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CRKL plays a pivotal role in tumorigenesis of head and neck squamous cell carcinoma through the regulation of cell adhesion

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ABSTRACT

The signaling adapter protein CRK is an indispensable molecule involved in regulating the malignant potential of human cancers. CRK-like (CRKL) is a hematopoietic cell-dominant homologue of CRK that is reported to be phosphorylated by BCR–ABL tyrosine kinase in chronic myelogenous leukemia patients, but its biological function in non-hematopoietic tumors remains unclear. In this study, we explored the tumorigenic role of CRKL in head and neck squamous cell carcinoma (HNSCC) *in vitro* and *in vivo*. Immunoprecipitation analysis of HNSCC cell line, HSC-3 cells, showed that the dominant binding partner for C3G was CRKL, not CRK. To clarify the molecular function of CRKL, we established lentiviral shRNA-mediated CRKL-knockdown HNSCC cell lines. In CRKL-knockdown HSC-3 and HSC-4 cells, cell growth and motility were diminished compared to control cells. Cell adhesion assays showed that cell attachment onto both fibronectin- and collagen-coated dishes was significantly suppressed in CRKL-knockdown HSC-3 cells, while no significant change was observed for poly-L-lysine-coated dishes. Immunofluorescence staining revealed that focal adhesion was reduced in CRKL-knockdown HSC-3 cells. With a pull-down assay, CRKL-knockdown HSC-3 cells showed decreased amounts of active Rap1 compared to control cells. Moreover, in an *in vivo* assay, tumor formation of CRKL-knockdown HSC-3 cells in nude mice was significantly abrogated. Our results indicate that CRKL regulates HNSCC-cell growth, motility, and integrin-dependent cell adhesion, suggesting that CRKL plays a principal role in HNSCC tumorigenicity.

1. Introduction

Signaling adaptor protein CRK, which carries Src homology (SH) 2 and SH3 domains, was originally identified as avian sarcoma virus CT10 (chicken tumor 10)-encoding oncogene product v-Crk [1], and was followed by isolation of its mammalian homologues, CRKI, CRKII, and CRKL (CRK-like) [2,3]. CRKI consists of one SH2 domain and one SH3 domain, while CRKII has an additional SH3 domain. CRK associates with $p130^{Cas}$ and paxillin through its SH2 domain and transmits signals to multiple downstream effectors by SH3 domain-binding proteins including C3G and DOCK180 [4–6], which are guanine nucleotide exchange factors (GEF) for small molecular-weight GTPases, including Rap1, R-Ras, and Rac [7–9]. CRK was shown to play an essential role in the malignant potential of various human tumors including ovarian cancer, synovial sarcoma, glioblastoma, and head and neck squamous cell carcinoma (HNSCC) [10–13].

The CRKL protein has high sequence identity within the SH2 and SH3 domains of CRKII, despite their distinct gene locations, and was identified as a major substrate of the BCR–ABL tyrosine kinase in chronic myelogenous leukemia [14,15]. A previous report showed that phosphorylated CRKL activates Ras and Jun kinase signaling pathways and transforms mouse fibroblasts in a BCR–ABL-dependent fashion [16]. Recently, Kim et al. [17] used siRNA and CRKL overexpression experiments to show that CRKL plays important roles in non-small-cell lung carcinoma cells *in vitro*. Wang et al. [18] reported that both CRK and CRKL presented with higher expression in ovarian cancer tissues than those in normal and benign ovarian tissue by immunohistochemical analysis. The detailed oncogenic function of CRKL including *in vivo* analysis in non-hematopoietic tumors has not been clarified.

Abbreviations: HNSCC, head and neck squamous cell carcinoma; KD, knockdown; GEF, guanine nucleotide exchange factor.

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Head and neck tumors include cancers of the upper aerodigestive tract, paranasal sinuses, and salivary glands, of which squamous cell carcinoma is the most common histological type. The predilection sites of HNSCC have crucial functions for speech, swallowing, taste, and smell, thus organ preservation is a key point for HNSCC treatment. Although HNSCC has been treated by surgical intervention, radiation, chemotherapy, and a combination of these therapies, the majority of HNSCC patients presents with locoregionally advanced disease, which leads to poor prognosis [19–22]. Therefore, defining the molecular mechanisms involved in HNSCC pathogenesis and identification of new drug targets is of critical importance.

