ORIGINAL ARTICLE

IMP1 3' UTR shortening enhances metastatic burden in colorectal cancer

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Abstract

The RNA-binding protein insulin-like growth factor 2 mRNA binding protein 1 (IMP1) is overexpressed in colorectal cancer (CRC); however, evidence for a direct role for IMP1 in CRC metastasis is lacking. IMP1 is regulated by let-7 microRNA, which binds in the 3' untranslated region (UTR) of the transcript. The availability of binding sites is in part controlled by alternative polyadenylation, which determines 3' UTR length. Expression of the short 3' UTR transcript (lacking all microRNA sites) results in higher protein levels and is correlated with increased proliferation. We used *in vitro* and *in vivo* model systems to test the hypothesis that the short 3' UTR isoform of IMP1 promotes CRC metastasis. Herein we demonstrate that 3' UTR shortening increases IMP1 protein expression and that this in turn enhances the metastatic burden to the liver, whereas expression of the long isoform (full length 3' UTR) does not. Increased tumor burden results from elevated tumor surface area driven by cell proliferation and cell survival mechanisms. These processes are independent of classical apoptosis pathways. Moreover, we demonstrate the shifts toward the short isoform are associated with metastasis in patient populations where IMP1-long expression predominates. Overall, our work demonstrates that different IMP1 expression levels result in different functional outcomes in CRC metastasis and that targeting IMP1 may reduce tumor progression in some patients.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide. In the USA, over 140 000 cases are diagnosed and nearly 55 000 people die of the disease each year (1). In its early stages, it is highly treatable and has a favorable prognosis by virtue of early detection and intervention. However, late stage and metastatic CRC has a <10% 5-year survival and fewer treatment options (2). Although the number of studies examining metastatic

mechanisms of CRC is growing, our understanding of the molecular mediators at each step of the metastatic cascade is incomplete (3).

The RNA-binding protein IMP1 (insulin-like growth factor 2 mRNA binding protein 1) modulates CRC pathogenesis (4–7). IMP1 is overexpressed in over 80% of CRC (8, 9). The role of IMP1 in the pathogenesis of CRC is dependent upon the compartment

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Abbreviations

APA	alternative polyadenylation
CDS	coding sequence
CRC	colorectal cancer
miRNA	microRNA
NEB	New England Biolabs
UTR	untranslated region

of expression (epithelial versus mesenchymal) (5, 6); whether one considers tumor initiation, progression, or metastasis; and the type of animal model (7, 10–12). In the context of advanced CRC, IMP1 expression is associated with severe tumor grade, poor patient outcomes (8, 11), and metastasis (8, 10, 11). Nonetheless, how IMP1 contributes mechanistically and functionally to CRC metastasis *in vivo* is lacking, thereby forming the basis for the current study.

IMP1 expression is regulated in part through microRNA (miRNA) binding sites, most notably let-7, located within the 3' untranslated region (UTR) of the transcript (13). The length of the UTR, and therefore the presence of these miRNA sites, is determined by polyadenylation. Many eukaryotic transcripts contain multiple polyadenylation signals, thereby resulting in generation of transcripts of variable length, depending upon which polyA site is used. The usage of multiple polyA sites is termed alternative polyadenylation (APA) (14). APA affects RNA stability, localization, translation rate and secondary structure (14). Usage of the proximal polyA site or 3' UTR shortening occurs in a number of solid tumors and can influence protein expression levels by altering transcript stability and conformation (15). Shorter 3' UTRs are associated with aggressive tumors in breast, lung (16) and colon (17). IMP1 transcripts can undergo APA, resulting in loss of let-7 and other miRNA regulatory sites. *In vitro* work suggests that the shorter 3' UTR isoform of IMP1 confers higher colony-forming potential when expressed in fibroblast cells (13); however, little is known about the role of APA and the 3' UTR isoforms of IMP1 *in vivo*.

This led us to ask whether IMP1 enhances CRC metastasis *in vivo* and whether metastatic potential is influenced functionally by protein levels controlled by the IMP1 3' UTR, thereby addressing a critical gap in the field.

Materials and methods

DNA cloning

All primer sequences and DNA duplexes used for cloning are listed in [Supplementary Table 1](#), available at *Carcinogenesis* Online. The PiggyBac-FLAG-IMP1-UTR constructs were generated using conventional cloning methods. The PiggyBac construct (PB513-1; System Biosciences; Mountain View, CA) contains a cytomegalovirus (CMV) promoter and green fluorescent protein (GFP) and puromycin selectable markers. Portions of the final constructs were cloned from pMSCV PIG (puro IRES GFP empty vector), pIS1-long UTR and pMSCV PIG Imp-1 short (13) generously provided by Dr. Bartel.

