

Rho GTPase activating protein DLC1 (deleted in liver cancer) suppresses cell proliferation and invasion in hepatocellular carcinoma

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Abstract

DLC1 is a candidate tumor suppressor gene recently isolated from human hepatocellular carcinoma (HCC). Structurally, DLC1 protein contains a conserved RhoGAP domain, which has been thought to regulate the activity of Rho family proteins. Previous studies indicated that DLC1 was frequently inactivated in cancer cells. In the present study, we aimed to characterize the tumor suppressor roles of *DLC1* in HCC. We demonstrated that *DLC1* significantly inhibited cell proliferation, anchorage-independent growth and *in vivo* tumorigenicity when stably expressed in HCC cells. Moreover, *DLC1* expression greatly reduced the motility and invasiveness of HCC cells. With RhoGAP deficient *DLC1* mutant (*DLC1-K714E*), we demonstrated that the RhoGAP activity was essential for *DLC1*- mediated tumor suppressor function. Furthermore, the 292-648 a.a. region and START domain played an auxiliary role to RhoGAP and tumor suppressor function of *DLC1*. Taken together, our findings demonstrated that *DLC1* functions as a tumor suppressor in HCC and provide the first evidence to support the hypothesis that *DLC1* suppresses cancer cell growth by negatively regulating the activity of Rho proteins.

(Total 170 words)

Introduction

Hepatocellular carcinoma (HCC) is the fifth commonest cancer worldwide and is the second leading fatal cancer in Southeast Asia and Hong Kong. Etiologically, HCC is closely associated with chronic hepatitis B and C virus infection, cirrhosis and aflatoxin B1 intake (1). However, the molecular mechanisms leading to the development and progression of HCC remain unclear. In HCC, deletions of chromosomal materials are common and of a non-random pattern, with recurrent deletions on chromosomes 1p, 4q, 8p, 13q, 16q and 17p. These strongly suggest that putative tumor suppressor genes may be located in these chromosome arms (2-7).

Recently, a candidate tumor suppressor gene, namely frequently deleted in liver cancer (*DLC1*), was identified in primary HCC sample (8) and mapped to 8p21.3-22, where chromosomal deletion is particularly common in human cancers (2, 9-11). *DLC1* mRNA encodes a protein with 1,091 amino acid residues and is ubiquitously expressed in human tissues (8). The amino acid sequence of *DLC1* shares 86% homology with rat p122RhoGAP and this strongly suggests that *DLC1* is a p122RhoGAP human ortholog. p122RhoGAP was first identified as a phospholipase C- δ 1 binding protein in a rat brain expression library screening (12).

Structurally, both p122RhoGAP and *DLC1* contain three conserved functional domains, namely SAM (sterile alpha motif), RhoGAP (GTPase activating protein for Rho family protein) and START (steroidogenic acute regulatory related lipid transfer) domains (13, 14). Proteins

containing a RhoGAP domain usually function to catalyze the hydrolysis of GTP that is bound to Rho family proteins. When the bound GTP is hydrolyzed to GDP, Rho proteins return to the inactive basal state. Thus, RhoGAPs negatively regulate Rho-mediated cellular processes, such as actin cytoskeleton organization, gene expression, and cell-cycle progression (15, 16).

Evidence has shown that hyperactivation of Rho proteins are oncogenic (17-20). Clinically, Rho proteins were found to be overexpressed in different human cancers and overexpression of Rho proteins was associated with more aggressive tumor behavior (21, 22). Several members of the RhoGAP family have been suggested to possess potential tumor suppressor function. For instance, anti-tumor function of *p190RhoGAP* has been demonstrated by *in vitro* studies.

Overexpression of antisense *p190RhoGAP* RNA is able to transform normal NIH3T3 fibroblasts. In contrast, overexpression of the wild-type *p190RhoGAP* domain can suppress v-Ha-Ras-induced transformation (23). Therefore, it is logical to hypothesize that *DLC1* might have tumor suppressor functions by negatively regulating tumor cell growth or tumor progression through down-regulation of oncogenic activity of Rho family proteins.

In this study, we demonstrated that *DLC1* reduced the proliferation rate, anchorage-independent growth ability, and *in vivo* tumorigenicity of HCC cells. Stable expression of *DLC1* also significantly suppressed the motility and invasiveness of HCC cells. Furthermore,

DLCI inhibited Rho dependent stress-fiber formation in fibroblasts and HCC cells, and this Rho inhibitory function was essential for the *DLCI*- mediated growth inhibition in HCC cells. Thus, our findings support the hypothesis that *DLCI* is a RhoGAP antagonizing the oncogenic activity of Rho proteins and serves as a tumor suppressor gene in human HCC.

Materials and Methods

Cell lines and plasmid construction

Human HCC cell lines, SMMC-7721 and BEL7402, which lack *DLCI* expression, were obtained from Shanghai Institute of Cell Biology (14, 24). The murine fibroblast cell line Swiss-3T3 was obtained from American Tissue Culture Collection (ATCC). The rodent fibroblast cell line Rat 6 was kindly provided by Dr. W.W. Hsiao (25). SMMC-7721 and BEL7402 were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and antibiotics. Swiss-3T3 and Rat 6 cells were cultured in DMEM supplemented with 10% (vol/vol) calf serum (CS) and antibiotics at 37°C in a humidified incubator with 5% CO₂.

Plasmid construction

A 3.5-kb fragment of full-length coding sequence of the *DLCI* gene was amplified from normal liver tissue and cloned into pcDNA3.1(-) vector (26). Truncated fragments were subcloned into eukaryotic expression vector, pCS2+MT, of Myc-tagged protein as follows:

DLCI-FL (1-1091 a.a.), *DLCI-SAM* (1-292 a.a.), *DLCI-GAP* (648-839 a.a.), *DLCI-START* (879-1091 a.a.), *DLCI-NΔC* (1-595 a.a.), *DLCI-CΔN* (648-1091 a.a.), *DLCI-Δ SAM* (292-1091 a.a) and *DLCI-ΔSAMΔSTART* (292-839 a.a.). The DNA sequences and the reading frame of the recombinant plasmids were confirmed by DNA sequencing.

