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RESEARCH ARTICLE

Inhibition of insulin-like growth factor 2 mRNA-binding protein 1 sensitizes colorectal cancer cells to chemotherapeutics

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Abstract

Although colorectal cancer (CRC) treatment has seen a remarkable improvement in the recent years, many patients will develop metastasis due to the resistance of cancer cells to chemotherapeutics. Targeting mechanisms driving the resistance of CRC cells to treatment would significantly reduce cases of metastasis and death. Induction of insulin-like growth factor 2 mRNA-binding protein 1 (IGF2BP1), a direct target of the Wnt/β-catenin signaling pathway, might promote resistance of CRC cells to treatment via activation of anti-apoptotic pathways and induction of the multidrug resistance (MDR1) membrane transporter that pumps drugs out of the cells. We hypothesized that inhibition of IGF2BP1 will sensitize CRC cells to chemotherapeutics. We used CRC cell lines with different status of activation of Wnt signaling to show that inhibition of IGF2BP1 potentiates the anti-growth and anti-proliferative effects of chemotherapeutics on CRC cells with activated Wnt/β-catenin signaling pathway. We observed that the inhibition of IGF2BP1 significantly increases apoptosis in the same cells. A remarkable reduction in the migratory capability of those cells was noted as well. We found that inhibition of IGF2BP1 is sufficient to decrease the resistance of chemotherapy-resistant cancer cells with activated Wnt/β-catenin signaling pathway. These findings portray IGF2BP1 as a good candidate for CRC therapy.

Abbreviations: IGF2BP1, Insulin-like growth factor 2 mRNA-binding protein 1; CRC, Colorectal cancer; MDR1, Multidrug resistance 1 membrane transporter; ABC, ATP-binding cassette transporter superfamily; ABCB1, ATP Binding Cassette Subfamily B Member 1; ABCG2, ATP Binding Cassette Subfamily G Member 2; HPRT1, Hypoxanthine Phosphoribosyltransferase 1; β-TrCP1, β-transducing repeat containing protein 1; NF-κB, Nuclear factor κB; GLI1, Glioma-associated oncogene 1; 5-FU, 5-fluorouracil; Hh, Hedgehog; ALCAM, Activated Leukocyte Cell Adhesion Molecule; AMIGO2, Adhesion Molecule With Ig Like Domain 2; MMP1, Matrix Metallopeptidase 1; MCAM, Melanoma Cell Adhesion Molecule; SynCAM, Synaptic adhesion molecule; HCT116, Human colon cancer cells; RKO, Human colon cancer cells; SW480, Human colon cancer cells; SW620, Metastatic human colon cancer cells; CCD-841-CoTr, Normal cell of the intestinal epithelium; LS174T-L8, Human colon cancer cells expressing doxycycline-inducible dominant negative Tcf4; DLD1D7Δ15, Human colon cancer cells expressing doxycycline-inducible dominant negative Tcf4; Tcf4, Transcription Factor 4; P/S, Penicillin/streptomycin.

KEYWORDS

5-FU, chemotherapeutics, CRC, IGF2BP1, irinotecan, oxaliplatin, resistance, Wnt, β-Catenin

1 | INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in men and women, after prostate or breast, and lung cancer. It is estimated that 151,030 new patients would be diagnosed with and 52,850 men and women would die of cancer of the colon and rectum in the United States in 2022.[1] Colorectal cancer is also one cancer that continues to demonstrate widening incidence and survival disparities between different groups of US population.[2–4] The advanced stage at diagnosis may explain up to 50% of survival disparity.[5]

Treatment for colorectal cancer may involve surgery, chemotherapy, biological therapy, radiation therapy, or a combination of treatments. The choice for treatment of CRC depends mainly on the location of the tumor and the stage of the disease. Chemotherapy is used for advanced cancers; however, advanced CRCs are notoriously resistant to drugs. Some of the FDA-approved drugs used for chemotherapy in CRCs include the antimetabolite 5-Fluorouracil (5-FU), the topoisomerase inhibitor Irinotecan, the alkylating agent Oxaliplatin, and Xeloda which is the oral version of 5-FU.

Two major mechanisms employed by CRC cells are thought to be responsible for insensitivity to therapeutics: (i) resistance to apoptosis usually achieved by activation of anti-apoptotic pathways, and (ii) induction of multidrug resistance (MDR) membrane transporters that pump drugs out of the cells. Targeting factors involved in these mechanisms should aid in the sensitization of CRC cells to drugs.

A better understanding of the biology of colorectal tumorigenesis, especially the role of different signal transduction pathways in the development of colorectal tumors would facilitate the identification of potential targets involved in the resistance of CRC cells to drugs and help in the design of better drugs for CRC treatment.

Constitutive activation of the Wnt/β-catenin signaling pathway is one of the central drivers of the development of CRC.[6] We previously showed that β-catenin stabilizes the mRNA encoding the F-box protein β-TrCP1 and identified the RNA-binding protein IGF2BP1 (Insulin- like growth factor 2 mRNA-binding protein 1) as a previously unknown target of β-catenin/Tcf transcription factor. β-catenin/Tcf complex induces IGF2BP1 transcription. IGF2BP1, in turn, binds to the coding region of β-TrCP1 mRNA, stabilizes it and elevates its levels, both in vitro and in vivo. One of the major physiological outcomes of β-TrCP1 mRNA stabilization is the β-TrCP1-dependent activation of the NF-κB signaling pathway and suppression of apoptosis in CRC cells.[7] Whereas absent or scarce in adult tissues, IGF2BP1 is de novo activated and/or overexpressed in various neoplastic and preneoplastic tumors, including primary CRCs.[8–16] Its expression was shown to be associated with the most aggressive form of some cancers.[9,11,17–19] IGF2BP1 also regulates a set of targets (ALCAM, AMIGO2, Collagen V, Alpha1, Dysadherin, Keratin 19, Lumican, MMP1, MCAM, and synCAM) involved in cellular adhesion, invasion, and extracellular matrix remodeling which are important in metastasis.[20] In addition, IGF2BP1 serves as a mechanistic link in the cross-talk between Wnt and Hh pathways by upregulating the transcriptional activator of the Hh pathway GLI1. Wnt/β-catenin signaling induces expression of IGF2BP1, which, in turn, binds and stabilizes GLI1 mRNA, causing an elevation of GLI1 expression and transcriptional activity. This mode of regulation of GLI1 appears to be important to several functions of Wnt, including survival and proliferation of CRC cells.[21] We have also demonstrated that IGF2BP1 is transcriptionally regulated by c-myc and is involved in several functions of c-myc including regulation of translation, cell size, cell cycle progression, cell proliferation, and cell survival.[22] Since IGF2BP1 was demonstrated to upregulate c-myc expression at the post-transcriptional level,[23] the transcriptional regulation of IGF2BP1 by c-myc suggests a positive feedback loop regulation between c-myc and IGF2BP1 that might be critical in cancer cell growth. The multidrug resistance-1 (MDR-1) membrane transporter was also shown to be regulated by IGF2BP1 at the post-transcriptional level.[24] MDR-1 gene is responsible for the resistance to a large variety of drugs in human cells.[25] It encodes the 170-kD transmembrane P-glycoprotein (P-gp) which belongs to the ATP binding-cassette (ABC) transporter superfamily.

