

**Downregulation of CRKL expression can inhibit tumorigenesis in colon cancer****Bin Lan<sup>1</sup>, Jian Zhang<sup>2</sup>, Jingxuan Shan<sup>3</sup>, Peng Zhang<sup>4</sup>, Weihong Zhang<sup>1</sup>, Yanchao Chen<sup>1</sup>, Wei Zhen<sup>1</sup>, Shugang Yan<sup>1</sup>**

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**1. ABSTRACT**

CRKL, as a "switch" factor on several oncogenic pathways, plays vital roles in multiple cancers. However, little is known about CRKL in gastrointestinal cancers. Here, we showed that CRKL is involved in colon cancer, which is the most common form of cancer of the digestive system. Immunohistochemistry analysis showed that CRKL expression in colon tumor tissue is significantly higher than normal tissue and CRKL level is associated with tumor differentiation. Suppression of CRKL in colon cancer cells inhibited cell proliferation, migration and invasion, while induced apoptosis. Colon cancer cells xenografts in nude mice showed that CRKL promoted tumorigenesis. Our results suggest that CRKL has the ability to regulate colon cancer malignancy and CRKL has the potential to serve as a diagnosis and prognosis marker and a therapy target of colon cancer.

**2. INTRODUCTION**

CRKL (v-crk sarcoma virus CT10 oncogene homolog (avian)-like), a member of the CRK family of adapter proteins, plays a role in multiple biological processes, including cell proliferation, adhesion and migration, as well as signal transduction (1-5). CRKL was once thought to be the substrate of BCR-ABL fusion protein, which mediating the inhibition of bcr-abl gene associated apoptosis in chronic myeloid leukemia (6). A growing body of evidences suggests that CRKL is also involved in solid tumor genesis by regulating tumor bio-behavior from various aspects, such as proliferation, differentiation, invasion and poor outcome (7-13). Recently, CRKL protein had been shown to overexpress in a subset of gastric cancers (12). To our knowledge, little is known about the contribution of CRKL to the colon cancer progression. CRK (v-crk sarcoma virus CT10 oncogene

homolog (avian)), another member of the CRK family, is significantly elevated in colon cancers (14). A high-throughput analysis of copy number alterations in cancer specimens identified the 22q11.21 amplicon as one of the most commonly amplified regions in colorectal cancers (15). *CRKL* is located at the center of the 22q11.21 amplicon. These findings suggest that *CRKL* acts as an oncogene in colon cancers and prompt us to investigate the expression pattern of *CRKL* in colon cancer and its functional significance in cancer cell behavior. In this study, colon cancer cell line-sw1116 treated with shRNA targeting *CRKL* (pGU6/GFP/Neo/sh-*CRKL*) was adapted to evaluate the association of *CRKL* with colon cancer.

### 3. MATERIALS AND METHODS

#### 3.1. Patients and tissue samples

Ninety-five paraffin-embedded colon cancer samples resected from 2009-2011 at Department of gastroenterology, the First Affiliated Hospital, Fujian Medical University were obtained. No patients received any chemotherapy before operation. Mean age 65.5 years; male 58; female 37; Pathological stage was performed according to the criteria of the international TNM staging for colon cancer.

#### 3.2. Immunohistochemistry

Immunohistochemistry was performed using the labeled streptavidin-biotin immunoperoxidase technique to determine the expression of *CRKL*. Four-micrometer thick sections of formalin-fixed and paraffin-embedded samples were mounted on saline-coated glass slides, deparaffinized in xylene and rehydrated through a graded series of ethanols. They were microwaved in 10 mM citrate-phosphate buffer (pH6.0) for antigen retrieval for 15 min for *CRKL*, then incubated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, followed by bovine serum albumin (BSA) for 10 min to block non-specific binding. Next, the sections were incubated with mouse anti-human *CRKL* monoclonal antibody (BD corporation) diluted 100-fold in Tris-buffered saline pH 7.6 (TBS) with 1%BSA, in a humidified chamber at 4°C overnight. All slides were incubated in sequence with secondary biotinylated antibody for 10 min and peroxidase-labeled streptavidin for 10 min using an LSAB kit (DAKO Corp). Finally, sections were visualized by 3,3'-diaminobenzidine (DAB, DAKO Corp) and counterstained with hematoxylin. A negative control was performed with serial sections, omitting incubation with the primary antibody.

