote malignancy by acting at many different levels; others can exert tumor-inhibiting functions that may restrict tumor growth (38). Therefore, it is possible that the equilibrium between chemokines with tumor-supporting functions and chemokines with an antimalignancy potential plays a key role in dictating the fate of tumor growth and its ability to metastasize. By scavenging the tumor-supporting chemokines and inhibiting subsequent chemokine-induced angiogenesis and TAM infiltration, D6 inhibits the growth and metastasis of breast cancer.

**Table 2. Patients' Clinical Characteristics (105 Cases, for Real-time PCR Analysis)**

	Cancer Tissues		Normal Tissues
	LN(-)	LN(+)	
No. of cases	35	37	32
Median age	51.23 ± 12.45	52.74 ± 10.28	45.70 ± 13.56
Status of menses			
Premenopause	16	14	18
Menopause	19	23	14
Tumor size (cm)	1.53 ± 0.72	3.46 ± 1.62	
Pathologic type			
Infiltrating ductal carcinoma	30	33	
Others	5	4	
Nuclear grades			
I	10	7	
II	16	20	
III	9	10	
Status of hormonal receptors			
ER and/or PR positive	21	27	
ER and PR negative	14	10	
Her-2			
Positive	16	17	
Negative	19	20	

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; Her-2, human epidermal growth factor receptor 2.

**Table 3. Relationship between D6 mRNA Expression and Clinical Characteristics**

	No. of Cases	Median (SE)	P
LN(+)	37	0.3267 (0.0125)	0.0424
LN(-)	35	0.3622 (0.0117)	
ER(+)	32	0.3470 (0.0129)	0.8996
ER(-)	22	0.3495 (0.0156)	
PR(+)	20	0.3504 (0.0151)	0.7283
PR(-)	34	0.3433 (0.0129)	
Her(+)	17	0.3384 (0.0213)	0.4478
Her(-)	38	0.3545 (0.0105)	
Stage I	19	0.3794 (0.0744)	0.0177
Stage II	50	0.3344 (0.0565)	
Cancer tissues	31	0.3491 (0.0134)	0.5257
Normal tissues	31	0.3362 (0.0151)	

In conclusion, our results show that human breast cancers express D6 and the expression could be regulated by some inflammatory cytokines. More importantly, D6 has an inhibitory function both *in vitro* and *in vivo*. In addition, D6 is negatively related with lymph node metastasis and clinical stages in breast cancer patients. Further studies are needed to clarify the role of D6 in human cancers, and it may provide a novel molecular target in cancer therapy. It is possible to overexpress D6 by engineering or cytokine stimulation and thus inhibit cancer progression. Clearly, a better understanding of D6 will lead to appropriate application of D6 in cancer treatment in the future.

## Materials and Methods

### Cell Lines and Reagents

Human breast cancer cell lines MDA-MB-231, MDA-MB-435, MDA-MB-468, ZR-75-30, ZR-75-1, MCF-7, and SK-BR-3 and normal mammary fibroblast-like cell line Hs578Bst were obtained from the American Type Culture Collection. MDA-MB-435HM cell lines with a high propensity to metastasize to the lung were established by subclone selection procedure in our laboratory. BCaP-37, a breast cancer cell line from a 48-year-old Chinese female patient, and a normal mammary epithelial-like cell line, HBL-100, were purchased from the cell bank of the Chinese Academy of Science. Cells were routinely maintained in the recommended medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 µg/mL streptomycin. The cultures were incubated at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

All the culture media and fetal bovine serum were obtained from Life Technologies, Inc. Real-time PCR reagents were obtained from TaKaRa Company. TNF-α and IFN-γ were purchased from PeproTech, Inc. Rat anti-human D6 monoclonal antibody was purchased from R&D Systems. The plasmid pcDNA3.0/D6 was kindly provided by Dr. Massimo Locati.

