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14. ABSTRACT

We have established a relationship between Crk protein expression and cell proliferation in basal breast cancer. Stable knockdown of Crk protein expression using shRNA in multiple basal breast cancer cell lines decreased both adhesion-independent growth *in vitro* and proliferation *in vivo*. Immunohistochemistry to CrkI/II, CrkL, and the proliferative marker Ki67 revealed a positive correlation between Crk protein levels and the proliferative index of human basal tumors in a tissue microarray. The association of a Crk gene expression signature with basal tumors and poor prognosis further supports a role for Crk-dependent signaling in the growth of basal tumors. These findings support a requirement for Crk protein expression in basal tumor progression, and identify Crk proteins as an attractive subject for further study and therapeutic intervention in basal tumors.

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Introduction

Crk, or CT10 regulator of kinase, was originally identified as an oncogene fusion product of the CT10 chicken retrovirus (v-Crk), of which there are three cellular homologues: CrkI, CrkII, and CrkL. Crk adaptor proteins contain SH2 and SH3 domains, which allow them to engage in multiple signaling pathways by mediating protein-protein complex formation. Crk proteins have been shown to act in many signaling pathways that are known to be important in breast cancer progression, and are overexpressed in multiple cancer types, including breast. Consequently, the purpose of this project is to establish the mechanistic role of Crk proteins in breast cancer progression. This is particularly important given that our recent data suggests that Crk proteins specifically contribute to tumorigenic growth of the basal subtype of breast cancer, which has the worst prognosis due to a lack of effective targeted therapy. Elucidating the role of Crk proteins in basal breast cancer could lead to a better understanding of this subtype and potential therapeutic targets. We will use stable shRNA-mediated knockdown of Crk in a panel of breast cancer cell lines to determine cellular processes that consistently require Crk proteins. We will use rescue and overexpression of individual Crk proteins and Crk protein mutants to determine the specific Crk proteins and Crk protein domains responsible for a tumorigenic phenotype. We will identify the Crk protein-protein interactions involved in mediating this phenotype, which could represent novel therapeutic targets. These studies will expand our current knowledge of adaptor protein function as well as basal breast cancer cell signaling. We will use multiple cell lines and focus on *in vivo* assays to maximize the applicability of our findings to understanding human breast cancer.

Body

Aim 1: Validate the role for Crk proteins in breast tumor growth and metastasis. (SOW months 1-6, Completed)

Task 1: All animal experiments received ethical approval (protocol #4170).

Task 2: Generate knockdown of all three Crk proteins in other basal breast cancer cell lines. We intended to use retrovirus to deliver shRNA targeting CRKI/II and CRKL into SUM1315 and BT-20 basal breast cancer cell lines. However, due to slow growth rates, stable BT-20 cell lines were difficult to produce using a retroviral approach. We were successful in producing stable knockdown of CrkI/II and CrkL in SUM1315 cells. In the future, we will increase the scope of our findings by expanding our study to other breast cancer cell lines using lentivirus to generate stable cell lines.

Task 3: Investigate whether Crk knockdown affects tumor growth, metastasis, and metabolism in SUM1315 and BT-20 cells.

SUM1315 and MDA-231 1833TR cells were used to study the effects of Crk knockdown on tumorigenic growth. Detailed findings are published in (*1*) and figures can be viewed in the attached manuscript reprint. A role for Crk proteins in breast tumorigenic growth was confirmed, accomplishing Aim1 of the SOW. *In vivo* growth assays were used to monitor tumor outgrowth of 1833TR cells, and showed delayed outgrowth with Crk knockdown. Tumors that did form showed re-expression of Crk proteins at endpoint, suggesting that Crk proteins are absolutely required for tumor formation. To better study the role of Crk in the developing tumor we analyzed *in vivo* growth of 1833TR and SUM1315 cells 3 days after injection into the mammary fat pad. Both cell lines showed decreased proliferation with Crk knockdown. Given that tumor outgrowth at the primary site is impaired by Crk knockdown, we elected not to perform cardiac injection metastasis assays using the SUM1315 stable cell lines, because interpretation of results would be confounded due to differential outgrowth at the metastatic site. We have established that Crk knockdown in the 1833TR cell line decreased formation of bone metastasis after cardiac injection, but this approach cannot differentiate between decreased outgrowth and impairment at other steps of the metastatic cascade. Although we have not yet performed cardiac injection with the SUM1315 cell line, Crk knockdown did decrease migration and invasion *in vitro*, which are important cellular processes in metastatic dissemination.(*1*) (figures and detailed methods in appended manuscript)

In addition to validating a requirement for Crk protein expression for *in vivo* proliferation and tumor formation of basal cell lines, we also expanded these findings to human tumors. Detection and quantification of Crk proteins levels and Ki67 proliferative marker by immunohistochemistry on two independent tissue microarrays of human breast tumors showed that Crk protein levels are higher in grade III tumors compared to grade I. Importantly, within high grade, basal tumors Crk protein levels exhibit a significant positive correlation with proliferation. This suggests that Crk-dependent signaling could function in basal tumors to contribute to proliferation. Further support of a role for Crk-mediated signaling specifically in basal breast cancer was obtained by comparing gene expression changes mediated by Crk to gene expression profiles of human breast tumors. A Crk gene expression signature was generated from the genes most differentially expressed in a luminal breast cell line, T47D, overexpressing CrkII. When this Crk signature was applied to previously published breast cancer datasets, there was a significant association with high grade, basal tumors and decreased disease-free survival. Therefore, we have shown that Crk protein expression is correlates with proliferation in human basal tumors, associates with gene expression patterns most similar to human basal tumors, and is required for *in vivo* tumor formation and bone metastasis of basal cell lines.(*1*) (figures and detailed methods in appended manuscript)

[Unpublished data] We are still in the process of studying the metabolic changes associated with Crk knockdown. In our original narrative we showed that Crk knockdown in the 1833TR cell line increases mitochondrial respiration, which is consistent with a reversion away from typical cancer cell metabolism. We hypothesized that Crk proteins may be critical to adhesion-independent growth and tumor formation by promoting a metabolic phenotype that is better able to support proliferation(*2*). We have since found that Crk knockdown also greatly increases production of reactive oxygen species (ROS), a major source of which is mitochondrial respiration (Figure 1). NMR metabolomics confirmed changes in metabolite levels, including decreased glucose and lactate levels with Crk knockdown. These findings support a role for Crk proteins in maintaining the primarily glycolytic metabolism that supports the biosynthetic requirements of rapidly proliferating cells. To study the mechanistic cause of this switch in metabolic phenotypes with Crk knockdown, the activation of proteins known to regulate cell metabolism were examined by Western blot. Preliminary data suggests that Crk knockdown decreases activation of an Akt/mTOR signaling pathway, which has been shown to mediate the glycolytic, proliferative metabolism commonly seen in cancer cells(*3*). Crk proteins are known to act in signaling from many different kinases, so kinase inhibitors were used to identify potential Crk signaling pathways involved in mediating adhesion-independent growth and the associated metabolic phenotype in 1833TR cells (Figure 2). Cells were grown as single cells in suspension on polyhema-coated dishes to prevent attachment, and Crk knockdown greatly diminished proliferation in these conditions. In control and CrkI/II rescue cells, inhibition of Met and Abl did not affect proliferation in suspension, but inhibition of PI3K and mTOR abrogated adhesion-independent growth of 1833TR cells. This finding confirms a requirement for PI3K/Akt/mTOR signaling in proliferation of 1833TR cells, but further study is necessary to determine whether Crk proteins promote adhesion-independent growth and glycolytic metabolism through activation of this pathway. Interestingly, hormone receptor-negative breast tumors exhibit a lower frequency of PI3K/Akt/PTEN mutations(*4*, *5*), leading to the hypothesis that Crk could be required for activation of this pathway, specifically in basal tumors. We will now expand these studies to a greater number of breast cell lines to validate both the role of Crk in activation of this pathway, and the requirement of Crk-dependent Akt/mTOR signaling in the ability of different breast cell lines to grow as tumors *in vivo* and under adhesion-independent conditions *in vitro*. Understanding the role of Crk in metabolism and mTOR signaling could identify a subset of Crk-overexpressing breast tumors with therapeutic response to inhibition of these pathways. Our work on the metabolic phenotype associated with levels of Crk protein expression has not differed from the intentions outlined in the SOW, but has been extended to include more mechanistic studies, which will continue as we perform experiments with Crk protein mutants and protein-protein interaction studies as outlined in the SOW.

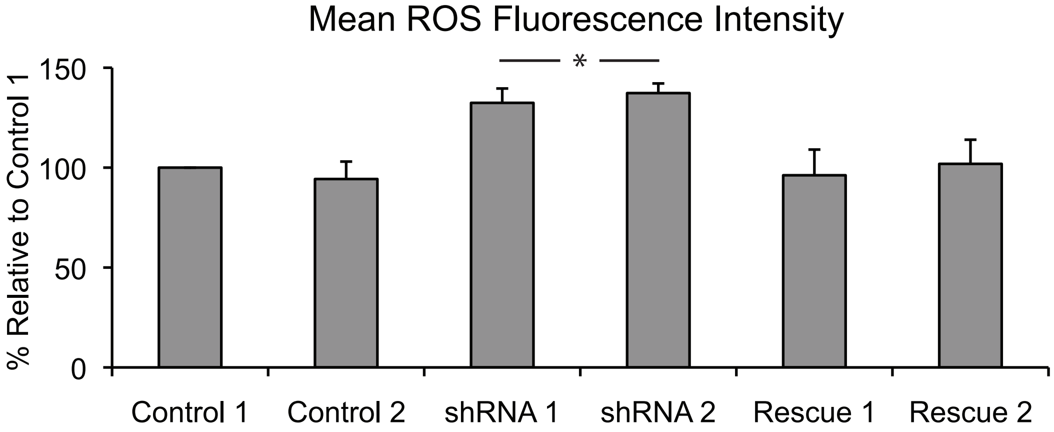

Figure 1. **Knockdown of Crk adaptor proteins increases levels of reactive oxygen species.** MDA-231 1833TR cells with control vector (Control), Crk knockdown (shRNA), or re-expression of CrkI/II (Rescue) were incubated at 37°C for 30 minutes in 10uM CM-H2DCFDA before analysis by flow cytometry. n=3, * indicates $p<0.05$. [Unpublished data]