In this study, we explored the alternative function of CRKL related to HNSCC carcinogenesis through *in vivo* and *in vitro* experiments.

2. Materials and methods

2.1. Cell lines

The HNSCC cell lines HSC-2, HSC-3, HSC-4, SAS, OSC-20, and Ca9–22 were kindly provided by Dr. Masanobu Shindo (Department of Oral Pathology & Biology, Hokkaido University Graduate School of Dental Medicine). All of the cells described above were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 100 U/ml penicillin and streptomycin.

2.2. Establishment of CRKL-KD HNSCC cell lines

We employed BLOCK-iT™ HiPerform™ Lentiviral Pol II miR RNAi Expression System with EmGFP (Invitrogen, Carlsbad, CA) for knockdown of CRKL expression. The target sequences (codons 1019-, 1064-, and 1205-) were determined by BLOCK-iT™ RNAi Designer (Invitrogen), and oligonucleotides were subcloned into the pLenti6.4/R4R2/V5-DEST MultiSite Gateway® vector. pcDNA™ 6.2-GW/EmGFPmiR-neg control (Invitrogen) was used as a negative control and contained random oligonucleotide that failed to target any known vertebrate gene. For generation of infectious lentiviral particles, pLenti6.4/R4R2/V5-DEST vectors were co-transfected with ViraPower packaging plasmids mixture: pLP1, pLP2, and pLP/VSV-G into 293 FT cells using lipofectamine 2000 (Invitrogen). Transduction of miRNA viruses to HNSCC cell lines HSC-3 and HSC-4 was conducted following the manufacturer's protocol.

2.3. Antibodies

Antibodies were obtained from the following sources: anti-CRKL (C20), C3G (C19), DOCK180 (H4) (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-phosphotyrosine (PY20), anti-paxillin, anti-p130^{Cas}, anti-CRK, anti-Rap1 (Transduction Laboratories, Lexington, KY, USA); and alpha-tubulin (B5-1-2) (Sigma, St. Louis, MO, USA).

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation, protein determination, SDS–PAGE, and immunoblotting were carried out as described previously [23]. Briefly, cells were lysed with lysis buffer [1% Triton X-100, 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate (Na_3VO_4), and protease inhibitor mixture (Complete, EDTA-free®, Roche Molecular Biochemicals, Germany)] for 30 min on ice and centrifuged at 20,600*g* for 10 min at 4 °C. Supernatants were incubated with the appropriate antibody at 4 °C with gentle shaking for 1 h followed by incubation with protein A/G Sepharose (Amersham Biosciences, Piscataway, NJ, USA) for 1 h at 4 °C. After washing with lysis buffer, the precipitants were analyzed by immunoblotting with antibodies.

2.5. Pulldown assay for Rap1 activity

Pulldown assay was performed as described previously [23]. Briefly, cells were lysed with buffer containing 50 mM Tris–HCl (pH 7.4), 500 mM NaCl, 10% glycerol, 1% NP40, 2.5 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor mixture. Lysates were centrifuged at 14,000*g* at 4 °C for 5 min, and the supernatants were incubated with 10 μg GST-RalGDS-RBD at 4 °C for 1 h and glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, England) at 4 °C for 1 h. The resulting precipitants were analyzed by immunoblotting with anti-Rap1 antibody.

2.6. Immunofluorescence staining of cultured cells

Subconfluent cells were plated on 35 mm glass-based dishes coated with collagen and fibronectin. Sixty minutes after plating on collagen and two hours after plating on fibronectin, cells were fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature, permeabilized with 0.1% Triton X-100 in PBS for 4 min at room temperature and incubated with 1% bovine serum albumin in PBS for 20 min at room temperature. To visualize focal adhesion, cells were stained with anti-paxillin overnight, then with Alexa Fluor 594-conjugated anti-mouse IgG antibodies (Molecular Probes, Invitrogen) for 1 h at room temperature and observed under a confocal laser scanning microscope (FV-300, Olympus, Tokyo, Japan) [10,24].

2.7. Cell adhesion assay

For analysis of cell adhesion, 4×10^4 cells were seeded on 96-well plates coated with collagen, fibronectin, or poly-L-lysine (PLL), and incubated for 10 and 20 min (collagen), and 20, 40, and 60 min (fibronectin and PLL) at 37 °C. The bound cells in each well were lysed, stained with 0.04% crystal violet, and quantified by spectrophotometry at OD 595 nm [10].

