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## **BCAM and LAMA5 Mediate the Recognition between Tumor Cells and the Endothelium in the Metastatic Spreading of KRAS Mutant Colorectal Cancer**

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**Running title:** BCAM in KRAS mutant metastatic CRC

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## TRANSLATIONAL RELEVANCE

Patients with KRAS mutant colorectal cancer (CRC) have a poor prognosis and are unresponsive to EGFR-targeted therapies. Therefore, novel approaches are urgently needed to prevent or reduce the metastatic progression in this patient subset. In this work, we have identified BCAM and LAMA5 as an unrecognized molecular system in KRAS mutant hepatic metastasis from CRC. In this pathological setting, BCAM was overexpressed in tumor epithelial cells and in tumor microenvironment (TME), while its ligand LAMA5 was specifically overexpressed in TME blood vessels. Two BCAM-mimic peptides showed preclinical efficacy against hepatic colonization by human KRAS mutant CRC cells. Inhibition of BCAM/LAMA5 interaction abrogated adhesion of KRAS mutant CRC cells to endothelial cells, suggesting that this system may be important in tumor-TME recognition events causative of the metastatic spreading. Together, our findings indicate that BCAM-targeted agents may provide novel prevention and/or early intervention strategies for KRAS mutant CRC metastasizing to the liver.

## ABSTRACT

**Purpose:** KRAS mutations confer adverse prognosis to CRC and no targeted therapies have shown efficacy in this patient subset. Paracrine, nongenetic events induced by KRAS mutant tumor cells are expected to result in specific deregulation and/or relocation of tumor microenvironment (TME) proteins, which in principle can be exploited as alternative therapeutic targets.

**Experimental Design:** A multimodal strategy combining *ex-vivo/in vitro* phage display screens with deep-sequencing and bioinformatics was applied to uncover TME-specific targets in KRAS mutant hepatic metastasis from CRC. Expression and localization of BCAM and LAMA5 were validated by immunohistochemistry in preclinical models of human hepatic metastasis and in a panel of human specimens (n=71). The anti-metastatic efficacy of two BCAM-mimic peptides was evaluated in mouse models. The role of BCAM in the interaction of KRAS mutant CRC cells with TME cells was investigated by adhesion assays.

**Results:** BCAM and LAMA5 were identified as molecular targets within both tumor cells and TME of KRAS mutant hepatic metastasis from CRC, where they were specifically overexpressed. Two BCAM-mimic peptides inhibited KRAS mutant hepatic metastasis in preclinical models. Genetic suppression and biochemical inhibition of either BCAM or LAMA5 impaired adhesion of KRAS mutant CRC cells specifically to endothelial cells while adhesion to pericytes and hepatocytes was unaffected.

**Conclusions:** These data show that the BCAM/LAMA5 system plays a functional role in the metastatic spreading of KRAS mutant CRC by mediating tumor-TME interactions, and as such represents a valuable therapeutic candidate for this large, currently untreatable patient group.



## INTRODUCTION

Despite major efforts to develop innovative bio-drugs, colorectal cancer (CRC) still remains largely an incurable disease in the metastatic setting (1, 2). A paradigmatic example is represented by EGFR targeted therapies, to which 85-90% patients are unresponsive. A large body of prior work has shown that mutations in EGFR downstream effectors, such as KRAS (3), NRAS (4), BRAF (5), and PI3K (6), lead to a constitutive activation of the signaling pathway, thus bypassing a therapeutic block of the receptor. The presence of a mutant RAS (either KRAS or NRAS) in the tumor is now a clinically approved criterion of exclusion from EGFR-targeted regimens. Historical attempts to directly target KRAS (e.g. by farnesyl transferase inhibitors) have so far failed (7), although recent innovative approaches appear to be more promising, at least from initial preclinical data (8, 9). Targeting single effectors downstream to KRAS, such as PI3K or MEK, showed little or no efficacy in CRC (10, 11). Alternative treatments are clearly needed for CRC patients with KRAS mutant tumors.

In this work, we hypothesized that an unexplored strategy to tackle KRAS would be to target proteins in the TME of KRAS mutant metastatic CRC. To identify such molecular targets, we set up preclinical models of human hepatic metastasis by implanting human CRC cell lines carrying monoallelic KRAS mutations (12) into the livers of immuno-suppressed mice. We used phage-displayed random heptapeptide libraries to profile exhaustive proteomic signatures selectively associated with these genetically controlled metastatic models. Unexpectedly, within these signatures we identified and characterized the transmembrane glycoprotein basal cell adhesion molecule (BCAM) (13) as being specifically associated to both the tumor and TME of KRAS mutant hepatic metastases. BCAM is a cell adhesion protein originally identified in the Lutheran blood group system and circulating sickle red cells, and a receptor for laminin  $\alpha 5$  (LAMA5) (14), the chain supporting many of the biological functions of laminin  $\alpha 5$ ,  $\beta 1$ ,  $\gamma 1$  (LM-511) in endothelial basement membranes.

Here, we demonstrate that BCAM is specifically overexpressed both in preclinical and clinical KRAS mutant hepatic metastasis from CRC. We further show that inhibition of the BCAM/LAMA5 pathway

leads to impaired adhesion of CRC cells to vascular endothelial cells, with consequent reduction of metastatic growth. We therefore propose BCAM as a specific TME marker and therapeutic target in KRAS mutant hepatic metastasis from CRC.