Mutagenesis

A *DLCI* fragment (1-804 a.a) was released from pcDNA3.1/*DLCI* plasmid by *EcoRI* digestion and subcloned into pBluescript II SK (+) vector. The site-specific mutation of K714E was introduced into *DLCI* cDNA by PCR mutagenesis with two consecutive PCR amplifications. For the first PCR amplification, PCR fragments contain *DLCI*-K714E mutation was generated from pSK+/*DLCI* (1-804 a.a.) with two set of PCR primers: 5'- GGA TGG ATG AGG AGA AGC TG -3' (set 1, forward), 5'- GAT CTC GAA AAT ACT GCT CCA - 3'(set 1, reverse), 5'- TGC TGG AGC AGT ATT TTC GAG -3' (set 2, forward) and 5'- TAA TAC GAC TCA CTA TAG GG -3' (set 2, reverse). Subsequently, an aliquot of the first PCR products were used as templates for the second PCR, which was designed to amplify the full-length insert with the desired mutation in the *DLCI* cDNA with the set 1 forward primer and set 2 reverse primer that have been used in the first PCR amplification. The final amplified product was excised and gel-purified. The *NciI/BamHI* fragment of *DLCI* cDNA in pSK+/*DLCI* (1-804 a.a) plasmid was then replaced by this PCR amplified fragment. The full length *DLCI*-K714E mutant was generated by releasing the *DLCI*-K714E fragment (1-804 a.a) from pSK+/*DLCI* K714E

plasmids by *EcoRI* digestion and subcloned into pCS2+MT/*DLC1* (805-1091 a.a.) plasmid, which was then verified by DNA sequencing.

Establishment of *DLC1* stably expressing cells

Two μg of pcDNA3.1(-)/*DLC1* plasmid was transfected into SMMC-7721 cells with FuGENE 6 Transfection Reagent as described by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). After 24 hours, the transfected cells were trypsinized and splitted onto 100 mm culture dish at a density of 1×10^4 /dish. Transfected cells were selected for 2 weeks in culture medium containing 0.75 mg/ml G418 (Invitrogen, Carlsbad, CA). Single G418-resistant clones were isolated from the culture dish using a Cloning cylinder (Bellco Biotechnology, Vineland, NJ). The expression of *DLC1* was determined by RT-PCR and Northern blotting as described previously (14).

Cell proliferation assay

Cells (2.5×10^4) were plated in each well of 12-well culture plates containing 1 ml DMEM medium supplemented with 10% FBS or 0.5% FBS. Cells were harvested at 24-hour intervals for 7 days. Cell numbers were counted by trypan blue exclusion assay with hemacytometer. The doubling time of proliferating cells was determined by nonlinear regression (exponential growth) model using GraphPad Prism version 3.00 (GraphPad Software, San Diego CA)

Anchorage-independent growth assay in soft-agar

Five ml of 0.5% agarose culture medium containing 10% FBS was used to coat the bottom of each of the wells of the 6-well culture plates. After hardening, 1×10^4 cells were suspended in 2 ml of 0.3% agarose culture medium containing 10% FBS warmed at 40°C and plated onto the bottom layer. The cell solution was allowed to set for 30 minutes at room temperature before moving into the 37°C CO₂ incubator. Colonies (≥ 10 cells) formed in the soft agarose culture were counted and photographed two weeks after inoculation.

Cell motility assays

For cell motility assay, 8×10^5 cells were seeded onto 60-mm dishes and grown for 24 hours. Cells were treated with 10 µg/ml mitomycin C (Sigma) for 3 hours prior to the wounding. A linear wound was made by scraping a pipette tip across the confluent cell monolayer. Cells were rinsed with phosphate buffered saline (PBS) and grown in DMEM supplemented with 10% FBS for additional 48 hours. The cell motility in terms of wound closure was measured by photographing at 3 random fields at the time of wounding (time zero), 24 and 48 hours after wounding.

***In vitro* cell invasion assay**

For *in vitro* cell invasion assay, 3×10^5 cells were suspended in 300 µl of serum-free DMEM medium and loaded onto the upper compartment of invasion chamber that contained a polycarbonate membrane with an 8 µm pore size and coated with a layer of extracellular matrix

(ECM) (Chemicon International, Temecula, CA). After 60 hours of incubation, the invasive cells which had migrated through the ECM layer to the complete medium in the lower compartment were stained, and the numbers of invasive cells were photographed and counted under the microscope.

***In vivo* tumorigenicity assay**

SMMC-7721 and SMMC-7721/*DLC1* cells were harvested and resuspended in PBS. One $\times 10^6$ cells were inoculated subcutaneously into the right flank of 6-week-old female BALB/C nude mice using a 25-gauge needle (5 mice for each group). Tumor size was monitored weekly by measure the largest and smallest diameters of tumor and estimated according to the formula: volume = $1/2 \times (\text{largest diameter}) \times (\text{smallest diameter})^2$. This experiment was performed following the Animals (Control of Experiments) Ordinance (H.K.) and Institute's guidance on animal experiments.

Immunofluorescence microscopy

Five $\times 10^4$ cells were seeded onto coverslips 16 hours prior to transfection. At 24 hours after transfection, cells were serum starved for an additional 24 hours and treated with 200 ng/ml lysophosphatidic acid (LPA) (Sigma) for 30 min. Cells were fixed in 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS for 10 min. Fixed cells were blocked with 3% bovine serum albumin (BSA) and incubated with 1:1,000 rabbit polyclonal anti-Myc antibody (A14) (Santa Cruz, CA) for 2 hours followed by 1:100 FITC conjugated goat

anti-rabbit antibody (Amersham) and 1:1,000 rhodamine conjugated phalloidin (Sigma) for an hour with extensive washing in between. Cells were counterstained with 4,6 diamidion-2-phenylindole (DAPI) (Calbiochem, San Diego, CA) and mounted in Vectashield antifade mountant (Vector Laboratory, Burlingame, CA). Images were captured under magnification of $\times 1,000$ by a fluorescence microscope equipped with a charge-coupled device (CCD) camera (Leica, Wetzlar, Germany).

Colony suppression assay

Two $\times 10^5$ cells were seeded onto 35 mm dish one day before transfection. Two μg each of the various pCS2+MT/*DLC1* constructs or the pCS2+MT empty vector were co-transfected with 0.2 μg pBABE-puro puromycin selection vector into the cells using FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals). After 24 hours, cells were seeded onto 10 cm culture dishes in 1:5 dilution and grown in culture medium containing 0.6 $\mu\text{g}/\text{ml}$ puromycin (Sigma) for 2 weeks. The puromycin-resistant colonies were fixed with 3.7% formaldehyde, and the colony formation efficiency was examined by Giemsa staining (Sigma).