Thus, IGF2BP1 may promote the resistance of CRC to chemotherapeutics by contributing to both inhibition of apoptosis and active drug efflux from cells. Inhibition of apoptosis is achieved by induction of expression and activity of β-TrCP1, c-myc, and GLI1, whereas active drug efflux from cells is performed via elevated expression of MDR-1 (Figure 1). This makes IGF2BP1 an attractive target to sensitize CRC cells to chemotherapeutics.

In this study, we used CRC cell lines with different activation status of Wnt signaling pathway to show that inhibition of IGF2BP1 potentiates the anti-growth and anti-proliferative effects of chemotherapeutics on CRC cells with

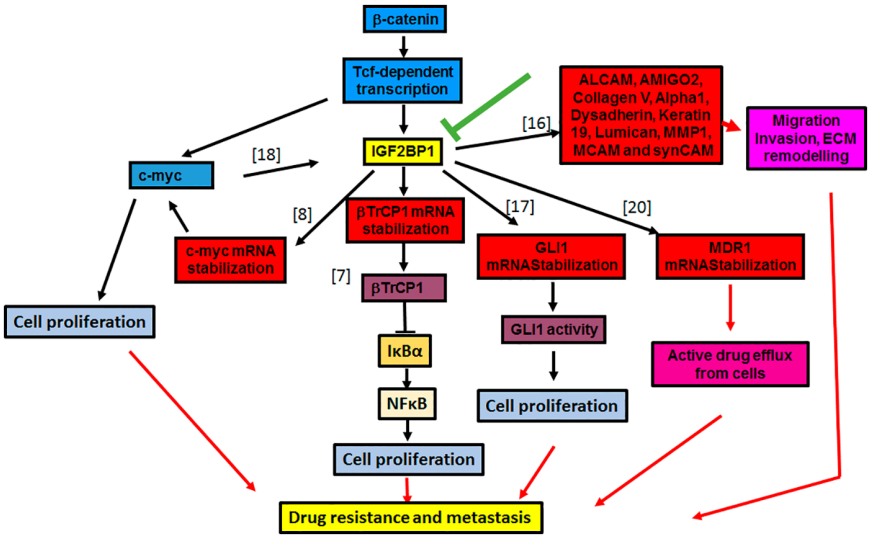

FIGURE 1 Proposed mechanisms through which IGF2BP1 induces drug resistance and metastasis. The red arrows represent the proposed mechanisms of action of IGF2BP1 in cancer cells with activated Wnt signaling. The green hammer represents the inhibition of IGF2BP1 as a potential mechanism to sensitize the cancer cells to chemotherapeutics.

activated Wnt/β-catenin signaling pathway. We also showed that the anti-growth and anti-proliferative effects are associated with a significant increase in apoptosis in the same cells and a reduction in the migratory capability of those cells.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

HCT116 (ATCC, Cat# CCL-247, RRID:CVCL_0291), SW480 (ATCC, Cat# CCL-228, RRID:CVCL_0546), SW620 (ATCC, Cat# CCL-227, RRID:CVCL_0547), RKO (ATCC, Cat# CRL-2577, RRID:CVCL_0504), and normal epithelial colon cell line CCD-841 CoTr (ATCC, Cat# CRL-1807, RRID:CVCL_2872) were obtained from ATCC and not passaged for more than 6 months. HCT116 cells were maintained in McCoy's 5a Medium Modified, MEM (Cat#16600082, Gibco, Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (FBS) (Cat# ES-009-B, MilliporeSigma), and 1% penicillin/streptomycin (P/S) (Cat# 17-602E, Lonza). SW480 and SW620 cells were maintained in Leibovitz's L-15 Medium (Cat#11415064, Gibco, Thermo Fisher Scientific Inc.), supplemented with 10% FBS and 1% P/S. CCD-841 CoTr cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Cat#12430054, Gibco, Thermo Fisher Scientific Inc.), supplemented with 10% FBS and 1% P/S. RKO cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Cat#11095080, Gibco, Thermo Fisher Scientific Inc.) and supplemented with 10% FBS and 1% P/S. All cells were maintained according to the manufacturer's recommendations.

LS174T-L8 and DLD1D7Δ15 were a gift from Dr. Hans Clevers. They were maintained in DMEM supplemented with 10% FBS and 1% P/S.

All cells were transfected using Lipofectamine 2000 (Invitrogen Corporation) or TransfeX™ Transfection Reagent (ATCC, Cat#ACS-4005™) according to the manufacturers' recommendations. The plasmids used were pTK-puro plasmid, IGF2BP1 shRNA, scrambled shRNA, β-catenin expression plasmid,[7] and pcDNA3.1 (Cat# V79020, Invitrogen Corporation). The IGF2BP1 shRNA construct used in this study was previously screened out of four constructs for its ability to selectively reduce IGF2BP1 in cells.[7]

2.2 | Generation of stable clones

To generate stable clones, cells grown in 100 mm plates were co-transfected with pTK-puro plasmid (0.5 μg) and scrambled shRNA (9.5 μg) or pTK-puro plasmid (0.5 μg) and IGF2BP1 shRNA (9.5 μg)[7] as indicated. 48 h after transfection, an equal number of cells from each plate was seeded in five 100 mm plates and treated with puromycin (Cat# A11138-02, Gibco, Thermo Fisher Scientific Inc.) (2–7 μg/ml) for 14 days. Isolated colonies were collected using cloning rings, and each clone was grown successively in 96, 24, 6 well plates, and 100 mm tissue culture plate. The clones were screened for IGF2BP1 expression by immunoblotting (Figure 2).

2.3 | Immunoblotting

Antibodies against IGF2BP1 (Thermo Fisher Scientific, Cat# PA5-23968, RRID:AB_2541468) and β-actin (Cell Signaling Technology, Cat# 4970, RRID:AB_2223172) were purchased as well as the secondary antibody conjugated with horseradish peroxidase (Cell Signaling Technology, Cat# 7074, RRID:AB_2099233). To obtain whole cell lysates for western blot analysis, the cells were

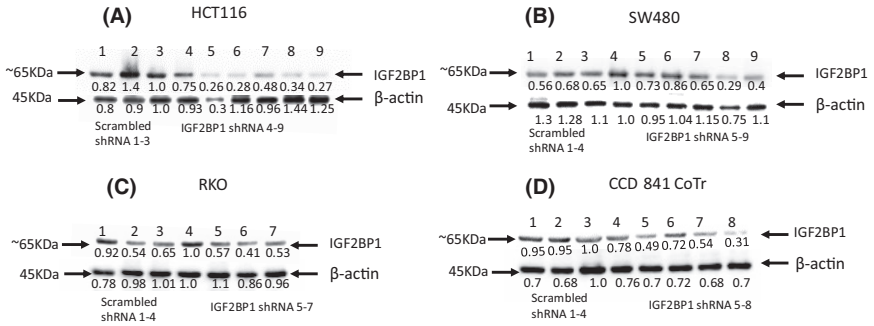

FIGURE 2 Immunoblots. Screening of stable clones. Each cell line was grown in 100 mm dish and co-transfected with IGF2BP1 shRNA expression plasmid[7] or the scrambled control plasmid (9.5 μg each) and the plasmid expressing the gene for resistance to puromycin (0.5 μg). 48 h after transfection, cells from each plate were sparsely seeded in multiple 100 mm plates and treated with puromycin (2–7 μg/ml) for 14 days. Isolated colonies were collected using cloning rings, and each clone was grown successively in 96, 24, 6 well plates, and 100 mm tissue culture plates. The clones were screened for IGF2BP1 expression by immunoblotting as described in Materials and Methods. (A) Clones 3 and 9 of HCT116 were used in our study. (B) Clones 4 and 9 of SW480 were used in our study. (C) Clones 4 and 6 of RKO were used in our study. (D) Clones 1 and 8 of CCD-841 CoTr were used in our study

lysed using RIPA buffer containing PBS (pH 7.4), 0.5% sodium deoxycholate, 0.1% SDS, 1% (v/v) IGEPAL, 100 mM sodium orthovanadate, and proteinase inhibitor cocktail (Cat# 539134, Sigma-Aldrich). Immunoblotting procedures were performed as described previously.[26]