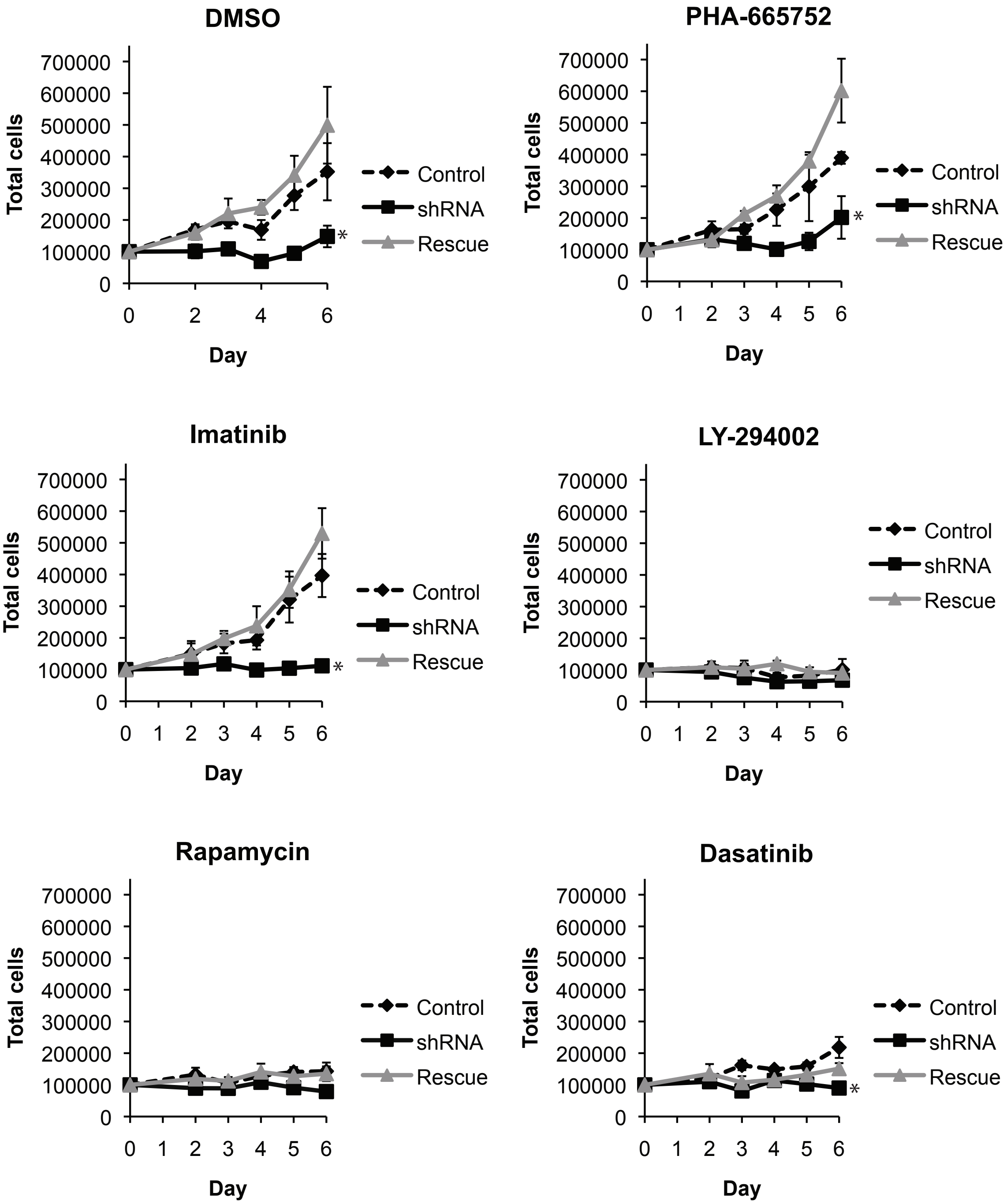

Figure 2. Affect of Crk knockdown and kinase inhibitors on MDA-231 1833TR proliferation in suspension. MDA-231 1833TR cells stably expressing vector control (Control), CrkI/II and CrkL shRNA (shRNA), or rescue of CrkI/II expression in shRNA cell lines (Rescue) were grown in suspension on dishes coated with polyhema to prevent attachment. Cells were collected and counted by hemacytometer at time points indicated. Treatments included DMSO control or inhibition of Met (0.2uM PHA-665752), Abl (4uM Imatinib), PI3K (20uM LY-294002), mTOR (0.1uM Rapamycin), and Src (0.1uM Dasatinib). n=3, * indicates $p<0.05$. [Unpublished data].

Aim 2: Determine which Crk proteins, and specifically which Crk protein domains, are required to mediate the ability of Crk to promote metastasis, primary tumor growth, and a transformation-associated metabolic phenotype. (SOW months 1-20, In progress)

Task 4: Rescue knockdown of each Crk protein individually in MDA-231 1833TR cells. Retroviral constructs were obtained to allow stable expression of Crk proteins. Site-directed mutagenesis was used to introduce silent mutations in the region targeted by the shRNA that was used to generate the Crk knockdown cell lines. CrkI and CrkII were successfully reintroduced into the Crk knockdown 1833TR cells(*1*). CrkL was not efficiently expressed, and so new lentiviral constructs were obtained to perform individual rescue of each Crk protein. CrkL is introduced efficiently with this new construct.

Task 5. Determine Crk proteins required for metastasis.

Metastasis studies have not yet been performed due to delays in establishing stable cell lines with rescue of each Crk protein. The impact of individual Crk proteins on metastasis will be assessed by monitoring liver and lung metastasis occurring from mouse mammary fat pad xenograft growth of breast cell lines containing knockdown, overexpression, and rescue of individual Crk proteins. This approach will combine studies of primary outgrowth with analysis of the ability of cells to complete the full metastatic cascade, and will minimize the number of animal subjects required for experiments.

Task 6. Determine Crk proteins required for primary tumor growth.

Reintroduction of CrkI and CrkII into the 1833TR Crk knockdown cell lines was sufficient to rescue *in vivo* proliferation and *in vitro* adhesion-independent growth(*1*). This was a key finding for demonstrating that defects in tumorigenic growth were specifically due to a lack of Crk proteins. Aim 2 is not yet complete, as we have determined that CrkI/II protein expression is sufficient to rescue *in vivo* proliferation, but have yet to determine which individual Crk proteins are required to mediate tumorigenesis and phenotypes associated with metastasis and metabolism. Due to difficulties with expression of CrkL, studies with rescue of individual Crk proteins were not conducted. Lentiviral constructs have since been obtained and used with high efficiency to introduce CrkL into cell lines. Currently, 12 different breast cancer cell lines have been infected with lentiviral constructs for knockdown of CrkI/II and CrkL, and overexpression of CrkI, CrkII, and CrkL. The use of multiple cell lines will allow more general conclusions about the roles of Crk proteins in breast cancer than could be obtained using only the 1833TR and SUM1315 cell lines.

Task 7. Determine Crk proteins involved in metabolic regulation.

Experiments studying the role of individual Crk proteins in mediating an aggressive cancer cell metabolic phenotype have not yet been conducted due to delays in establishing stable cell lines with rescue of each Crk protein. However, re-expression of CrkI/II restored levels of reactive oxygen species to those seen in control 1833TR cells (Figure 1). This suggests that the metabolic reprogramming of these cells is both reversible and sensitive to Crk levels. The Crk proteins able to promote a proliferative metabolic phenotype will be determined by rescue with individual Crk proteins.

Task 8. Determine which domains of Crk proteins of interest are required to rescue metastatic, tumorigenic, and metabolic phenotypes. (methods will be better defined based on findings in milestones 5- 7)

The Crk proteins found to be required and sufficient to rescue tumorigenic growth and metabolism in Crk knockdown cells will be further studied to determine which specific domains are necessary to mediate this phenotype. Mutated regions tested will include the SH2 and SH3 domains, and previously identified proline-rich and phospho-tyrosine motifs(*6*-*8*).

Aim 3: Determine key protein-protein interactions important in mediating the effects of Crk proteins on metastasis, tumor growth, and metabolism. (SOW months 20-36, In progress)

Task 9. Set up the TAP method to study Crk proteins.

Cloning required to set up experiments has been initiated. Further work on Aim 3 will be based on results obtained during the completion of Aim 2. Identification of Crk-binding partners interacting with the full length Crk protein, but not with Crk mutants that are unable to rescue tumorigenic phenotypes, will identify Crk signaling proteins with a potential role in promoting tumor growth. The role of these proteins in Crk-mediated tumorigenic growth will be validated using shRNA, and possibly blocking peptides to disrupt binding to Crk(*9*).

Training Program

- Attended workshop on ethics in science (to understand ethical issues and associated protocols for research involving human material)
- Attended knowledge translation workshop (to improve ability to communicate scientific results to people in other fields and occupations)
- Attended bioinformatics workshop (to understand the basic principles and capabilities of working with R software for analysis of data)
- Attended numerous seminars from visiting lecturers in oncology and other areas of biochemistry (Seminars given by job candidates applying to the department were of particular interest to see the type and extent of work presented by early career scientist applying for a position)
- Course work required for the McGill biochemistry program is complete
- Attended McGill Annual Research day
- Presented progress to my Research Advisory Committee (received advice and approval to move forward with experiments)
- Presented a “Work in Progress” seminar to the McGill Goodman Cancer Centre
- Involved in the process of writing, submitting, and revising a scientific paper, which was then accepted for publication

Key Research Accomplishments

1. Crk protein expression in breast tumors is associated with high grade.
2. Crk protein expression positively correlates with proliferative index within high grade basal breast tumors.
3. A Crk gene expression signature is associated with high grade and basal subtype breast tumors.

4. Stable shRNA-mediated knockdown of CrkI/II and CrkL in basal breast cell lines decreases cell migration, cell invasion, and adhesion-independent growth.
5. Crk knockdown in basal breast cancer cell lines decreases *in vivo* proliferation and tumor formation.
6. Crk protein levels are a consistent determinant of p130Cas phosphorylation in breast cancer cell lines and human breast tumors.

Reportable Outcomes

Manuscript published in *Breast Cancer Research* **14**, R74 (2012).
<u>Crk adaptor proteins act as key signaling integrators for breast tumorigenesis.</u>
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[†] Authors contributed equally

Conclusions

Crk adaptor protein expression is elevated in many cancer types, drawing attention to potential roles for these proteins in oncogenic signaling(*10*). We have demonstrated that Crk proteins play a key role in adhesion-independent growth and *in vivo* proliferation of breast cancer cell lines, and have established a correlation between Crk protein level and proliferation in human basal breast tumors(*1*). This work has extended previous studies of the role of Crk proteins in cell migration and invasion (*11*) to include an unexpected requirement for Crk proteins in tumor formation of the aggressive basal 1833TR cell line(*12*). Interestingly, recent work in non-small cell lung cancer has identified CrkL amplification in a subset of tumors and cell lines, and cell lines harboring this amplification exhibit oncogene addiction to CrkL for cell proliferation(*13*). Importantly, in response to EGFR inhibition, overexpression of CrkL promotes resistance and maintenance of Erk and Akt activation. This suggests that Crk proteins are able to initiate or amplify oncogenic signals in the absence of an upstream activation event. Determining whether Crk proteins also play this role in breast cancers will have implications in breast cancer therapy and treating resistance. Our initial data implies that Crk proteins are important oncogenes in basal breast cancer, and our study of Crk knockdown and overexpression in a panel of breast cell lines will provide a system to identify the relationship between Crk protein levels, Akt activation, and response to inhibitors. Our preliminary data suggests a requirement for Crk proteins for Akt activation in adhesion-independent growth of the basal 1833TR cell line [unpublished data]. Basal tumors have a lower frequency of mutations that activate PI3K/Akt(*4*, *5*), which led us to hypothesize that Crk proteins are important for activation of this pathway specifically in the basal breast cancer subtype.