## **MATERIALS AND METHODS**

### **Peptides and cell lines**

All the peptides [control (scrambled): SLSTSKLTVASSLDRG; pep-BCAM1: ASGLLSLTSTLY; pep-BCAM2: SSSLTLKVTSALS RDG] were from New England Peptides (Gardner, MA), provided with >95% purity. The LIM1215 parental cell line (15) was obtained from Prof. Robert Whitehead, Vanderbilt University, Nashville, TN, with permission from the Ludwig Institute for Cancer Research Ltd, New York, NY. The metastatic variant of HCT-116 cells (16), and SW-48 and LIM1215 cell lines isogenic for KRAS mutant alleles (G12V, G12D and G13D) have been described (12). To obtain fluorescent cells for *in vitro* experiments, each cell line was individually infected with a lentiviral vector based on pLVX-IRES-ZsGreen1 (Clontech, Mountain View, CA). Human umbilical cord endothelial cells (HUVECs) were extracted and cultured as described (17). Human brain vascular pericytes (HBVP) were from ScienceCell Research Laboratories (Carlsbad, CA). All other cell lines were from LGC-Promochem (Sesto San Giovanni, Italy) and were cultured in specific media and standard supplements (Sigma-Aldrich, Milan, Italy). All cells were proven negative for mycoplasma and characterized by proliferation, morphology evaluation, and multiplex short tandem repeat profiling.

### **Animal models**

Experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and by the Italian Ministry of Health. Six-week-old female CD1-nude mice were purchased from Charles River (Lecco, Italy). All surgical procedures were performed under deep general anesthesia by isoflurane inhalation. For intrahepatic transplantation, a midline incision was performed, the median lobule of the liver was gently exposed (18) and  $5 \times 10^6$  suspended cells were injected. The wound was closed by a double suture, and each animal was given 0.1 mg caprofen (Rymadil®, Pfizer, Milan, Italy) in physiological solution to allow post-operative pain relief and rehydration. Ampicillin was administered for 5 days after surgery. For phage display screens, tumor-bearing mice were sacrificed as soon as the masses became visible and/or before appearance of any sign of distress. For pharmacological

studies, cells were admixed with either targeting or control peptide (100  $\mu$ M) immediately before intrahepatic injection. Explanted livers were photographed with a PL-200 camera (Samsung Electronics, Milan, Italy) and external areas of metastatic masses were quantified with ImageJ (16).

### **Phage display**

Tissues and cell lines were processed (16) and maintained in binding medium [Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2% fetal calf serum (FCS)] at 4°C for the duration of the experiments.  $10^{10}$  transducing units of a  $X_7$  ( $X$  = any amino acid) phage library (Ph.D.<sup>TM</sup>-7 Phage Display Peptide Library Kit, New England Biolabs, Ipswich, MA) was added to  $5 \times 10^5$  target cell suspensions in binding medium, and incubated for 4 h at 4°C (first round). For successive rounds, phage was first pre-adsorbed on control cells/tissues for 1 h at 4°C, and subsequently incubated with target or control for 2 h at 4°C. After 5 washes in binding medium, bound phage was recovered and amplified by infection of K91Kan *Escherichia coli* in log-phase. Phage particles were purified by precipitation in PEG-NaCl (polyethylene glycol-800 20%, NaCl 2%).

### **Deep-sequencing and bioinformatics analysis**

Phage DNA was extracted in iodide buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA, 4 M NaI). Multiplexing barcodes (Illumina, San Diego, CA) were inserted by PCR in each individual sample and amplicons were purified by gel extraction (QIAquick® Gel Extraction Kit, QIAGEN, Hilden, Germany). DNA was quantified with Quant-iT<sup>TM</sup> PicoGreen® dsDNA Assay Kit (Invitrogen/Life Technologies, Monza, Italy), and purified DNA samples were multiplexed and sequenced with an Illumina HiSeq2000 instrument. The derived 101-bp paired-end reads were first de-multiplexed to separate single samples; successively, inserts were extracted based on the known flanking regions to give 21-bp oligonucleotide sets that were finally translated into the corresponding heptapeptides. Each peptide set was subjected to a similarity analysis with the repertoire of annotated protein sequences contained in the Ensembl database (19) via custom Perl scripts. Output proteins were accepted only if they shared at least 5 matches with a complete 7 residue sequence. To extract only the extracellular or

transmembrane proteins, the output datasets were filtered by Gene Ontology\_Cell Component (GO\_CC) annotations through the DAVID Bioinformatics Resource Functional Annotation tool (20) with default settings. The complete list of BCAM-mimic peptides has been submitted to the BDB: Biopanning Data Bank (21) and is accessible with the Dataset ID #2970.

### **Human samples and mutational analysis of KRAS**

Formalin-fixed paraffin-embedded (FFPE) specimens from CRC patients were collected by the Units of Surgical Oncology and Pathology at Candiolo Cancer Institute-IRCCS, Mauriziano and Molinette Hospitals (Turin, Italy). Collection and manipulation of human samples were approved by the Institutional Review Board (IRB). Informed written consent was obtained from each patient in accordance with the Declaration of Helsinki. Genomic DNA was purified with the QIAamp DNA FFPE Tissue Kit (QIAGEN). PCR primers (from Sigma-Aldrich) were designed to amplify the selected exon with products ~250 bp in length. PCRs were performed in 96-well formats in 25- $\mu$ l reaction volumes, in the presence of 0.25 mM deoxynucleotides (dNTPs), 1  $\mu$ M each primer, 6% dimethyl sulfoxide (DMSO), PCR buffer, 0.05 U/ $\mu$ l Platinum Taq (Invitrogen/Life Technologies), and with a touchdown PCR program (Peltier Thermocycler, PTC-200, MJ Research, Bio-Rad). Products were purified with AMPure (Agencourt Bioscience Corp., Beckman Coulter, Milan, Italy). Sequencing was carried out with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and products were purified with CleanSeq (Agencourt Bioscience, Beckman Coulter) and evaluated on a 3730 DNA Analyzer (Applied Biosystems). Traces were analyzed with Mutation Surveyor software package (SoftGenetics, State College, PA).