2.4 | Cell treatment

Cells were treated for 72 h with 5-FU (Cat# F6627, Sigma-Aldrich) (5, 30, and 80 μM), Irinotecan (Cat# I1406, Sigma-Aldrich) (0.1, 0.5, and 1 μM), or Oxaliplatin (Cat# O9512, Sigma-Aldrich) (0.25, 0.5, 2, and 4 μM). The drugs were used at concentrations below or within the range of typically achieved plasma concentration during the course of chemotherapy which are 10–80, 2–20, and 1–8 μM for 5-FU, Irinotecan, and Oxaliplatin, respectively.

2.5 | Cell growth

2.5.1 | Clonogenic assay

HCT116, SW480, SW620, RKO, and CCD-841 CoTr cells were grown in 100-mm plates and co-transfected with pTK-puro plasmid (0.5 μg) and IGF2BP1 shRNA expression plasmid (9.5 μg) or the scrambled control plasmid (9.5 μg each). 48 h after transfection, an equal number of cells from each plate was seeded in four other 100 mm dishes. 24 h after splitting the cells, they were treated for 72 h with different concentrations of 5-FU (5, 30, 80 μM), irinotecan (0.1, 0.5, 1 μM), or oxaliplatin (0.25, 2, 4 μM). Puromycin (2–7 μg/ml) was used for selection. The cells were allowed to grow and form colonies for an additional 10 days in the puromycin media. The colonies formed were fixed with 10% formalin for 10 min at room temperature and subsequently stained with 0.5% crystal violet for 30 min. The colonies were counted using a light microscope.

2.5.2 | MTS assay

The MTS assay was performed on the stable clones generated from HCT116, RKO, SW480, and CCD-841 CoTr Cells. The cells were counted using the TC10™ automated cell counter from BioRad, and an equal number of viable cells expressing scrambled shRNA or IGF2BP1 shRNA was plated in 96 well plates. 24 h after the seeding of the cells, they were treated with 5-FU, irinotecan, or oxaliplatin at different concentrations for 72 h. To measure cell proliferation, the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Cat # G3580, Promega) was used. The intensity of the chromogenic substrate was measured at 490 nm according to the manufacturer's recommendations.

2.5.3 | Detection of apoptosis

Apoptosis was accessed on the stable clones generated from HCT116, RKO, SW480, and CCD-841 CoTr cells. The cells were seeded in 96 well white plates with a clear bottom (Cat# 165306, Thermo Fisher Scientific Inc.) and treated with the drugs as for the MTS assay. Apoptosis was detected using the Caspase-Glo 3/7 assay kit (Cat# G8091, Promega). The luminescence was recorded according to the manufacturer's recommendations.

2.5.4 | Wound healing assay

The migration assay (wound healing assay) was performed on the stable clones using the Ibidi Culture-Insert 3 Well

(Cat # 80366, Ibidi Inc.). 5–8.5 × 10^4 cells in 70 µl media were seeded in each well and allowed to attach and grow to 100% confluency at 37°C in a 5% CO_2 incubator (24–48 h). The inserts were removed using sterile tweezers, creating wounds. Images of wounds for time 0 h were recorded under a microscope (Olympus ck X53, magnification 4X). Each plate was treated with 5-FU, irinotecan, or oxaliplatin at different concentrations, and images of wounds were recorded every 24 h. The size of wounds was measured using the NIH ImageJ software.

2.5.5 | Real time PCR

Total RNA was isolated from cells transiently transfected with scrambled shRNA control (10 µg) or IGF2BP1 shRNA (10 µg) using TRIzol (Cat# 15596-026) from Life Technologies. Real-time PCR for quantitative measurement of ABCB1, ABCG2, βTrCP1, c-myc, GLI1, and IGF2BP1 was performed using One Step TB Green PrimeScript RT-PCR Kit II (Cat# RR086A) from Takara. Predesigned PrimeTime qPCR primers (Table 1) were obtained from IDT. HPRT1 was used as the reference gene.

2.5.6 | Luciferase assay

Stable clones of HCT116, SW480, RKO, and CCD-841 CoTr expressing scrambled shRNA control or IGF2BP1 shRNA were used in this assay. 20,000 cells were seeded in the 96-well white plates with clear bottom. 24 h after seeding, they were transfected by lipofection with pGL4.74(hRLuc/TK)

TABLE 1 sequence of primers used for the real-time PCR

Primers	Forward	Reverse
ABCB1	5′GATTGACTGAATGCTGATTCCTC3′	5′ACTCACTTCAGGAAGCAACC3′
ABCG2	5′ACTGGAAGACATCTGGAGAGT3′	5′CTTCGTATTACCACTGTCTCTGC3′
βTrCP1	5′GCTGTTGTATGTCTGTCTAAGTGA3′	5′GCGATGCCTGTATAACCCA3′
c-Myc	5′TCTTCCTCATCTTCTTGTTCCTC3′	5′TCCTCGGATTCTCTGCTCTC3′
GLI1	5′CATTGCCAGTCATTTCCACAC3′	5′CATCAGGGAAAGCAGAC3′
IGF2BP1	5′AGTTCTCCTCCTTGAGTTTGC3′	5′CCTCCATCAAGATTGCACCA3′
HPRT1	5′GCGATGTCAATAGGACTCCAG3′	5′TTGTTGTAGGATATGCCCTTGA3′