All general cloning steps were performed as follows: digests were performed for 2 h at 37°C using the indicated enzymes (New England Biolabs (NEB), Ipswich, MA) and following manufacturer instructions. Vectors were phosphatase treated to prevent religation using calf intestinal alkaline phosphatase (NEB) and following manufacturer instructions. Isolation of desired DNA fragments was performed using a GeneJet Gel Extraction kit or GeneJet PCR Purification kit (ThermoFisher Scientific) and following manufacturer instructions. Ligations were performed for a minimum of 2 h up to overnight at 16°C using T4 DNA ligase (NEB) and following manufacturer instructions. Vector:insert ratios were calculated using NEB Ligation Calculator (<http://nebiocalculator.neb.com/#/ligation>). Ligation product (2 µl) was transformed into competent cells following manufacturer instructions. Competent cells were plated on Luria-Bertani (LB) broth +

ampicillin (50 µg/ml) plates. DH5α subcloning efficiency cells (Invitrogen) were used for all subcloning steps except those involving the PiggyBac vector where One-Shot TOP10 chemically competent cells (Invitrogen) were used. Ampicillin-resistant colonies were individually picked, grown overnight in a shaker at 37°C in LB + ampicillin broth (50 µg/ml); DNA was isolated using GeneJet Plasmid Mini Prep Kit (ThermoFisher, Waltham, MA) following manufacturer instructions. DNA was eluted from the column in 1:10 elution buffer diluted in water. DNA sequencing confirmed the correct ligation product using seq primers listed in [Supplementary Table 2](#), available at *Carcinogenesis* Online (University of Pennsylvania DNA Sequencing Facility).

The IMP1-long UTR construct was generated as follows: the multiple cloning site of pMSCV PIG and the PiggyBac vector were replaced with a new multiple cloning site DNA duplex containing the following digest sites from 5' to 3' *Sall*, *BamHI*, *SwaI*, *AgeI*, *PmeI*, *NotI*, *XhoI*, *MluI* with *BglIII*/*AgeI* and *XbaI*/*NotI* sticky ends, respectively. The sticky ends destroyed the cut sites following ligation. The pMSCV PIG with the new multiple cloning site is referred to as pMSCV* and the PiggyBac as PB*. The IMP1 coding sequence (CDS) was amplified from pMSCV PIG Imp-1 short using primers that added *AgeI* and *BamHI* cut sites to the ends of the CDS. pMSCV* and the *AgeI*-IMP1 CDS-*BamHI* fragment were digested with *AgeI*-HF/*BamHI*-HF, purified, ligated and transformed into competent cells. Ampicillin-resistant colonies were selected, screened and sequenced for the IMP1 CDS insert. The sequence of the entire CDS was confirmed and point mutations were repaired using the QuikChange II XL Site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and following manufacturer instructions. XL10 Gold Ultracompetent cells (included with the kit) were used for all transformations following site-directed mutagenesis. The confirmed pMSCV*-IMP1 clone was then digested with *BamHI*/*BglIII* and a DNA duplex containing a Kozak sequence-3X FLAG-proximal IMP1 CDS upstream of the *BglIII* site with *BamHI*/*BglIII* sticky ends was ligated in and transformed into competent cells. Ampicillin-resistant colonies were selected, screened and sequenced for the presence of the 3X FLAG duplex. The long 3' UTR was then added by digesting the pMSCV*-FLAG-IMP1 and the pIS1-long UTR constructs with *AgeI*-HF/*NotI*-HF. The desired product was gel purified, ligated and transformed into competent cells. Ampicillin-resistance colonies were selected and confirmed by sequencing. The pIS1-long UTR construct contained the five mutated APA sites as described in (13), which were confirmed by sequencing. Finally, the FLAG-IMP1-long UTR construct was subcloned into the PB* vector by digestion with *Sall*-HF/*MluI*-HF. Ampicillin-resistant colonies were screened and sequenced to confirm the presence of the FLAG-IMP1-long UTR construct.

The IMP1-short UTR construct was generated as follows: The pMSCV*-FLAG-IMP1-long UTR and pMSCV PIG Imp-1 short constructs were digested with *MfeI*-HF/*XhoI* to swap the long UTR with the short UTR. Ampicillin-resistant colonies were sequenced for the presence of the short UTR. The FLAG-IMP1-short UTR construct was then subcloned into the PB* by digestion of pMSCV*-FLAG-IMP1-short UTR and PB* with *Sall*-HF/*MluI*-HF/*NcoI*-HF to aid in gel isolation of the appropriate fragment. The *Sall*/*MluI* fragment was then ligated into the *Sall*-HF/*MluI*-HF digested PB* vector. Following transformation, ampicillin resistant clones were selected and sequenced for the FLAG-IMP1-short UTR construct.

Transfection

SW480 and HT-29 cell lines were obtained directly from the American Type Culture Collection (ATCC) and all experiments were performed within 6 months of receipt or resuscitation. The ATCC authenticates all human cell lines through short tandem repeat analysis. All cell lines were confirmed to be mycoplasma negative every 3 weeks (MycAlert Mycoplasma Detection Kit, Lonza). IMP1 was deleted in HT-29 and SW480 cells by cotransfecting cells with IMP1 CRISPR/Cas9 KO Plasmid (h) (Santa Cruz; sc-401703) and IMP-1 HDR Plasmid (h) (Santa Cruz; sc-401703 HDR) followed by sorting and expansion of red fluorescent protein (RFP)+ve cells. Knockout was verified by western blotting for IMP1. SW480 and HT-29 IMP1-/- cells were subsequently transfected with one of the PiggyBac IMP1 UTR constructs (PB-empty, PB-IMP1-long, PB-IMP1-short) using lipofectamine-3000 in a 5:1 ratio with Hyperactive Piggybac Transposase. Transfection reagents were prepared in Opti-MEM and incubated for 10 min before dropwise addition to the cells. The media were changed 24 h post-transfection. The cells were fluorescence-activated cell sorting

sorted for dual RFP/GFP-positive cells (IMP1^{-/-} and PB-IMP1 construct) and grown in the presence of 2 µg/ml puromycin to maintain selection for CRISPR/PB vectors. PB-IMP1-expressing cells were validated by western blot for IMP1 and FLAG.