### **Histological procedures**

Immunohistochemistry (IHC) was performed on 2- $\mu$ m FFPE tissue sections. Briefly, after deparaffinization and rehydration, antigens were retrieved by incubating at sub-boiling temperature in Tris-EDTA pH 9.0, followed by peroxidase inactivation and blocking of endogenous biotin. Sections were stained with antibodies specific for BCAM (1:250, Abcam, Prodotti Gianni, Milan, Italy), LAMA5

(1:100, Millipore, Milan, Italy) and Ki67 (1:100, Thermo Scientific, Milan, Italy), diluted in Antibody Diluent with Background Reducing Components (Dako, Cernusco sul Naviglio, Italy) for 1.5 h at room temperature (RT). Visualization of the staining was performed with avidin-biotin-peroxidase 3,3'-diamino-benzidine (DAB, Dako REAL™ Detection System Peroxidase/DAB+, Rabbit/Mouse, Dako). Sections were counterstained with hematoxylin, dehydrated and mounted in xylene-based mounting medium (Richard-Allan Scientific™ Cytoseal™ XYL, Thermo Scientific). The Mallory's trichrome stain was performed with the Mallory Trichrome Special Stains kit (Bio-Optica, Milan, Italy). Visible images were acquired with an ICC50HD camera (Leica, Milan, Italy); all human samples were also scanned in an Aperio ScanScope ® XT System (Leica). DAB signal was isolated by color deconvolution and the positive areas were quantified with ImageJ.

### ***In vitro* assays**

For cell adhesion assays, HUVECs, HBPV or THLE-3 cells were seeded to confluence in replicate wells of 24-well plates. In a first set of experiments, fluorescent cells ( $5 \times 10^4$ /well) were allowed to adhere in 5% CO<sub>2</sub> at 37°C for 30 minutes, in the presence of control peptide, pep-BCAM1 or pep-BCAM2 (100 µM), as well as of the specific antibodies against BCAM or LAMA5 (1 µg/mL). In a second set of experiments, fluorescent HCT-116m cells were silenced for the expression of BCAM by specific Trilencer-27 siRNA Knockdown Duplexes (OriGene, Tema Ricerca, Bologna, Italy) in Lipofectamin RNAiMAX reagent (Life Technology). At 72 h post-transfection, adhesion on HUVECs was assayed in 5% CO<sub>2</sub> at 37°C for 30 minutes. In both experimental settings, plates were successively washed three times with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde-containing PBS for 10 minutes at RT. Adhered cells were photographed under a fluorescence microscope and cell nuclei were quantified with ImageJ.

### **Immunoblot**

Proteins were extracted in lysis buffer [150 mM NaCl, 50 mM Tris pH 7.4, 1 mM phenylmethane sulfonylfluoride (PMSF) protease inhibitor cocktail, Sigma-Aldrich] supplemented with 1% Nonidet NP-

40, and were separated by SDS-PAGE (Mini-PROTEAN® TGX Gels 4-10%, Bio-Rad, Segrate, Italy) followed by blotting onto PVDF membranes (Trans-Blot® Turbo™ Transfer Pack, Bio-Rad). The BCAM-specific antibody (Abcam) was used 1:1,000 overnight at 4°C. Detection of specific signals was performed with a peroxidase-conjugated secondary anti-rabbit antibody and revealed by enhanced chemi-luminescence (Western Lightning® Plus-ECL, PerkinElmer, Waltham, MA).

### **Retrotranscription and Real-time PCR**

Total RNA was extracted in QIAzol Lysis Reagent, purified with the RNeasy kit (both Qiagen, Hilden, Germany) and quantified with a Nanodrop instrument (Thermo Scientific). One microgram RNA was subjected to retrotranscription with the High-Capacity cDNA Reverse Transcription Kit, and amplified in Power SYBR® Green PCR Master Mix with a 7900HT Real-Time PCR System (all Applied Biosystems). BCAM expression was evaluated with the dCt method, normalized against 3 independent housekeeping genes (Hypoxanthine Phosphoribosyltransferase 1, HPRT1; Succinate dehydrogenase complex, subunit A, SDHA; TATA Box Binding Protein, TBP), and expressed as fold increase ( $2^{\Delta\Delta Ct}$ ) over the control (WT KRAS) for each cell line panel. The following primers were used for the Real-time PCR amplification: BCAM\_FW, CCTTCAGGATGAGCAGGAG; BCAM\_REV, CCACTCTGCAGCCATAGGT; HPRT1\_FW, TCAGGCAGTATAATCCAAAGATGGT; HPRT1\_REV: AGTCTGGCTTATATCCAACACTTCG; SDHA\_FW: TGGGAACAAGAGGGCATCTG; SDHA\_REV: CCACCACTGCATCAAATTCATG; TBP\_FW: CACGAACCACGGCACTGATT; TBP\_REV: TTTTCTTGCTGCCAGTCTGGAC.

### **Statistics**

All the analyses were performed with the Prism 5 software (GraphPad, La Jolla, CA): two-tailed t-test (C.I. 95%) and Fisher's exact test were used to compare selected experimental points; asterisks indicate the following p-value ranges: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .

## RESULTS

**BCAM and LAMA5 are a candidate receptor/ligand system in KRAS mutant hepatic metastasis from CRC.** To reproduce the molecular diversity at the tumor-TME interface in hepatic metastasis from CRC, we set up mouse models derived by intrahepatic implant of human CRC cell lines (SW-48 and LIM1215) in which a single KRAS allele had been replaced by either a WT or one of three mutant (G12D, G12V, G13D) KRAS coding sequences by site-specific recombination (12). Such cells recapitulate genetic events observed in patients and represent a more physiological model of CRC epithelial cells in comparison to mutant KRAS-overexpressing cells. In addition, we observed that both SW-48 and LIM1215 generate hepatic masses with a substantial TME component, making them suitable preclinical models of metastatic CRC. To identify TME markers in KRAS mutant hepatic metastasis, we designed a high-throughput proteomic approach based on low-stringency parallel screens of phage-displayed random heptapeptides on all the experimental metastases (*ex-vivo*) and cell lines (*in vitro*) (**Figure 1A**), each screen providing  $10^4$ - $10^6$  unique heptapeptide ligands (**Supplemental Material**, details of the procedure; **Figure S1**, quality control). From these massive datasets, we derived heptapeptide ligands with dual specificity for (i) mutant KRAS [mutant vs. WT] and (ii) TME [experimental metastases (tumor cells + TME) vs. cell lines (tumor cells only)]. Corresponding native protein networks were reconstructed by high-stringency sequence identity analysis, leading to the identification of candidate TME-binding proteins for each KRAS mutant setting. Such proteins were assigned a TME score, based on the percent experimental points in which they had been identified (**Figure 1B**). By definition, a TME-binding protein is a receptor expressed by tumor and/or TME cells that binds TME-specific ligands. In the described *in vivo* models of hepatic metastasis, tumor and TME components are human and murine, respectively. On this basis, we refined our search on candidate receptors (i) associated with all three mutant KRAS settings, (ii) retrieved in both human (tumor epithelial cells) and mouse (TME) proteomes, and (iii) whose recognized ligand(s) was retrieved exclusively in the mouse proteome (TME). BCAM was identified as the only protein fulfilling all these criteria (**Figure 1C**).