### Treatment of Cells with IL-1β, TNF-α, and IFN-γ

Human MDA-MB-231 breast cancer cells were cultured at the exponential phase of growth. The medium was withdrawn and replaced with fresh serum-free medium containing IL-1β (0.5, 1.0, and 2.0 ng/mL), TNF-α (10, 20, and 40 ng/mL), and IFN-γ (10, 20, and 40 ng/mL). The cells were maintained at

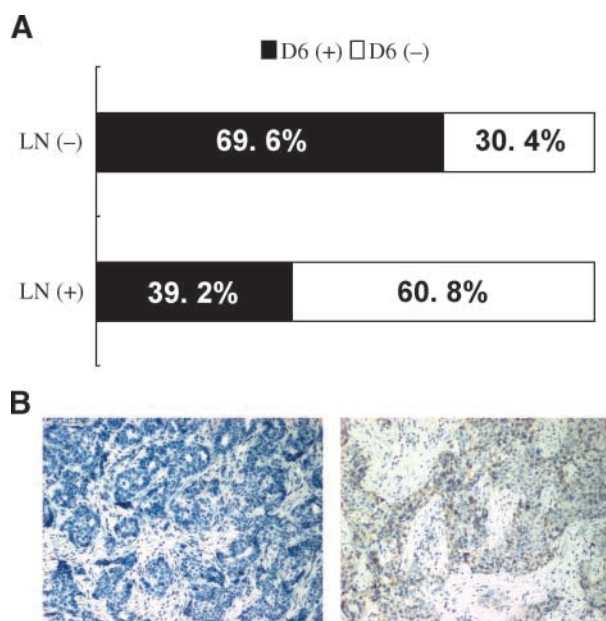
37°C with 5% CO<sub>2</sub> in a humidified atmosphere for 24 h. Then, the cells were harvested for the analysis of D6 mRNA expression by real-time PCR.

### RNA Extraction, Reverse Transcription-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. In brief, 3.0 µg of total RNA were used for reverse transcription in 20 µL. The cDNA was diluted to 100 µL with Milli-Q water, and 5.0-µL cDNA was used for each PCR in 25 µL. Each reaction condition, including annealing temperature and PCR cycle, was carefully optimized through pilot experiments to make sure that the amplification reaction was in linear range. Gene-specific primers for human *D6*, *CCL2*, *CCL4*, *CCL17*, and *GAPDH* and their annealing temperatures are listed in Table 5. All experiments were repeated thrice, and the mRNA levels of each gene were normalized to that of *GAPDH* as an internal control.

### Real-time Quantitative PCR

Total RNA extraction and reverse transcription were done as described above. The quantification of mRNA levels was carried out using DNA Engine Opticon 2 real-time PCR detection system (MJ Research) with SYBR Green. Two microliters of diluted cDNA were used as a template; 10 µL × 2 SYBR Premix Ex Taq (TaKaRa) were mixed with template and primers. The total reaction volume was 20 µL. Cycling conditions were denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 45 s. Plate reading



**FIGURE 10.** The protein levels of D6 negatively correlated to lymph node status in breast cancer patients. **A.** Percentage of D6-positive cases from immunohistochemical staining using goat anti-human D6 polyclonal antibody in a total of 97 invasive breast cancer patients with (45 cases) or without (52 cases) lymph node metastasis (\*,  $P < 0.05$ ). **B.** Typical immunohistochemical staining photogram in a D6-negative patient (left) and a D6-positive patient (right).