2.8. Wound healing and cell growth assay

For analysis of cell motility, cells were seeded onto 10 cm diameter plates in DMEM with 10% FBS overnight. The injury line was made with a blue tip 1 mm in width on the confluent cell monolayer. At 4, 8, and 12 h, the lengths of the movement were measured [25]. In order to measure growth rate, 1×10^5 cells were seeded onto 60 mm diameter plates with DMEM with 1% FBS, and the numbers of cells were counted 1, 3, and 5 days after seeding using a hemocytometer (Fisher Scientific, Japan).

2.9. In vivo HSC-3 cell tumor formation assay in nude mice

pcDNA™ 6.2-GW/EmGFPmiR-neg transduced negative control cells (Mock), and CRKL-KD cells (CRKLi1064) were employed and 3×10^6 cells were subcutaneously injected into 6-week-old female nude mice, BALB/cA Jcl-nu/nu (Clea Japan, Inc., Tokyo, Japan). Three mice were euthanized 19 days after cell injection and tumors were removed and weighted.

3. Results

3.1. Head and neck squamous cell carcinoma expresses CRKL, which is a dominant binding partner for C3G

Immunohistochemical analysis of human HNSCC specimens revealed CRKL and CRK overexpression in tumor cells compared to normal squamous epithelium (Supplementary Fig. 1A–L). The positive rate of CRKL and CRK were recognized 74% (26 of 35) and 66% (23 of 35) (Supplementary Fig. 1M). The staining intensity and proportion of CRKL and CRK showed no correlation with clinical parameters, including clinical stage, T classification, and N classification (data not shown). To investigate the functions of CRKL in HNSCC, we analyzed the protein levels of CRKL and its related molecules such as CRKI, CRKII, DOCK180, and C3G in HNSCC-derived cell lines, including HSC-2, HSC-3, HSC-4, SAS, OSC20, and Ca9–22 (Fig. 1A). In addition to the histological analysis, CRKL and CRKI/II expression was found in all HNSCC cell lines examined. No significant differences in the protein expression levels of CRKL, CRKI, and CRKII were observed in these cell lines, while the expression levels of DOCK180 and C3G, downstream molecules for CRK and also CRKL, were relatively increased in HSC-3 and HSC-4 cell lines. The tyrosine-phosphorylation status of estimated $p130^{Cas}$ was increased in HSC-3, Ca9–22, and SAS cell lines (Fig. 1B). To explore the contribution of CRK-associated molecules in signal transduction, we performed co-immunoprecipitation using anti-CRKL and CRK antibodies. The amount of C3G bound with CRKL was significantly higher than that with CRK in the HSC-3 cell line, while $p130^{Cas}$ was precipitated in equal amounts with both CRKL and CRK (Fig. 1C). Taken together, a preferential role for CRKL in HNSCC is suggested, especially in association with C3G.

3.2. CRKL-knockdown HSC-3 cells showed decreased cell motility and adhesion

To investigate the molecular function of CRKL, we established CRKL-knockdown (KD) HSC-3 and HSC-4 HNSCC cells using shRNA for human CRKL. CRKL was significantly depleted in both cell lines, although CRKI and CRKII levels were not affected (Fig. 2A). Since CRK was already known to contribute to cell adhesion and motility [10–12], we investigated whether CRKL has similar biological mechanisms. A cell adhesion assay revealed that cell attachment onto both fibronectin- and type I collagen-coated dishes was significantly suppressed in CRKL-KD HSC-3 cells compared to control cells, while no significant change was observed for poly-L-lysine-coated dishes (Fig. 2B). Immunofluorescence staining using anti-paxillin antibody showed that the formation of focal adhesions was diminished in CRKL-KD cells compared to control cells (Fig. 2C). Furthermore, when cells were plated onto fibronectin-coated dishes and incubated for 30, 60, and 120 min, a decrease in the levels of active Rap1 was observed in CRKL-KD cells (CRKLi1064) compared to control cells (Fig. 2D). These results suggest that CRKL regulates integrin-dependent cell adhesion through the C3G–Rap1 pathway. To clarify the association of CRKL with cell motility, a wound healing assay was next performed. Wound recovery was significantly decreased in both CRKL-KD HSC-3 and HSC-4 cells (Fig. 3), which suggests a significant role for CRKL in regulating cell motility.