**BCAM and LAMA5 are overexpressed in KRAS mutant hepatic metastasis from CRC.** As an initial step toward the characterization of these new candidate markers, we validated the levels and distribution of BCAM and LAMA5 in hepatic metastases with different KRAS mutational status. Tissues from the described mouse models were IHC-stained, revealing that BCAM was overexpressed in both epithelial cells and TME (stroma and vasculature) of KRAS mutant experimental metastases, compared to WT tumors where it was undetectable. Staining for LAMA5 was barely detectable in epithelial cells but strong in the TME of KRAS mutant tumors (**Figure 2**, upper panels). *In vitro*, BCAM protein amounts were consistent with those observed *in vivo*, with the exception of SW-48 WT and LIM1215 WT cell clones, which expressed BCAM at medium levels (**Figure S4A**). Overall, there was no correlation between protein and mRNA levels in cultured cell lines (**Figure S4B**). Together, these data suggest that *in vivo* protein levels are influenced by the microenvironment and are possibly regulated by post-transcriptional events related to the occurrence of a mutant KRAS gene in cancer cells.

We next evaluated the presence of BCAM and LAMA5 in hepatic metastases from CRC patients. Overexpression of BCAM and LAMA5 was confirmed in KRAS mutant metastases, with only a difference in the staining pattern compared to the mouse models: in human TME, BCAM and LAMA5 were confined to the vasculature and barely detectable in stromal cells (**Figure 2**, lower panels), a feature possibly related to the more organized and/or less inflammatory phenotype of clinical tumors compared to experimental models. BCAM expression was investigated in patient samples (n=29 WT and 42 KRAS mutant) also with a routine scanner set on default parameters (**Table 1**, patient cohort; **Figure 3A**, representative BCAM staining; **Figure S2**, complete panel). Although this cohort was relatively limited, our analysis confirmed BCAM overexpression in KRAS mutant vs. WT tumors ( $P < 0.0001$ , **Figure 3B**). The same approach was applied to a panel of primary CRCs (n=18 WT and 16 KRAS mutant, **Figure S3**), revealing a non-significant trend of increased BCAM expression in KRAS mutant vs. WT tumors (**Figure 3C**). In hepatic metastases from the same patients, however, this trend was highly significant ( $P = 0.0098$ , **Figure 3C**), suggesting that the overexpression of BCAM in KRAS

mutant tumors is specifically acquired or enhanced during the metastatic spreading.

These data show that increased levels of BCAM and LAMA5 are present in KRAS mutant hepatic metastases, and suggest BCAM-LAMA5 as a receptor-ligand system for targeted intervention.

**BCAM-mimic peptides inhibit the intrahepatic growth of KRAS mutant human CRC cells.**

Several peptide sequences retrieved by phage display shared similarity with portions of both human and mouse BCAM (**Figure 4**, peptides used in this study; **Table S1**, complete list of BCAM-mimic peptides identified in the phage display screens). Most of them overlapped in two conserved regions, i.e. residues 212-223 (ASGLLSLTSTLY) and 504-519 (SSSLTLKVTSALS RDG) of the human protein, corresponding to portions of the immunoglobulin-like V-type 2 (also named D2) and C2-type 3 (D5) domains, respectively. Although the function of these phage display-identified portions of BCAM remains uncharacterized, both the D2-D3 linker and the D5 domain are required for LAMA5 binding (22, 23) (**Figure 4**). We therefore evaluated whether corresponding synthetic peptides could block any functional interaction involving BCAM in the experimental metastasis models.

Because both SW-48 and LIM1215 give rise to experimental metastases very slowly (SW-48, 72-127 days; LIM1215, 109-284 days) and with incomplete engraftment rates, we evaluated alternative human CRC-derived cell lines. Among the WT (n=7) and KRAS mutant (n=11) cell lines investigated, only HT-55 (WT), HCT-116m and DLD-1 (both mutant) were capable of producing experimental metastases in a relatively short period of time (28-35 days) and with high (75-100%) engraftment rates (**Table S2**). *In vitro*, these cell lines completely recapitulate the correlation between KRAS mutational status and BCAM expression levels observed in the isogenic xenograft models (**Figure S4A**, compare with **Figure 2**). For the *in vivo* pharmacological studies, each cell line was co-injected with either pep-BCAM1 (ASGLLSLTSTLY), pep-BCAM2 (SSSLTLKVTSALS RDG), or control peptide (100 µM each), and the ability to colonize the hepatic parenchyma was evaluated after 28 days on the explanted livers. When co-administered with the KRAS mutant cell lines, both pep-BCAM1 and pep-BCAM2 inhibited the occurrence and extent of experimental metastases compared to a control peptide. Neither peptide interfered with the engrafting of WT cells (**Figure 5**, **Figure S5**).

Together, these data demonstrate that BCAM-mimic peptides inhibit the intrahepatic growth of human CRC cells specifically in KRAS mutant settings where BCAM and LAMA5 are overexpressed.