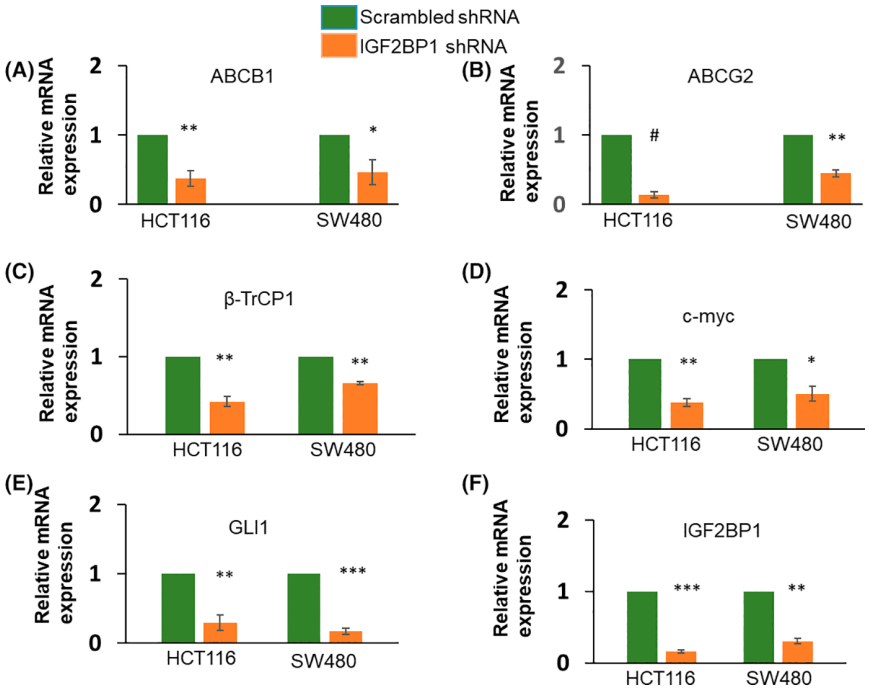

FIGURE 3 Real-time PCR. Inhibition of IGF2BP1 reduces the expression of ABCB1, ABCG2, β-TrCP1, c-myc, and GLI1 in HCT116 and SW480 cells. Quantitative RT-PCR was performed on total RNA isolated from HCT116 and SW480 cells transiently transfected with scrambled shRNA control (10 µg) or IGF2BP1 shRNA (10 µg) for the measurement of ABCB1 (A), ABCG2 (B), β-TrCP1 (C), c-myc (D), GLI1 (E), and IGF2BP1 (F) using One Step TB Green PrimeScript RT-PCR Kit II. HPRT1 was used as the reference gene. Each experiment was done at least twice and in triplicates. Each value represents Mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $^{\#}p < 0.00001$

(5 ng) (Cat# E692A) and M50 Super 8x TOPFlash (0.05 μg) (Cat# 12456) or M51 Super 8xFOPPFlash (0.05 μg) (Cat# 12457) (Addgene). 24 h after transfection, the cells were treated with each drug for 24 h, and the luciferase activity was measured using the Dual-Glo luciferase assay system (Cat# E2920) by Promega. Renilla luciferase was used for normalization.

2.5.7 | Transwell migration assay

The transwell assay was performed on stable clones of HCT116, SW480 expressing scrambled shRNA control or IGF2BP1 shRNA using the CytoSelect 24-well cell migration assay (Cat# CBA-101) from Cell Biolabs. 300,000 cells suspended in low serum media (1% FBS) containing each drug were added to the insert and incubated for 24 h in the 5% CO_2 humidified incubator. 10% FBS in the culture media in the lower chamber served as chemo-attractant. After 24 h, the migrating cells were dislodged, lysed, and stained with the CyQuant GR dye according to the manufacturer recommendations. The samples were transferred into a 96-well white plate with clear bottom and the fluorescence intensity was read using a plate reader (POLAR Star Omega) at 480 nm/520 nm.

2.5.8 | Statistics

Comparison between any two groups was performed using the *t*-test, while ANOVA differentiated more than two treatment groups with further classifications by multiple range tests. These tests were conducted at $p < 0.05$ to attain statistical significance.

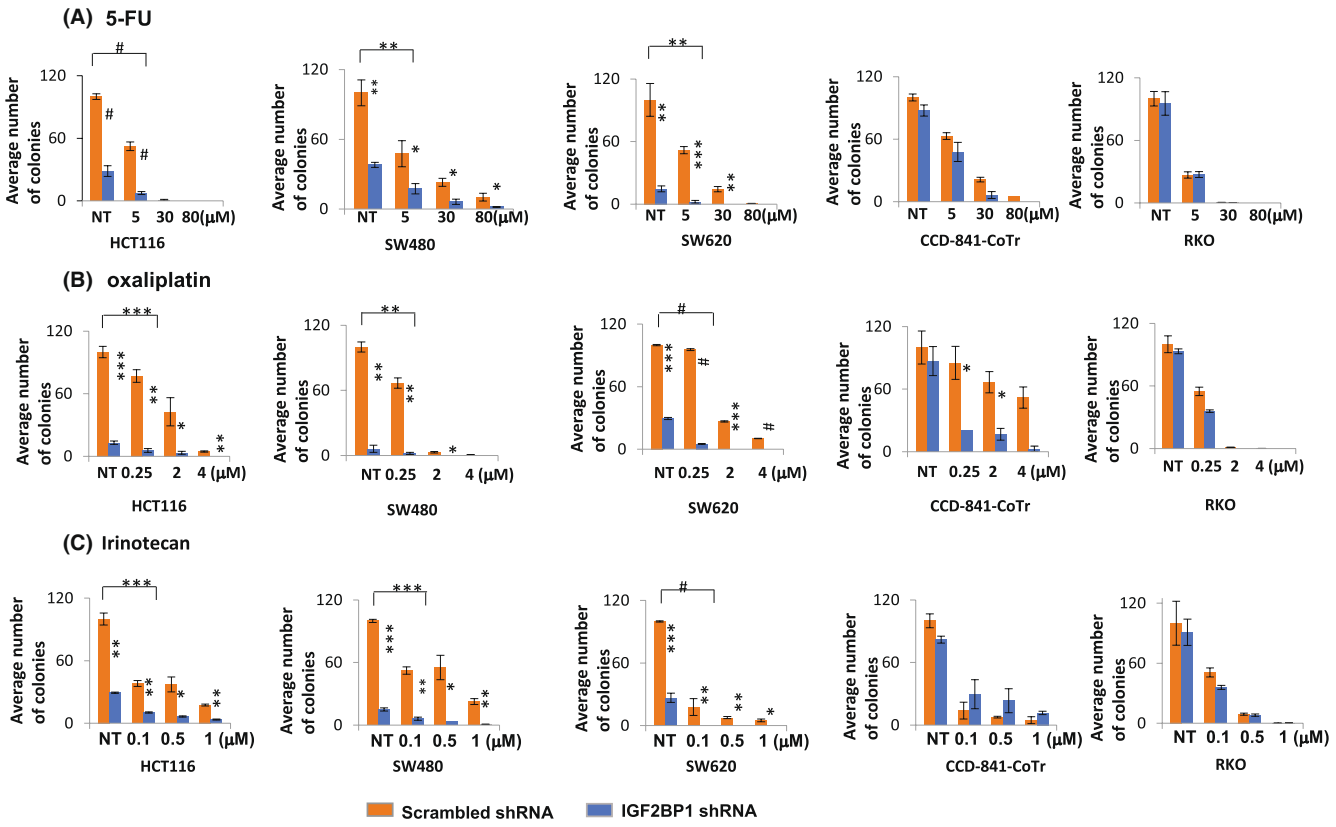

FIGURE 4 Colony formation assay. Colorectal cancer cells as well as the normal epithelium control cells, were grown in 100 mm dish and co-transfected with IGF2BP1 shRNA expression plasmid or the scrambled control plasmid (9.5 μg each) and the plasmid expressing the gene for resistance to puromycin (0.5 μg). 48 h after transfection, an equal number of cells from each plate was seeded in four other 100 mm dishes. 24 h after splitting the cells, they were treated with (A) 5FU, (B) Oxaliplatin, and (C) Irinotecan at the concentrations indicated for 72 h. Puromycin (2–7 μg/ml) was used for selection. The cells were allowed to grow and form colonies for an additional 10 days in the puromycin media. The colonies formed were fixed with 10% formalin for 10 min and subsequently stained with crystal violet. The colonies were counted using a light microscope. Each experiment was done at least twice and in triplicates. Each value on *Y* axis represents the number of colonies per plate relative to control plates. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.00001$