#### 3.3. Animals and cells

SW1116 cell line was purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Science (Shanghai, China). The SW1116 cell line and cells stably transfected with pSilencer-CRKL and pSilencer-NC were cultured routinely in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin and 100U/ml Penicillin in a humidified cell incubator with an atmosphere of 5% CO<sub>2</sub> at 37°C (16, 17). 4-week-old male nude mice were purchase from Shanghai Experimental Animal Center of Chinese Academy of

Sciences (Shanghai, China). SW1116 cells (2×10<sup>6</sup> cells/mouse) stably transfected with pSilencer-CRKL and pSilencer-control vector were subcutaneously injected into 4-week-old male nude mice. The mice were euthanized 21 days after injection, and the tumors were removed and weighed.

#### 3.4. Western blotting

Cells were lysed in lysis buffer AM1 (Active Motif corporation), protein concentration was measured by BCA Protein Assay kit (PIERCE corporation), 50µg protein was loaded onto 12.5%SDS-PAGE, transferred onto PVDF membranes after electrophoresis. After being blocked in TBST (20mM Tris/137mM, NaCl/0.1%, Tween20, pH7.6) with 5% skim milk for 2h at room temperature, membranes were incubated with primary antibody *CRKL* (1:200) (Santa Cruz, USA) (1:1000), and GAPDH (Santa Cruz, USA) (1:5000). Then the membranes were further probed with horseradish peroxidase-conjugated secondary antibody (Abcam, USA). Immobilon Western chemiluminescent HRP Substrate (Millipore, USA) was used to detect the protein quantity and GAPDH was used for loading control.

#### 3.5. Cell proliferation assay

Cells stably transfected with pSilencer-CRKL and pSilencer-NC (Shanghai, GenePharma) were cultured in 96-well microtiter plates 4×10<sup>3</sup> cells/well with an atmosphere of 5% CO<sub>2</sub> at 37°C. At 0 hour, 24 hour, 48 hour, 72 hour and 96 hour after cell culture, according to the protocol of CCK8 Assay Kit (Dojindo, Japan).

#### 3.6. Cell migration and invasion assay

Migration of the transfected cells was carried out by using the QCM™ 24-Well Colorimetric Cell Migration Assay Kit (Millipore, USA) according to the production instructions. Cells transfected with pSilencer-CRKL and pGU6/GFR/Neo/NC Cells were incubated in serum free medium for 24 hours. 3×10<sup>4</sup> cells in 300µl serum free medium were added to the upper chamber, and 10% FBS-containing medium was used as chemoattractant in the lower chamber. After another 24 hours, cells migrated to the bottom of the membrane were stained for 20 minutes, and then, were checked and counted within at least three random field at 100×magnification of microscope. For cell invasion assay, a Cell Invasion Assay Kit (Millipore, USA) was used and the cells were treated similar to the cell migration assay, with precoated ECMatrix™ added to the upper chamber. After an incubation of 48 hours at 37°C, the invasive cells were fixed, stained and counted as mentioned above.