**BCAM drives the adhesion of KRAS mutant CRC cells to the vascular endothelium.** There was no difference in the numbers of proliferating cells (evaluated by Ki67 staining) or the levels of BCAM (consistent with those observed in cultured cell lines, **Figure S4A**) and LAMA5 between pep-BCAM1, pep-BCAM2 or control peptide-treated experimental metastases (**Figure 3**). These data indicate that the BCAM-mimic peptides do not interfere with tumor growth rates or with BCAM/LAMA5 expression once the metastatic cascade has occurred. Rather, we hypothesized that an earlier step in metastatic progression of CRC, e.g. the recognition and/or binding between tumor epithelial cells and TME components, might be affected by the block of BCAM/LAMA5 binding.

To test this hypothesis, we investigated whether the BCAM-mimic peptides could inhibit the interaction of KRAS mutant cancer cells with cell types representative of the TME. For this purpose, we evaluated the adhesion of HCT-116m cells (the only cell line suitable for this assay, **Figure S6**) on human endothelial cells (HUVECs), pericytes (HBVP) and hepatocytes (THLE-3). For prompt visualization, HCT-116m were stably transduced with a lentiviral construct to express high amounts of a human codon-optimized variant of the reef coral *Zoanthus sp.* green fluorescent protein. HUVECs, HBPV and THLE-3 cells were seeded to confluence, and fluorescent HCT-116m cells were allowed to adhere in the presence of either control, pep-BCAM1 or pep-BCAM2 peptide, or in the presence of a specific anti-BCAM or anti-LAMA5 antibody. Targeted peptide- and antibody-treated HCT-116m cells showed substantially impaired adhesion on endothelial cells compared to the control, whereas adhesion on pericytes and hepatocytes was unaffected (**Figure 6A**). To confirm the role of BCAM in the interaction of CRC with endothelial cells, we silenced the corresponding gene with 3 different siRNA duplexes (**Figure 6B**). The down-modulation of BCAM also impaired adhesion of fluorescent HCT-116m to the endothelium (**Figure 6C**).

Collectively, these data strongly suggest a role for the BCAM/LAMA5 receptor-ligand interaction in the mutual recognition between CRC cells and the vascular endothelium.

## DISCUSSION

Whilst anti-EGFR antibodies have prolonged survival in metastatic CRC (25), they have proven ineffective for 85-90% of patients (26) due to primary (e.g., related to RAS mutations) or secondary pharmacological resistance. Patient stratification according to mutational backgrounds (27) and drug combination to restrain the emergence of secondary resistance (28) may improve EGFR-targeted treatments, but not for patients with KRAS mutant tumors. Therapies that target the TME in addition to cancer cells should result in synergistic anti-tumor activity; such therapies are also expected to be less susceptible to development of resistance, because TME cells are genetically stable. However, the advantage of combining angiogenesis inhibitors with EGFR-targeted drugs has proven poor overall (29, 30), and absent in patients with KRAS mutant tumors (31). On the other hand, the availability of TME-targeting drugs other than angiogenesis inhibitors or immunomodulators remains disappointingly limited: a single agent addressed to cancer-associated fibroblasts (CAFs), sonidegib, has been approved by FDA for basal cell carcinoma (32), while a few others are undergoing preclinical evaluation in different tumor types including CRC (33, 34). The recent characterization of CAF-specific gene expression signatures in primary CRC (35, 36) may also be promising in the light of novel therapeutic strategies. Whether all these approaches would be translatable to patients with KRAS mutant tumors remains to be elucidated.

In KRAS mutant CRC, the tumor/TME crosstalk is altered, and specific molecules [among which EphA2 (37) in tumor cells, fascin-1 (38) and adrenomedullin (39) in the stroma] acquire a functional role, mediating cancer aggressiveness toward an accelerated metastatic spreading (40). Although pro-metastatic signaling pathways and signatures have been successfully identified in primary CRC, important players in the metastatic cascade might not be detectable in the primary tumor and should be sought in the actual TME of a secondary tumor. Taking all these points into consideration, we designed an approach aimed at the identification of molecular markers/pathways in the hepatic metastasis of KRAS mutant CRC, by the use of a clinically relevant mouse model that combines human KRAS mutant CRC cells with a murine TME. This system was explored by high-throughput

proteomics and deep-sequencing, to identify TME-binding proteins in KRAS mutant tumors. We focused our attention on the only receptor-ligand system that emerged from our high-stringency analysis, i.e. BCAM and LAMA5. Other players have as well been identified that might be worth of further characterization, among which laminin chains, Sema4A, integrins ( $\alpha_8$ ,  $\alpha_x$ ,  $\beta_5$ ), Tie1, and Smoothed. The latter is a CAF-associated molecule targeted by sonidegib (30), confirming the reliability of our screening procedure and suggesting that this FDA-approved drug should be also explored in KRAS mutant CRC metastasizing to the liver.

BCAM and LAMA5 act at the interface between tumor cells and the TME, allowing a dual targeting of non-tumor and tumor components, which in principle should be more effective compared to standard combined therapies. Similarly, an allosteric inhibitor of IGF-1R (NT157) that acts both on tumor cells and CAFs by blocking IGF-1R/IRS1 and STAT3 signaling has showed preclinical efficacy in primary CRC (34). The uniqueness of BCAM and LAMA5, however, goes further: being a player in the tumor/vasculature crosstalk, this molecular system is likely involved in an early step of the metastatic cascade, i.e., the recognition between circulating CRC cells and hepatic sinusoids. We suggest a paracrine mechanism in which KRAS mutant CRC cells induce increased vascular levels of LAMA5 during metastatic colonization. In turn, high levels of LAMA5 would favor its interaction with BCAM on cancer cells, leading to their accumulation in sinusoids toward the metastatic colonization of the liver. Consistently, we demonstrated that blocking either BCAM or LAMA5 leads to impaired adhesion of CRC cells to the vascular endothelium, and inhibition of metastatic colonization.

The only curative approach against hepatic metastasis is surgery. However, ~50% of surgically-removed metastases relapse, possibly due to the spreading and growth of either single cells or micro-metastases that were already present but undetectable at the time of surgical intervention. This regrowth is favored by the pro-inflammatory environment elicited by the surgery itself, and takes places in a very limited period of time, ranging from a few minutes to a maximum of two weeks (41). We propose BCAM as an intervention target in a potential metastasis prevention scheme that targets KRAS mutant CRC cells disseminated to the liver in this postoperative inflammation phase. A similar

targeting of early metastatic phases has been proposed for the inhibition of Notch signaling (42), and we believe that these two approaches might be efficiently combined.