Xenograft animal models

All animal studies were performed in accordance with University of Pennsylvania IACUC (protocol # 805829) guidelines.

Subcutaneous xenograft model

NCr nude mice aged 7–8 weeks (CrTac:NCr-Foxn1^{nu}; Taconic, Germantown, NY) were given 5 Gy of gamma irradiation prior to injection. Animals were anesthetized with ketamine (70–100 mg/kg) and xylazine (5–10 mg/kg) prior to injection of 1–2.5 × 10⁶ cells (HT-29 empty vector, HT-29 IMP1-long, or short 3' UTR) in 100 µl 50:50 Matrigel and Dulbecco's modified Eagle's medium (DMEM)-high glucose in the left and right flank. Tumor volume was measured weekly using calipers and tumor mass was measured following tissue harvest at weeks 4–5. Data presented are an average of both two independent experiments and normalized to the respective empty vector control for that experiment, *n* = 4–12 animals per UTR group.

Splenic injection liver metastasis model

Eight-week-old NCr nude mice (CrTac:NCr-Foxn1^{nu}; Taconic, Germantown, NY) were given 5 Gy of gamma irradiation prior to surgery. Animals were administered subcutaneous 1 mg/kg BW of sustained-release buprenorphine (Zoopharm, Windsor, CO) and 50 µl 0.25% bupivacaine. Animals were anesthetized with 2–3% isoflurane. The skin was cleaned with iodine prior to making a small incision in the left abdominal wall. The spleen was externalized and 1 × 10⁶ cells (HT-29 empty vector, HT-29 IMP1-long, or short 3' UTR) in 50 µl of phosphate-buffered saline (PBS) were injected into the splenic capsule with a 31G needle. All cell lines were confirmed to be mycoplasma negative prior to injection (Lonza). A drop of VetClose tissue glue (Henry Schein Animal Health) was applied at the injection site and allowed to dry prior to placing the spleen back into the abdominal cavity and suturing the incision closed. Body weight was monitored daily for the first 72 h and weekly thereafter until tissue harvest at 6 weeks. Two independent experiments were performed with a total of *n* = 7–9 animals per UTR group.

Tissue harvest

Six weeks after splenic injections, animals were euthanized by CO₂ asphyxiation and livers were removed. Gross metastases were quantified and measured using a dissecting microscope by a researcher blinded to the tumor IMP1 status. Metastases were lysed for western blot, snap frozen, dissociated for tumor-derived cell lines and/or fixed in 10% zinc-buffered formalin.

Tumor-derived cell lines

Tumors were removed from surrounding liver tissue and minced in DMEM High glucose (Gibco), 10% penicillin/streptomycin (Gibco), 10 ml/l Fungizone (Gibco; DMEM media) using sterile scissors in a sterile hood. Tissue was washed several times in DMEM media prior to digestion with Type IV collagenase (2 mg/ml, Gibco) in DMEM media. Digestion performed on a rotator at 37°C for 25–30 min. Tissue was further digested using 0.05% Trypsin-ethylenediaminetetraacetic acid (Gibco) for 5 min in 37°C water bath. Trypsin digestion was neutralized with DMEM-10% fetal bovine serum and contents were filtered over 40 µm filter prior to plating. Cells were cultured in the presence of 2 µg/ml puromycin to select for CRISPR/PB vector-expressing cells and cell lines were sorted for GFP to enrich for the PB-IMP1 vector prior to experimentation.

Histology

Subcutaneous tumors and livers containing metastases were fixed in 10% zinc-buffered formalin for 24 h at 4°C. Tissues were washed 3× in 1× PBS and placed in 70% ethanol prior to paraffin embedding. Sections (5–7 µm) were mounted on glass slides and stained with hematoxylin and eosin (H&E), Ki67 (Abcam ab16667, 1:1000). Samples were imaged on a bright field microscope (Nikon E600) by a pathologist blinded to tumor IMP1 status. The percent of necrosis was calculated in each H&E stained slide using the planimetric function for area determination (image analysis

software, NIS elements). The total area of the tumor section and the area corresponding to necrotic tissue were determined using the mouse cursor. The software calculates percent of necrotic area.

Ki67 scoring was performed by counting the number of Ki67-positive versus Ki67-negative nuclei in 10–40× fields taken at random within the tumors present in a single stained section, *n* = 4 animals per tumor genotype. Images were taken and counted by an observer blinded to the tumor genotype. Counting was performed using the ImageJ Cell Counter plugin.

Western blot

Cells or tumors were lysed on ice in 1× (cells) or 2× (tumors) cell lysis buffer [Cell Signaling Technologies (CST)] containing 2 mM of phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche) or 1× radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technologies) containing 0.1% sodium dodecyl sulfate, and protease inhibitor cocktail. Cells were lysed using a 21G needle. Tumors were homogenized using a hand-held tissue homogenizer, passed through a 21G needle, and sonicated 2×, 30 s. Protein concentration was quantified by BCA. 4–12% NuPage Bis-tris gels (ThermoFisher Scientific) were loaded with 15 µg protein and run at 100 V for 2 h using 1× (N-morpholino) propanesulfonic acid sodium dodecyl sulfate running buffer (ThermoFisher Scientific). Protein was transferred to polyvinylidene difluoride membrane (Immobilon-FL, Millipore, Temecula, CA) and detected using the following primary antibodies: CTNNB1 (BD Transduction 610153, 1:2000); FLAG M2 (Sigma F1804; 1:5000); GAPDH (Chemicon Mab374; 1:10 000); GSK3β (CST 9332, 1:1000); IMP1 (MBL RN007P; 1:1000); LRP6 (CST 2560, 1:1000); MCL1 (CST D35A5, 1:1000); cMYC (Santa Cruz sc-764, 1:250); RAC (Upstate 05-389, 1:500); XIAP (CST 14334, 1:1000). Secondary antibodies conjugated to horseradish peroxidase or fluorophores were used prior to exposure to film or reading on the LI-COR Odyssey.