3.3. Knockdown of CRKL diminished cell growth in vitro and suppressed tumor-forming ability in vivo

Since CRK was reported to play an important role in cell growth [10,11], we investigated the role of CRKL in cell proliferation using CRKL-KD HSC-3 and HSC-4 cells. Compared to control cells, growth rates of both CRKL-KD HSC-3 and HSC-4 cells were significantly diminished 3 and 5 days after seeding (Fig. 4A and B), suggesting that CRKL regulates cell growth *in vitro*. For further investigation of the tumor-forming potential of CRKL-KD cells, we injected control and CRKL-KD (CRKLi1064) HSC-3 cells into the subcutis of nude mice (the number of injected cells = 3×10^6 each). Nineteen days after cell injection, the tumor size produced by CRKL-KD HSC-3 cells was significantly smaller than that of control HSC-3 cells, which suggests that CRKL-KD suppresses tumor-forming ability *in vivo* ($n = 3$, Fig. 4C and D).

4. Discussion

Previously, we showed that CRK is overexpressed in various human cancers and sarcomas by immunohistochemical analysis [26]. Furthermore, we clarified that multiple cellular functions including adhesion, motility, growth, and tumor-forming ability *in vivo* were decreased in CRK-KD malignant tumor cells [10–12]. In this study, we demonstrated that CRKL-KD-HNSCC cells showed suppressed

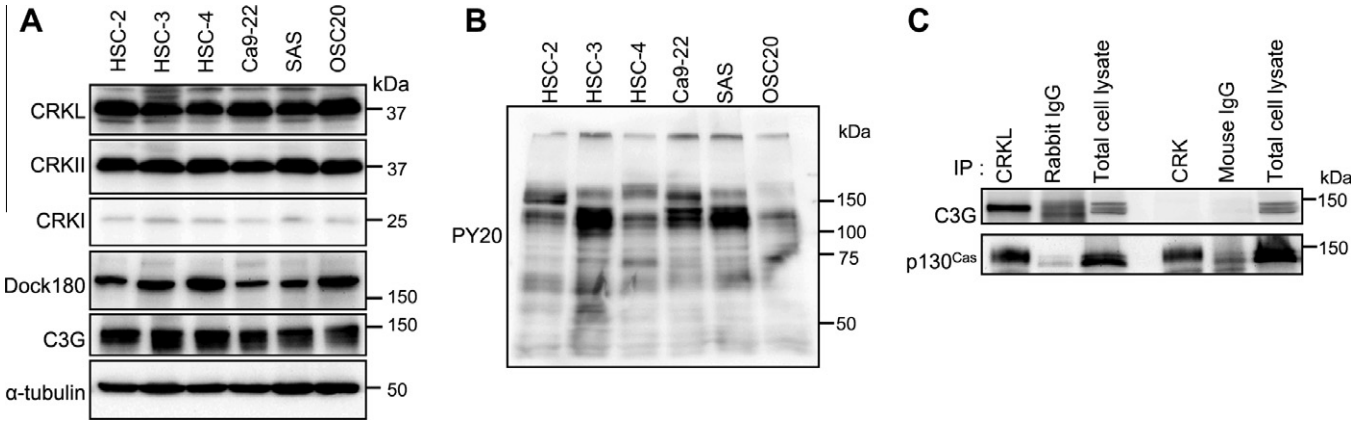

Fig. 1. Head and neck squamous cell carcinoma expresses CRKL, a dominant binding partner for C3G. (A) Expression of CRKL and its related molecules in head and neck squamous cell carcinoma (HNSCC) cell lines. No significant differences in the protein expression levels of CRKL, CRKI, and CRKII were observed in the six HNSCC cell lines, HSC-2, HSC-3, HSC-4, Ca9–22, SAS, and OSC20. The expression levels of DOCK180 and C3G, downstream molecules for CRK and CRKL, were relatively increased in HSC-3 and HSC-4 cell lines. (B) Immunoblotting analysis with anti-tyrosine-phosphorylation antibody (PY20) revealed the tyrosine-phosphorylation status of estimated $p130^{Cas}$ to be increased in HSC-3, Ca9–22, and SAS cell lines. (C) HSC-3 cell extracts immunoprecipitated with anti-CRKL and anti-CRK antibodies were analyzed by immunoblotting using anti-C3G and anti-$p130^{Cas}$. The amount of C3G bound with CRKL was significantly higher than those with CRK in HSC-3 cells. (Abbreviations: IB, immunoblotting; IP, immunoprecipitation.)