#### 3.7. Apoptosis detection

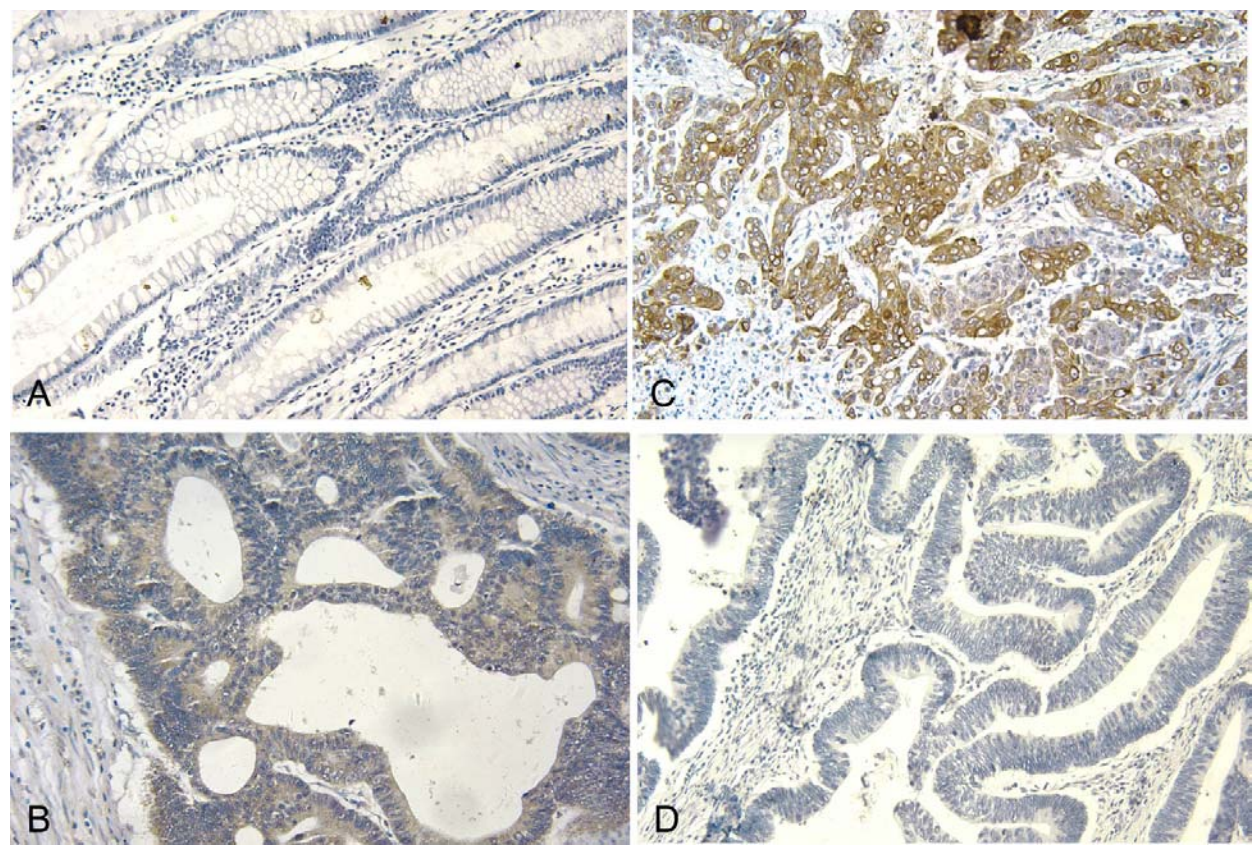
Cell apoptosis was assessed by using Annexin V kit (BD corporation); cells were harvested, washed in PBS and labeled with Annexin V antibody and propidium iodide according to the manufacturer's protocol. Finally, they were subjected to flow cytometry to determine the extent of cell apoptosis.

#### 3.8. Statistical analysis

All above experiments were repeated three times. Data were analyzed with SPSS, version16.0. *t*-test and *X*<sup>2</sup>

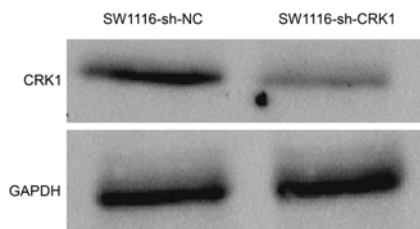
**Table 1.** Correlation between patient’s clinicopathological characteristics and the expression level of CRKL measured by immunohistochemistry

Parameters	CRKL expression		<i>P</i> value
	negative	positive	
Age(years)			
<60	36	8	0.681
>60	40	11	
Gender			
Male	46	12	0.833
Female	30	7	
Size(cm)			
<10	50	13	0.828
>10	26	6	
Depth of wall invasion			
T1	11	1	0.381
T2	20	3	
T3	25	7	
T4	20	8	
Lymph node metastasis			
negative	42	11	0.836
positive	34	8	
Pathologic stage			
Stage I	11	3	0.964
Stage II	33	7	
Stage III	21	6	
Stage IV	11	3	
Tumor differentiation			
Well differentiation	47	6	<0.001
Bad differentiation	29	13	

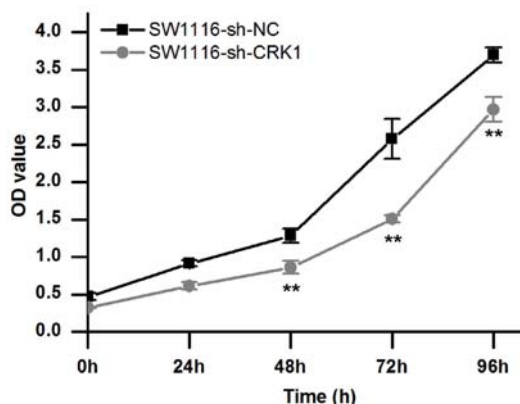


**Figure 1.** Immunohistochemistry showed CRKL expression level is associated with certain clinicopathological parameters of colon cancer. A. and B. Immunohistochemical staining of CRKL positive expression and in human colon cancer tissue and negative expression in matching noncancerous mucosa (x200). C. CRKL positive staining in a bad-differentiated colon cancer (x200). D. CRKL negative staining in a well-differentiated colon cancer (x200).