Flow cytometry

Cells were released from the plate using trypsin, 96 h post-plating and washed twice in PBS. Cells were resuspended in Annexin-V binding buffer (0.1 M HEPES pH = 7.4, 1.4 M NaCl, 25 mM CaCl₂), APC-Annexin-V (BioLegend, San Diego, CA) and 7-AAD (1 µg) and incubated for 15 min in the dark at room temperature. Samples were diluted 1:5 with Annexin-V binding buffer prior to quantification using the Accuri C6 (BD Biosciences, Ann Arbor MI). Experiment was conducted twice on three independent tumor-derived cell lines per group, each originating from a different mouse. GFP-positive cells (possessing the PiggyBac vector plasmid) were analyzed.

Immunofluorescence staining

Tumor-derived cell lines (*n* = 3 independent cell lines per group) were grown on coverslips and fixed for 15 min at room temperature in 4% paraformaldehyde. Following three washes in 1× PBS, cells were stained following the immunostaining protocol from Cell Signaling Technologies. The following antibodies were used: anti-FLAG (1:200; Sigma F1804), anti-phalloidin-Cy3 (1:500; ThermoFisher Scientific A12380), anti-mouse-Cy2 (1:600; Jackson Immunolabs, West Grove, PA). Coverslips were mounted using Vectashield anti-fade mounting media with 4',6-diamidino-2-phenylindole (Vector Labs, Burlingame, CA) and imaged using a Leica confocal microscope (Leica, Buffalo Grove, IL).

Apoptotic protein array

Protein lysates from empty vector and IMP1-short tumor-derived cell lines (*n* = 4 per genotype) were isolated in 1× cell/tissue lysis buffer (RayBiotech, Norcross, GA) supplemented with 2 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche). Samples were hybridized to G-series apoptosis array (AAH-APO-G1, RayBiotech) and analysis was performed by RayBiotech staff.

WST1 Assay

Tumor-derived cell lines (*n* = 3 independent cell lines per group) were plated in 96-well plates at a density of 2000 cells/well. Cell viability was measured by WST-1 (Roche) assay at 24 h (baseline), 48, 72 and 96 h post-plating. Data represent the average of two independent experiments that are expressed relative to the empty vector control. All values are normalized to the corresponding baseline reading.

RNA isolation and quantitative polymerase chain reaction

RNA was isolated from cell lines or subcutaneous xenograft tumors using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and following the manufacturer protocol. Tumor samples were homogenized with an electric mortar and pestle prior to homogenization with a Qias shredder column (Qiagen). All samples were DNase treated (Qiagen) and quality was confirmed by gel electrophoresis. cDNA was generated using the High-Capacity Reverse Transcription kit with RNase inhibitor (Applied Biosystems, Foster City, CA) and following the manufacturer's protocol. Quantitative polymerase chain reaction (qPCR) was performed using the TaqMan Fast Universal PCR Master Mix (ThermoFisher) and the following primer/probe sets: IGF2BP1 (Hs 00198023_m1; ThermoFisher); PPIA (Hs99999904_m1; ThermoFisher).

APA analysis in RNA microarray datasets

Expression ratio indices were calculated from published microarray datasets using published methods (16, 18) and R version 3.4.1 (19). Datasets were generated using human patient samples and the Affymetrix HG-U133_Plus_2 array platform and are available from GEO repository accession: GSE17538 (20); GSE14333 (21); GSE39582 (22). Local disease was defined as stages 1–2; metastatic disease was defined as stages 3–4.

Statistical methods

Unpaired t-test analyzed differences between two groups. One-way analysis of variance analyzed differences between endpoint measurements across all three experimental groups. Two-way repeated measures analysis of variance compared proliferation values in the WST1 assay over time. t-Test and analysis of variance analyses were performed using Prism 6 Software (GraphPad Software, La Jolla, CA). Linear mixed effects

models compared lesion surface area between experimental groups (i.e. Empty, Long, Short). Most animals had numerous lesions within the liver and thus, contributed multiple surface area measurements to the analysis. Mixed effects models allow for group comparisons, while accounting for the correlation among multiple measurements within an animal. The *mixed* command in STATA v15 (StataCorp, College Station, TX) was used. An unstructured covariance structure was assumed. Probability plots were employed to assess whether lesion surface area was normally distributed. Natural log transformation was subsequently applied to normalize the distribution of lesion surface area, prior to modeling.

Results

IMP1 expression correlates inversely with 3' UTR length

To define the role of the IMP1 3' UTR in regulating protein expression, we generated two FLAG-tagged IMP1 3' UTR constructs and the corresponding empty vector control (13). The IMP1-long construct (Long) contains the full 6.7kb 3' UTR with all regulatory miRNA binding sites, including the six predicted let-7 binding sites. However, the APA sites were mutated to ensure use of the most distal APA site and generation of transcripts containing only the full length 3' UTR (Figure 1A). The IMP1-short construct (Short) contains only the most proximal polyadenylation site, resulting in an mRNA transcript lacking all miRNA regulation (Figure 1A).

