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Conditional expression of fascin increases tumor progression in a mouse model of intestinal cancer

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ABSTRACT

While absent from normal epithelia, an actin bundling protein, fascin, becomes expressed in invasive carcinoma of different origins. It is highly enriched at the tumors' invasive front suggesting that it could play a role in cancer invasion. Multiple studies have shown that fascin, through its role in formation of cellular protrusions such as filopodia and invadopodia, enhances cancer cell migration and invasion *in vitro*. However, the role of fascin *in vivo* remains unknown. We have generated a compound transgenic mouse model that allows expression of fascin in the intestinal epithelium in the Apc-mutated background. Conditional expression of fascin led to decrease in mice survival and increase in tumor burden compared to control animals. Induction of fascin expression in adult tumor-bearing animals accelerated tumor progression and led to formation of invasive adenocarcinoma. Altogether, our study shows that fascin can promote tumor progression *in vivo*, but also unravels an unexpected role of fascin in tumor initiation.

Introduction

Colorectal cancer (CRC) is the third most common cancer in the world. Mutations in the gene *Apc* and subsequent activation of the Wnt pathway is one of the initial events in CRC tumor formation (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1998; Radtke and Clevers, 2005). Characterization of the genetic alterations involved in CRC progression has been done by several groups and highlights among others the key roles of KRAS, p53 and Notch pathways (Fearon and Vogelstein, 1990; Fre et al., 2009; Radtke and Clevers, 2005). However, the mechanisms driving invasion and metastasis formation are still poorly understood.

We and others have shown that fascin is expressed in several human tumors, among them CRC (Hashimoto et al., 2004; Hashimoto et al., 2005; Jawhari et al., 2003; Pelosi et al., 2003; Puppa et al., 2007; Vignjevic et al., 2007; Yoder et al., 2005). While fascin is not expressed in normal intestinal tissue and tumor bulk, it becomes expressed at the tumor invasive front (Vignjevic et al., 2007). Its expression correlates with advanced tumor grade and poor prognosis in human colorectal cancer (Hashimoto et al., 2006; Vignjevic et al., 2007). Fascin is an actin-bundling protein, that plays a role in cell migration by controlling the dynamics of focal adhesions (Elkhatib et al., 2014) and formation of finger-like protrusions called filopodia (Vignjevic et al., 2006). Moreover, fascin is necessary for the formation of invasive protrusions called invadopodia and increases cell invasion *in vitro* (Li et al., 2010; Schoumacher et al., 2010; Vignjevic et al., 2007). Finally, fascin increases cell dissemination *in vivo* (Vignjevic et al., 2007), suggesting that fascin may promote metastasis development. However, the above studies were performed *in vitro* or after tail vein injection of human cells in immunodeficient mice, conditions that do not fully recapitulate the environment and constraints present in human tumors. There is so far no evidence that fascin is necessary and sufficient to promote invasion and metastasis in intestinal tumors.

In this study, we asked whether fascin expression in a mouse model of colorectal cancer could (1) increase tumor aggressiveness and (2) promote invasion and metastasis formation. We generated a genetically modified mouse model in which fascin expression could be specifically induced in the mouse intestine. These mice were then crossed with animals that spontaneously develop intestinal tumors due to a mutation in the *Apc* gene (Fodde et al., 1994). We found that mice with constitutive fascin expression lived significantly shorter and developed a higher number of tumors, providing

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evidence of a novel unexpected role of fascin in tumor initiation. To mimic fascin expression in human colon tumors more closely, we also generated inducible mouse model in which fascin expression was induced in adult tumor-bearing animals. Those animals show accelerated tumor progression and formation of invasive adenocarcinoma, supporting the hypothesis that fascin could promote tumor progression *in vivo*.

Materials and methods

Animal care

All mice were obtained from Charles Rivers (Orléans, France) and kept in the Curie Institute specified-pathogen free animal area for mouse breeding and experimental purposes (facility licence #B75-05-18) under the authority of the veterinarian Virginie Dangles-Marie from the Institute Curie. The care and use of animals used in this facility is strictly applying European and national regulation for the protection of vertebrate animals used for experimental and other scientific purposes (Directive 86/609 and 2010/63).

Transgene construction

We started from pFascin-IRES-GFP construct (plasmid 1). The vector pCAG-CAT-LacZ was obtained from J. Miyazaki (Osaka University, Japan). pCAG-CATLacZ and pENTRY (Invitrogen) were used to insert in plasmid 1 a stop cassette (CAT) flanked by two loxP sites. The LacZ sequence of the pCAG-CAT-LacZ vector was first removed by BamHI digestion. The chicken-actin promoter (pCAG) and the CAT cassette flanked by two loxP sites were cut out form the pCAG-CAT pBluescript vector and inserted into pENTRY by KpnI/NotI digestions. The plasmid obtained was named pENTRY-pCAG-CAT (plasmid 2). Finally, the Fascin-IRES-GFP sequence of the plasmid 1 was amplified by PCR in which SpeI and NotI sites were added at the 3′ and 5′ ends, respectively. The PCR fragment was then inserted in the plasmid 2, previously linearized by SpeI/NotI digestion, to obtain the plasmid used for transgenesis: pENTRY-CAG-loxCATlox-Fascin-Ires-GFP.