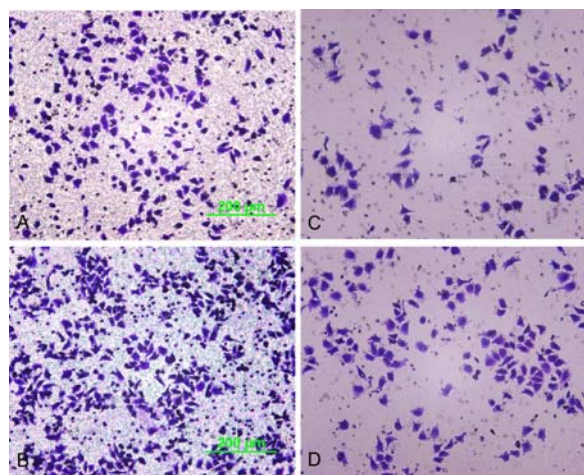




**Figure 2.** CRKL expression level decreased in pSilencer-CRKL transfected cell line. Western blotting showed that CRKL level decreased obviously at 72 hour after transfection in pSilencer-CRKL colon cells line.



**Figure 3.** Down-regulation of CRKL expression inhibits the colon cancer cell proliferation. CCK8 assay suggested that SW1116 cells transfected with pSilencer-CRKL grew significantly slower than the control group (\*\* $P < 0.001$ ).



**Figure 4.** Down-regulation of CRKL expression inhibits migration and invasion of colon cancer cells *in vitro*. A-B. The number of migrating SW1116 cells in pSilencer-CRKL group was less than that of pSilencer-NC group, \*\* $P < 0.001$ . C-D. The number of invading SW1116 cells in pSilencer-CRKL group was less than that of pSilencer-NC group, \*\* $P < 0.001$ . test was applied to evaluate the difference of pSilencer-CRKL and pSilencer-NC group.  $P$  value less than 0.05 was considered statistically significant.

## 4. RESULTS

### 4.1. Immunohistochemistry showed CRKL expression level is associated with certain clinical pathological parameters of colon cancer

Immunohistochemistry was applied to investigate the CRKL protein level in colon cancer, the results showed that, in whole selected 95 cases, the positive rate of CRKL expression in tumor tissues is 20.00%(19/95), compared with 8.42% in adjacent noncancerous tissues (8/95) ( $P = 0.022$ ). In 19 cases of CRKL positive staining tumor tissue, all the cases showed only cytoplasmic staining, clinicopathological characteristics of all 95 patients included in this study are listed in table 1 to reveal their association with the CRKL protein expression: CRKL was shown to have linkage to tumor differentiation. ( $P < 0.001$ ) (Table 1, Figure. 1).

### 4.2. CRKL expression level decreases in pSilencer-CRKL transfected cell line

To determine the effect of shRNA on CRKL depletion, we collected cell samples at 72h after transfection, extracted protein and performed standard western blotting. CRKL protein level was reduced 61.36% compared with the control group, which indicated the CRKL expression or its function was blocked by shRNA in most of cells (Figure. 2)..

### 4.3. Down-regulation of CRKL expression inhibits the colon cancer cell proliferation

CCK8 assay was applied to determine if the CRKL depletion can affect the tumor cell proliferation, the proliferation curves (represent three kind of treatments) suggested the CRKL depleted cells divided slower than control group ( $P = 0.0003$ ) (Figure.3).