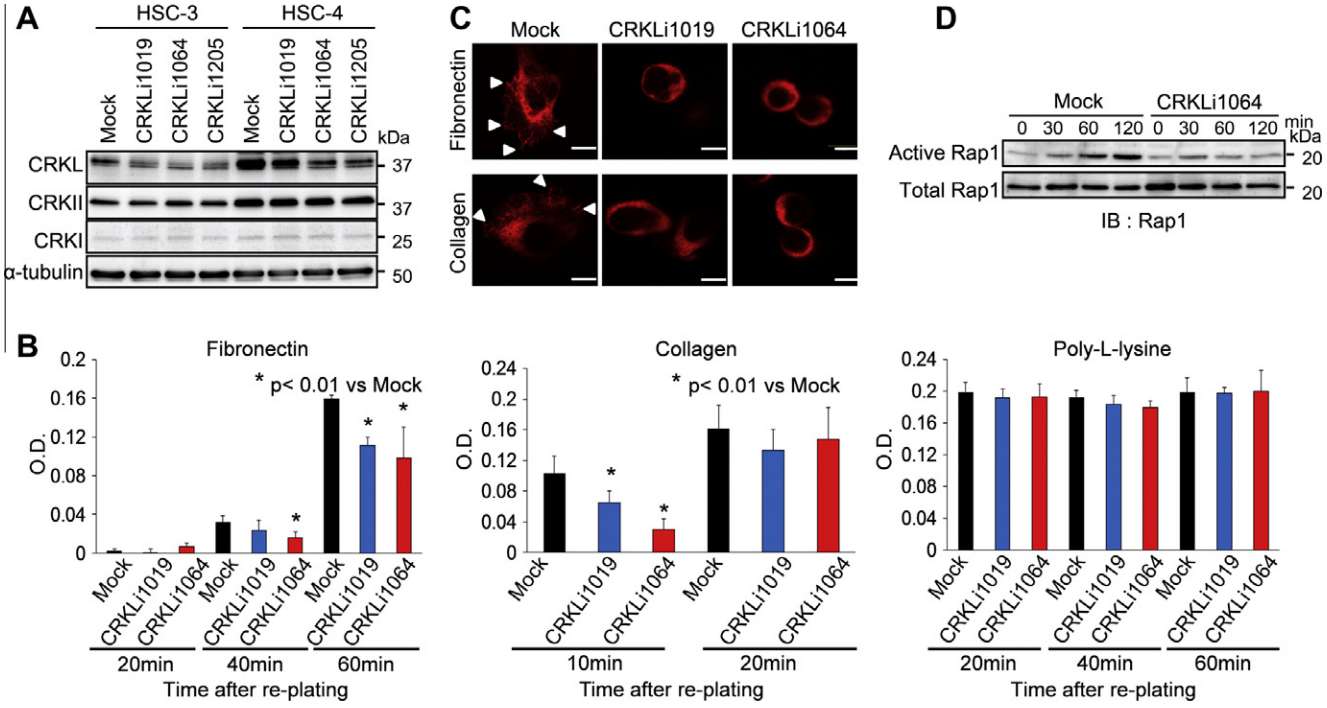

Fig. 2. shRNA-mediated CRKL-knockdown HNSCC cells showed suppressed integrin-dependent focal adhesion. (A) CRKL-knockdown HSC-3 and HSC-4 cells were established using short-hairpin RNA for human CRKL (CRKLi1019, 1064, 1205). CRKL was depleted in both cells, although CRKI and CRKII were not affected. (B) HSC-3 cells were plated on dishes coated with fibronectin, collagen or poly-L-lysine. After 10, 20, 40, or 60 min incubation, the number of attached cells was quantified as described in Section 2. Cell attachment onto both fibronectin- and type I collagen-coated dishes was significantly suppressed in CRKL-knockdown HSC-3 cells compared to control cells, while no significant change was observed on the poly-L-lysine-coated dish. (C) Cells cultured on glass-based dishes coated with fibronectin or collagen were subjected to immunofluorescence staining with anti-paxillin antibody. The formation of focal adhesions (white arrowheads) was diminished in CRKL-knockdown HSC-3 cells compared to control HSC-3 cells (original magnification 600×, Scale bars = 10 μm). (D) Rap1 activity was measured by pulldown assay. A decrease in the levels of active Rap1 was observed for CRKL-knockdown cells (CRKLi1064) compared to control cells.