We then expressed these constructs in two CRC cell lines, HT-29 and SW480, in which endogenous IMP1 had been deleted using CRISPR/Cas9 (Supplementary Figure 1, available

Figure 1. IMP1 expression levels are modulated by 3' UTR length. (A) PiggyBac vectors containing 3X-FLAG-tagged IMP1 CDS followed by either the full length (long) or truncated (short) 3' UTR were generated. The long form contains all 6 putative let-7 binding sites, whereas the short form lacks all miRNA (including let-7) regulation. All but the most distal polyadenylation (APA) sites are mutated in the long construct to ensure usage of the most distal APA site and expression of exclusively the long 3' UTR. (B) Following transfection into cell lines lacking endogenous IMP1, IGF2BP1 mRNA levels were confirmed by qPCR. Data are normalized to housekeeping gene PPIA. **P* < 0.05 versus empty by one-way ANOVA. (C) Western blotting demonstrated an inverse relationship between IMP1 protein expression and 3' UTR length. *n* = 3 biological replicates per cell line. (D) Western blotting for FLAG protein validated the inverse relationship between IMP1 protein levels and 3' UTR length. *n* = 3 biological replicates per cell line. APA, alternative polyadenylation; CDS, coding sequence; UTR, untranslated region

at Carcinogenesis Online). Wild-type HT-29 cells express negligible IMP1, whereas wild-type SW480 cells exhibit robust IMP1 expression (7). Expression of the long construct resulted in low-moderate levels of IMP1 expression as measured by qPCR (Figure 1B), FLAG-IMP1 and IMP1 western blotting (Figure 1C and D). The short construct, which lacks all miRNA regulation, expressed >5-fold more IMP1 than the long isoform-expressing cells (Figure 1C), suggesting that IMP1 protein expression is influenced by 3' UTR length.

IMP1 enhances tumor mass and limits necrosis

To determine whether low-moderate IMP1 expression (long isoform) or high IMP1 expression (short isoform) had functional effects on tumor growth or metastasis *in vivo*, we performed subcutaneous xenografts and splenic injections. Both IMP1-expressing cell lines formed tumors with greater mass relative to the empty vector control (Figure 2A); however, there was no difference in tumor volume relative to IMP1 expression (Figure 2B). Notably, a significant reduction in necrosis was observed in the IMP1-short-expressing tumors (Figure 2C and D). This suggests that expression of moderate-to-high levels of IMP1 protein driven by either the long or short isoform enhances tumor density. In the case of the short isoform, this is in part through reduced necrosis. We confirmed maintenance of IMP1 expression in the tumors by qPCR (Supplementary Figure 2A, available at Carcinogenesis Online).

We next used the splenic injection model to assess the effects of the IMP1 3' UTR length on liver metastasis. HT-29 cells expressing empty vector, long, or short IMP1 3' UTR construct were injected into the spleens of athymic nude mice and metastatic burden measured 6 weeks later. Maintenance of FLAG-IMP1 expression within the tumors was confirmed by western blot (Supplementary Figure 2B, available at Carcinogenesis Online). Animals injected with IMP1-short-expressing cells had livers with greater mass (Figure 2E) due to the formation of larger tumors (Figure 2F). However, there was no difference in the total number of metastases (Figure 2G). The majority of the metastases were moderately differentiated (Figure 2H). This suggests that only high levels of IMP1 expression, driven by IMP1-short are sufficient to enhance the metastatic burden to the liver.

IMP1 enhances cell proliferation

To understand the mechanism by which IMP1, particularly IMP1-short, enhances metastatic burden in the liver, we generated tumor-derived cell lines from xenograft liver metastases. We confirmed FLAG-IMP1 expression by western blot (Supplementary Figure 2C, available at Carcinogenesis Online). Localization signals within the 3' UTR can influence transcript and in turn protein localization following translation (14). To rule out differences in protein localization between IMP1-long and IMP1-short contributing to the phenotypic differences in tumor growth, we performed immunofluorescence staining for FLAG-IMP1. FLAG-IMP1 was present throughout the cytoplasm of all cells expressing the construct and did not appear to be differentially localized based upon the length of the 3' UTR of the construct of origin (Figure 3A).

We measured cell proliferation relative the IMP1-3' UTR status using a WST1 assay (Figure 3B) and found that IMP1 expression significantly enhanced cell proliferation relative to the empty vector control regardless of 3' UTR status; however, high IMP1 expression driven by the short isoform also enhanced proliferation compared with the long isoform, indicating a positive correlation between IMP1 protein levels

and proliferation rate. Furthermore, this suggests that increased proliferation is one factor contributing to increased tumor mass and enhanced metastatic burden. There was also a trend for an increase in Ki67+ staining in tumors derived from IMP1-short-expressing cells (Figure 2H and I).

WNT signaling is upregulated in most CRC, promoting unchecked cell growth and survival (23). Studies have implicated a role for IMP1 in regulating expression of several WNT pathway proteins (24–27) and recent work from our group suggests a novel role for IMP1 in reducing WNT pathway gene expression (12).