**Table 4. Patients' Clinical Characteristics (97 Cases, for Immunohistochemistry Analysis)**

	LN(-)	LN(+)
No. of cases	45	52
Median age	56.9 ± 10.85	52.4 ± 11.66
Status of menses		
Premenopause	15	23
Menopause	31	28
Tumor size (cm)	2.50 ± 0.87	2.60 ± 0.98
Pathologic type		
Infiltrating ductal carcinoma	35	46
Others	11	5
Status of hormonal receptors		
ER and/or PR positive	38	40
ER and PR negative	8	11
Her-2		
Positive	21	27
Negative	25	24

was at 72°C and 82°C. Experiments were done in triplicate for each sample. The amplification plots of the PCR reaction were used to determine the  $C_t$ . For the expression of D6 in the cell lines and under stimulation by cytokines, we used the formula  $2^{-\Delta\Delta C_t}$  to determine the D6 mRNA expression levels. For the expression of D6 in the tissues, D6 mRNA expression was normalized ( $\Delta C_t$ ) to *GAPDH* mRNA by dividing the  $C_t$  value of *GAPDH* mRNA by the  $C_t$  value of the D6 mRNA. The relative D6 gene expression was calculated by the formula  $2^{-\Delta C_t}$ . To ensure that the correct product was amplified in the reaction, all the PCR products were also separated on 1.2% agarose gel electrophoresis.

#### Stable Transfections

For stable transfection of D6 cDNA, MDA-MB-435 and MDA-MB-231 cells were initially plated at a density of  $3 \times 10^5$  in six-well plates for 24 h before transfection in regular medium. Two micrograms of the expression plasmid pcDNA3.0 with or without D6 were transfected using the Lipofectamine reagent (Life Technologies). After 48 h, the

medium was replaced and G418 was added to select stable transfectants. G418 sulfate (geneticin)-resistant colonies were selected in medium and expanded. The D6-positive colonies were identified by reverse transcription-PCR and Western blot.

#### Proliferation Assay

Cell proliferation was done by using Cell Counting Kit-8 (CCK-8, Dojindo). Cells were plated in 96-well plates at 2,000 per well (100  $\mu$ L) and cultured in growth medium. The number of the cells was counted according to CCK-8 manipulation.

#### Analysis of Cell Cycle Distribution

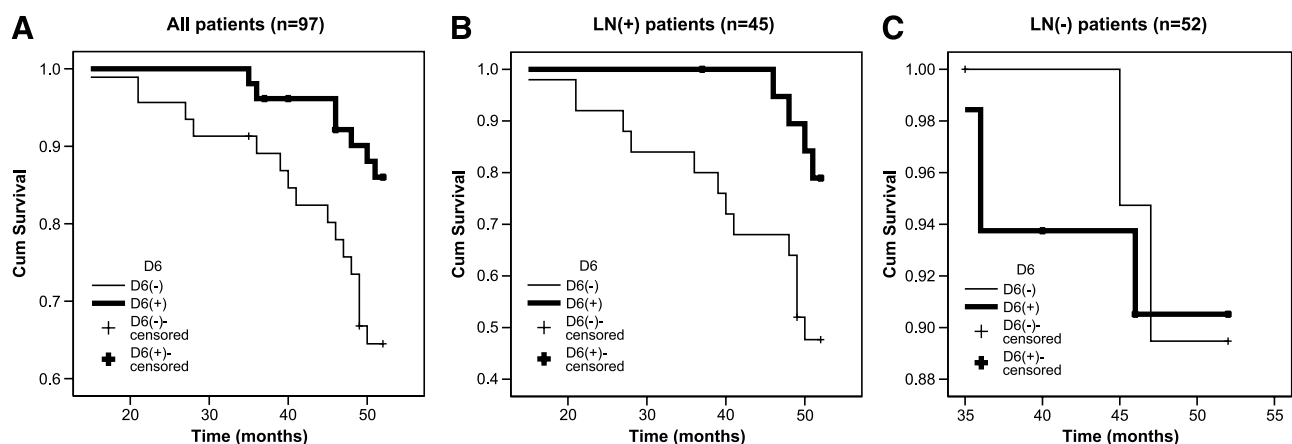
Flow cytometry analysis of DNA content was done to assess the cell cycle phase distribution as described previously. D6, mock-transfected, and parental cells were harvested and stained for DNA content using propidium iodide fluorescence. The computer program Multicycle from Phenix Flow System was used to generate histograms, which were used to determine the cell cycle phase distribution.