Since the discovery of v-Crk(*14*), the central mystery surrounding the role of Crk as an oncogene has been to understand how a protein with no catalytic activity can act in transformation and tumor formation. In a traditional view of Crk signaling, Crk proteins bind through their SH2 domain to phosphorylated tyrosine motifs in response to growth factor receptor or integrin activation, and Crk SH3-binding partners are then recruited into signaling complexes. This allows Crk proteins to have highly pleiotropic roles in signal transduction(*15*). Crk proteins have been shown to play downstream roles in a variety of signalling pathways known to be important for breast cancer progression, such as Met, EGFR, ErbB2, and integrins(*16*-*19*). However, novel functions of Crk proteins, such as integrin activation by extracellular Crk-binding(*20*), continue to be discovered, suggesting that there is still much to learn about the role of these proteins in normal and cancer cell signaling. Our approach to identifying proteins binding to full length Crk and Crk mutants could discover new binding partners, and will shed light on the Crk protein-protein interactions involved in mediating particular cellular processes. Understanding the mechanisms through which Crk adaptors can promote aggressive cancer cell phenotypes is of relevance not only to breast cancer, but also to other cancer types in which Crk proteins are overexpressed.

The molecular events contributing to development of the basal subtype of breast cancer are still poorly understood, identifying a particular need to understand the role of Crk in this system.

Recent advances have also been made in understanding the structural organization of Crk proteins. Despite a high degree of homology within the functional domains of CrkII and CrkL, the linker regions are highly variable and affect protein conformation and binding. In contrast to the relatively open conformation of CrkL, CrkII adopts a compact structure that partially blocks binding to the SH3 domain (*21*, *22*). These findings suggest that although CrkII and CrkL are very similar proteins they may have distinct roles in signaling pathways. Given that both proteins are overexpressed in different human cancers, there is a need to define the cellular processes and signaling pathways that each protein promotes. We will determine the primary roles of individual Crk proteins in breast cancer by shRNA-mediated knockdown of all Crk proteins in a panel of breast cell lines followed by rescue with individual Crk proteins. We will be able to determine the phenotypes and cell signaling mediated by each Crk protein. Differences in function between CrkI, CrkII, and CrkL will be of great interest to the field of adaptor proteins, and will identify which protein(s) would be best to explore as therapeutic target(s). The ability of CrkI/II re-expression to rescue *in vivo* proliferation of Crk knockdown 1833TR cells lends support for this approach, and suggests that either CrkI or CrkII may be able to compensate for loss of other Crk proteins and promote tumorigenic growth.

Our data has demonstrated the importance of Crk proteins in basal breast cancer cell proliferation. We will extend these studies to a panel of cell lines in order to make a more general statement about the role of Crk proteins in specific breast cancer subtypes. We will also determine the cellular processes and signaling promoted by individual Crk proteins. The identification of specific Crk protein-protein interactions that are required for an aggressive cancer cell phenotype could identify new therapeutic targets for basal breast cancer treatment. Our studies will answer key questions that have recently emerged in the field of Crk adaptor proteins, and provide a better understanding of basal breast cancer cell signaling.

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RESEARCH ARTICLE **Open Access**

Crk adaptor proteins act as key signaling integrators for breast tumorigenesis

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Abstract

Introduction: CT10 regulator of kinase (Crk) adaptor proteins (CrkI, CrkII and CrkL) play a role in integrating signals for migration and invasion of highly malignant breast cancer cell lines. This has important implications, as elevated CrkI/II protein levels were observed in a small cohort of breast cancer patients, which identified a potential role for Crk proteins in breast cancer progression. Numerous *in vitro* studies identified a role for Crk proteins in cell motility, but little is known about how Crk proteins contribute to breast cancer progression *in vivo*.

Methods: The clinical significance of Crk proteins in human breast cancer was assessed by analyzing published breast cancer datasets using a gene expression signature that was generated following CrkII over-expression and by examining Crk protein expression in tissue microarrays of breast tumors ($n = 254$). Stable knockdown of Crk (CrkI/CrkII/CrkL) proteins was accomplished using a short hairpin RNA (shRNA)-mediated approach in two basal breast cancer cell lines, MDA-231 1833TR and SUM1315, where the former have a high affinity to form bone metastases. Both *in vitro* assays (cell migration, invasion, soft agar growth) and *in vivo* experiments (intra-cardiac, tibial and mammary fat pad injections) were performed to assess the functional significance of Crk proteins in breast cancer.

Results: A gene signature derived following CrkII over-expression correlated significantly with basal breast cancers and with high grade and poor outcome in general. Moreover, elevated Crk immunostaining on tissue microarrays revealed a significant association with highly proliferative tumors within the basal subtype. RNAi-mediated knockdown of all three Crk proteins in metastatic basal breast cancer cells established a continued requirement for Crk in cell migration and invasion *in vitro* and metastatic growth *in vivo*. Furthermore, Crk ablation suppressed anchorage independent growth and *in vivo* orthotopic tumor growth. This was associated with diminished cell proliferation and was rescued by expression of non-shRNA targeted CrkI/II. Perturbations in tumor progression correlated with altered integrin signaling, including decreased cell spreading, diminished p130Cas phosphorylation, and Cdc42 activation.

Conclusions: These data highlight the physiological importance of Crk proteins in regulating growth of aggressive basal breast cancer cells and identify Crk-dependent signaling networks as promising therapeutic targets.

Introduction

Crk, or CT10 regulator of kinase, was originally isolated as the oncogene fusion product of the CT10 chicken retrovirus (v-Crk) [1]. Cellular homologues of v-Crk include the c-Crk gene, which encodes two alternatively spliced mRNAs that give rise to two proteins (c-CrkI and c-CrkII) and a second gene, c-CrkL [2]. Crk adaptor proteins contain Src homology 2 (SH2) and Src homology 3 (SH3) domains, which mediate the formation of protein-protein complexes. As adaptor proteins, Crk proteins are considered highly pleiotropic, as they have been proposed to regulate cell migration, invasion, and survival downstream of integrins and various receptor tyrosine kinases [3]. For instance, in response to hepatocyte growth factor, Crk adaptor proteins are required for the dispersal of organized epithelial colonies, formation of lamellipodia and the breakdown of adherens junctions, events that are critical

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to tumor cell dispersal and, hence, cancer progression [4]. Furthermore, over-expression of Crk promotes an invasive phenotype regardless of upstream signaling, which identifies Crk as a potential key regulator of cell invasion [5]. In support of this, transient knockdown of CrkI/II resulted in significant inhibition of migration and invasion of multiple malignant breast (MDA-231, MDA-435s) and other human cancer cell lines (HeLa, H1299, KB) [6], confirming that CrkI/II are critical integrators of upstream signals for cell migration and invasion and highlighting a potential role for these adaptor proteins in metastatic spread.

Elevated expression of Crk proteins has also been linked to cell transformation, with CrkI exhibiting the highest transforming potential. CrkI, CrkII and CrkL over-expressing fibroblasts proliferate in soft agar, although only CrkI over-expressing fibroblasts form tumors in nude mice [2,7,8]. However, a mouse model over-expressing CrkL exhibited an increased incidence of hematopoietic and epithelial cancers and a mammary mouse model over-expressing CrkII was associated with altered mammary gland development and accelerated tumor development [9,10]. Although tumor incidence was low in the latter study, the ability of CrkII to induce a branching phenotype during a normally quiescent state suggests that CrkII may play an active role in mammary epithelial proliferation and remodeling [9]. This precocious development, coupled with the fact that CrkII is downstream of several signaling proteins involved in breast cancer development, highlights the potential consequence of elevated Crk proteins in the human disease.

Elevated levels of CrkI and CrkII mRNA and protein are found in various human tumors, including glioblastoma [11], gastric and breast cancers [6,12,13]. CrkI/II mRNA expression was increased predominantly in advanced lung tumors and those associated with poor survival, implying that Crk proteins may play a key role in epithelial human cancers [14].

The complexity and multi-step nature of tumor initiation, maintenance and metastasis has hindered the elucidation of key molecular processes defining breast cancer progression. Although Crk proteins have been characterized in the context of cellular signaling, particularly through over-expression studies, and demonstrate aberrant expression in multiple cancer types, including breast, few studies have addressed the precise role of Crk proteins in breast cancer-related processes *in vivo*. Since Crk is proposed to be involved in many aspects of tumorigenesis, dissecting whether Crk is required in tumor initiation and progression is an important step towards developing targeted therapeutics for breast cancer. Here, we demonstrate that elevated Crk is significantly associated with highly proliferative breast tumors of triple negative subtype and a gene expression signature derived following CrkII over-expression correlated with basal breast cancer, implicating Crk signaling in an aggressive breast cancer phenotype. To confirm the functional significance of Crk proteins within basal breast cancer, we demonstrate that Crk proteins are critical for the growth of breast cancer cells *in vivo* at both orthotopic and metastatic sites, highlighting the physiological importance of Crk proteins in regulating cancer signaling. This data implicates Crk proteins not only in the enhanced malignancy of breast cancer, but also in an aggressive breast cancer phenotype.

Methods

Reagents, cell lines and retroviral transduction

The MDA-231 1833TR cell line was generously provided by Joan Massagué in 2004. This cell line and its triple reporter (TR) were previously described [15,16] and cells used in this study were expanded from freeze downs from 2004. The SUM1315 cell line was purchased from Asterand (Detroit, MI, USA) in 2009 and cultured as in [17] from freeze downs from 2009. Both cell lines underwent standard mouse antibody production (MAP) testing for pathogens, as well as mycoplasm testing. CrkL short hairpin RNA (shRNA) (V2HS_43900, Open Biosystems, Huntsville, AL, USA) was subcloned into LMP according to [18]. pSuperCrkI/II constructs were made according to the manufacturer's protocol. The CrkI/II shRNA sequence was based on the duplex published in [6]. Retrovirally-generated LMP or LMP-CrkL shRNA cell lines were subsequently infected with pSuper or pSuperCrkI/II. CrkI/II rescue cell lines were made using pLXSP-CrkI and pLXSH-CrkII constructs containing silent mutations in the shRNA-targeted region from site directed mutagenesis using the QuickChange Multi Site-Directed Mutagenesis Kit from Stratagene (La Jolla, CA, USA). Antibodies included: CrkI/II, Rac1 and p130Cas (BD Transduction Laboratories, Mississauga, ON, Canada), CrkII (Novus Biologicals, Oakville, ON, Canada), CrkL, Cdc42 and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-p130Cas (Cell Signaling Technology, Mississauga, ON, Canada), paxillin and alpha-tubulin (Sigma-Aldrich, Oakville, ON, Canada), and AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR, USA).

Cellular assays

Cell spreading, migration and invasion assays are described in [6]. Analysis was performed with ImageScope (Aperio, Vista, CA, USA). A minimum of 300 cells were counted per condition for cell spreading. For soft agar assays, cells were plated as in [19], except grown in 5% serum and quantified using Infinity Analyze Software (Lumenera Corp., Ottawa, ON, Canada). Immunohistochemistry and immunofluorescence were performed according to [6,9]. Human breast tumor sections for immunofluorescence were obtained courtesy of the Breast Cancer Functional Genomics Group at McGill University.