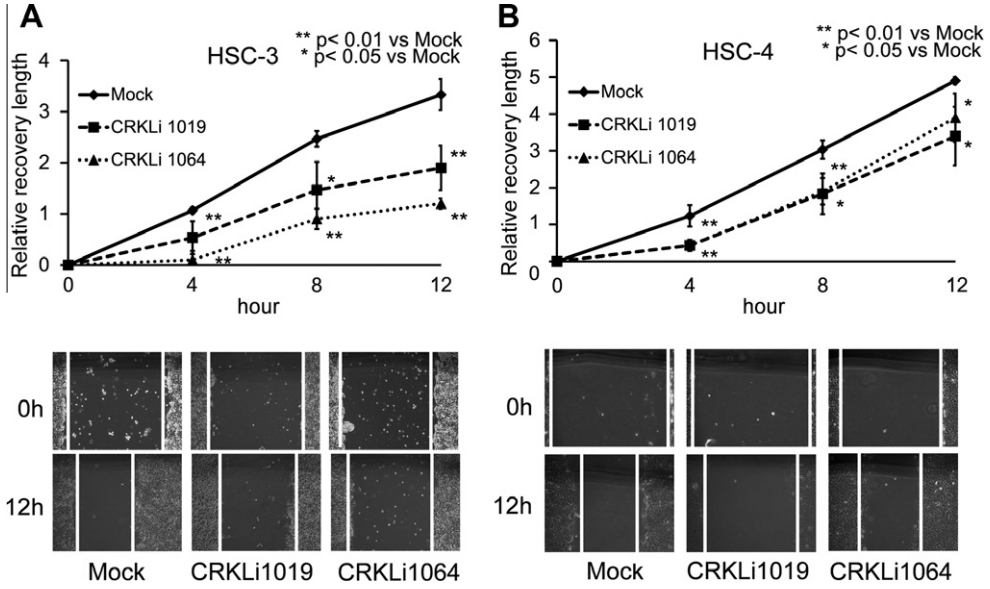

Fig. 3. CRKL-knockdown suppressed cell motility of HSC-3 and HSC-4 cells. (A and B) Cell motility was examined 0, 4, 8, and 12 h after scraping, and results are displayed as the mean ± SE of three independent experiments. The recovery of the wound was significantly decreased in both CRKL-knockdown HSC-3 (A) and HSC-4 cells (B).

cell motility, cell adhesion, cell growth, and tumor-forming ability *in vivo*, indicating that CRKL has cellular functions similar to CRK. In fact, CRKL contains one SH2 and two SH3 domains that have high sequence similarity to those of CRKII [3]; thus the structures and functions of these molecules through their SH domains should be similar. Meanwhile, immunoprecipitation analysis revealed that in HSC-3 cells the dominant binding partner for C3G was CRKL, not CRK. These results indicate that the functional priority for CRK and CRKL may be determined by tissue- or cell-specific molecular selection in addition to expression profile. Since the gene loci of CRKL and CRK are completely distinct [3,27], the functional priority for CRKL is likely to be important for HNSCC-carcinogenesis at a