3 | RESULTS AND DISCUSSION

3.1 | IGF2BP1 inhibition potentiates the anti-growth response to 5-FU, Irinotecan (CPT-11), and Oxaliplatin of CRC cells with activated Wnt signaling

To delineate the effects of IGF2BP1 inhibition on the sensitivity of CRC cells to chemotherapeutics, we used CRC cell lines (HCT116, SW480, SW620, and RKO) with different status of activation of Wnt signaling pathway, and diverse degrees of aggressiveness. The HCT116 and SW480 cells exhibit constitutively active Wnt signaling pathway with high expression levels of IGF2BP1, whereas the Wnt signaling is not activated in RKO cells.[21] This cell line also expresses low levels of IGF2BP1. Since chemotherapy regimens are used to treat advanced cancers, we also used the metastatic CRC cell line SW620. This cell line is also characterized by a constitutively activated Wnt signaling pathway with high expression levels of IGF2BP1 (Supplemental Figure 5a,b).[21] The normal epithelial colon cell line CCD-841 CoTr served as a control in our study. We analyzed the ability of those cells to form colonies when IGF2BP1 was inhibited and when cells were exposed to various concentrations of chemotherapeutics. We used the FDA-approved drugs 5-FU, irinotecan, and oxaliplatin. All the concentrations used in our study are below or within the range of typically achieved plasma concentrations during the course of chemotherapy which are 10–80, 2–20, and 1–8 μM for 5-FU, Irinotecan, and Oxaliplatin, respectively. We transiently co-transfected the cells with either IGF2BP1 shRNA expression vector or scrambled shRNA control and the plasmid expressing the gene for resistance to puromycin. The cells were then treated with different concentrations of either 5-FU, irinotecan or oxaliplatin and puromycin as described in materials and methods. The IGF2BP1 shRNA construct used in this study was previously screened out of four constructs for its ability to selectively reduce IGF2BP1 in cells.[7] Inhibition of IGF2BP1 using the IGF2BP1 shRNA construct significantly reduces the expression of IGF2BP1 in the cells at both RNA ($p < 0.01$) (Figure 3) and protein levels (Supplemental Figure 5c,d). We observed a significant decrease in the ability of HCT116, SW480, SW620 cells to form colonies when IGF2BP1 was inhibited (Figure 4) ($p < 0.01$). When the cells were treated with each of the drugs (5-FU, oxaliplatin and, irinotecan), the decrease in colony formation was concentration-dependent and

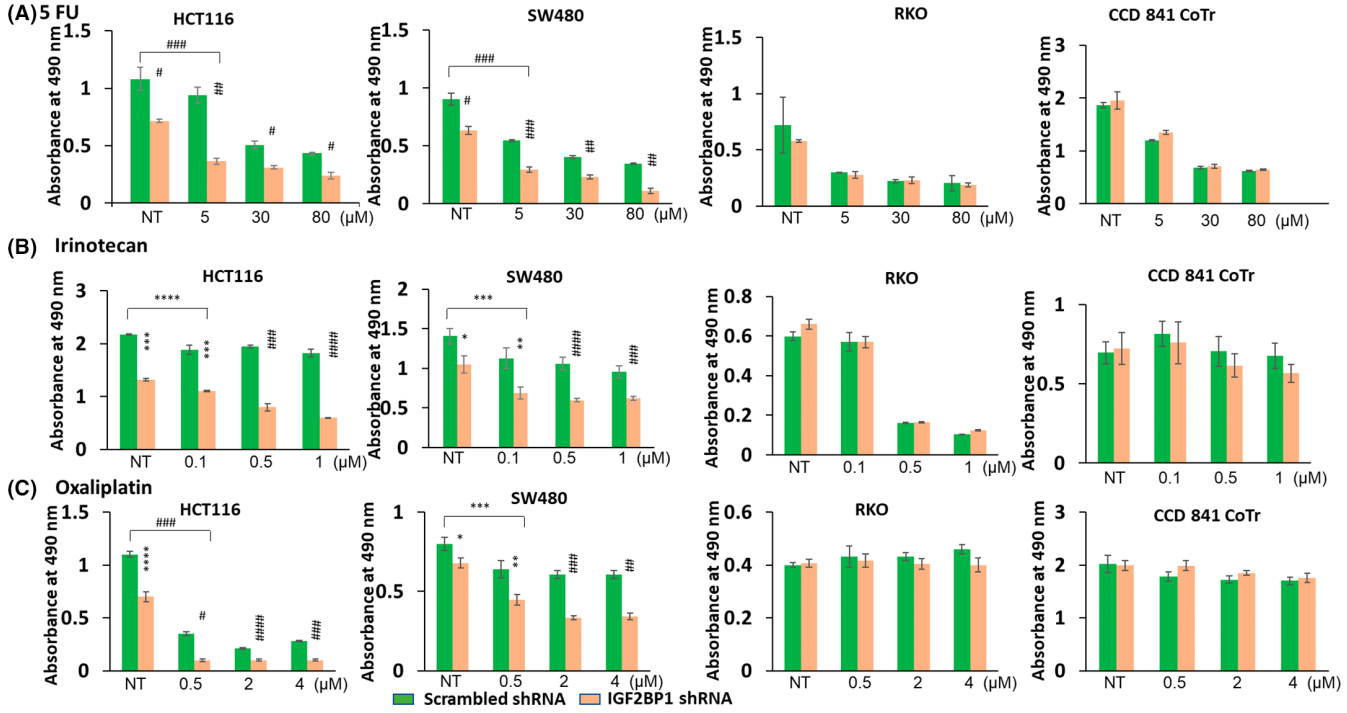

FIGURE 5 MTS assay. Inhibition of IGF2BP1 potentiates the anti-proliferative effect of chemotherapeutics on cells with an activated Wnt signaling pathway. Stable clones of each cell line expressing scrambled shRNA or IGF2BP1 shRNA plasmids were seeded in 96 well plate in triplicates at the density of 5000–7000 cells. 24 h after seeding, the cells were treated with 5-FU (A), Irinotecan (B), or Oxaliplatin (C) at the indicated concentrations for 72 h. The media was changed to media without drug for 24 h and the MTS assay was performed as described in Materials and Methods. Each experiment was performed at least three times and in triplicates. The *Y* axis represents the OD at 490 nm and the *X* axis, the concentrations of the drugs. Each value represents Mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; $^{\#}p < 0.00001$; $^{\#\#}p < 0.000001$; $^{\#\#\#}p < 0.0000001$; $^{\#\#\#\#}p < 0.00000001$

more pronounced across all cell lines (HCT116, SW480, SW620) even at very low concentration of each drug when IGF2BP1 was inhibited compared to control cells ($p < 0.001$) (Figure 4). This is an order of magnitude decrease in colony formation. This drastic decrease in colony formation was not observed in RKO or CCD-841 CoTr cells, although CCD-841 CoTr cells showed some sensitivity to oxaliplatin treatment. However, this sensitivity was not observed in the MTS assay (Figure 5).