To determine whether IMP1 might modulate WNT signaling pathway components, we overlaid the GO Pathway for WNT signaling with IMP1 targets from three unbiased approaches performed in different cell types and conditions: PAR-CLIP in HEK293T cells (28), eCLIP in human embryonic stem cells (29) and ribosomal profiling in IMP1 null and expressing SW480 CRC cells (12). Data from studies suggest that IMP1 may bind transcripts or modulate expression of nearly half (43%) of the 414 WNT pathway proteins, including many critical signaling mediators, such as CTNNB1, GSK3 β and β TRCP1 (Figure 3C). To determine the effect of IMP1 on WNT signaling gene expression, we performed western blots in SW480 and HT-29 IMP1-long and IMP1-short-expressing cell lines. We found that select WNT signaling pathway protein levels were not changed by IMP1 expression (Figure 3D) in these CRC cell lines.

IMP1-short enhances cell survival

Cell proliferation is probably not the only mechanism driving the enhanced metastatic burden in IMP1-expressing cells, because both IMP1-long and IMP1-short showed elevated proliferation, but only IMP1-short showed significantly increased metastatic burden. We next assessed cell survival as another potential mechanism using Annexin-V-7AAD staining and flow cytometry to measure relative apoptosis levels between tumor-derived cell lines (Figure 4A). We demonstrated that cells expressing the IMP1-short construct had significantly reduced levels of apoptosis, suggesting that differences between IMP1-long and IMP1-short metastatic burden are also in part due to increased cell survival. Interestingly, enhanced cell survival is not mediated through classic apoptotic pathways, as no changes in pro- or anti-apoptotic protein expression were observed by apoptotic protein array (Supplementary Table 3, available at Carcinogenesis Online) or western blot for select anti-apoptotic targets XIAP or MCL1 (Figure 4B). Notably, BCL2 was not detectable (data not shown).

Taken together, these data suggest that moderate and high levels of IMP1 protein expression enhance cell proliferation, whereas high levels of IMP1 increase cell survival. The observed increase in proliferation and survival in the IMP1-short cells results in higher metastatic burden to the liver, highlighting a potentially important role for IMP1 3' UTR shortening in metastatic CRC, as well as distinct functional outcomes resulting from different levels of IMP1 expression.

IMP1 3' UTR isoforms are expressed in patient samples

Although IMP1 is highly expressed in over 80% of primary colorectal tumors (8, 9), the APA status of the expressed transcripts is not known. Because our *in vivo* data suggest that high levels of IMP1 expression mediated by IMP1-short may worsen metastatic tumor burden, we sought to determine if different patterns of APA are present within the IMP1 transcript pool. We compared expression ratio indices for IMP1 APA usage using published protocols (16, 18) and microarray datasets from human CRC

Figure 2. IMP1 expression enhances tumor mass and metastasis surface area. Cells were injected subcutaneously into the flanks of athymic, nude mice, $n = 4$ –12 animals, 2 tumors per animal. Tumor mass (A) and tumor volume (B) were measured at the time of sacrifice and are expressed as fold change relative to the empty vector control. H&E sections from the subcutaneous tumors (C) were scored for % necrosis (D). Data represent $n = 4$ –5 mice, 2 tumors per mouse, 2 sections per tumor (one from the interior and one from the exterior of the tumor). Scale bar = 500 μ m. Liver metastases were assessed following injection of 1 million cells into the spleens of athymic nude mice, $n = 7$ –9 animals per group. Livers were harvested 6 weeks postinjection and liver mass (E), tumor size (F) and tumor number (G) were quantified. * $P < 0.05$ versus empty vector control by one-way ANOVA. ** $P < 0.05$ versus empty vector control by linear mixed effects modeling. Representative images of gross liver metastases (scale bar = 1 cm), H&E images of tumors sections (scale bar = 500 μ m) and Ki67 to assess proliferation (scale bar = 50 μ m) are shown in (H). The majority of the metastases were moderately differentiated, although 3 of 25 metastases (12%) derived from the IMP1-short cells were classified as poorly differentiated. (I) % Ki67+ cells were calculated by counting Ki67+ cells versus total using ImageJ. An observer blinded to tumor genotype scored 10–40 $\times$ images randomly taken throughout all tumors present in a single section. $n = 4$ animals per group.

Figure 3. IMP1 increases cell proliferation. Tumor-derived cell lines were generated from the liver metastases of xenograft mice and fluorescence-activated cell sorting isolated to enrich for the GFP-expressing Piggybac vector. (A) Confocal images of immunofluorescence staining assessed intracellular FLAG-IMP1 protein localization in empty, IMP1-long and IMP1-short-expressing tumor-derived cell lines. Images are representative from $n = 3$ cell lines stained per IMP1 genotype and were taken at $\times 40$ magnification. Scale bar = $50 \mu\text{m}$. (B) A WST1 assay was performed to measure cell proliferation 48, 72 and 96 h after plating. Data are normalized to 24 h post-plating to control for differential seeding. $n = 3$ tumor-derived cell lines per group. Data represent average of two independent experiments and are expressed relative to empty vector cell lines. (C) Predicted IMP1-mediated regulation of WNT pathway transcripts was determined by overlaying the WNT GO Pathway with IMP1 binding data from PAR-CLIP (28), eCLIP (29) and regulation data from Ribo-seq (12) datasets. Select genes were predicted targets in one or more datasets. (D) Western blotting assessed the effect of IMP1 expression on WNT pathway protein levels in HT-29 and SW480 cells expressing differing levels of IMP1 protein. * $P < 0.05$ versus empty by one-way ANOVA; $^{\#}P < 0.05$ versus empty by two-way ANOVA; $^bP < 0.05$ versus long by two-way ANOVA