Generation of transgenic mice (classical transgenesis)

The DNA fragment containing the actin promoter, the CAT cassette and the Fascin-IRES-GFP cDNA (pCAG-CAT-Fascin-IRES-EGFP) was prepared by digestion of pENTRY-CAG-loxCATlox-Fascin-Ires-GFP with the enzymes AfeI/EcoRV. After purification by elutips (Schleicher and Schull, Keene, NH), the transgene were injected into pronuclei of fertilized oocytes prepared from the hybrid line B6D2 (C57Bl/6 × DBA/2). Germline transmission was detected by polymerase chain reaction (PCR) from DNA isolated from mouse tails with the following primers: *5′-CCAGACCGTTCAGCTGGATATTACGGCCTTT-3′* (forward) and *5′-CCTGAATCGCCAGCGGCATCAGCA-3′* (reverse). These primers are amplifying the CAT cassette in order to distinguish the transgene from the endogenous *fascin* gene.

Generation of transgenic mice (targeted transgenesis)

This mouse model was generated using the "speedy mouse" technique developed by NUCLEIS (Lyon, France), which uses direct–targeted insertion of the transgene in the *hprt* (Hypoxanthine PhosphoRibosylTransferase) locus. This technique uses specialized embryonic stem cells (BPES) which lack a part of the *hprt* locus and can therefore accept the construct of interest recombined with the deleted portion of *hprt* gene. The vector expressing the transgene contains homologous sequences of the *hprt* gene and allows the homologous recombination between the transgene and the *hprt* locus.

Genotyping

DNA was extracted from mouse tails pieces in lysis buffer (67 mM Tris HCl pH 8.8; 16 mM $(NH_4)_2SO_4$; 0.5% Tween20; 0.4 mg/ml proteinaseK) overnight at 55 °C. After inactivation of the enzyme for 20 min at 95 °C, genotyping was performed using standard procedures with the primers listed below:

Gene	Forward primer	Reverse primer
Apc^{1638N}	5′-TCAGCCATGCCAA-CAAAGTCA-3′	5′-GCCAGCTCATTCC-TCCACTC-3′
Fascin	5′-CCAGACCGTTCAGCTG-GATATTACGGCCTT-3′	5′-CCTGAATCGCCAGCGG-CATCAGCA-3′
Cre	5′-CAAGCCTGGCTCGA-CGGCC-3′	5′-CGCGAACATCTTCA-GGTTCT-3′

Analysis of tumor grades

Animals were killed at the ages indicated in the text. Macroscopically visible tumors were resected and embedded in paraffin according to standard procedures. Paraffin sections were stained with hematoxylin and eosin and tumors were classified according to standard World Health Organization (WHO) histopathologic criteria.

Antibodies

The following antibodies were used to detect Cre (polyclonal from Novagen), Fascin (clone 55K-2, Dako), Ki67 (polyclonal from Abcys Rabbit), Villin (polyclonal homemade antibody).

Immunofluorescence and immunohistochemistry staining on paraffin-embedded tissues

Tissues were fixed for 2 h in 4% paraformaldehyde in PBS, washed in PBS and embedded in paraffin. 4 μm-thick sections were cut and proceed for immunostaining as followed. Antigen retrieval was performed for 20 min in a boiling antigen unmasking solution (Vector Laboratories). Sections were blocked with 5% fetal calf serum in PBS, incubated with primary antibodies in the blocking solution for 2 h at RT or overnight at 4 °C, and followed by incubation with secondary antibodies for 1 h at RT. Sections were mounted in AquaPolymount (Polysciences). Images were acquired with a wide-field fluorescence microscope (DM6000 B/M, Leica) equipped with a CCD camera (CoolSNAP HQ; Photometrics) and Apotome system with a 40× water Plan – Apochromat lens (Zeiss). Fascin expression in tumors was quantified by measuring fluorescence intensity of fascin staining on whole images taken from $n = 2$–6 tumors normalized to fascin fluorescence intensity in A/C mice.

Western blot analysis

Snap-frozen mouse tissues were lysed in ice-cold Laemmli buffer using a 1 ml Dounce Homogenizer. After centrifugation (15 000 × *g*, 15 min, 4 °C), supernatants were collected and protein concentration was determined using the BCA kit (Pierce). Equal amounts (30 g) of protein lysates were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and blocked in 5% nonfat dried milk for 30 min. The membranes were incubated with primary antibodies for 1 h at RT or overnight at 4 °C, and followed by incubation with horseradish peroxidase-conjugated secondary