In vivo experiments

Female nude mice (six to eight weeks old, Charles Rivers) were injected with 10^6 cells into the mammary fat pad; tumor volumes were monitored bi-weekly and calculated as described [20]. Cardiac injections were performed using 10^5 cells as described [15]. Mice were sacrificed six to eight weeks post-injection. Tibial injections were performed using 10^4 cells injected directly into the marrow space. Ten mice were used per cell line and were sacrificed four weeks post-injection. Tumor outgrowth within bone was monitored according to [15]. Osteolytic lesions were blindly scored using X-ray radiographs. Total tumor area versus total tumor/bone interface was scored using ImageJ. All animal experiments were carried out in accordance with the guidelines of the McGill University Animal Ethics Committee and the Canadian Council on Animal Care as a protocol approved by the Facility Animal Care Committee (Protocols #4170 and #5562). The human tumor samples used in this study were collected from breast cancer patients at time of surgery. Tissue collection was conducted under protocols approved by the McGill University Health Centre (MUHC) Research Ethics Board (Protocols SUR-99-780 and SUR-00-966). All subjects provided written, informed consent.

Western blot analysis

Cells were serum starved for four hours, plated on collagen for 30 minutes and protein lysates were resolved by SDS-PAGE [6]. Detection of GTP-bound endogenous Rac1 or Cdc42 was performed according to [21]. Densitometry was performed using ImageJ or Odyssey (LI-COR, Guelph, ON Canada).

Crk signature and microarray profiling

A Crk signature was derived from expression analysis of T47D-CrkII cell lines [9] by selecting genes significantly differentially expressed between T47D and T47D-CrkII cell lines (FDR corrected *P*-value < 0.05 and fold change > 2, GSE ID: GSE30731). Expression of this signature was examined in breast cancer datasets and a breast cancer cell line dataset [22-27]. Molecular subtypes were determined using PAM50 centroids [23]. Patients were ordered based on correlation with the expression of the CRK signature in tumor samples. The association between patient ordering, molecular subtype and grade was determined using a Krusal-Wallis rank sum test. Analysis was conducted using Bioconductor [28].

Tissue microarrays

Tissue microarray 1 (TMA-1) consisted of 113 patients diagnosed with breast cancer at the Centre Hospitalier de l'Universite de Montreal, Hôtel Dieu, and Notre Dame and consisted of low grade (Grade I, n = 15) and high grade (Grade III, n = 74) ductal carcinomas and typical (n = 9) and atypical (n = 15) medullary carcinomas [29], approved by the research ethics committee (Centre Hospitalier de l'Université de Montréal). TMA-2 consisted of 141 cases selected from consenting patients who had undergone surgical resection for breast cancer at the Centre Hospitalier de l'Université de Montréal, Hôtel Dieu. Representative tumor areas were annotated by a pathologist, and two single core TMAs were constructed in parallel. The TMA-2 clinical parameters included samples from grade 1 (n = 17), grade 2 (n = 39), and grade 3 (n = 83) patients. Tumors were of ductal (n = 102), lobular (n = 20) or other (n = 19) morphology. Immunohistochemistry for HER2, ER and PR identified 29 triple negative breast tumors. Crk positivity and the percentage of Ki67 positive cells were assessed using ImageScope. Crk staining intensity was also reported by a pathologist and an independent observer.

Statistical analysis

A two-sample variance student's t-test was used to assess for statistical significance. Kendall's tau non-parametric statistical analysis was used to assess significance between Crk expression and Ki67 within TMA-1 and TMA-2.

Results

A CrkII-associated transcriptional signature and Crk protein expression correlate with a proliferative basal breast cancer subtype

Crk over-expression has been linked to various aspects of tumorigenesis, including cell migration, invasion and anchorage independent growth [2,7,30]. Although elevated CrkI/II proteins have been reported in a small cohort (n = 20) of breast cancer samples [6], no studies have addressed if elevated Crk, or its signaling pathways, are associated with known clinical variables or breast cancer subtype. Gene expression profiling is a valuable tool to search for links between signaling networks and defined steps of tumor progression. Thus, to investigate an association between Crk-mediated signaling with breast cancer outcome, we constructed a gene expression signature composed of 151 genes that are most differentially expressed following CrkII over-expression (Additional file 1) [9]. This CrkII transcriptional signature was derived following gene expression profiling of the breast cancer cell line T47D versus T47D cells over-expressing CrkII [9]. Expression of 'Crk signature' genes was examined in five independent datasets (Figure 1a, Additional file 2) for a total of 1,469 breast cancer patients [22-26]. In each dataset, patients were ordered by expression of Crk signature genes (Additional file 1). Notably, the 'Crk gene signature' strongly correlated with the basal molecular subtype ($P <$ 6.7e-14) in the NKI dataset (Figure 1a) [31]. In addition, there was a significant correlation with the basal subtype within the four additional breast cancer datasets examined

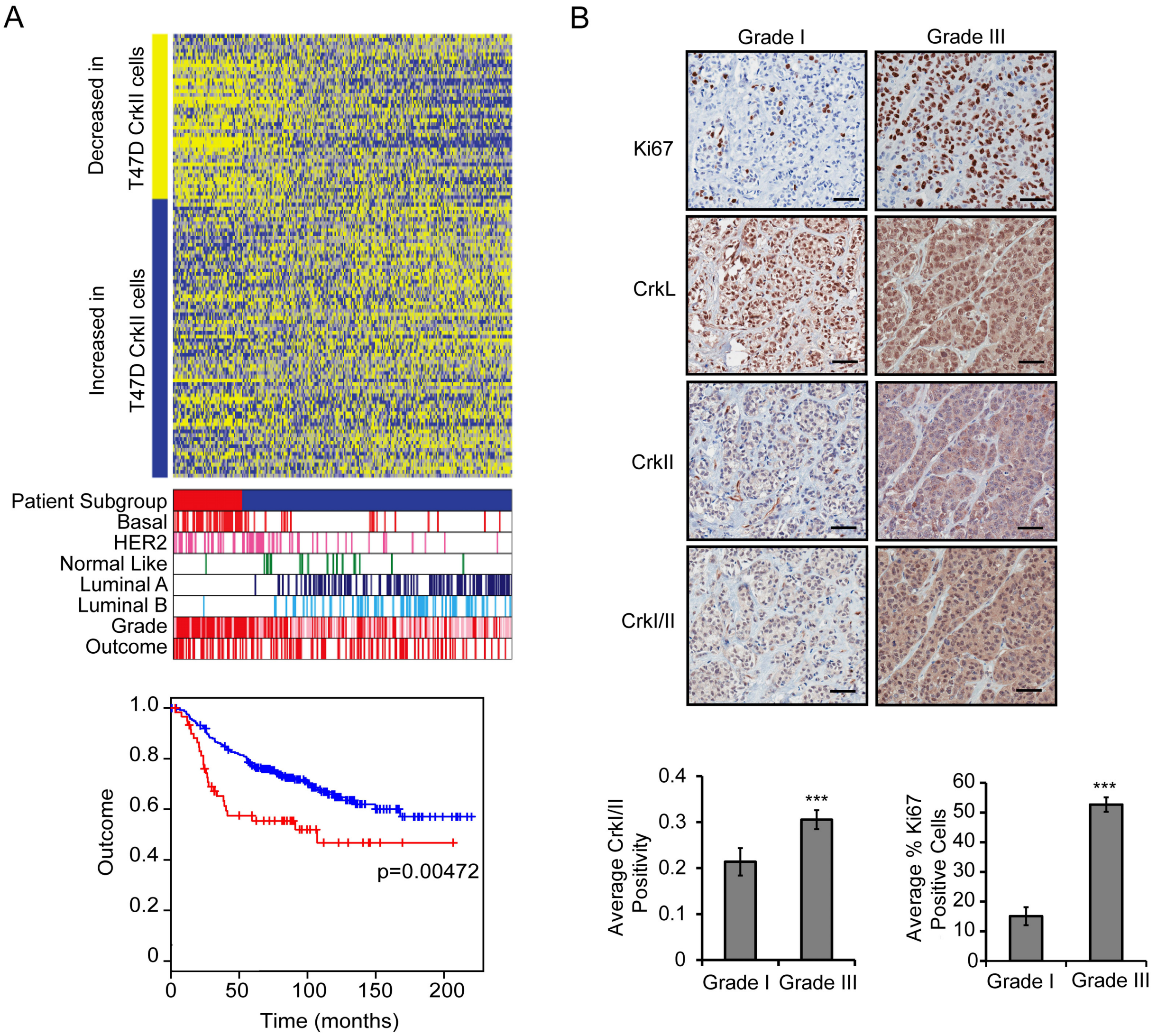

Figure 1 Crk pathway signature in breast cancer is associated with basal subtype and tumor grade. Heatmap of Crk signature in NKI breast cancer dataset. Bars on the left indicate if genes increased or decreased expression in the T47D-CrkII cell line compared to control. The patients are ordered by correlation of the expression of these 151 genes in the patients sample to the Crk signature. Patients were separated into two subgroups: high correlation (correlation > 0.17, red) and low correlation (correlation < 0.17, blue). On heatmap, blue indicates increased expression; yellow indicates decreased expression. The Crk signature was used to examine overall 20 year outcome; the red line indicates patients who exhibit the Crk gene signature, whereas the blue line represents patients whose tumors do not express the signature (**A**). TMA-1 was stained with Ki67, CrkI/II, CrkII or CrkL specific antibodies in high (Grade 3) and low (Grade 1) grade tumors. Images taken using ImageScope; scale bar represents 50 μm. TMA-1 was utilized to examine CrkI/II and Ki67 expression in relation to tumor grade. Errors bars represent standard error of the mean (SEM) (**B**). Statistically significant data is illustrated as (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

(Ivshina, Loi, Parker and Wang, Additional file 2). The Crk signature also significantly associated with high tumor grade ($P < 6.4\text{e-}12$) and poor outcome ($P = 0.00472$) within the NKI (Figure 1a) and the additional four breast cancer datasets examined (Additional file 2). Thus, a CrkII-dependent gene signature is associated with high grade, basal breast cancers and poor outcome. The T47D cancer cell line used to generate the Crk gene signature is identified as a luminal subtype [27], thus, a dataset derived from a panel of breast cancer cell lines [27] was examined to confirm that the Crk signature is indeed more common within the basal subtype. Similar to the data obtained using datasets derived from human breast cancer patients, the Crk signature more closely correlated with the two basal subtypes (Basal A and Basal B) typically found within a subset of breast cancer cell lines (Additional file 3). Since an expression signature associated with high grade basal tumors and cell lines could be generated from the luminal

T47D cell line over-expressing CrkII, it provides evidence for a role for Crk in promoting key signaling events in the progression of basal breast cancer.

To confirm whether elevated Crk proteins are associated with a breast cancer subtype or outcome, we also performed immunohistochemical staining using various Crk antibodies (CrkI/II, CrkII, CrkL C-20, CrkL H-62) on two independent human breast cancer tissue TMAs (Figure 1b, Additional file 4). TMA-1 consisted of 113 patients while the second, TMA-2, consisted of 141 primary breast cancer tissue cores. TMA-1 consisted of both low grade (Grade 1, n = 15) and high grade (Grade 3, n = 74) ductal carcinomas [29]. Within this dataset, CrkI/II protein was significantly elevated in Grade 3 tumors versus Grade 1 tumors (Figure 1b). As expected, Ki67 antigen, a measure of proliferative index, also correlated strongly with Grade 3 tumors (Figure 1b). Importantly, within the high grade basal tumors in both TMA datasets, CrkI/II and CrkL protein levels correlated with proliferation, as assessed using Kendall's tau non-parametric statistical analysis (Table 1), demonstrating a strong link between elevated Crk protein and an aggressive tumor phenotype. Together, these observations identify a significant correlation of elevated Crk protein with high grade and poor outcome subtypes in human breast cancer.