Interestingly, LAMA5 has a documented role in breast cancer cell migration and invasion (43), as well as in self-renewal of breast cancer stem cells (44). These studies are focused on laminin-integrin interactions; we suggest that similar functions of LAMA5 are possibly activated in metastatic CRC, where protein networks that include BCAM might be responsible for mechanisms that go beyond the simple tumor/TME recognition here described. Furthermore, because in hepatic metastasis BCAM is co-expressed with LAMA5 in vascular cells, additional autocrine mechanisms are possible, including a role in tumor angiogenesis. Although these aspects need further investigation, it is reasonable to hypothesize that a concomitant targeting of (i) cancer cell attachment to, and potentially invasion of the hepatic vasculature, (ii) sustenance of cancer stem cells, and/or (iii) angiogenesis would have a deep impact on the metastatic cascade, because it might be active also on established secondary tumors.

Finally, despite its characterization as adhesion protein in the Lutheran blood group system and circulating sickle red cells, BCAM has been shown upregulated in skin (45), brain (46), and endometrial-ovarian (47) tumors, in hepatocellular carcinoma (48), and in breast cancer (49), where it represents an independent marker of response to neoadjuvant chemotherapy (50). These data suggest that BCAM-targeted agents might have broad application in different tumor types besides the specific approach against KRAS mutant CRC metastasis described in the present work.

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**TABLES**

**Table 1. Patient cohort analyzed**

	<b>Category</b>	<b>Number</b>	<b>Percent</b>
<b>Sex</b>	M	47/71	66.2%
	F	24/71	33.8%
<b>Age at diagnosis</b>	Mean	63	NA
	Median	65	NA
	>60-y old	42/71	59.2%
	<60-y old	29/71	40.8%
<b>Primary tumor grade at diagnosis*</b>	T1	3/68	4.4%
	T2	5/68	7.4%
	T3	39/68	57.3%
	T4	21/68	30.4%
<b>Lymph node involvement at diagnosis*</b>	Y	49/68	72.1%
	N	19/68	27.9%
<b>Metastasis presentation</b>	Synchronous	33/71	46.5%
	Metachronous	38/71	53.5%
<b>KRAS status</b>	WT	29/71	40.8%
	Mutant	42/71	59.2%
* staging unavailable for 3/71 patients			

## FIGURE LEGENDS

**Figure 1. Identification of BCAM as a candidate receptor at the interface between tumor and TME in KRAS mutant hepatic metastases. (A) Experimental design:** for each cell line, two WT or mutant (G12D, G12V, or G13D) KRAS clones were individually screened with a phage display library *in vitro* (cell culture) or *ex-vivo* (explanted tissues from the intrahepatic mouse models). For each condition, the phage display screens provided 5 experimental points (details in **Suppl. Methods**). **(B) Data analysis flowchart:** phage DNAs extracted from a total of 120 experimental points were deep-sequenced. Phage-displayed heptapeptides were inferred from all sequenced 21-bp oligonucleotide inserts and were subjected to BLAST analysis. Proteins with regions identical to at least 5 heptapeptides were scored based on their enrichment in mutant KRAS and *ex-vivo* experimental points, compared to WT and *in vitro*. **(C) Graph,** TME scores for human proteins retrieved in at least two, or in all three KRAS mutant settings. Scores are the mean of the 3 KRAS mutant settings. **Table,** TME scores (both human and mouse) for BCAM and LAMA5 in each mutant KRAS setting.

**Figure 2. BCAM and LAMA5 are similarly overexpressed in KRAS mutant experimental metastases and patient specimens.** 2- $\mu$ m FFPE sections of (i) experimental metastases from intrahepatic implant of isogenic SW-48 cells in immuno-suppressed mice and (ii) surgically removed human liver metastases were stained with specific anti-BCAM and anti-LAMA5 antibodies and counterstained with hematoxylin. Images were acquired with a microscope-connected digital camera; representative pictures of KRAS mutant or WT tumors are shown. Black arrows, blood vessels; red arrows, stroma. BCAM and LAMA5, human proteins; Bcam and Lama5, mouse proteins.

**Figure 3. BCAM overexpression is associated with KRAS mutant tumors in clinical settings. (A)** BCAM expression in representative human samples (n=10 WT and 10 mutant KRAS) of liver metastasis. 2- $\mu$ m FFPE tissue sections were stained for BCAM with routine hospital protocols and counterstained with hematoxylin. Images were acquired with an Aperio microscope-connected scanner set on default settings. **(B)** Quantitative evaluation of BCAM expression in the complete panel

of human samples (n=29 WT and 42 mutant KRAS). (C) Quantitative evaluation of BCAM expression in a subset (n=18 WT and 16 mutant KRAS) of matched primary CRCs and hepatic metastases. In B and C, DAB signals were isolated by deconvolution and quantified with ImageJ, and are expressed as percent positive area. \*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.01$