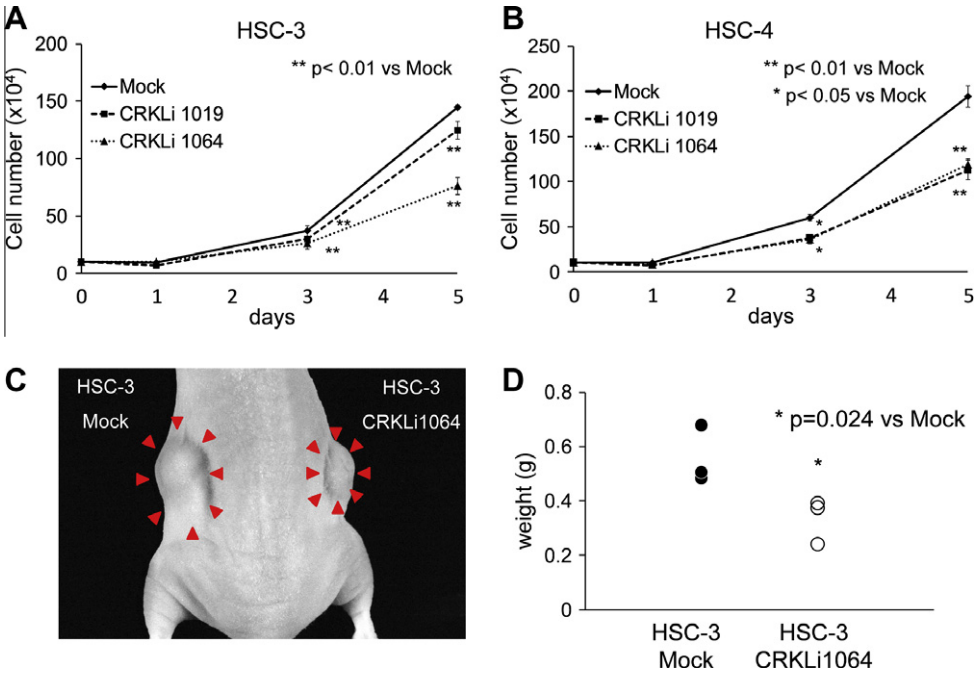

Fig. 4. CRKL-knockdown HNSCC cells showed not only diminished cell growth *in vitro* but also suppressed tumor-forming ability *in vivo*. (A and B) Cell numbers were counted 1, 3, and 5 days after plating and results are expressed as the mean ± SE of three independent experiments. Compared to control cells, growth rates of CRKL-knockdown HSC-3 (A) and HSC-4 (B) cells were significantly diminished at 3 and 5 days after seeding. (C and D) *In vivo* tumor formation assay in nude mice using HSC-3 cells (Mock, $n = 3$; CRKLi1064, $n = 3$). Nineteen days after injection of 3×10^6 cells into the subcutis of nude mice, the tumor size derived from CRKL-knockdown HSC-3 cells (C, right, red arrowheads) was smaller than that from control cells (C, left, red arrowheads). The tumor weight of CRKL-knockdown HSC-3 cells was significantly smaller than that of control HSC-3 cells (D). The results are represented as the mean ± SE of three experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

genetic level. Here we also explored similar expression profiles of both CRKL and CRK in HNSCC by immunohistochemistry, although Yamada et al. [13] indicated the prominent expression and function of CRKII in HNSCC. They employed the CRKII-specific antibody which does not recognize CRKI and revealed the clinicopathological significance of CRKII in HNSCC, including clinical stage and patient's survival. However, in our analysis, we failed to show any statistical correlation between the expression of CRK and clinical parameters (data not shown). This discrepancy might be explained that we evaluated the total expression level of both CRKI and CRKII using the antibody against CRK which recognize both CRKI and CRKII.

C3G was identified as one of two major proteins bound to the SH3 domain of the CRK oncogene product [6]. C3G is a guanine nucleotide exchange factor (GEF) for Rap1, and is activated via CRK adaptor protein. Rap1 was shown to contribute to integrin-mediated cell adhesion [28,29], and C3G-dependent Rap1 activation promotes cell adhesion and cell spreading, but represses cell migration [17]. In this study, CRKL-KD decreased the levels of active Rap1, and suppressed not only cell adhesion on fibronectin- and collagen-coated dishes, but also cell growth. These results suggest that the integrin-p130^{Cas}–CRKL–C3G–Rap1 signaling pathway plays a pivotal role in cell growth through the regulation of cell adhesion in HNSCC. In contrast, we observed that levels of active Rac1 were not decreased in CRKL-KD HSC-3 cells compared to control cells (data not shown). This result supports the immunoprecipitation analysis results, which revealed that DOCK180, the other of the two major proteins bound to the SH3 domain of the CRK oncogene product and a specific GEF for Rac, did not bind to CRKL or even CRK in HSC-3 cells (data not shown). These results suggest: (1) there is a minimal contribution of DOCK180 to CRKL-dependent tumorigenesis in HSC-3 cells; and (2) the presence of CRK-independent signaling pathway that regulates DOCK180-Rac activation in these cells.

In summary, we explored the pivotal role of CRKL in HNSCC tumorigenesis and obtained results that suggest that CRKL may be a potential molecular target for HNSCC diagnosis and novel therapeutic strategies as well as CRK.

Disclosure

The authors have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.12.142.

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