The MTS assay was performed on the stable clones of HCT116, SW480, RKO, and CCD-841 CoTr cells expressing IGF2BP1 shRNA or scrambled shRNA and treated with different concentrations of 5-FU, irinotecan, and oxaliplatin. We observed a significant reduction in the proliferation of HCT116 and SW480 cells when IGF2BP1 was inhibited ($p < 0.001$ and $p < 0.05$, respectively) (Figure 5). Addition of the drugs to IGF2BP1 inhibition further reduced cell proliferation ($p < 0.0001$ and $p < 0.001$ for HCT116 and SW480, respectively). This reduction in proliferation was concentration-dependent across both cell lines and for all the drugs tested (Figure 5). IGF2BP inhibition did not affect the proliferation of RKO or CCD-841 CoTr cells (Figure 5). Collectively, our results suggest a potentiation of the anti-colony forming ability and anti-proliferative effects of the drugs by IGF2BP1 inhibition in cells with activated Wnt signaling. This supports our hypothesis that targeting IGF2BP1 sensitizes CRC cells with activated Wnt signaling to chemotherapeutics. The significance of this finding is that CRC cells could be killed with lower doses of chemotherapeutics if IGF2BP1 is inhibited, therefore potentially reducing the harmful side effects of drugs and achieving a better cancer treatment. In addition, inhibition of IGF2BP1 does not seem to have significant effects on the growth and proliferation of the normal cell line CCD-841 CoTr projecting it as an attractive target for cancer treatment.

3.2 | Inhibition of IGF2BP1 is sufficient to decrease the resistance of chemotherapy-resistant cancer cells with activated Wnt/β-catenin signaling pathway

A subset of cells called side populations has been described in many tumors and cancer cell lines, and are believed to be responsible for cancer metastasis.[27–31] These cells are chemoresistant, exhibit high regenerative capabilities, and express highly activated ABC transporters.[30,31] Previous studies showed that inhibition of β-catenin reduces the transcription of the ABC transporter genes ABCB1 and ABCG2 in these cells.[29] Silencing of the Wnt transcription factor TCF4 was also shown to sensitize CRC cells to

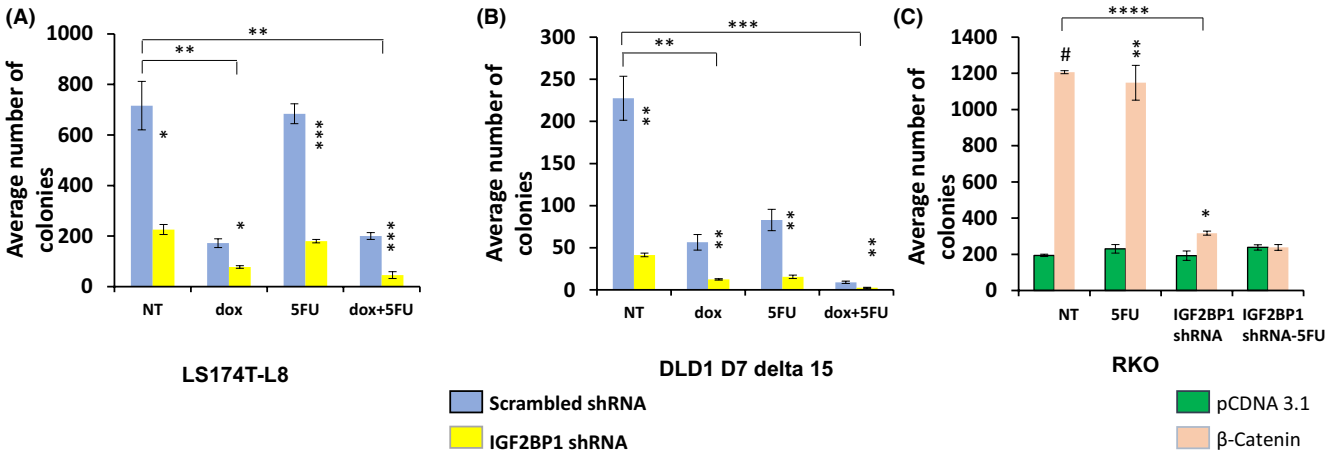

FIGURE 6 Colony formation assay. Inhibition of IGF2BP1 prevents Wnt-induced cell growth and proliferation. (A, B) LS174T-L8 and DLD D7 delta 15 cells were grown in 100 mm dish and co-transfected with pTK-puro plasmid (0.5 μg) and scrambled shRNA (9.5 μg) or pTK-puro plasmid (0.5 μg) and IGF2BP1 shRNA (9.5 μg) expression plasmids. 48 h after transfection, an equal number of cells from each plate was seeded in four other 100 mm dishes. 24 h after splitting the cells, they were treated with doxycycline (1 μg/ml) and 5-FU for 72 h. Puromycin (7–10 μg/ml) was used for selection. The cells were allowed to grow and form colonies for an additional 10 days in the puromycin media. The colonies formed were fixed with 4% PFA for 10 min and subsequently stained with crystal violet. The colonies were counted using a light microscope. (C) RKO cells were grown in a 100 mm dish and co-transfected with pTK-puro plasmid (0.5 μg), IGF2BP1 shRNA expression plasmid, and β-catenin expression plasmid or pTK-puro plasmid (0.5 μg), scrambled shRNA and pCDNA 3.1 plasmid. 48 h after transfection equal number of cells from each plate was seeded in four other 100 mm dishes. 24 h after splitting the cells, they were treated with 5-FU as indicted for 72 h. Puromycin (4 μg/ml) was used for selection. The cells were allowed to grow and form colonies for an additional 10 days in the puromycin media. The colonies formed were fixed and stained as above. The colonies were counted using a light microscope. Each experiment was done at least twice and in triplicates. Each value represents Mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; $^{\#}p < 0.00001$