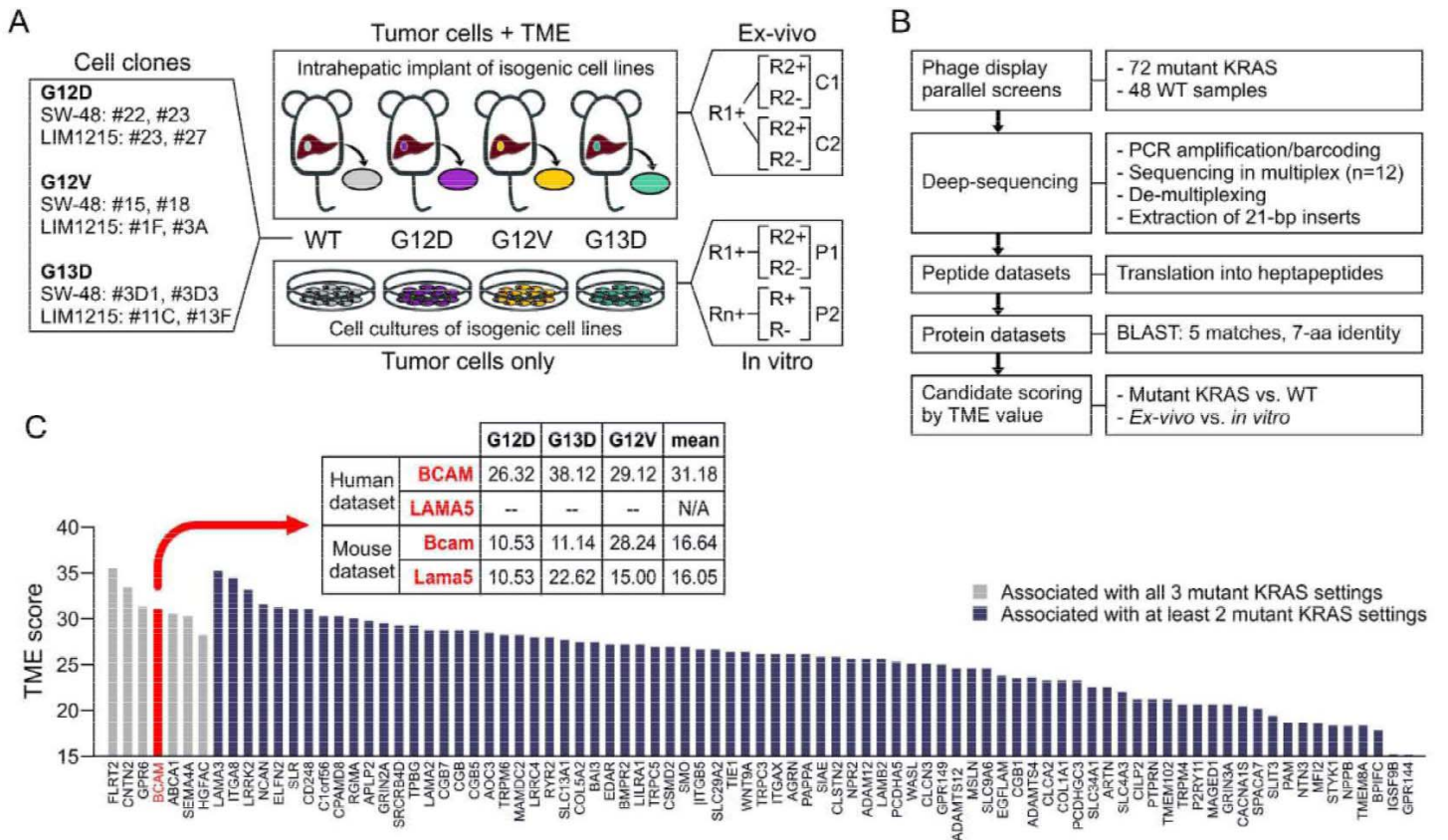
**Figure 4. Human BCAM protein structure and BCAM-mimic peptides.** Schematic illustration of BCAM (i) domains, (ii) regions described as involved in the interaction with LAMA5, and (iii) regions identical to the phage display-identified peptides. For each peptide, the numbers of identity matches and of experimental points in which the sequence has been retrieved are indicated in parenthesis.

**Figure 5. BCAM-mimic synthetic peptides inhibit the intrahepatic growth of human KRAS mutant CRC cells.** Human CRC cell lines with WT (HT-55) or mutant (HCT-116m, DLD-1) KRAS ( $5 \times 10^6$  cells/mouse) were injected into the livers of immuno-suppressed mice (n=6-12), in the presence of either control or BCAM-mimic (pep-BCAM1, pep-BCAM2) peptides (100  $\mu$ m each). Twenty-eight days after the implant, animals were sacrificed, and their livers were explanted and photographed for quantification of external tumor areas. Depending on the number of mice/group, differences between experimental points were evaluated by t-test or Fisher's exact test. FFPE tissue sections were subjected to Mallory's trichrome stain or immunostained with specific anti-Ki67, anti-BCAM and anti-LAMA5 antibodies, followed by counterstaining with hematoxylin. Representative pictures from one mouse/group are shown. BCAM and LAMA5, human proteins; Bcam and Lama5, mouse proteins. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$

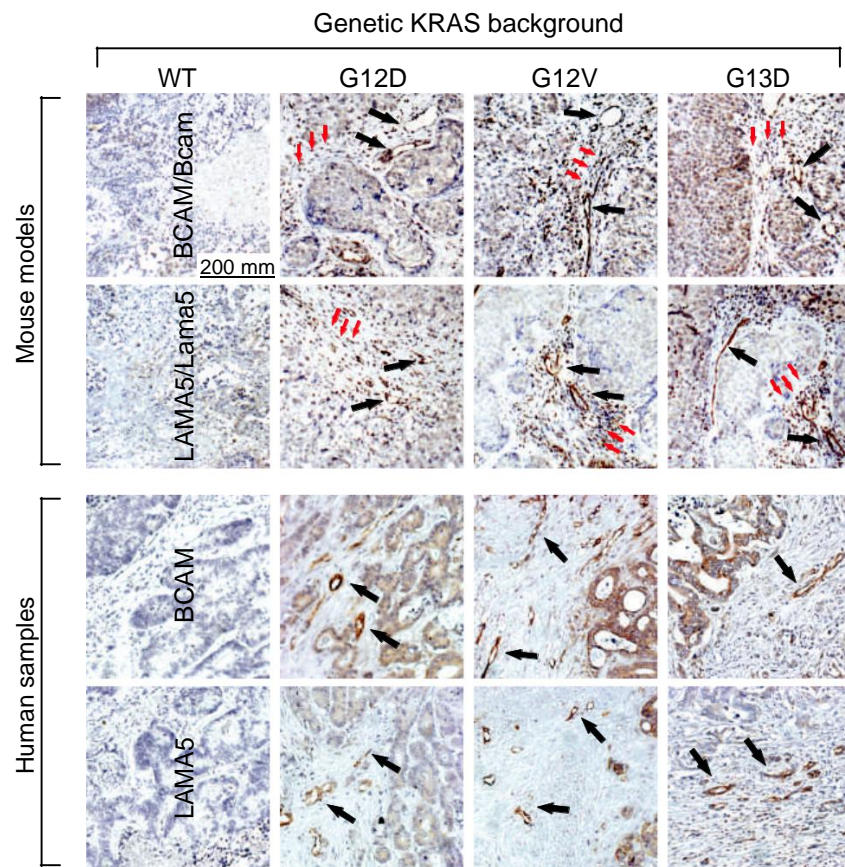
**Figure 6. BCAM and LAMA5 mediate the adherence of KRAS mutant CRC cells to the vascular endothelium.** (A) Fluorescent HTC-116m cells ( $5 \times 10^4$ /well) were subjected to adhesion assays on confluent layers of endothelial cells (HUVECs), pericytes (HBVP) or hepatocytes (THLE-3), in the presence of (i) control, pep-BCAM1 or pep-BCAM2 peptide, or (ii) specific anti-BCAM or anti-LAMA5 antibody. Adhered cells were revealed and counted under a fluorescence microscope. Fluorescent HCT-116m cells were silenced for BCAM expression with 3 different siRNA duplexes (B) and