### 4.4. Down-regulation of CRKL expression inhibits migration and invasion of colon cancer cells *in vitro*

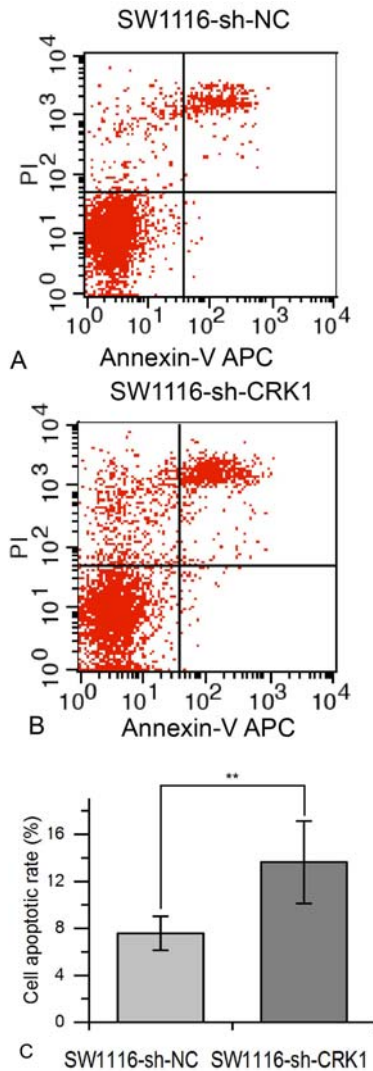
Cell migration and invasion assay indicated that the number of migrating SW1116 cells in pSilencer-CRKL group was  $48.20 \pm 5.07$ , while  $171.20 \pm 20.02$  in pGU6/GFR/Neo/NC ( $P < 0.001$ , Figure.4A-B). The number of invading SW1116 cells in pSilencer-CRKL group was  $97.40 \pm 4.34$ , while  $42.60 \pm 11.59$  in pGU6/GFR/Neo/NC (\*\* $P < 0.001$ , Figure.4C-D).

### 4.5. Down-regulation of CRKL expression induces colon cancer cells apoptosis

To evaluate the role of CRKL in tumor cell fate, CRKL depleted cells labeled with Annexin V antibody were subjected to FCM to assay the apoptosis degree. The results showed that, at 72h, more cells tend to apoptosis in CRKL depleted cells than the control group. (Apoptosis rate:  $13.63 \pm 3.5$  vs  $7.59 \pm 1.45$ , \*\* $P < 0.001$ , Figure.5).

### 4.6. Down-regulation of CRKL expression inhibits tumorigenicity *in vivo*

After injecting SW1116 cells, tumor size was measured at 7 th、14th and 21th day. The mean weight of pSilencer-CRKL group was  $1.16 \pm 0.36g$ , obviously lower than the control group  $2.14 \pm 0.58g$  (\* $P < 0.01$ , Figure. 6D). Accordingly, based on the mean volume of tumor, the



**Figure 5.** Down-regulation of CRKL expression induces colon cancer cells apoptosis. A-B. Representative histograms depicting apoptosis of SW1116 cells transfected with pSilencer- pSilencer-NC. Cells were stained with PI and Annexin V-FITC at 48h post-transfection. C. The percentage of apoptotic cells of three independent experiments  $\pm$  S.D. are shown \*\* $P < 0.001$ .

growth curve displayed the reduction of pSilencer-CRKL group (\* $P < 0.01$ , Figure. 6C).

## 5. DISCUSSION

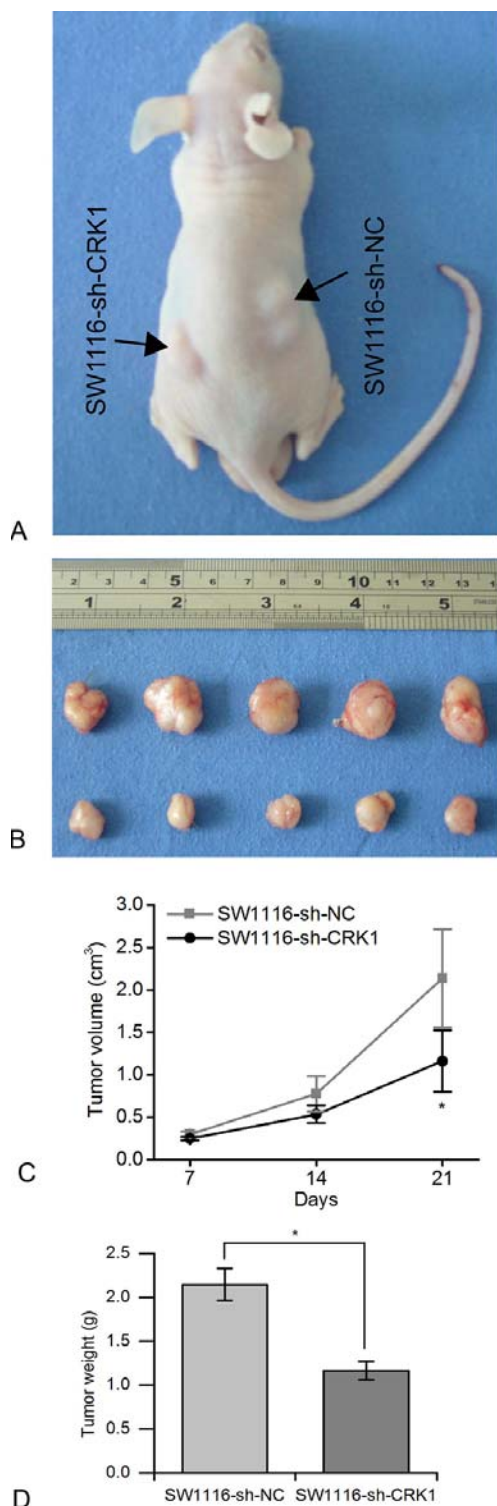
Colon cancer is still one of the most common cancer in many areas of the world, Like other malignant tumor, the origin and progression of colon cancer are mostly concerned to the abnormal cell signal transduction pathways-Ras/MAPK, JAK/STAT, PI3K, etc. (18-20). Human CRKL was proved to be the “switch” and the adaptor of these pathways (1-3). The variation of CRKL in these tumors was illustrated to contribute to tumorigenesis even affect tumor biobehaviours including breast, ovarian, lung and gastric tumors (7, 10-12). Furthermore, CRKL