Crk proteins are required for cancer cell cytoskeletal organization, migration and invasion

As elevated Crk proteins and a Crk gene signature are associated with an aggressive and proliferative basal breast cancer phenotype as well as basal breast cancer cell lines, we sought to examine the functional significance of Crk proteins in integrating signals for breast cancer progression. Thus, shRNAs were utilized to knock down all three Crk proteins in MDA-231 1833TR cells, a metastatic subpopulation of the basal MDA-231 cell line, that has a high affinity to form bone metastases [15]. Using CrkI/II and CrkL shRNA in pSuper and LMP vectors, respectively, multiple independent 1833TR cell lines were established with stable knockdown of Crk proteins (Figure 2a).

To first assess whether loss of Crk proteins affected proliferation, an Alamar Blue assay was performed. No significant differences in cell proliferation were observed (Figure 2b). In contrast, there was a significant decrease in cell migration and invasion towards 10% fetal bovine serum (FBS) in stable Crk knockdown cells compared to control cells (Figure 2c, d). Transient knockdown of CrkI/II and CrkL in combination resulted in a greater decrease in migration and invasion than single knockdown (CrkI/II or CrkL) alone (Additional file 5), supporting that Crk proteins have an additive role in promoting cell migration and invasion, and demonstrating a requirement to knock down all three Crk proteins within the 1833TR aggressive breast cancer cell line.

Since Crk proteins are implicated in integrating signals downstream of integrins, the importance of Crk for integrin engagement was assessed by plating cells on fibronectin. When cells were grown on fibronectin, staining of paxillin, a marker of focal adhesions, demonstrated that paxillin-positive adhesions were present within control and Crk knockdown cells (Figure 2e). However, whereas lamellipodia were identified in control cells, Crk knockdown cells failed to develop polarized membrane ruffles or lamellipodia and instead, dense cortical actin was observed (Figure 2e). In addition, delayed cellular spreading was found in Crk knockdown cells 0.5 and one hour post-plating (Figure 2f), establishing that diminished Crk expression decreased integrin-mediated spreading.

As Crk has been shown to regulate Rho GTPases involved in cell spreading and migration, we examined the activity of Rac1, which is known to regulate lamellipodia formation and cell migration [32]. Using a pull-down assay, small decreases in endogenous Rac GTP levels were consistently observed in Crk knockdown cells upon plating on collagen or upon stimulation with serum (Additional file 6). In contrast, a substantial decrease in Cdc42 activation was observed following serum stimulation (Additional file 6). Thus, knockdown of Crk proteins does not significantly disrupt Rac-dependent signals, but is required for activation of Cdc42 upon serum stimulation. In conclusion, alterations in cell morphology, diminished cellular spreading, and significant decreases in cell migration and invasion *in vitro* are caused by loss of Crk

Table 1 Crk protein expression correlates with Ki67 within the basal subtype

Antibody	Correlation to Ki67 proliferation index (% cells Ki67 positive)	
CrkI/II (TMA-1)	Correlation Coefficient	0.289
	P-value (2 tailed)	0.019
	number	43
CrkI/II (TMA-2)	Correlation Coefficient	0.323
	P-value (2 tailed)	0.016
	number	28
CrkII (TMA-2)	Correlation Coefficient	0.483
	P-value (2 tailed)	0.00024
	number	29
CrkL (C-20) (TMA-2)	Correlation Coefficient	0.483
	P-value (2 tailed)	0.00024
	number	29
CrkL (H-62) (TMA-2)	Correlation Coefficient	0.547
	P-value (2 tailed)	0.00003
	number	29

CrkI/II expression was examined in relation to Ki67 expression within 43 basal tumors using TMA-1. TMA-2 was utilized to examine Crk protein expression (using four different Crk antibodies recognizing CrkI/II, CrkII or CrkL) relative to Ki67 within 29 basal tumors. Kendall's tau non-parametric statistical analysis was used to assess for significance. TMA, tissue microarray.

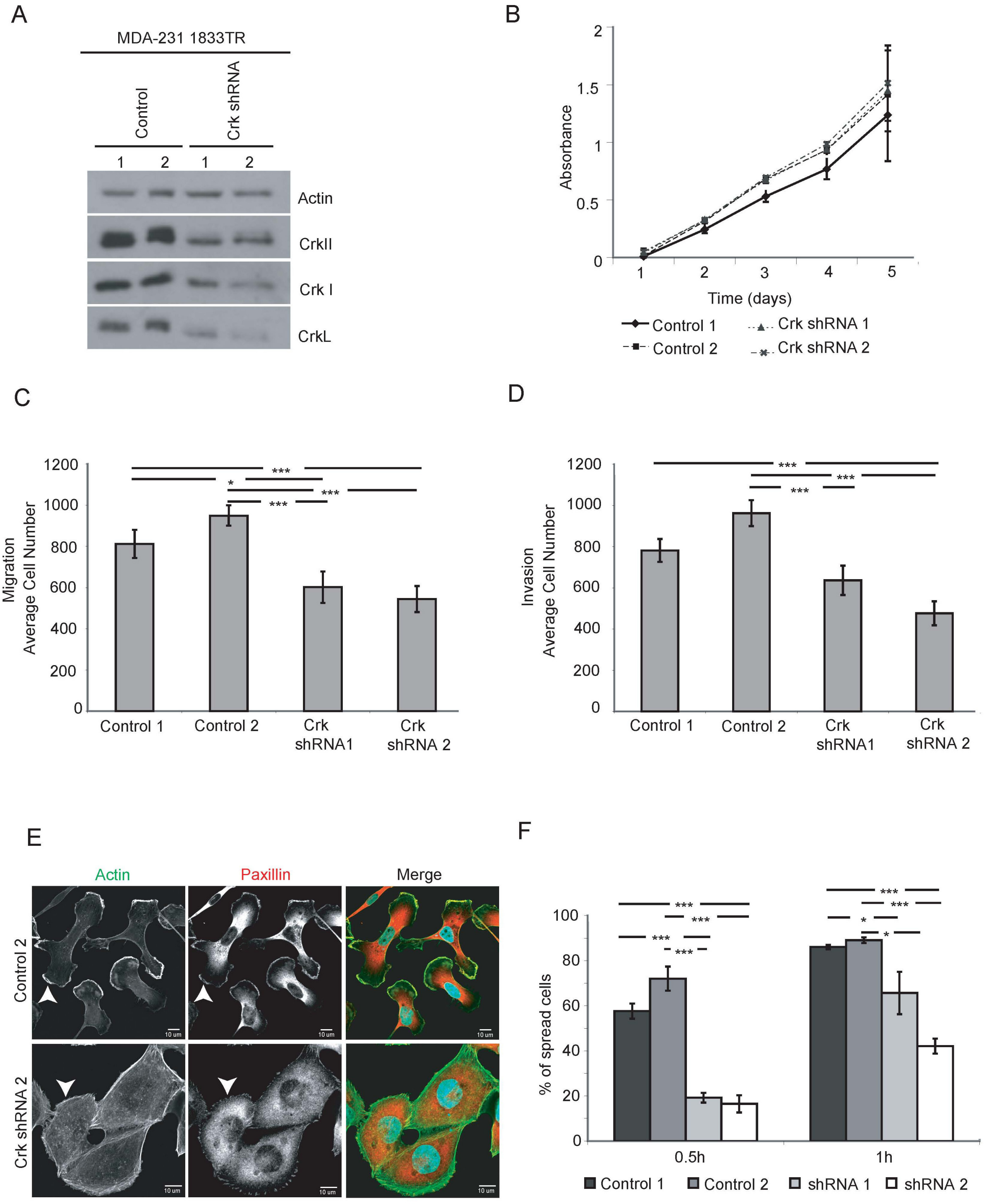

Figure 2 Knockdown of Crk alters migration and invasion, cell morphology and adhesion. Western blot of Crk proteins from whole cell lysates with actin as loading control (**A**). Alamar blue assay ($n = 3$) of control and Crk shRNA cells (**B**). Control and Crk shRNA cells were analyzed for migration (**C**) and invasion (**D**) using transwells ($n = 3$). Control and Crk shRNA cells plated on fibronectin coated coverslips (63x). Control cells exhibit lamellipodia (white arrowheads) compared to Crk shRNA cells, which lack these structures (white arrowheads). Cells co-stained with anti-paxillin and phalloidin. Scale bars represent 10 μm (**E**). Cell spreading ($n = 3$) was quantified by counting the total number of spread cells versus total cell number (**F**). Error bars represent SEM. Statistically significant data illustrated as (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

proteins and are associated with perturbations in Cdc42 activation.

Crk proteins are required for breast cancer growth in the bone microenvironment

Loss of Crk proteins impaired the ability of metastatic breast cancer cells to migrate, invade and adhere *in vitro*, thus, we examined how loss of Crk would affect bone metastasis *in vivo* following intra-cardiac injection, which takes into account several aspects of the metastatic cascade, including survival in the bloodstream, extravasation and secondary outgrowth. As stated previously, the MDA-231 1833TR cell line is a highly metastatic subpopulation when compared to the parental MDA-231 cell line and has a high affinity to form bone metastases [15]. Following intra-cardiac injection, cells are dispersed body-wide, and based on the observations that loss of Crk affects cell migration and invasion, we hypothesized that Crk proteins may modulate the ability of cells to extravasate and initiate early colonization steps. The 1833TR cells express luciferase, allowing for imaging of metastatic capacity [16]. Ablation of Crk significantly diminished the growth of metastatic bone lesions as assessed by bioluminescence, which correlated with decreases in the overall number and size of osteolytic lesions, as determined by X-ray analysis (Figure 3a-c). Interestingly, several of the animals injected with Crk knockdown cells failed to develop any metastatic lesions, relative to control cells in which 100% of the animals developed metastatic bone lesions (Table 2).