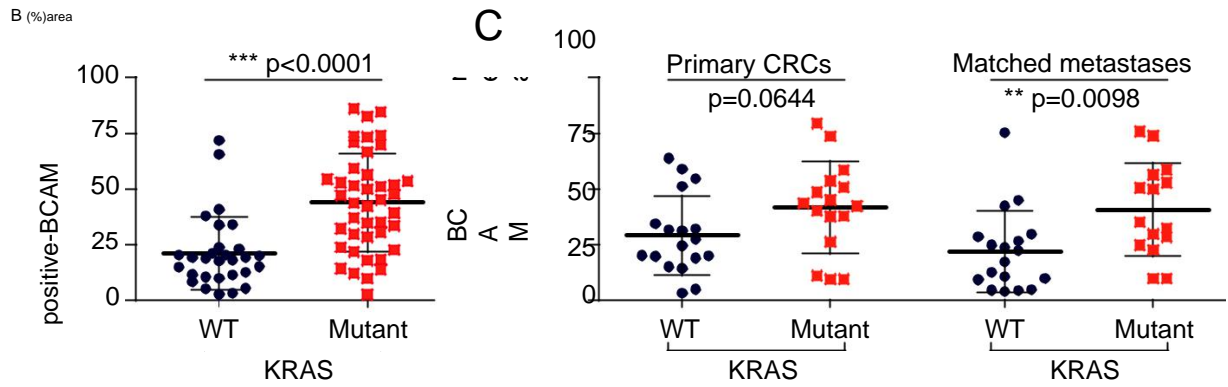
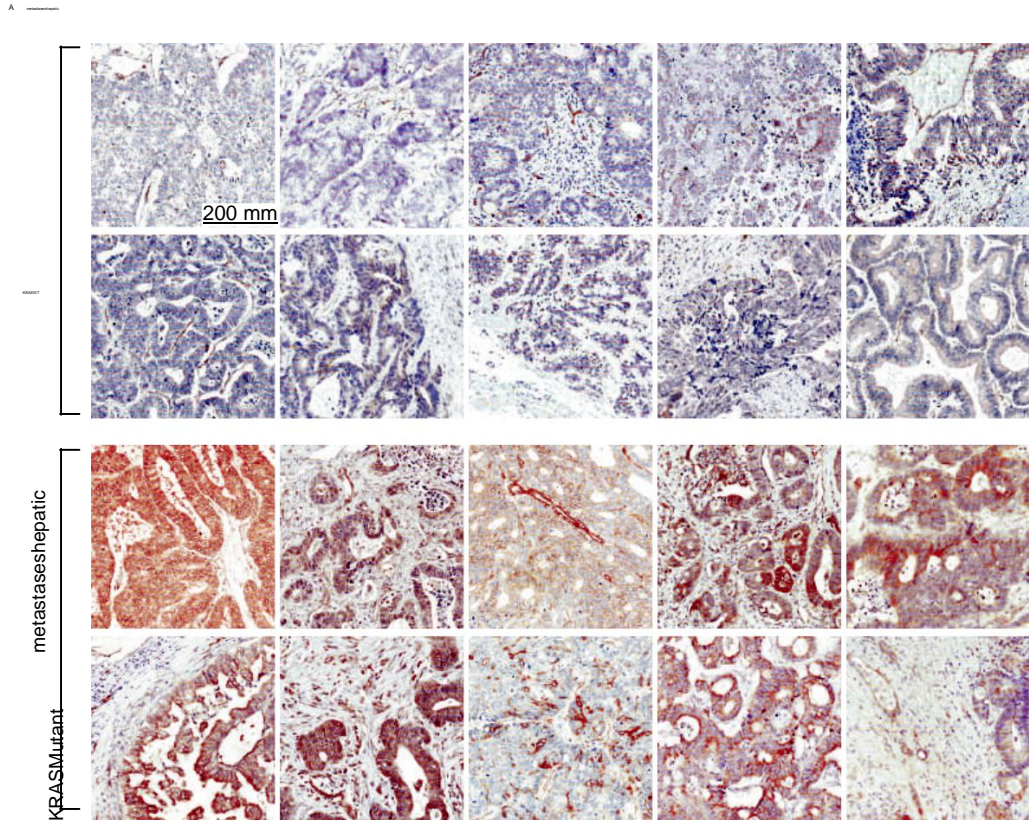
subjected to adhesion assays on HUVECs (C). Graphs indicate mean  $\pm$  S.D. of sextuplicate wells and differences between samples were evaluated by t-test. \*\*\*,  $P < 0.001$





Bartolini et al\_Fig2







Bartolini et al\_Fig4