Results

***DLC1* inhibited cell proliferation, anchorage-independent growth and tumorigenicity of**

HCC cells

We have previously demonstrated that ectopic overexpression of *DLC1* cDNA can suppress cancer cell growth in colony suppression assay (26). In order to further characterize the tumor suppressor function of *DLC1* in HCC, in this study, we established a cellular model in which *DLC1* cDNA was stably expressed in SMMC-7721 cells that lack endogenous *DLC1* expression. The ectopic expression of *DLC1* in stably transfected cells was confirmed by semi-quantitative RT-PCR and Northern blot analysis (Figure 1A). To determine the effect of *DLC1* on cell proliferation, we monitored the proliferation rate of *DLC1* stably expressing SMMC-7721 cells (SMMC-7721/*DLC1*) for 7 days in a mitogen-dependent (10% FBS) and a mitogen-independent (0.5% FBS) manner. In both culture conditions, we observed that SMMC 7721/*DLC1* exhibited a significantly slower growing phenotype. The doubling time of SMMC-7721/*DLC1* cells in mitogen-dependent and mitogen-independent conditions were, 37.8 hrs (95% CI: 34.4 - 41.8 hrs) and 55.0 hrs (95% CI: 46.3-67.8 hrs), respectively. In contrast, the doubling time of parental SMMC-7721 cells were 29.5 hrs (95% CI: 27.5 - 31.8 hrs) and 41.1 hrs (37.6-45.4 hrs), respectively (Figure 1B). These results indicated that *DLC1* exerts growth inhibitory effects on HCC cells.

The capability of anchorage independent growth is an important indicator of malignant transformation. To substantiate the tumor suppression function of *DLC1*, we compared the anchorage-independent growth ability of SMMC-7721/*DLC1* and SMMC-7721 cells in soft agar culture. As expected, the *DLC1* stably transfected cells exhibited a dramatically reduced

ability to form colonies on soft agar. Both the number and size of colonies formed by *DLC1* stably expressing cells were significantly reduced as compared with those of the parental SMMC-7721 cells ($P = 0.011$, Figure 1C). Our findings suggest that ectopic expression of *DLC1* is sufficient to suppress anchorage-independent growth in SMMC-7721 cells.

Next, we determine the effects of *DLC1* expression on the *in vivo* tumorigenicity in nude mice xenograft model. One $\times 10^6$ cells were inoculated into the right flank of nude mice and the formation of solid tumor was monitored for 63 days. We observed that growth rate of *DLC1* expressing SMMC-7721 cells was significantly suppressed in nude mice (Figure 2). The above finding strongly suggests that *DLC1* gene plays a tumor suppression role in human carcinogenesis.

***DLC1* suppressed cell motility and invasiveness of HCC cells**

To test whether *DLC1* affects the motility of HCC cells, wound healing assay was performed on SMMC-7721 and SMMC-7721/*DLC1* cells in the absence or presence of a cell division inhibitor, mitomycin C. Our results showed that the motility of *DLC1* stably expressing cells was significantly reduced. Similar results were also obtained when the cells were pretreated with mitomycin C, which indicated that the reduction of cell motility in *DLC1* expressing cells was not due to the difference in proliferation rate (Figure 3A). Consistently, the cell motility of SMMC-7721/*DLC1* was significantly reduced in cell motility assay using invasion chamber without ECM coating, and this confirmed that expression of *DLC1* inhibited cell motility

(Supplementary Figure 1). Furthermore, we asked whether *DLC1* could suppress the invasiveness of HCC cells. To this end, we performed *in vitro* cell invasion assay using invasion chamber coated with an ECM layer (27). In sharp contrast with the parental SMMC-7721 cells, the number of invasive cells was dramatically reduced in *DLC1* stably expressing cells (Figure 3B). Thus, our results clearly demonstrated that *DLC1* suppressed the cell motility and invasiveness of HCC cells.

Expression of *DLC1* inhibited Rho-mediated stress fiber formation

The existence of RhoGAP domain on *DLC1* protein strongly suggests that *DLC1* may function as a negative regulator toward the activity of Rho family proteins. Previously, we had demonstrated that bacterially expressed DLC1 protein significantly enhanced the intrinsic GTP hydrolysis activity of two well characterized Rho family proteins, RhoA and Cdc42 (14). This observation implied that DLC1 facilitated the conversion of the active GTP-bound Rho proteins to the GDP-bound inactive form. It is well known that Rho proteins play a critical role in regulating cytoskeleton organization and mediates actin stress-fiber formation upon extracellular stimulations (28, 29). Actin stress fibers are disassembled in serum starved cells but restored by LPA treatment through activation of Rho proteins. In this study, we further tested the *in vivo* RhoGAP activity of *DLC1* by transiently expressing myc-tagged *DLC1* cDNA in HCC cell lines and fibroblast cell lines. The effects of *DLC1* on the activity of Rho proteins were examined under fluorescence microscope with regard to morphological changes and actin

stress fiber formation. As shown in Figure 4B, LPA induced the formation of actin stress fibers in serum starved SMMC-7721 cells. However, in *DLC1* overexpressing cells, the formation of actin stress fibers was significantly suppressed. Furthermore, overexpression of *DLC1* resulted in extensive cell rounding and cortical retraction. It is of note that these morphological changes were remarkably similar to that caused by the Rho inhibitor, C3 exoenzyme, in HeLa cells (30) suggesting that these morphological changes were due to inhibition of Rho proteins. Consistent observation was obtained from both HCC cell lines SMMC-7721 and BEL7402 as well as fibroblast cell lines Swiss-3T3 and Rat6 suggesting that the effects of *DLC1* on actin-stress fiber formation and cell morphological changes were not cell line specific response.

In silico analysis had revealed that several lysine and arginine residues are highly conserved in various mammalian RhoGAPs. A previous study on the rat p122RhoGAP (*DLC1* ortholog) has provided evidence that Lys-706 residue is involved in stimulating the GTP hydrolysis of active GTP-bound Rho proteins (13). To further confirm the RhoGAP function of *DLC1*, we constructed a RhoGAP deficient mutant by converting the Lys-714 into glutamate (K714E), which corresponds to the Lys-706 in rat p122RhoGAP. Expression of *DLC1-K714E* mutant abolished the *DLC1* induced cell rounding and cortical retraction. In addition, upon LPA induction, *DLC1-K714E* mutant expressing cells formed actin stress fibers as efficiently as the empty vector control (Figure 4B). Similar findings were also reproduced in another *DLC1* null HCC cell line (BEL7402) as well as in fibroblast cell lines (Swiss-3T3 and Rat 6) (data not

shown). Taken together, our results strongly suggest that *DLC1* functions as a RhoGAP and negatively regulates the activity of Rho proteins *in vivo*.