Since intra-cardiac injection encompasses multiple steps of the metastatic cascade, we wanted to establish whether Crk knockdown affects tumor outgrowth in the bone microenvironment. The tibial injection model [33] was chosen to focus our investigation on the importance of Crk proteins in mediating tumor cell interactions with the microenvironment, thereby excluding any possible effects that loss of Crk may have on tumor cell survival in circulation, endothelial adhesion, and extravasation. When compared to control cells, loss of Crk decreased tumor

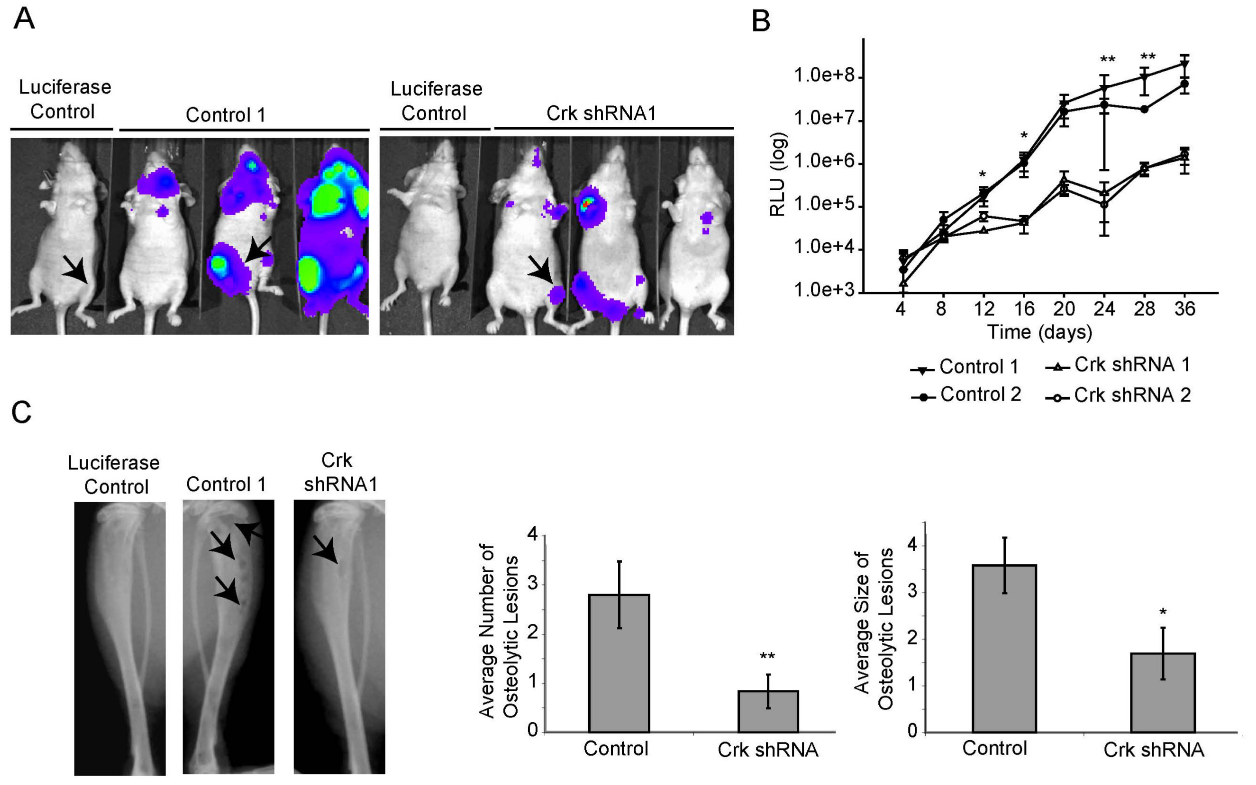

Figure 3 Crk proteins are required for efficient breast cancer outgrowth in bone. Outgrowth of 1833TR control (*n* = 7 for control 1, *n* = 4 for control 2) or Crk shRNA cells (*n* = 6 for Crk shRNA1, *n* = 6 for Crk shRNA 2) was measured by bioluminescence. Representative luciferase images at Day 40 are shown, closely resembling the mean bioluminescence values (**A**). Bioluminescence of positive limbs was quantified as relative luciferase units (RLU) over time. Averages from each cell line were plotted on a log scale +/- SEM (**B**). Representative X-ray images from negative control, control 1833TR and Crk shRNA mice. The average number and size of osteolytic lesions was blindly scored. Both control (*n* = 8) and Crk knockdown cohorts (*n* = 6) that exhibited osteolytic lesions were pooled for analysis (**C**). Statistically significant data illustrated as (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

Table 2 Comparison of 'take rates' between the control 1833TR cells and the Crk shRNA cells from the various *in vivo* experiments

In vivo assay	Control 1	Control 2	Crk shRNA1	Crk shRNA 2	Controls (Total)	Crk shRNA (Total)
Cardiac	7/7	4/4	5/6	3/6	11/11 (100%)	8/12 (66.7%)
Tibial	8/16	7/16	10/16	4/16	15/32 (46.9%)	14/32 (43.8%)
Mammary Fat Pad	9/10	8/10	6/8	3/8	17/20 (85%)	9/16 (56.3%)

outgrowth in the tibia, as measured by bioluminescence imaging (Additional file 7), which was associated with a moderate decrease in the overall number and size of osteolytic lesions (Additional file 7). Thus, Crk proteins are required for outgrowth of 1833TR breast cancer cells in the bone microenvironment. Since differences observed between control and Crk knockdown 1833TR cells were more dramatic in the intra-cardiac model, loss of Crk may also contribute to bone metastasis.

Crk proteins are required for mammary tumor growth

Since a significant delay in the outgrowth of 1833TR cells was observed in the bone microenvironment, we investigated whether loss of Crk would affect outgrowth in the primary site, the mammary fat pad. Similar to the intra-cardiac injection, seven out of the 16 animals injected with Crk knockdown cells failed to develop mammary tumors that reached a detectable size and the tumors that did derive were significantly delayed, taking twice as long as control cells to reach 500 mm^3 (Figure 4a, Table 2). Importantly, endpoint mammary tumors derived from control and Crk shRNA cells expressed similar levels of Crk protein (Additional file 8), indicating that Crk knockdown cells fail to form tumors and selective pressures exist within tumors to lose expression of Crk-targeting shRNAs, allowing re-expression of Crk proteins for tumor progression.

To investigate how mammary tumor outgrowth is altered by Crk knockdown, 1833TR cells were analyzed three days post-injection for proliferation and apoptosis. Relative to control cells, Crk knockdown significantly decreased proliferation, as assessed through Ki67 staining (Figure 4b) but not apoptosis (Additional file 8). Importantly, re-expression of non-shRNA targeted CrkI/II rescued *in vivo* proliferation, indicating a specific requirement of CrkI/II for sustained tumor growth (Figure 4b, Additional file 8). Analysis of microscopic lesions eight days post-injection revealed smaller lesions derived from Crk knockdown cells relative to control and CrkI/II rescue cells, highlighting the importance of Crk for initiation and maintenance of 1833TR induced tumors (Figure 4c). These data were unexpected considering Crk knockdown cells grow at similar rates to control cells *in vitro* (Figure 2b). Hence, we examined whether diminished Crk expression affects anchorage-independent growth. Loss of Crk significantly impaired the ability of 1833TR cells to grow in soft agar compared to control cells and re-expression of CrkI/II proteins rescued this phenotype (Figure 4d), consistent with a specific requirement for Crk in anchorage-independent growth.

To address whether the phenotypes observed upon Crk knockdown are specific to 1833TR cells, Crk knockdown was established in another basal breast cancer cell line, SUM1315. In a similar manner to 1833TR, Crk knockdown significantly attenuated anchorage-independent growth and perturbed *in vivo* proliferation of SUM1315 cells when injected into the mammary fat pad, confirming that Crk is required for tumor progression in other basal breast tumor cell lines (Figure 4e, f, Additional file 8). Loss of Crk also significantly impaired the ability of SUM1315 cells to migrate and invade (Additional file 8), meaning that Crk knockdown severely impairs the growth, migration, invasion, and proliferation of several basal breast tumor cells.

Loss of Crk leads to decreased p130Cas phosphorylation

As previously mentioned, Crk adaptor proteins are known downstream integrators of integrin-mediated signaling and several studies have linked integrin signaling to tumor progression [34-36]. To understand the biochemical consequences of the diminished cell spreading and migration following Crk ablation, as well as the perturbations in tumor outgrowth, we examined known Crk-dependent signaling pathways associated with integrin activation. Integrin signaling induces phosphorylation of p130Cas (Crk-associated substrate), a scaffolding protein implicated in actin cytoskeleton reorganization, cell migration and spreading [37]. p130Cas was originally identified as a Crk SH2 domain-binding protein whose tyrosine phosphorylation is elevated upon v-Crk transformation of fibroblasts [37]. Immunofluorescence staining and confocal imaging of cells, one hour post-plating on fibronectin, revealed reduced phospho-p130Cas staining in Crk knockdown 1833TR cells relative to control cells. Interestingly, phospho-p130Cas was found predominantly in the cytoplasm rather than at membrane protrusions, as illustrated in control cells (Figure 5a). By Western blot analysis and quantification using digital software (Odyssey), phosphorylation of p130Cas was significantly reduced in Crk knockdown cells that demonstrated delayed cell spreading (Figure 5b). Thus, the delayed cell spreading correlated with decreased levels of phosphorylated p130Cas upon Crk knockdown

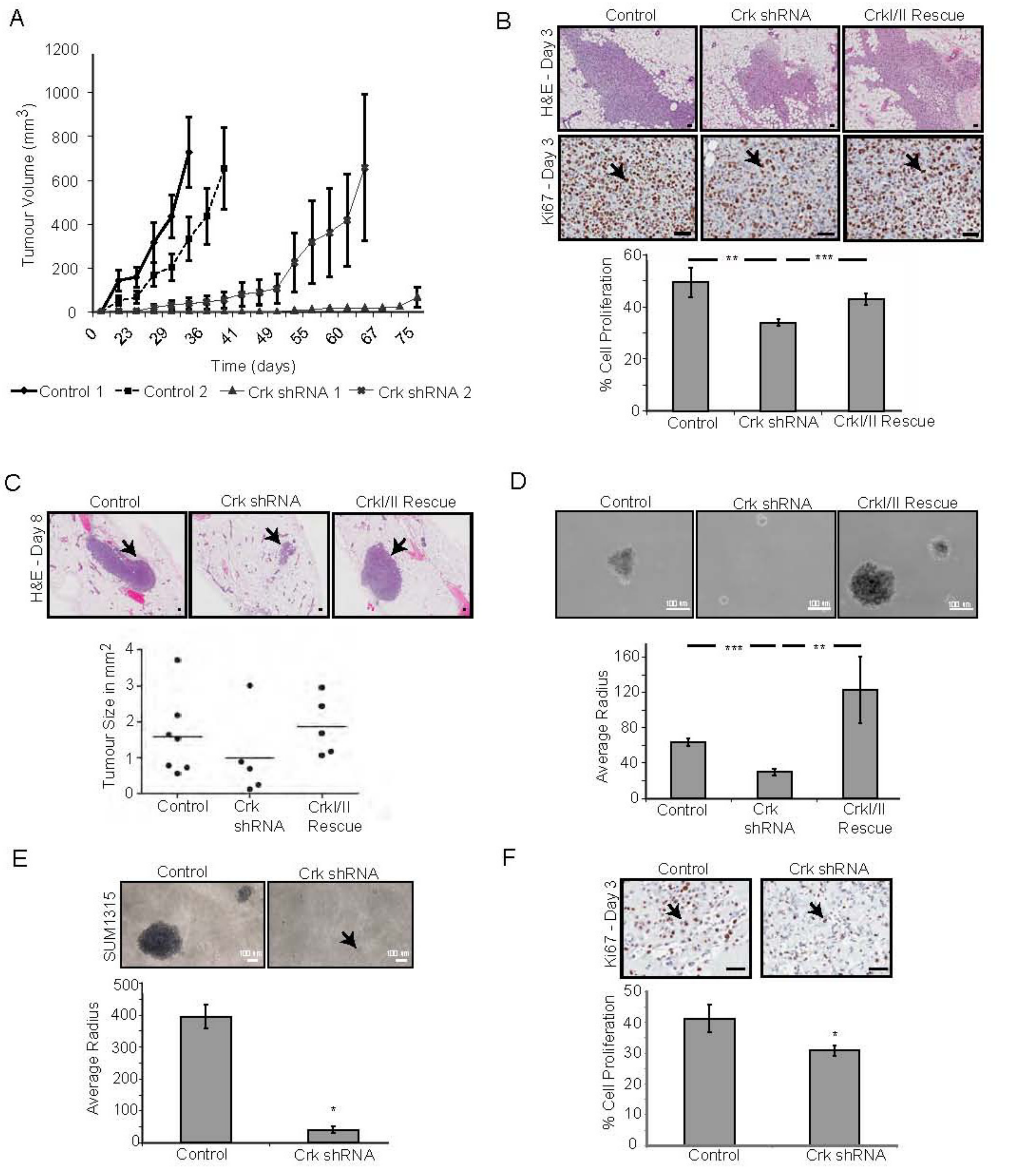

Figure 4 Crk proteins are essential for outgrowth in the primary site. Mammary tumor outgrowth was measured and the average volume of control (*n* = 10 per cell line) and Crk shRNA tumors (*n* = 8 per cell line) plotted over time +/- SEM (**A**). H & E (5x) and Ki67 (20x) staining were performed on sections from control, Crk shRNA and CrkI/II rescue samples three days post-injection. Scale bars 50 µm. Average cell proliferation +/- SEM was quantified using ImageScope (**B**). Representative H&E staining (2x) of lesion size eight days post-injection are shown. Scale bars 10 µm. Average lesion size eight days post-injection was plotted +/- SEM (**C**). Soft agar growth of control, Crk shRNA and CrkI/II rescue 1833TR cell lines; mean +/- SEM. Scale bars 100 µm (**D**). Soft agar growth of control and Crk shRNA SUM1315 cell lines; mean +/- SEM. Scale bars 100 µm. Soft agar quantified using Infinity Analyze (**E**). Ki67 (20x) staining of sections from control and Crk shRNA SUM1315 tumor samples three days post-injection. Scale bars 50 µm. Average cell proliferation +/- SEM quantified using ImageScope (**F**). Statistically significant data illustrated as (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$).