#### In vitro Assays of Invasion

Invasion experiments were conducted with a Matrigel invasion chamber (BD Labware). Each well insert was layered with 120  $\mu$ L of 1:3 mixture of Matrigel/DMEM (1,400 mg Matrigel/cm<sup>2</sup>). An amount of  $10^5$  cells were added to the top of this Matrigel layer. The wells were incubated at 37°C for 36 h. Invasion was assessed by counting the cells that had traveled across the filter and were attached to the bottom side of the filter. Then, the filters were fixed in 10% formalin and stained with H&E. Cells that had invaded through the Matrigel and reached the lower surface of the filter were counted under a light microscope at a magnification of  $\times 200$ . Five fields were counted for each sample.

#### Western Blot

Cells were washed twice with ice-cold PBS and scraped into 1.0 mL of ice-cold NP40 lysis buffer [10 mmol/L Tris-HCl



**FIGURE 11.** The protein levels of D6 positively correlated to overall survival rate in all breast cancer patients (**A**;  $n = 97$ ; \*,  $P < 0.05$ ), in lymph node-positive patients (**B**;  $n = 45$ ; \*,  $P < 0.05$ ), and in lymph node-negative patients (**C**;  $n = 52$ ; \*,  $P > 0.05$ ).

**Table 5. Primers for RT-PCR and Their Annealing Temperatures**

<i>GAPDH</i>	Up	5-GGGAGCCAAAAGGGTCATCATCTC-3	353 bp	60°C
	Down	5-CCATGCCAGTGAGCTCCCGTTC-3		
<i>D6</i>	Up	5-CCTGCTCCTTGCTACCATAGTATGG-3	232 bp	60°C
	Down	5-CACCAAGACACAACCAATACGGGAG-3		
<i>CCL2</i>	Up	5-ACTGAAGCTCGCACTCTCGCCTC-3	389 bp	58°C
	Down	5-TGTCTGGGGAAAGCTAGGGGAAAAT-3		
<i>CCL4</i>	Up	5-TGTCCTGTCTCTCCTCATGCTAGTA-3	264 bp	55°C
	Down	5-GCTCAGTTCAGTCCAGGTCATACA-3		
<i>CCL17</i>	Up	5-TCTCTGCAGCACATCCACGC-3	241 bp	58°C
	Down	5-GGCTTCAAGACCTCTCAAGG-3		

(pH 8.0), 150 mmol/L NaCl, 1% (v/v) NP40, 1.0 mmol/L EDTA, 50 mmol/L NaF, 5.0 mmol/L NaPPI, 1.0 mmol/L phenylmethylsulfonyl fluoride, 1.0 mg/mL leupeptin, and 1.0 mg/mL pepstatin A]. Cells were then sonicated for 5 s at 5 W. Insoluble debris was removed by centrifugation at 12,000 rpm for 30 min. Total proteins (50 µg) were analyzed by 10% SDS-PAGE. Western blot with rat anti-human D6 monoclonal antibody was done according to standard protocols. Blot quantification was done with a Molecular Dynamics Laser Densitometer and the ImageQuant version 1 software.

#### *In vivo Study with Animal Experiment*

Female athymic BALB/c nu/nu mice, 4 to 6 wk old, were obtained from the Shanghai Institute of Material Medical, Chinese Academy of Science, and housed in laminar-flow cabinets under specific pathogen-free conditions. All studies on mice were conducted in accordance with the NIH "Guide for the Care and Use of Laboratory Animals." The study protocol was approved by the Shanghai Medical Experimental Animal Care Committee.