RhoGAP activity was essential for *DLC1* mediated tumor suppressor function

We hypothesized that *DLC1* suppressed cancer cell growth through negatively regulating the activity of Rho proteins. In order to test this hypothesis, colony suppression assays were performed on SMMC-7721 cells transfected with wild-type and RhoGAP deficient mutant *DLC1*. We found that ectopic overexpression of wild-type *DLC1* significantly suppressed the colony formation in HCC cells (Figure 5A). Indeed, this finding was highly consistent with that found in cancer cell lines of different tissue types as reported by us and others (26, 31, 32). In contrast to wild-type *DLC1*, expression of the RhoGAP-deficient mutant had no effect on the colony formation ability of SMMC-7721 cells. The number of colony formed by *DLC1*-K714E transfected cells was comparable to that of the empty vector (Figure 5A). Thus, our data suggest that the RhoGAP activity is essential for *DLC1* mediated tumor suppressor function and lend further support to the notion that *DLC1* exerts tumor suppressor function by negatively regulating the activity of Rho proteins.

Cooperation among the multi-functional domains of *DLC1* was required for its growth inhibition of HCC cells

DLC1 contains three conserved functional domains. Apart from the RhoGAP domain, SAM and START domains are located at the N-terminal and C-terminal of the *DLC1* protein,

respectively (Figure 4A). We have demonstrated in this study that the RhoGAP domain of DLC1 is essential for negative regulation of Rho activity and suppression of cancer cell growth. However, the roles of the SAM and START domains in DLC1 functions are largely unclear. To address this question, we expressed different truncated *DLC1* cDNAs in SMMC-7721 cells (Figure 4A). The expression of the truncated DLC1 proteins was confirmed by western blotting using anti-Myc antibody (Figure 5B). The RhoGAP activity and tumor suppressor function of these truncated constructs were determined by their ability to inhibit Rho mediated actin stress fiber formation (Figure 4B) and to suppress colony formation (Figure 5A) in HCC cells, respectively. Since RhoGAP is essential for *DLC1*-mediated tumor suppressor function, we first asked whether the RhoGAP domain alone was sufficient to inhibit the activity of Rho proteins and suppress colony formation in HCC cells as efficiently as the wild-type *DLC1*. In this regard, we transiently expressed the *DLC1* fragment containing the RhoGAP domain (*DLC1-GAP*) in SMMC-7721 cells. Surprisingly, the *DLC1-GAP* failed to induce morphological change and inhibit actin stress fiber formation when overexpressed in HCC cells (Figure 4B). Moreover, we did not find any significant difference in the colony formation ability between HCC cells expressing *DLC1-GAP* and those transfected with empty vector alone (Figure 5A). Similarly, the colony formation ability of HCC cells expressing *DLC1* fragment containing either the SAM domain (*DLC1-SAM*) or START domain (*DLC1-START*) alone was also comparable to that of the empty vector control (Figure 5A). *DLC1-SAM* and

DLC1-START also had no effect on HCC cell morphology as well as stress fiber formation (data not shown). These observations suggest that SAM, RhoGAP or START domain on their own is insufficient to inhibit the activity of Rho proteins or suppress colony formation in HCC cells. Thus, cooperation among multi-functional domains in *DLC1* is required for RhoGAP and tumor suppressor functions.

Next we tested the RhoGAP and tumor suppressor functions of C-terminal (*DLC1-CAN*) and SAM domain-deleted (*DLC1-ΔSAM*) fragments of *DLC1* in SMMC-7721 cells. C-terminal of *DLC1* showed no suppressive effects on the stress fibers and colony formation, while expression of *DLC1-ΔSAM* resulted in extensive cell rounding and cortical retraction accompanied with loss of actin stress fiber formation (Figure 4B). In addition, *DLC1-ΔSAM* also significantly suppressed colony formation in HCC cells (Figure 5A). These findings indicate that SAM domain is not necessary for the RhoGAP and tumor suppressor function of *DLC1* while an unidentified functional domain located between SAM and RhoGAP (i.e. amino acid residues 292-639) may play an important role in regulating *DLC1* functions. To test the role of START domain in *DLC1*-mediated functions, we further expressed the N-terminal (*DLC1-NAC*) and the SAM and START domain-deleted fragment (*DLC1-ΔSAMΔSTART*), respectively, in HCC cells. Like the C-terminal fragment, expression of the N-terminal fragment of *DLC1* also had no effects on the cell morphology and colony formation efficiency of HCC cells. However, compared with *DLC1-ΔSAM*, further deletion of the START domain

resulted in loss of the inhibitory effect on actin stress fiber formation and colony formation in HCC cells (Figure 4B & 5A). These findings indicate that the START domain is indispensable for *DLC1*-mediated RhoGAP and growth inhibitory function. The concurrence of RhoGAP domain and growth inhibitory functions among these various truncated *DLC1* constructs provides further evidence to support the notion that the RhoGAP activity is essential for *DLC1* mediated tumor suppression (Figure 4A). Similar results were also obtained when these experiments were performed in BEL7402 and Rat 6 cell lines, indicating that these findings were not merely due to a cell line specific phenotype (data not shown).

Discussion

DLC1 is a candidate tumor suppressor gene recently isolated from PCR-based subtractive hybridization in human HCC (8). Follow-up studies indicate that inactivation of *DLC1* is implicated in hepatocarcinogenesis (14, 26, 33, 34). Although somatic mutations in *DLC1* gene are rare (14, 35, 36), loss of heterozygosity is present in approximately half of primary HCCs (8, 14, 35). *DLC1* mRNA expression is significantly down-regulated in primary HCC samples (14, 26). Earlier *in vitro* studies using hepatoma cell lines not expressing *DLC1* have revealed that epigenetic alterations, including promoter methylation, are also responsible for *DLC1* silencing in HCC (14, 34). In addition, methylation of the *DLC1* promoter was detected in about 24% of primary HCC samples (14). These findings suggest that epigenetic silencing of

DLC1 is not merely an artifact introduced by *in vitro* tissue culture but may substantially contribute to human carcinogenesis (14). The frequent inactivation of the *DLC1* gene in HCC prompted us to further investigate its tumor suppressor functions. We previously showed that transfection of *DLC1* cDNA into HCC cell lines resulted in inhibition of colony formation (26). Recently, inactivation of the *DLC1* gene has also been observed in human breast, gastric and lung cancers (31, 32, 37, 38), suggesting that *DLC1* functions as a bona fide tumor suppressor gene. Although several lines of evidence support the tumor suppression role of *DLC1*, little is known about the functions of the *DLC1* and how it is implicated in human carcinogenesis.