Figure 4. IMP1-short expression promotes cell survival. (A) Tumor-derived cell lines were labeled with 7AAD and AnnexinV-APC to assess apoptosis. Analysis was performed on GFP+ cells from $n = 3$ tumor-derived cell lines per genotype. Experiment was performed in duplicate. * $P < 0.05$ versus empty by one-way ANOVA; # $P < 0.05$ versus long by one-way ANOVA. Western blotting assessed anti-apoptotic protein expression for XIAP and MCL1 (B). Data are normalized to GAPDH and expressed as relative expression to empty vector control. Representative GAPDH is shown for each set of blots.

tumor samples (GSE17538 (20); GSE14333 (21); GSE39582 (22)) in localized versus metastatic primary human CRC (Figure 5A and B). We found that IMP1 APA occurs in CRC patient tumor samples. In two of three datasets surveyed, there was no significant difference in APA across tumor stages (data not shown) or between localized and metastatic primary tumors (Figure 5C), whereas one dataset showed that the short 3' UTR was enriched in primary tumor samples that formed metastases (Figure 5D). The patient populations where IMP1 3' UTR shortening was not associated with metastatic spread had overall higher levels of IMP1-short (Figure 5C) compared with patients where APA was associated with metastasis (Figure 5D). These data suggest that 3' UTR shortening for IMP1 correlates with metastasis in a subset of patient samples.

Taken together, these data indicate that different levels of IMP1 expression lead to different functional outcomes in CRC metastasis (Figure 6). Moderate IMP1 expression (IMP1-long) increases proliferation and subcutaneous tumor mass, but is not sufficient to enhance the metastatic burden to the liver, whereas high IMP1 expression (IMP1-short) increases metastatic tumor burden via enhanced cell proliferation, but also increased survival.

Discussion

The ability of IMP1 to enhance CRC tumor formation, growth and progression is controversial. The current studies used a combination of *in vivo* and *in vitro* systems to demonstrate that high levels of IMP1 expression (driven by 3' UTR shortening) enhance the growth of both primary and metastatic tumors via modulation of cell proliferation and survival.

IMP1 expression has been associated with poor prognosis, reduced survival and metastasis in CRC patients (8, 10, 11); however, the direct role of IMP1 in the metastatic progression of CRC had not been examined. Herein we directly tested the affect of IMP1 expression on CRC cell metastasis using the splenic injection xenograft model. Our results demonstrate that IMP1 is not required for metastatic seeding of the liver (Figure 2G); however, high IMP1 expression (IMP1-short) significantly enhanced the total metastatic burden (Figure 2E) via enhanced tumor size (Figure 2F). It is possible that IMP1 expression also increases metastatic seeding of the liver; however, at 6 weeks postinjection it was not possible to distinguish many small tumors merged together from a single large tumor derived from a single CRC cell. Taken together, the results from both xenograft models indicate that IMP1 enhances tumor size, both in primary and metastatic tumors, with higher IMP1 expression driven by 3' UTR shortening having the most profound effect on the size of metastatic tumors.

We next sought to determine the mechanism(s) by which IMP1 may enhance tumor size. We found that IMP1 expression enhanced cell proliferation at both moderate (IMP1-long) and high (IMP1-short) expression levels, suggesting that even moderate increases in IMP1 protein are sufficient to increase cellular proliferation. In our system, we did not find IMP1-mediated changes in select WNT pathway protein expression, although our bioinformatics analysis suggests that the WNT signaling pathway maybe an IMP1 posttranscriptional regulon (30) (Figure 3C). It is possible that IMP1 may modulate other WNT signaling mediators than we examined or that signaling changes are occurring posttranslationally. IMP1 may also regulate WNT

Figure 5. Shortening of the IMP1 3' UTR correlates with metastasis in patients where the long isoform predominates. (A) The expression ratio index (ERI) is a measure of alternative polyadenylation [alternative polyadenylation (APA)] site usage. ERI was calculated using methods published in (16, 18). The microarray probe sets for IMP1 were binned based on location. 5' probe sets detect the short and long isoform whereas the 3' probe set detects only the long isoform. (B) The workflow for the data analysis pipeline. The ERI was determined as a ratio of the signal from 5' to 3' probe set where the signal for the 5' probe set is $S_5 = \alpha_L e_L + \alpha_S e_S$ where $e_{L,S}$ is the expression level of the long versus short isoform and $\alpha_{L,S}$ is the affinity of the long and short forms for the 5' probe set. Similarly, the signal from the 3' probe set is $S_3 = \beta_L e_L$ where β_L is the affinity of the long form to the 3' probe set. The IMP1 ERI was calculated using data generated on the Affymetrix HG-U133_Plus_2 platform for three different microarray datasets (GSE17538 (20); GSE14333 (21); GSE39582 (22)) from human CRC patient primary tumor samples. Two datasets showed enrichment of the short isoform across all tumor samples (C), whereas one dataset showed higher expression of the long isoform (D). The n for each dataset is shown on the histograms. Comparisons in IMP1 ERI were made between localized (stages 1–2) or metastatic (stages 3–4) primary CRC tumors. *P < 0.05 versus local by unpaired t-test.