The tumorigenesis and spontaneous metastatic capability of the cell lines were determined by injection into the mammary fat pad. Cells ( $1 \times 10^6$ ) in 0.1 mL of culture medium were inoculated into the anesthetized mouse. Animals were monitored every 2 d for up to 6 wk for tumor growth and general health. The rate of primary tumor growth of different cells was determined by plotting the means of two orthogonal diameters of the tumors, measured at 7-d intervals. The volume of tumor was calculated by the following formula:  $\text{volume} = 1/2ab^2$ , where  $a$  is the long diameter and  $b$  is the short diameter. Animals were sacrificed and autopsied at 7 wk for MDA-MB-435 cell lines and 4 wk for MDA-MB-231 cell lines after inoculation. Metastasis formation was assessed by macroscopic observation of all major organs for secondary tumors and confirmed by histologic examination of organs. Tissue samples harvested for histologic analysis were either fixed and embedded in paraffin wax or snap-frozen in liquid nitrogen.

#### *ELISA*

The protein levels of human CCL2, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL14, CCL17, and CCL22 present in conditioned cell supernatants were determined with a sandwich ELISA kit (BioSource International). The absorbance of the test sample was compared with the standard curve. The amounts of mouse CCL2 and CCL5 in the serum and of CCL2, CCL4,

CCL5, and CCL13 in the supernatant of MDA-MB-435 and MDA-MB-435/D6 xenografts (six samples in each group) were also determined with ELISA kit. The concentrations were determined in duplicate according to the instruction booklet supplied by the manufacturer.

#### *Immunohistochemical Staining for Animal Xenograft*

Ten formalin-fixed, paraffin-embedded sections from xenografts of MDA-MB-435, MDA-MB-435/D6-1, and MDA-MB-435/D6-2 cells were used for D6 protein, CD34, and CD68 detection. After the slides were deparaffinized with xylene and rehydrated through ethanol series, microwave antigen retrieval was carried out by placing the slides in 0.1 mol/L sodium citrate buffer (pH 6.0) in a microwave oven at 98°C for 8 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol for 10 min. The sections were incubated in a 1:100 dilution of rat anti-human D6 monoclonal antibody (R&D Systems), 1:50 dilution of rat anti-mouse CD34 serum, and 1:100 dilution of rat anti-mouse CD68 serum. Primary antibody was detected with biotinylated secondary antibodies followed by incubation with streptavidin-conjugated horseradish peroxidase and colorimetric detection with 3,3'-diaminobenzidine.

#### *Immunohistochemical Staining for Clinical Samples*

Ninety-seven formalin-fixed, paraffin-embedded breast cancer tissue sections were used for D6 protein detection by immunohistochemistry. In brief, the sections were incubated in a 1:250 dilution of goat anti-human D6 polyclonal antibody (PA1-21614, ABR-Affinity BioReagents). EnVision+ System-horseradish peroxidase-3,3'-diaminobenzidine (DakoCytomation) was used for visualization (i.e., horseradish peroxidase-labeled polymer-conjugated rabbit anti-goat IgG was used as secondary antibody and 3,3'-diaminobenzidine as the chromogen). Informed consent forms from all patients have been obtained. This study has been approved by the Institutional Review Board of the Cancer Hospital, Fudan University in advance.

#### *Statistical Analysis*

ANOVA and Student's  $t$  test were used to determine the statistical significance of differences between experimental groups.

#### **Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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## References

1. Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998;392:565–8.
2. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000;12:121–7.
3. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* 2000;18:593–620.
4. Muller A, Homey B, Soto H, et al. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–6.
5. Balkwill F. Cancer and the chemokine network. *Nat Rev Cancer* 2004;4:540–50.
6. Rollins BJ. Inflammatory chemokines in cancer growth and progression. *Eur J Cancer* 2006;42:760–7.
7. Zlotnik A. Chemokines and cancer. *Int J Cancer* 2006;119:2026–9.
8. Balkwill F. Chemokine biology in cancer. *Semin Immunol* 2003;15:49–55.
9. Horuk R, Chitnis CE, Darbonne WC, et al. A receptor for the malarial parasite *Plasmodium vivax*: the erythrocyte chemokine receptor. *Science* 1993;261:1182–4.
10. Bonini JA, Martin SK, Dralyuk F, Roe MW, Philipson LH, Steiner DF. Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3. *DNA Cell Biol* 1997;16:1249–56.
11. Nibbs RJ, Wylie SM, Yang J, Landau NR, Graham GJ. Cloning and characterization of a novel promiscuous human  $\beta$ -chemokine receptor D6. *J Biol Chem* 1997;272:32078–83.
12. Gosling J, Dairaghi DJ, Wang Y, et al. Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK. *J Immunol* 2000;164:2851–6.
13. Mantovani A, Bonocchi R, Locati M. Tuning inflammation and immunity by chemokine sequestration: decoys and more. *Nat Rev Immunol* 2006;6:907–18.
14. Comerford I, Litchfield W, Harata-Lee Y, Nibbs RJ, McColl SR. Regulation of chemotactic networks by “atypical” receptors. *BioEssays* 2007;29:237–47.
15. Lentsch AB. The Duffy antigen/receptor for chemokines (DARC) and prostate cancer. A role as clear as black and white? *FASEB J* 2002;16:1093–5.
16. Shen H, Schuster R, Stringer KF, Waltz SE, Lentsch AB. The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth. *FASEB J* 2006;20:59–64.
17. Bandyopadhyay S, Zhan R, Chaudhuri A, et al. Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression. *Nat Med* 2006;12:933–8.
18. Addison CL, Belperio JA, Burdick MD, Strieter RM. Overexpression of the Duffy antigen receptor for chemokines (DARC) by NSCLC tumor cells results in increased tumor necrosis. *BMC Cancer* 2004;4:28.
19. Wang J, Ou ZL, Hou YF, et al. Enhanced expression of Duffy antigen receptor for chemokines by breast cancer cells attenuates growth and metastasis potential. *Oncogene* 2006;25:7201–11.
20. Nibbs RJ, Kriehuber E, Ponath PD, et al. The  $\beta$ -chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors. *Am J Pathol* 2001;158:867–77.
21. Daibata M, Matsuo Y, Machida H, Taguchi T, Ohtsuki Y, Taguchi H. Differential gene-expression profiling in the leukemia cell lines derived from indolent and aggressive phases of CD56<sup>+</sup> T-cell large granular lymphocyte leukemia. *Int J Cancer* 2004;108:845–51.
22. Martinez de la Torre Y, Buracchi C, Borroni EM, et al. Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6. *Proc Natl Acad Sci U S A* 2007;104:2319–24.
23. Nibbs RJ, Gilchrist DS, King V, et al. The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors. *J Clin Invest* 2007;117:1884–92.
24. Hansell CA, Simpson CV, Nibbs RJ. Chemokine sequestration by atypical chemokine receptors. *Biochem Soc Trans* 2006;34:1009–13.
25. Li DQ, Hou YF, Wu J, et al. Gene expression profile analysis of an isogenic tumour metastasis model reveals a functional role for oncogene AF1Q in breast cancer metastasis. *Eur J Cancer* 2006;42:3274–86.
26. Li DQ, Wang L, Fei F, et al. Identification of breast cancer metastasis-associated proteins in an isogenic tumor metastasis model using two-dimensional gel electrophoresis and liquid chromatography-ion trap-mass spectrometry. *Proteomics* 2006;6:3352–68.