To further substantiate the tumor suppressor function of *DLC1*, we stably expressed *DLC1* cDNA in SMMC-7721 cells. Expression of *DLC1* cDNA significantly suppressed cell proliferation in both mitogen-dependent and mitogen-independent cultures. In addition, stable expression of *DLC1* resulted in reduction of anchorage-independent growth capability. *DLC1* expression also suppressed the tumorigenicity of SMMC-7721 cells in nude mice xenograft model. Consistent with our findings, Yuan et al. recently demonstrated that introduction of the *DLC1* cDNA into other cancer cell lines also abolished the *in vivo* tumorigenicity in nude mice (32, 33). The above findings, when taken together, strongly suggest the *DLC1* gene functions as a tumor suppressor in human carcinogenesis.

Our previous *in vitro* study indicated that *DLC1* negatively regulated the activity of Rho family proteins by promoting the GTP hydrolysis (14). In the present study, we further demonstrated

that *DLC1* expression could significantly suppress Rho-dependent actin stress fibers formation in HCC and fibroblast cell lines. Thus, our findings strongly imply that overexpression of *DLC1* can inhibit the activity of Rho proteins and further support the notion that *DLC1* functions as RhoGAP *in vivo*.

Cell motility is tightly regulated by the activity of Rho proteins through actin cytoskeletal rearrangements (29). Recently, a growing body of evidence has suggested that deregulation of Rho proteins are implicated in cancer cell invasion and metastasis. For instance, overexpression of wild-type RhoA in rat hepatoma cells induced cell invasion in both *in vitro* and *in vivo* conditions (39). On the other hand, dominant negative RhoA (RhoA N19) was sufficient to inhibit the invasiveness of human prostate cancer cells (40). It has also been documented that RhoA specific inhibitor C3 exoenzyme greatly suppressed the cell motility of HCC cell lines, Li7 and KYN-2. In sharp contrast, the cell motility of these cell lines was strongly induced by LPA treatment (41). Based on the above evidence, we hypothesize that *DLC1*, a negative regulator of Rho proteins, will reduce cell motility. Indeed, our data clearly showed that expression of *DLC1* significantly inhibited cell motility and invasiveness of HCC cells.

More than 30 potential down-stream effectors of Rho family proteins have been identified (42). These effectors may be responsible for the different specific cellular functions mediated by the Rho proteins. ROCK is one of the best characterized down-stream effectors which selectively

binds to active GTP bound Rho proteins and regulates cell cytoskeletal reorganization through phosphorylating a wide range of cytoskeletal proteins (43, 44). Recent studies indicated that deregulation of Rho/ROCK signaling pathway is responsible for cancer invasion and metastasis (22, 41, 45). It is tempting to speculate that *DLC1* suppresses cell motility and invasiveness through down-regulation of the Rho/ROCK signaling pathway. Further experiments are warrant to clarify the functional relationship between *DLC1* and Rho/ROCK signaling pathway.

To further characterize the Rho inhibitory and tumour suppressor activity of *DLC1*, various *DLC1* truncated mutants was generated. RhoGAP deficient mutant abolished *DLC1* mediated growth inhibition when expressed in HCC cells. Thus, our results have demonstrated for the first time that the RhoGAP activity of *DLC1* is essential for its tumor suppressor function.

Surprisingly, ectopic expression of the *DLC1* RhoGAP domain alone did not inhibit stress fiber and colony formation. These observations indicate that, apart from the RhoGAP domain, other functional domains of the *DLC1* protein may also play some cooperative role in regulating the *DLC1* function *in vivo*. Indeed, deletion of START domain of *DLC1* resulted in impaired inhibition on actin stress fiber and colony formation, suggesting that the START domain may play an auxiliary role to *DLC1* in inhibiting Rho activity and tumor cell growth. The exact function of START domain is largely unknown, but a previous study on the other START domain-containing proteins indicated that this domain might be involved in membrane

targeting and lipid-binding (46). We speculate that START domain in *DLC1* may be responsible for targeting *DLC1* to particular subcellular localization where its specific substrates locate. We also noticed that the expression of C-terminal of *DLC* (648-1091 a.a), which contains intact RhoGAP and START domain, did not affect the actin stress fiber formation and was unable to suppress colony formation in HCC cells. However, *DLC1-ΔSAM* (292-1091 a.a.) significantly inhibited the activity of Rho proteins and colony formation ability of HCC cells. This result strongly suggests that an unidentified functional domain located at 292-638 may be important for the *DLC1* mediated RhoGAP and tumor suppressor functions. Thus, we propose that both the RhoGAP and START domains and an unidentified domain located within the region of 292-638 amino acid residues are required for *DLC1*-mediated growth and stress fiber inhibition, and further investigations are warranted in this regard. Furthermore, similar with the RhoGAP deficient mutant, we noticed a close association between RhoGAP activity and tumor suppressor function of various truncated *DLC1* cDNAs. Thus our results suggest that *DLC1* tumor suppressor function are related to its Rho protein inhibitory activity.

In conclusion, our findings thus far have indicated that *DLC1* significantly inhibited Rho-mediated actin stress fiber formation, and the RhoGAP activity of *DLC1* was essential for tumor suppressor function. Expression of *DLC1* suppressed cell proliferation, anchorage-independent growth, tumorigenicity and invasiveness of HCC cells.

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Legend for figures

Figure 1. Stable expression of *DLC1* inhibited cell proliferation and

anchorage-independent growth. (A) Stable expression of *DLC1* in SMMC-7721, left panel:

RT-PCR, right panel: Northern blotting. (B) *DLC1* inhibited cell proliferation in SMMC-7721.

Cells were harvested and counted at 24-hr intervals for 7 days. Data represent the mean and

S.D of three independent experiments. (C) *DLC1* expression suppressed

anchorage-independent growth of HCC cells ($P = 0.011$, t -test). Cells were grown in soft agar

culture for 2 weeks and the efficiency of colony growth was examined under the microscope.

The data represent the mean and SD of three independent experiments.

Figure 2. *DLC1* suppressed tumorigenicity in nude mice. One $\times 10^6$ cells were inoculated

subcutaneously into the right flank of 6-week-old female BALB/C nude mice (5 mice for each

group). Tumor size was estimated according to the formula: volume = $1/2 \times$ (largest diameter)

\times (smallest diameter)².