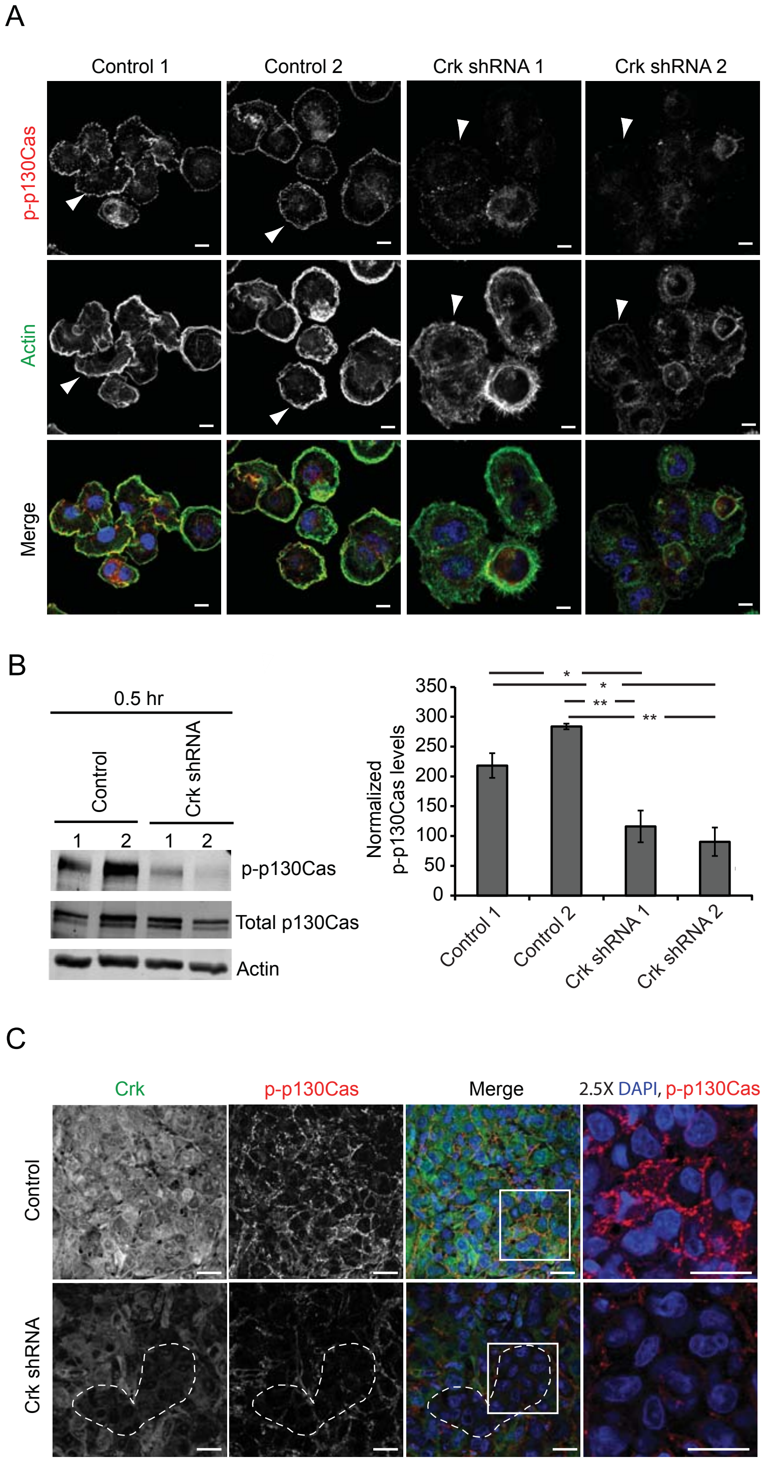

Figure 5 Crk knockdown alters p130Cas phosphorylation. Control and Crk shRNA MDA-231 1833TR cells plated on fibronectin one hour before fixation; stained for phospho-p130Cas and phalloidin. Phospho-p130Cas is prominent at membrane ruffles in control cells (white arrowheads), but cytosolic in Crk shRNA cells, with decreased accumulation of phalloidin at cell periphery (white arrowheads). Scale bars 10 μm (**A**). Western blot of proteins from control and Crk shRNA MDA-231 1833TR cells plated for 0.5 hour on fibronectin were quantified using Odyssey software. Phospho-p130Cas levels were normalized to total p130Cas levels (*n* = 3) (**B**). Immunofluorescence of CrkI/II and phosphorylated p130Cas was performed on sections from control and Crk shRNA MDA-231 1833TR samples three days post-injection. Scale bars 20 μm (**C**).

and a diminished ability of phospho-p130Cas to engage in adhesions, supporting that Crk ablation impairs integrin signaling at least in part through reduced p130Cas phosphorylation and localization.

In order to assess whether impaired integrin signaling correlated with the decreases observed in tumor outgrowth, 1833TR and SUM1315 tumor lesions derived three days post-injection were immunostained for phospho-p130Cas and CrkI/II protein expression. Diminished levels of phospho-p130Cas were detected within Crk knockdown lesions relative to control lesions, supporting a perturbation in downstream integrin signaling *in vivo* as well as *in vitro* (Figure 5c, Additional file 9). A similar correlation was also observed in human breast cancer tumors. Breast tumors expressing low levels of CrkI/II, as visualized by confocal microscopy, exhibited diminished phosphorylation of p130Cas (Additional file 10). Conversely, breast tumors expressing high levels of CrkI/II demonstrated robust tyrosine phosphorylation of p130Cas (Additional file 10). Hence, in human breast cancers, high Crk protein expression is associated with tyrosine phosphorylation of p130Cas and supports our *in vitro* data illustrating that loss of Crk is associated with reduced p130Cas phosphorylation.

Discussion

Although elevated Crk proteins are documented in several human cancers, Crk expression and signaling had not been well characterized in human breast cancer. Thus, it is of particular significance that a gene signature derived following CrkII over-expression correlates with the basal subtype in both human patients and breast cancer cell lines, and is associated with poor prognosis and decreased survival [31]. Using immunohistochemistry, we observed elevated CrkI/II and CrkL proteins in both high-grade tumors and proliferative breast cancers of the triple negative subtype, consistent with elevated Crk proteins being associated with aggressive breast cancer. The transcriptional Crk signature was also associated with high tumor grade and poor 20-year survival in multiple gene expression datasets from breast cancers. In support of this, CrkI/II are over-expressed in poorly differentiated and highly proliferative lung tumors [14], implicating Crk proteins in the progression from early to advanced states of disease.

The molecular mechanisms governing the induction and progression of basal breast cancer are poorly understood, and targeted therapies are lacking. Notably, the aggressive MDA-231 1833TR and SUM1315 cells used in this study are derived from the Basal B breast cancer subtype, which is associated with the Crk gene signature [27]. Using these basal breast cancer cell lines as a model, we demonstrate that Crk proteins are critical for growth in the mammary fat pad and bone. Interestingly, all mammary tumors that form with long latency from Crk knockdown MDA-231 1833TR cells expressed Crk proteins at levels that were similar to tumors derived from control cells, indicating that strong selective pressures exist within tumors for re-expression of Crk proteins for tumor progression.

Loss of Crk proteins abrogated the ability of aggressive MDA-231 1833TR breast cancer cells to grow in the bone after both intra-cardiac and tibial injections. Since loss of Crk diminished outgrowth in the tibia as well as the mammary fat pad, Crk proteins are likely required for tumor outgrowth regardless of the microenvironment. This is consistent with observations that loss of CrkI/II decreased the ability of ovarian cancer cells to grow within the peritoneal cavity and that ectopic expression of the microRNA miR-126, whose putative target is Crk, prevented outgrowth of gastric cancer cells in the lung and outgrowth of breast cancer cells in the lung, bone and mammary fat pad [13,38,39]. These data have important implications, as Crk proteins are elevated in many cancer types, including breast [6,11,12,14].

Since differences in bioluminescence observed between Crk knockdown and control cells following tibial injection were not as dramatic as those observed after intracardiac injection, the effect on metastasis formation may not be solely attributed to the effect on tumor outgrowth. The progression of cancer to metastasis is dependent, in part, on the deregulation of signaling pathways involved in cell migration and invasion. Downstream of integrins, Crk proteins can mediate migration through their association with the scaffold protein, p130Cas and the Rac exchange factor Dock180 [34]. Through these interactions, a role for Crk has been proposed in the regulation of cell migration, invasion, and phagocytosis, several of which have been demonstrated *in vivo* in *Caenorhabditis elegans* [3]. In the basal breast tumor cells examined here, loss of Crk reduces cell migration and invasion. However, although Crk has been well characterized upstream of Rac [34], we do not observe a strong correlation between Crk knockdown and overall Rac GTP levels, as assessed through pulldown assays. This suggests that Crk may be unlinked from Rac activation in the MDA-231 1833TR cell line or that Crk may affect the subcellular localization of Rac1 rather than its activation [40]. MDA-231 cells express mutant Ras (KRas G13D) [41], which is known to activate PI3K signaling, that can subsequently activate the Rac guanine nucleotide exchange factors, Sos and Vav1 [42] providing an alternative mechanism to activate Rac. However, these are insufficient to promote cell migration and invasion following Crk knockdown. A similar small reduction in Rac activation has been observed upon CrkI/II knockdown in MCAS ovarian cancer cells [38], further supporting the idea that Crk represents only one of several pathways that can activate Rac1.