27. Mantovani A, Allavena P, Sozzani S, Vecchi A, Locati M, Sica A. Chemokines in the recruitment and shaping of the leukocyte infiltrate of tumors. *Semin Cancer Biol* 2004;14:155–60.
28. Fra AM, Locati M, Otero K, et al. Cutting edge: scavenging of inflammatory CC chemokines by the promiscuous putatively silent chemokine receptor D6. *J Immunol* 2003;170:2279–82.
29. Locati M, Torre YM, Galliera E, et al. Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines. *Cytokine Growth Factor Rev* 2005;16:679–86.
30. Graham GJ, McKimmie CS. Chemokine scavenging by D6: a movable feast? *Trends Immunol* 2006;27:381–6.
31. Bonocchi R, Locati M, Galliera E, et al. Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor. *J Immunol* 2004;172:4972–6.
32. Weber M, Blair E, Simpson CV, et al. The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines. *Mol Biol Cell* 2004;15:2492–508.
33. Galliera E, Jala VR, Trent JO, et al.  $\beta$ -Arrestin-dependent constitutive internalization of the human chemokine decoy receptor D6. *J Biol Chem* 2004;279:25590–7.
34. Jamieson T, Cook DN, Nibbs RJ, et al. The chemokine receptor D6 limits the inflammatory response *in vivo*. *Nat Immunol* 2005;6:403–11.
35. Martinez de la Torre Y, Locati M, Buracchi C, et al. Increased inflammation in mice deficient for the chemokine decoy receptor D6. *Eur J Immunol* 2005;35:1342–6.
36. Whitehead GS, Wang T, DeGraff LM, et al. The chemokine receptor D6 has opposing effects on allergic inflammation and airway reactivity. *Am J Respir Crit Care Med* 2007;175:243–9.
37. Borroni EM, Buracchi C, de la Torre YM, et al. The chemoattractant decoy receptor D6 as a negative regulator of inflammatory responses. *Biochem Soc Trans* 2006;34:1014–7.
38. Ben-Baruch A. The multifaceted roles of chemokines in malignancy. *Cancer Metastasis Rev* 2006;25:357–71.
39. Kulbe H, Levinson NR, Balkwill F, Wilson JL. The chemokine network in cancer—much more than directing cell movement. *Int J Dev Biol* 2004;48:489–96.
40. Bieche I, Lerebours F, Tozlu S, Espie M, Marty M, Lidereau R. Molecular profiling of inflammatory breast cancer: identification of a poor-prognosis gene expression signature. *Clin Cancer Res* 2004;10:6789–95.
41. Luboshits G, Shina S, Kaplan O, et al. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. *Cancer Res* 1999;59:4681–7.
42. Niwa Y, Akamatsu H, Niwa H, Sumi H, Ozaki Y, Abe A. Correlation of tissue and plasma RANTES levels with disease course in patients with breast or cervical cancer. *Clin Cancer Res* 2001;7:285–9.
43. Ueno T, Toi M, Saji H, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. *Clin Cancer Res* 2000;6:3282–9.
44. Robinson SC, Scott KA, Wilson JL, Thompson RG, Proudfoot AE, Balkwill FR. A chemokine receptor antagonist inhibits experimental breast tumor growth. *Cancer Res* 2003;63:8360–5.
45. Salcedo R, Ponce ML, Young HA, et al. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 2000;96:34–40.
46. Stormes KA, Lemken CA, Lepre JV, Marinucci MN, Kurt RA. Inhibition of metastasis by inhibition of tumor-derived CCL5. *Breast Cancer Res Treat* 2005;89:209–12.
47. Azenshtein E, Luboshits G, Shina S, et al. The CC chemokine RANTES in breast carcinoma progression: regulation of expression and potential mechanisms of promalignant activity. *Cancer Res* 2002;62:1093–102.
48. Saji H, Koike M, Yamori T, et al. Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma. *Cancer* 2001;92:1085–91.
49. Leek RD, Harris AL. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia* 2002;7:177–89.
50. Pollard JW. Tumour-educated macrophages promote tumour progression and metastasis. *Nat Rev Cancer* 2004;4:71–8.
51. Flanagan K, Kaufman HL. Chemokines and cancer. *Cancer Invest* 2002;20:825–34.
52. Vicari AP, Caux C. Chemokines in cancer. *Cytokine Growth Factor Rev* 2002;13:143–54.