Figure 3. *DLC1* inhibited cell motility and invasiveness in HCC. (A) The ability of

SMMC-7721 and SMMC-7721/*DLC1* grown in a confluent monolayer to migrate into a linear

wound created by a pipette tip in the absence (left) or presence (right) of the cell division

inhibitor, mitomycin C (10 μ g/ml), was monitored for 48 hrs. (B) The invasiveness of the HCC

cells was assayed with ECM-coated invasion chamber. Invasive cells were stained,

photographed and counted under the microscope. The data represent the mean and S.D of three

independent experiments. Expression of *DLC1* significantly inhibited cell invasion of SMMC-7721 ($P < 0.001$, *t*-test).

Figure 4. The effect of DLC1 on cell morphology and Rho-dependent stress-fiber

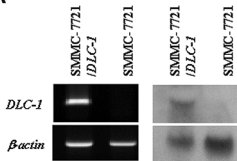
formation (A) Schematic representation of the various constructs of *DLC1*. The full-length *DLC1*, *DLC1-FL* (1-1091), contains three major functional domains, namely SAM, RhoGAP, and START. Various fragments of *DLC1* cDNA were subcloned into Myc-tagged expression vector, pCS2+MT. +: significant inhibition of actin-stress fiber formation or colony formation in HCC cells; -: no significant inhibition of actin-stress fiber formation or colony formation in HCC cells. (B) The morphological change and actin stress fiber formation of SMMC-7721 cells transfected with various *DLC1* mutants. SMMC-7721 cells grown on glass cover slips were transfected with Myc-tagged *DLC1* mutants or empty vector. Cells were serum starved for 18 hr and the formation of stress-fibers was induced by LPA. The expression of *DLC1* was determined by polyclonal anti-myc antibody (A14) followed by FITC-conjugated goat anti-rabbit antibody. The actin stress fibers were stained with rhodamine conjugated phalloidin. Arrow heads: Cells expression of *DLC1-FL* and *DLC1-ΔSAM* significantly inhibited stress fiber formation and induced cell rounding and cortical retraction.

Figure 5. Tumor suppressor function of DLC1 mutants (A) Colony suppression assays of SMMC-7721 cells transfected with *DLC1* mutants. The data represent the mean and S.D of three independent experiments. Similar findings were also obtained from another HCC cell line BEL7402 lacking endogenous *DLC1* expression (data not shown). (B) Expression of *DLC1* mutants in SMMC-7721 cells. *DLC1* expression were detected by Western blotting and confirmed that all *DLC1* mutants were at expected size and were expressed at similar level. NS: non-specific bands.

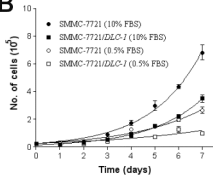
Supplementary Figure 1. Stably expression of *DLC1* inhibited cell motility. Cell motility of SMMC-7721 and SMMC-7721/*DLC1* were examined by in-vitro cell motility assay using invasion chamber without ECM coating. 0.4×10^5 cells were suspended in 300 μ l of serum-free DMEM medium and loaded onto the upper compartment of invasion chamber that contained a polycarbonate membrane with an 8 μ m pore size. After 16 hours of incubation, the invasive cells which had migrated through the membrane to the complete medium in the lower compartment were stained, and the numbers of invasive cells were photographed and counted under the microscope. The data represent the mean and S.D of three independent experiments. Expression of *DLC1* significantly inhibited cell motility of SMMC-7721 ($P < 0.001$, *t*-test).

Figure 1

A



B



C

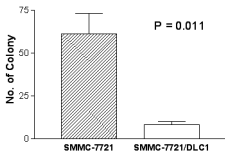
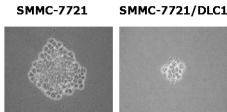


Figure 2

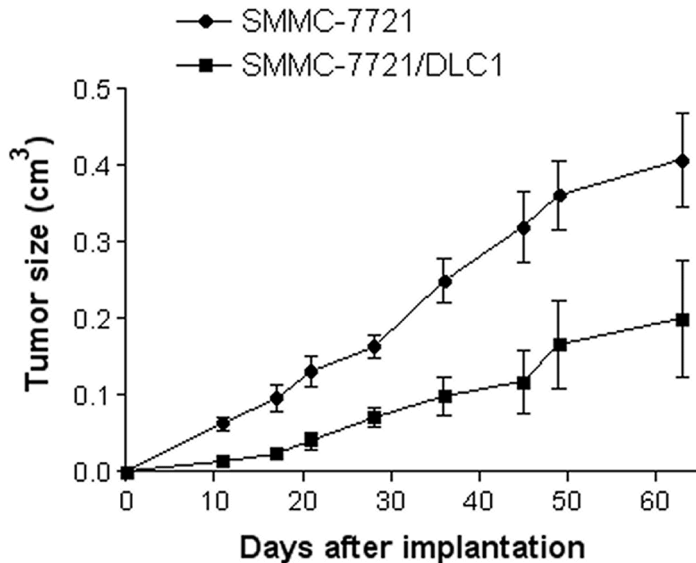
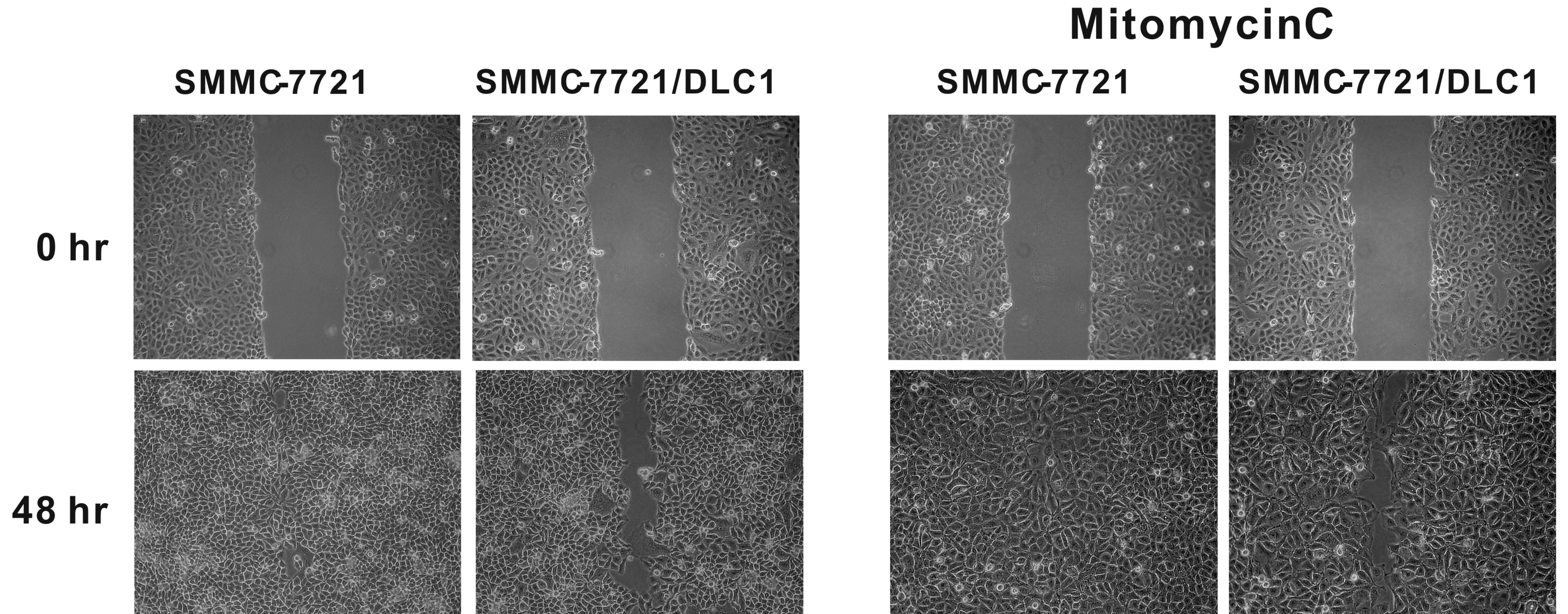