Despite the small decreases in Rac activity, we observed a significant decrease in Cdc42 GTP levels in Crk knockdown cells upon serum stimulation. Cdc42 is involved in filopodia formation, as well as cell polarity and invadopodia [43] and has also been implicated in regulating Ras-mediated cell transformation [44]. Thus, our data support studies that identify Crk in signaling upstream of Cdc42 [45] and demonstrates that 1833TR breast tumor cells are dependent, at least in part, on Crk for activation of this GTPase, providing a potential mechanism for diminished *in vivo* tumor growth, as well as cell migration and invasion that are required for metastatic spread.

A role for integrin signaling in breast tumor progression is supported by studies demonstrating that β1 integrin inhibitory antibody decreased the number and size of mammary tumors and suppressed the growth of MDA-231 cells cultured in 3D extracellular matrix through decreased proliferation and enhanced apoptosis [35,36]. Additional studies examining metastases determined that impairment of integrin signaling is required, not for extravasation, but secondary outgrowth, highlighting the importance of integrin dependent signals for proliferation and survival *in vivo* [46,47]. Our studies identify a key role for Crk in this process. Following Crk knockdown, integrin-dependent cell spreading and tyrosine phosphorylation of p130Cas, a downstream target following integrin engagement, are impaired, and both MDA-231 and SUM1315 cells with Crk knockdown have decreased ability to grow in soft agar and impaired tumor outgrowth associated with diminished proliferation *in vivo*.

Crk proteins may be necessary to sustain the phosphorylation status of their SH2 domain-binding partners. Cells transformed with v-crk show elevated levels of tyrosine phosphorylation of Crk SH2 domain-binding proteins, including p130Cas [1,48-51] and CrkI/II is required for sustained phosphorylation of SH2 domain-binding protein Gab1 in response to growth factor stimulation [52]. In support of a requirement for Crk proteins for p130Cas signaling, we observe decreased tyrosine phosphorylation and mislocalization of p130Cas from the plasma membrane in basal breast cancer cells *in vitro*, as well as short term tumor assays *in vivo* following Crk knockdown. Decreased phosphorylation of p130Cas was also observed in human breast cancer tissue biopsies that displayed low levels of CrkI/II proteins, whereas elevated levels of p130Cas tyrosine phosphorylation correlated with high levels of Crk supporting the idea that Crk levels modulate signaling through p130Cas in human breast cancer *in vivo*.

Conclusions

Elevated levels of Crk proteins (CrkI, CrkII, CrkL) are observed in human cancers, including those of epithelial origin, such as breast. Despite *in vitro* studies on cell transformation, migration and invasion, the precise role of Crk adaptor proteins in epithelial derived breast cancers and their correlation to human clinical parameters *in vivo* is poorly understood. We demonstrate the clinical significance of Crk proteins in human breast cancer using TMAs (n = 254), revealing a significant association between Crk protein expression with highly proliferative tumors and basal breast cancers of poor outcome. Moreover, a gene expression signature derived following CrkII over-expression significantly correlated with basal breast cancer and poor outcome. As a model to study the functional significance of Crk adaptor proteins within basal breast cancer, we utilized two basal breast cancer cell lines and demonstrated that Crk proteins are important for cellular adhesion, migration, invasion and *in vivo* proliferative breast tumor outgrowth. The data generated from this study identify Crk proteins, or Crk-dependent signaling pathways, such as p130Cas, as potential targets for the basal subtype of breast cancer.

Additional material

Additional file 1: The Crk gene signature is composed of 151 genes that are differentially expressed following CrkII over-expression in T47D cells.

Additional file 2: Additional microarray datasets illustrating an association of the Crk gene signature with basal molecular subtype and high tumor grade. Heatmaps of the Crk signature in the Ivshina, Loi, Parker and Wang breast cancer datasets. The Crk signature is derived from CrkII over-expressing T47D cell lines. The bars on the left indicate if the gene had increased or decreased expression in the CrkII cell line compared to the control. The patients are ordered by correlation of the expression of these 151 genes in the patients sample to the Crk signature. Blue indicates increased expression whereas yellow indicates decreased expression. The association between patient ordering, molecular subtype and grade was determined using a Krusal-Wallis rank sum test.

Additional file 3: There is an association with the Crk gene signature and the basal molecular subtypes found within breast cancer cell lines. Heatmap of the Crk signature in the Neve breast cancer cell line dataset. The Crk signature is derived from CrkII over-expressing T47D cell lines. The bars on the left indicate if the gene had increased or decreased expression in the CrkII cell line compared to the control T47D cell line. The breast cancer cell lines are ordered by correlation of the expression of these 151 genes in the cell line to the Crk signature. Blue indicates increased expression whereas yellow indicates decreased expression. Within the gene cluster category, blue represents luminal, pink represents Basal A and dark red represents Basal B subtypes.

Additional file 4: CrkI/II positivity within human breast cancer tissue microarrays. A representative image of CrkI/II immunohistochemical staining within TMA#1. Image taken using ScanScope software.

Additional file 5: All 3 Crk proteins are required for sufficient decreases in cell migration and invasion. MDA-231 1833TR cells were transiently transfected and analyzed for their migration (A) and invasion (B) capacity in the presence of either mock transfected, scramble siRNA, CrkI/II, CrkL siRNA or both CrkI/II and CrkL siRNA in combination. Image analysis of these assays was carried out using Scion Image software. A minimum of three experiments were performed. Error bars represent the standard error of the three experiments (A, B). Western blot analysis of proteins from whole cell lysates (MDA-231 1833TR) with an anti-CrkI/II or anti-CrkL sera was performed and actin protein levels were used as a loading control (C). All data that are statistically significant is illustrated as follows (* represents $P < 0.05$), ** represents $P < 0.01$, *** represents $P < 0.001$).

Additional file 6: Crk knockdown alters Cdc42 activation but does not significantly impact Rac activation. Rac1 pulldown experiments were performed after a four hour serum starvation, followed by lysis 30 minutes post-plating on collagen. All samples were analyzed at the same time, allowing for comparisons. The GTP bound form of endogenous Rac was precipitated by GST-CRIB and probed for anti-Rac1 sera. Total endogenous levels of Rac were visualized by immunoblot. Activated levels of Rac1 were quantified and compared to total Rac1 levels ($n = 6$) (A). In response to serum, Rac and Cdc42 activation was assessed utilizing GST-Pak-PBD or GST-WASP as binding partners for GTP loaded Rac and Cdc42 respectively. Pulldown experiments were performed after a four hour serum starvation, followed by 15 minutes serum stimulation. All samples were analyzed at the same time, allowing for comparisons. The GTP bound form of endogenous Rac was precipitated by GST-Pak-PBD and probed for anti-Rac1 sera. Total endogenous levels of Rac were visualized by immunoblot. Activated levels of Rac1 were quantified and compared to total Rac1 levels ($n = 4$) (B). The GTP bound form of endogenous Cdc42 was precipitated by GST-WASP and probed for anti-Cdc42 sera. Total levels of endogenous Cdc42 were visualized by immunoblot. Activated levels of Cdc42 were quantified as described above (C). Error bars represent SEM. All data that are statistically significant is illustrated as follows (* represents $P < 0.05$), ** represents $P < 0.01$, *** represents $P < 0.001$).

Additional file 7: Crk proteins are required for efficient outgrowth of breast cancer cells in the tibia. MDA-231 1833TR control cells or those expressing Crk shRNA were injected directly into the tibia and tumor outgrowth was measured by bioluminescence imaging. Tumor outgrowth within the tibia was quantified using limbs positive for bioluminescent activity, measured as relative luciferase units (RLU) over time and plotted on a log scale as the mean +/- SEM (A). The average number and size of osteolytic lesions from pooled control ($n = 9$) and Crk knockdown samples ($n = 5$) was quantified and plotted with SEM (B). All data that is statistically significant is illustrated as follows (* represents $P < 0.05$), ** represents $P < 0.01$, *** represents $P < 0.001$).

Additional file 8: Crk expression and growth kinetics *in vitro* and *in vivo*. Western blot analysis of Crk protein expression levels from tumor endpoint (A). Paraffin embedded sections of mammary tumors derived from 1833TR control and Crk shRNA cells were stained for Ki67 (positive staining represented by black arrows) and TUNEL (positive staining represented by black arrows). No significant differences were observed between control and Crk knockdown tumors for Ki67 and TUNEL. All images taken at 20x where the scale bar represents 50 μm. Arrows represent area of insert (B). CrkI/II rescue was examined via Western blot analysis of whole cell lysates (MDA-231 1833TR) with an anti-CrkI/II or anti-CrkL sera. Actin protein levels were used as a loading control (C). Paraffin embedded sections of mammary tumors derived from 1833TR control, Crk shRNA and CrkI/II rescue cells were stained for apoptosis via TUNEL staining and quantified using ImageScope (D). Crk knockdown of SUM1315 cells was examined via Western blot analysis of whole cell lysates with an anti-CrkI/II or anti-CrkL sera. Alpha-tubulin protein levels were used as a loading control (E). Control and Crk shRNA SUM1315 cells (8×10^4 cells) were plated on transwells and were analyzed for migration (F) and invasion (G) towards 10% FBS 24 hours post-plating then quantified using ImageScope software ($n = 3$). All data that is statistically significant is illustrated as follows (* represents $P < 0.05$), ** represents $P < 0.01$, *** represents $P < 0.001$).

Additional file 9: Phosphorylated p130Cas is diminished in lesions derived from Crk knockdown cell lines. Immunofluorescence of CrkI/II and phosphorylated p130Cas was performed on paraffin embedded sections from control and Crk shRNA MDA-231 1833TR samples three days post-injection. Scale bars 20 μm (A). Immunofluorescence of CrkI/II and phosphorylated p130Cas was performed on paraffin embedded sections from control and Crk shRNA SUM1315 samples 3 days post-injection. Scale bars 20 μm (A).

Additional file 10: Phosphorylated p130Cas expression correlates with CrkI/II expression in human breast cancer tumors. Immunofluoresence of CrkI/II and phosphorylated p130Cas was performed on frozen human basal breast cancer tissue. CrkI/II mRNA expression from these patients was also assessed by microarray analysis. TN = triple negative, ER = estrogen receptor. Scale bars 20 μm (A).

Abbreviations

Cas: Crk-associated substrate; Cdc42: cell division cycle 42; Crk: CT10 regulator of kinase; Crk-L: Crk-Like; Dock180: dedicator of cytokinesis 1; ER: estrogen receptor; FBS: fetal bovine serum; GTP: guanosine triphosphate; H & E: hematoxylin and eosin; HER2: human epidermal growth factor receptor 2; LMP: MSCV-LTR-mir30-PIG; mRNA: messenger ribonucleic acid; MUHC: McGill University Health Centre; PI3K: phosphoinositide 3-kinase; PR: progesterone receptor; Rac1: ras-related C3 botulinum toxin substrate 1; RLU: relative luciferase units; RNAi: RNA interference; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SEM: standard error of the mean; SH2: Src homology 2; SH3: Src homology 3; shRNA: short hairpin RNA; TMA: tissue microarray; TN: triple negative; TR: triple reporter.

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Authors' contributions

KEF designed and performed the research, analyzed data and wrote the paper. ESB designed and performed experiments and analyzed data. CVR, AM, HZ, DZ, AM and AAG performed experiments. JM, AMM, LG, SC and MH analyzed data. PS contributed reagents and analyzed data. MP analyzed data and wrote the paper. All authors contributed to the editing of the manuscript.

Competing interests

The authors declare that they have no competing interests.

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