(chemo-) radiotherapy by inducing cell cycle arrest at the G2/M phase, and impairing DNA double-strand break repair.[32] This indicates that inhibition of the Wnt signaling enhances the sensitivity of CRC cells to drugs. To better probe this effect of Wnt signaling inhibition, we used two CRC cell lines LS174T-L8 and DLD1D7Δ15. These cell lines are characterized by constitutive activation of the β-catenin/Tcf signaling. Additionally, these cells carry a doxycycline-inducible dominant negative Tcf4 (dnTcf4).[33] When treated with doxycycline, the Wnt signaling is inhibited in these cells. Downregulation of Wnt signaling leads to inhibition of IGF2BP1 expression.[21] Treatment of these two cells with doxycycline significantly reduced their ability to form colonies (Figure 6A,B) ($p<0.05$ and $p<0.01$ for LS174T-L8 and DLD1D7Δ15, respectively). This reduction was more significant when IGF2BP1 was inhibited ($p<0.01$) (Figure 6A,B). The addition of 5-FU further decreased the ability of cells to form colonies when IGF2BP1 was inhibited ($p<0.01$ and $p<0.001$ for LS174T-L8 and DLD1D7Δ15, respectively). Activation of the Wnt signaling in RKO cells by overexpressing a mutant form of β-catenin significantly increased their ability to form colonies ($p<0.00001$) even when they were treated with 5-FU ($p<0.01$) (Figure 6C). However, inhibition of IGF2BP1 abrogated the Wnt/β-catenin-induced ability of the cells to form colonies ($p<0.0001$). We also showed that inhibition of IGF2BP1 significantly reduces the mRNA expression of the ATP binding cassette members ABCB1 and ABCG2 in HCT116 and SW480 cells ($p<0.01$) (Figure 3). In addition, IGF2BP1 inhibition reduced TCF-dependent luciferase activity in HCT116 and SW480 cells ($p<0.05$) (Figure 7; Supplemental Figure S4). That inhibition was further pronounced when the cells were treated with the drugs. Together, our data suggests that IGF2BP1 inhibition might be sufficient to decrease the resistance of chemotherapy-resistant cancer cells with an activated Wnt/ β-catenin signaling pathway.

3.3 | Inhibition of IGF2BP1 increases apoptosis and reduces migration of cells with activated Wnt/β-Catenin signaling pathway treated with chemotherapeutics

We previously showed that IGF2BP1 promotes survival mechanisms in cells through up-regulation of its targets

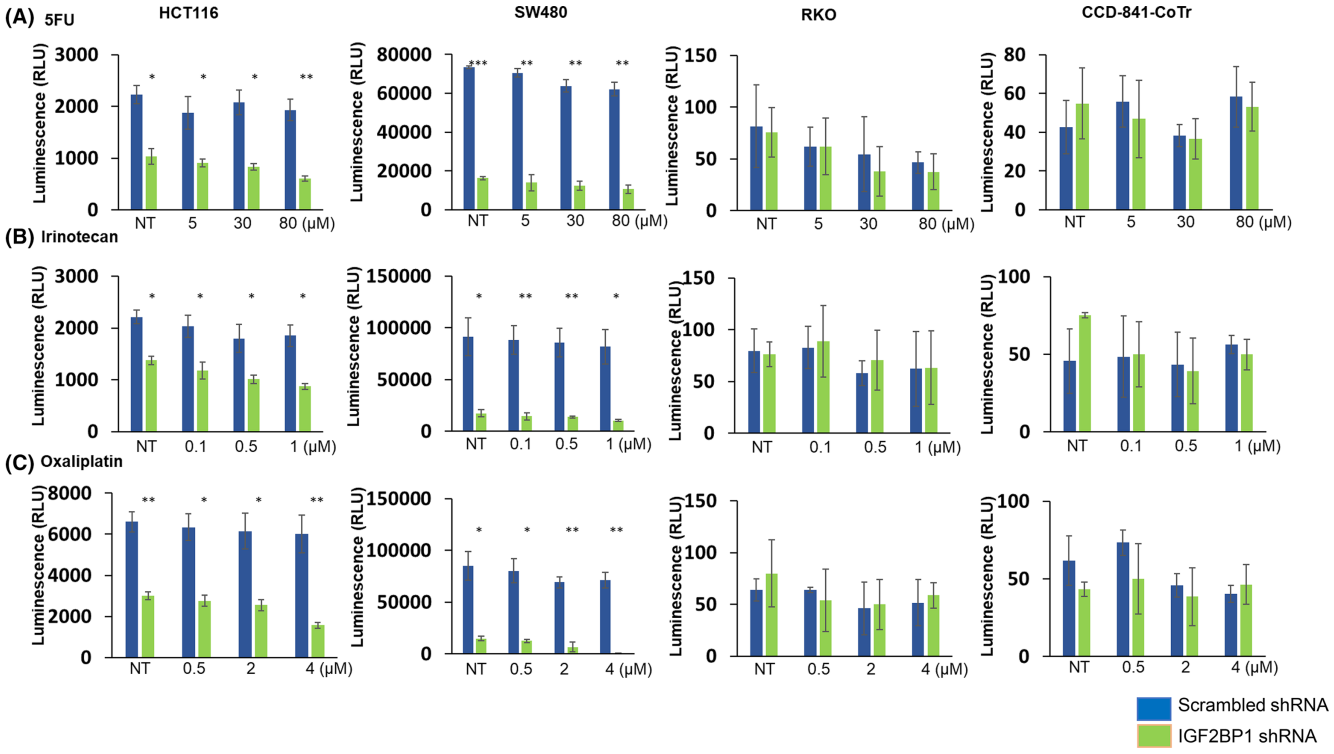

FIGURE 7 TOPFlash luciferase assay. Inhibition of IGF2BP1 reduces TCF-driven luciferase activity in HCT116 and SW480 cells treated with 5FU, Irinotecan, or Oxaliplatin. 20,000 cells of each stable clone were seeded in the 96 well white plates with clear bottom. 24 h after seeding, they were transfected by lipofection with pGL4.74(hRLuc/TK) (5 ng) and M50 Super 8x TOPFlash (0.05 μg) or M51 Super 8xFOPPFlash (0.05 μg). 24 h after transfection, the cells were treated with 5FU (A), Irinotecan (B), or Oxaliplatin (C) at the indicated concentration for 24 h, and the TCF-dependent luciferase activity was measured and normalized to Renilla luciferase using the Dual-Glo luciferase assay system. Experiments were performed at least three times and in duplicates. Each value represents Mean ± SD. *$p<0.05$; **$p<0.01$; ***$p<0.001$

β-TrCP1, c-myc, and Gli1.[7,21,22] IGF2BP1 was also shown to upregulate MDR1.[24] Downregulating the Wnt/β-catenin signaling pathway has been shown in some studies to inhibit survival mechanisms and promote drug-induced apoptosis of CRC cells.[34–37] Since the induction of IGF2BP1 is responsible for a variety of pleiotropic effects of the Wnt/β-catenin signaling pathway in CRC cells, we hypothesized that inhibition of IGF2BP1 would enhance drug-induced apoptosis in CRC cells with activated Wnt signaling. Apoptosis was assessed by measuring caspase activity in stable clones of HCT116, RKO, SW480, and CCD-841 CoTr cells expressing either IGF2BP1 shRNA or scrambled shRNA control and treated with different concentrations of 5-FU, irinotecan, or oxaliplatin for 72 h as described in the Materials and Methods section. We observed that in cells with activated Wnt/β-catenin signaling, the caspase activity increased with the inhibition of IGF2BP1 as we previously demonstrated ($p < 0.01$ and $p < 0.001$ for HCT116 and SW480, respectively). This increase in apoptosis was concentration-dependent and more pronounced when IGF2BP1 inhibition was combined with 5-FU, irinotecan, or oxaliplatin even at lower concentrations of these drugs (Figure 8) ($p < 0.001$). Caspase activity was not affected by IGF2BP1 inhibition or drug treatment in RKO cells, whereas in CCD-841 CoTr, we observed a decrease in caspase activity with 5FU treatment and a slight increase in caspase activity with irinotecan and oxaliplatin; however, these changes were not significant.