Figure 6. Model of IMP1 and metastatic colon cancer to the liver. Our data indicate that different levels of IMP1 expression result in different functional outcomes in colon cancer metastasis. 3' UTR shortening of IMP1 results in higher mRNA and protein expression. High IMP1 levels in turn result in larger subcutaneous tumors and a higher metastatic burden to the liver, which is driven at least in part by enhanced cell proliferation and survival.

signaling in other types of model systems that examine tumor initiation or progression (12, 24, 25, 27).

Both SW480 and HT29 cell lines have truncating mutations in APC (31, 32), which activate β -catenin and promote WNT

signaling. It is possible that these mutations may mask any changes in WNT signaling that occur as a result of IMP1 manipulation. Moreover, HT29 cells possess a V599E mutation in BRAF, which inhibits WNT signaling and could also mask IMP1 modulations of WNT (33–35). It is conceivable that alterations in cellular metabolism (36, 37) may influence IMP1-mediated cell proliferation in our system.

Because differences in proliferation did not explain the enhanced tumor burden originating from IMP1-short cells, we examined cell survival as a distinguishing factor. Our flow cytometry data indicate that high IMP1 levels (IMP1-short) significantly reduced cell death in the tumor-derived cell lines, probably contributing to decreased necrosis and increased tumor burden, although classical apoptotic pathway proteins do not appear to be involved (Supplementary Table 3, available at Carcinogenesis Online, and Figure 4B). Other signaling pathways that affect cell survival could be mediating these effects, including the ER stress or autophagy pathways, as well as altered metabolic signaling.

HT-29 cells are considered a moderate-well differentiated cell line, whereas SW480 cells are poorly differentiated (38). In xenograft models, wild-type HT-29 cells form larger tumors and metastasize more readily than wild-type SW480 cell lines, despite differences in differentiation status (38, 39). Interestingly HT-29 cells do not normally express detectable levels of IMP1, whereas SW480 cells express IMP1 highly (Supplementary Figure 1, available at Carcinogenesis Online). Our results provide

strong evidence for the metastatic potential of IMP1 expression in specific contexts, as forced IMP1 expression was able to enhance the metastatic potential of the already metastatic HT-29 cell lines. Because SW480 cells are naturally less metastatic than HT-29 cells (39), despite their endogenous, high expression of IMP1, we predict that enhancing IMP1 expression further may not have the same stark effect on metastasis seen in HT-29 cells. We believe that IMP1 is one contributing factor to metastatic potential, which is influenced by numerous variables, including intracellular gene expression and microenvironment. When studying the molecular mechanism behind IMP1-mediated, enhanced metastasis, comparisons between HT-29 and SW480 cells may yield different results because SW480 cells may contain more or different IMP1-regulated transcripts than HT-29 given their normally high IMP1 expression.

RNA-binding proteins, transcription factors, promoter methylation and miRNAs, most notably *let-7* (10, 13, 40, 41), regulate IMP1 expression. CpG island methylation can modulate IMP1 expression levels in a variety of tissues (25, 42–44). In our system, IMP1 expression is driven by the CMV promoter and is therefore refractory to differential promoter methylation. We cannot exclude the possibility that differential promoter methylation occurs in human patient tissues as this was not examined in our study. miRNA regulation is highly dependent upon the availability of binding sites within the 3' UTR of the IMP1 transcript (13). Loss of miRNA regulation via shortening of the 3' UTR through APA confers altered mRNA stability and protein expression and is often associated with more aggressive tumors in a number of solid tumor cancers, including CRC (14–17). Studies in fibroblasts suggest that shortening of the IMP1 3' UTR confers enhanced colony formation (13); however, to the best of our knowledge, our study provides the first *in vivo* evidence that IMP1 3' UTR shortening enhances CRC metastasis. We used published methods (16, 18) for quantifying and comparing 3' UTR isoform length in human patients to determine whether shortening of the IMP1 3' UTR is favored in metastatic CRC. We found that primary colon tumors express both IMP1-long and IMP1-short isoforms (Figure 5C and D). In patient tumors where IMP1-short generally predominates (Figure 5C), expression is not further enriched in metastatic tumors compared with primary tumors. This could be due to already high levels of IMP1 short or due to a smaller overall sample size in these two datasets. However, in patients where IMP1-long is the predominant isoform, there is a significant enrichment in IMP1-short associated with tumor metastasis (Figure 5D). This suggests that shifts in the balance between IMP1 APA can affect liver metastasis in patients where IMP1-long is the predominant isoform.

The differential functional consequences of IMP1-long versus IMP1-short with regard to metastasis may be driven in part by protein expression levels (rather than changes to the IMP1 protein itself). We speculate that the cumulative effects of IMP1-mediated changes in downstream translation may be affected by the amount of IMP1 protein available to bind IMP1 target transcripts. In the case of the long 3' UTR isoform, there is less IMP1 protein available to modulate translation relative to IMP1-short-expressing cells. We surmise that these differences in protein expression will change the overall translational landscape within the tumor cells ultimately impacting metastatic ability.

Taken together, our study demonstrates that the level of IMP1 expression, mediated by APA and 3' UTR shortening, is important to the functional outcome in CRC progression. High IMP1 levels, driven by 3' UTR shortening, enhance the metastatic tumor burden to the liver via increased tumor size. These effects

on tumor metastasis are in part mediated by enhanced cell proliferation and survival. In human CRC patients, expression of the IMP1-short:long isoform ratio is associated with metastasis in patients where the long isoform is dominant, suggesting a shift toward 3' UTR shortening in this subset of patients. Targeting IMP1 in this patient population may be useful for reducing tumor progression.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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