Figure 3

A



B

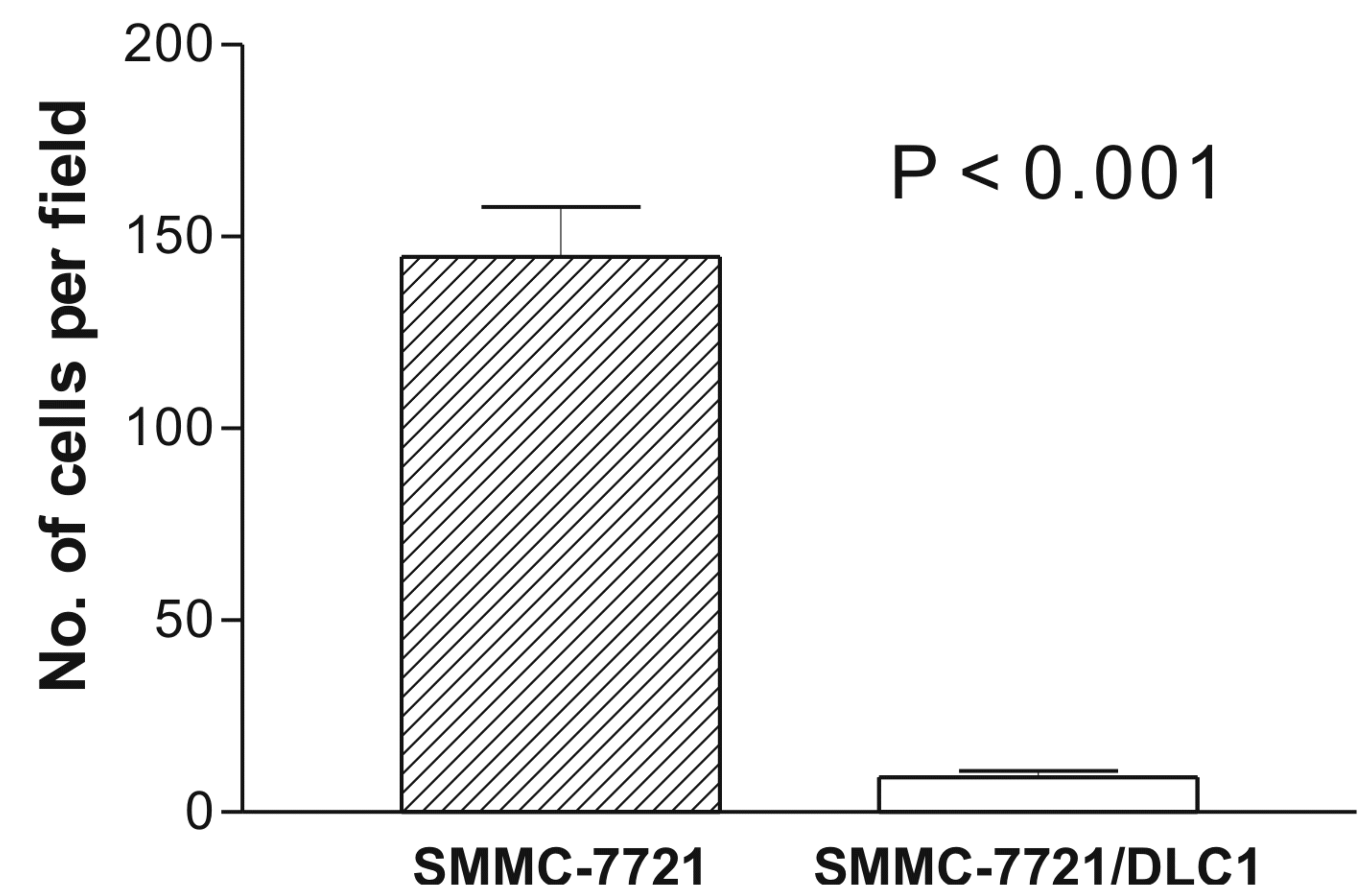
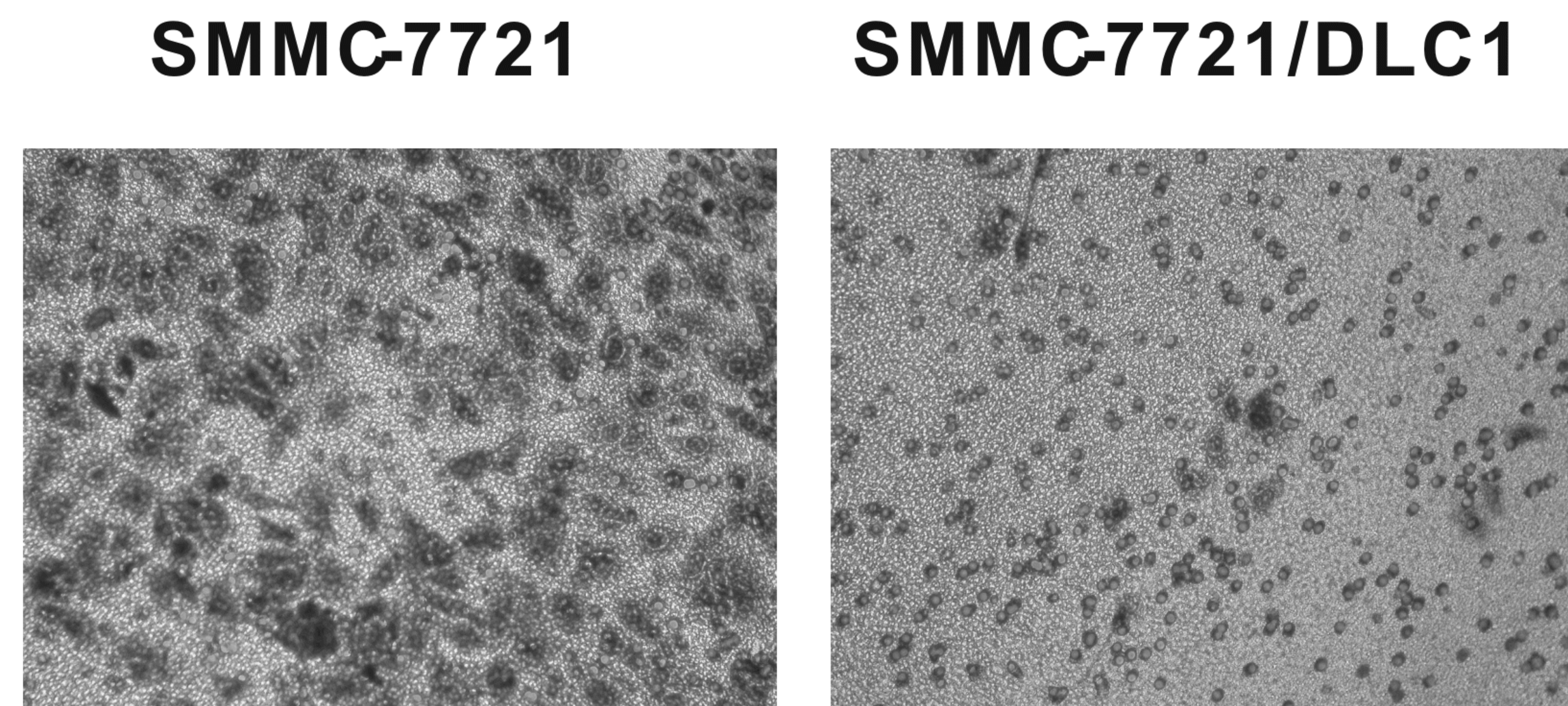
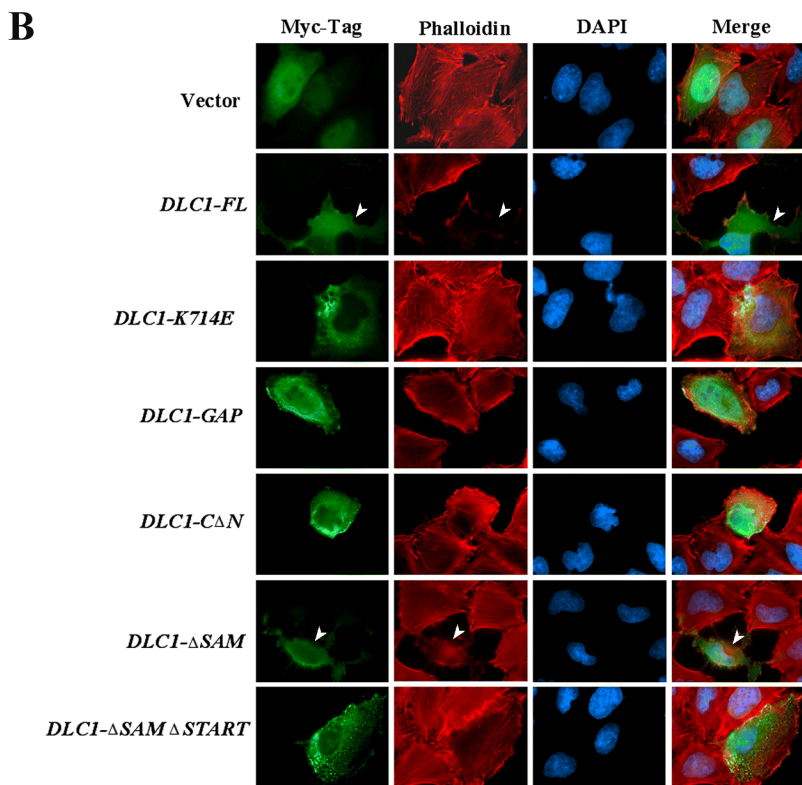
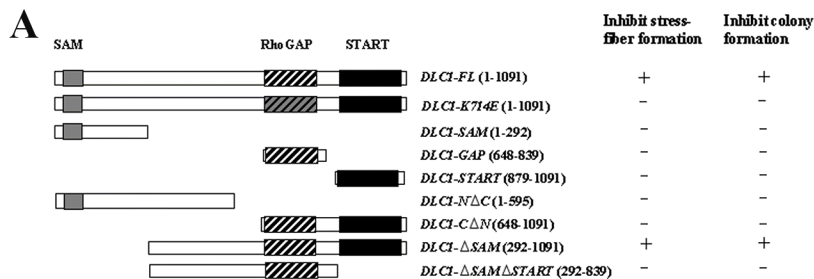