was also proved to be involved in the construction of tumor microenvironments and finally affected the origin and progression of tumor (21, 22). But up to now, whether and how it exerts its effect on colon cancer remains unclear. In this study, experiments *in vitro* and *in vivo* were performed to identify whether CRKL be involved in the colon cancer.

There were studies so far in multiple tumors, including non-small cell lung cancer (11), gastric cancer (12), squamous cell carcinomas of the head and neck (13), epithelial ovarian carcinoma (10), which proved the overexpression of CRKL in these tumor tissues. In our experiment, quantitative detection of protein by immunohistochemistry showed CRKL was also overexpressed in colon cancer tissue compared with the normal epithelial mucosa, which is in line with previous studies. So we speculate that CRKL overexpression may globally exist in solid malignant tumors. Further analysis in our study indicated that the expression of CRKL was correlated with the tumor differentiation. Consistent with previous studies, CRKL also seems to be related to particular pathological index and presents its certain oncogene characteristic. The most important functions of a clinical staging, tumor invasion and Lymph node metastasis are to estimate the prognosis accurately and to guide treatment decision-making, so the relationship between CRKL expression and pathological parameter in our study would make CRKL beneficial on judging colon cancer biological behavior.

In order to assess functions of CRKL in tumor progression, CRKL knockdown cell line (pSilencer-CRKL) model was established. CRKL protein level decreased 72h after addition of siRNA targeting CRKL, indicating that CRKL was wiped off effectively and continuously in the pSilencer-CRKL cells and did benefit to a serial of subsequent observations.

Being the “switch” and adaptor of multiple cell signal transduction pathways, Recent investigations proved that variation of CRKL can lead to the downstream abnormality of cell signal transduction even tumorigenesis (7-9), but whether and how CRKL exerts its effect on colon cancer cell remain unclear. Our *in vitro* and *vivo* experiments showed while CRKL gene was knocked down, growth, motion and invasion of colon cancer cells-sw1116 was markedly inhibited, this results confirmed our speculation that CRKL is involved in the colon cancer progression. Besides, more apoptosis rate was also observed in FCM assay and the rate of tumor growth in nude mice slowed down. Up to now, the relation between CRKL and apoptosis mostly focus on the leukemia cells (23, 24), CRKL was reported to be the adaptor of RAS/MAPK、JAK/STAT、PI3K pathways, which up-regulate the expression of apoptosis-inhibited gene bcl-2 (6, 25), so we speculate that once the pathways were “switched off” or attenuated by the CRKL downregulation, the expression of bcl-2 was inhibited respectively and brought about the occurrence of apoptosis. The association of CRKL with apoptosis in our study suggested CRKL may also be pivotal to the colon cancer survival and it is worthwhile to clear up weather this phenomenon widely



**Figure 6.** Down-regulation of CRKL expression inhibits tumorigenicity *in vivo*. A-B. Photographs of tumors derived for pSilencer-CRKL or pGU6/GFR/Neo/NC transfected SW1116 cells in nude mice. C. Growth kinetics of tumors in nude mice. Tumor diameters were measured every 7 days \* $P < 0.01$ . D. Average weight of tumors in nude mice. Means  $\pm$  S.D. were shown, \* $P < 0.01$ .

exists in other solid tumors and the pathway via which the apoptosis occurred.

Our study indicates CRKL may be vital to the progression and survival of colon cancer cells and provides a new candidate target gene in solid tumors for cancer research and clinical practice.

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**Key Words:** Colon Cancer Cells, CRKL, Cell Growth, Migration, Invasion; Apoptosis

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