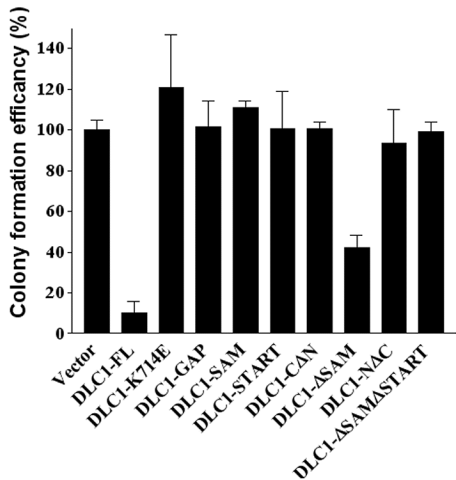
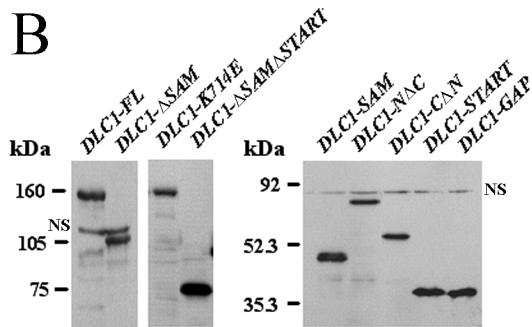


Figure 4



A**B****Figure 5**

Supplementary figure 1

SMMC-7721

SMMC-7721/DLC-1