CRC treatment has seen a remarkable improvement in recent years; however, a significantly high number of patients with CRC will develop metastasis due to the localization of the tumor and the resistance to chemotherapeutics. The five-year survival rate is around 65%.[38] IGF2BP1 was shown to regulate a set of targets (ALCAM, AMIGO2, collagen V, alpha1, Dysadherin, Keratin 19, Lumican, MMP1, MCAM, and synCAM) involved in cellular adhesion, invasion, and extracellular matrix remodeling indicating its role in metastasis.[20] Its expression was associated with aggressive forms of many cancers as well.[9,11,17,18] We tested whether inhibition of IGF2BP1 would augment the inhibitory effect of chemotherapeutics on the migratory capability of CRC cells. For this experiment, we performed the wound healing assay using the Ibidi Culture-Insert 3 Well as described in the Materials and Methods section. Stable clones of HCT116, RKO, SW480, and CCD-841 CoTr cells

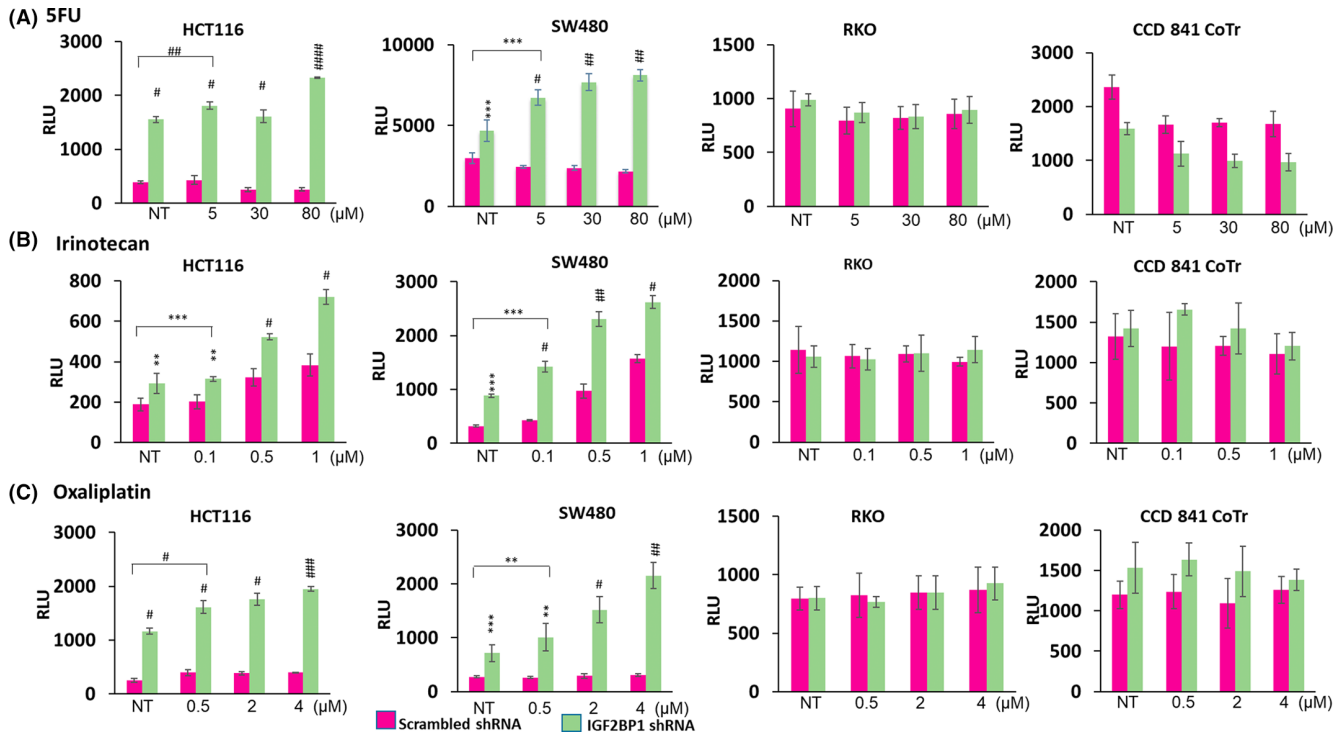

FIGURE 8 Caspase assay. Inhibition of IGF2BP1 increases the apoptotic effect of chemotherapeutic drugs on cells with an activated Wnt signaling pathway. Stable clones of each cell line expressing scrambled shRNA or IGF2BP1 shRNA plasmids were seeded in 96 well white plates with a clear bottom at the density of 5000–7000 cells. 24 h after seeding, the cells were treated with 5-FU (A), Irinotecan (B), or Oxaliplatin (C) at the indicated concentrations for 72 h. Media were changed to media without the drug for 24 h and the caspase assay was performed as described in Materials and Methods. Each experiment was performed at least three times and in triplicates. The *Y*-axis represents the RLU and the *X*-axis represents the concentrations of the drugs. Each value represents Mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{\#}p < 0.00001$; $^{\#\#}p < 0.000001$; $^{\#\#\#}p < 0.0000001$; $^{\#\#\#\#}p < 0.00000001$

expressing either IGF2BP1 shRNA or scrambled shRNA control were seeded in the wells of the Ibidi plates and allowed to attach and grow. When the cells reached 100% confluency, the inserts were removed to create wounds. The cells were treated with different concentrations of 5-FU (Figure 9, Supplemental Figure S1), Irinotecan (Supplemental Figure S2), or Oxaliplatin (Supplemental Figure S3), and images of the wounds were taken every 24 h. Cell migration was assessed by measuring the width of wounds using ImageJ. We observed that inhibition of IGF2BP1 significantly reduces migration of CRC cells with activated Wnt/β-catenin signaling pathway over time (Figure 9, Supplemental Figure S1-3a,b,e,f). Combination of IGF2BP1 inhibition and either drug treatment reduced further migration of HCT116, and SW480 cells. This reduction in migration was also concentration-dependent (Figure 9, Supplemental Figure S1-3a,b,e,f). Migration of RKO and CCD-841-CoTr cells was not significantly affected by IGF2BP1 inhibition (Figure 9, Supplemental Figure S1-3c,d,g,h). The wound-healing assay was supported by the transwell migration assay performed on the stable clones of HCT116 and SW480 cells (Figure 10). We observed a significant reduction in the migration of the cells when IGF2BP1 was inhibited, and further reduction when the drug treatments were associated with IGF2BP1 inhibition ($p < 0.05$) (Figure 10). Our data supports the contribution of IGF2BP1 to the migration and invasion of CRC cells exhibiting an activated Wnt/β-catenin signaling pathway.

In this study, we tested whether inhibition of the Wnt/β-catenin target IGF2BP1 could sensitize CRC cells to chemotherapeutics. We observed that inhibition of IGF2BP1 significantly reduces the ability of HCT116 and SW480 cells to form colonies and proliferate. This was paralleled by a significant increase in apoptosis as measured by the caspase assay and a reduction in migratory capability as measured by the wound healing and transwell migration assays. RKO and CCD-841 CoTr cells were not significantly affected by IGF2BP1 inhibition. Using DLD1 D7Δ15, LS174T-L8, and RKO cells, we showed that inhibition of IGF2BP1 was sufficient to decrease the resistance

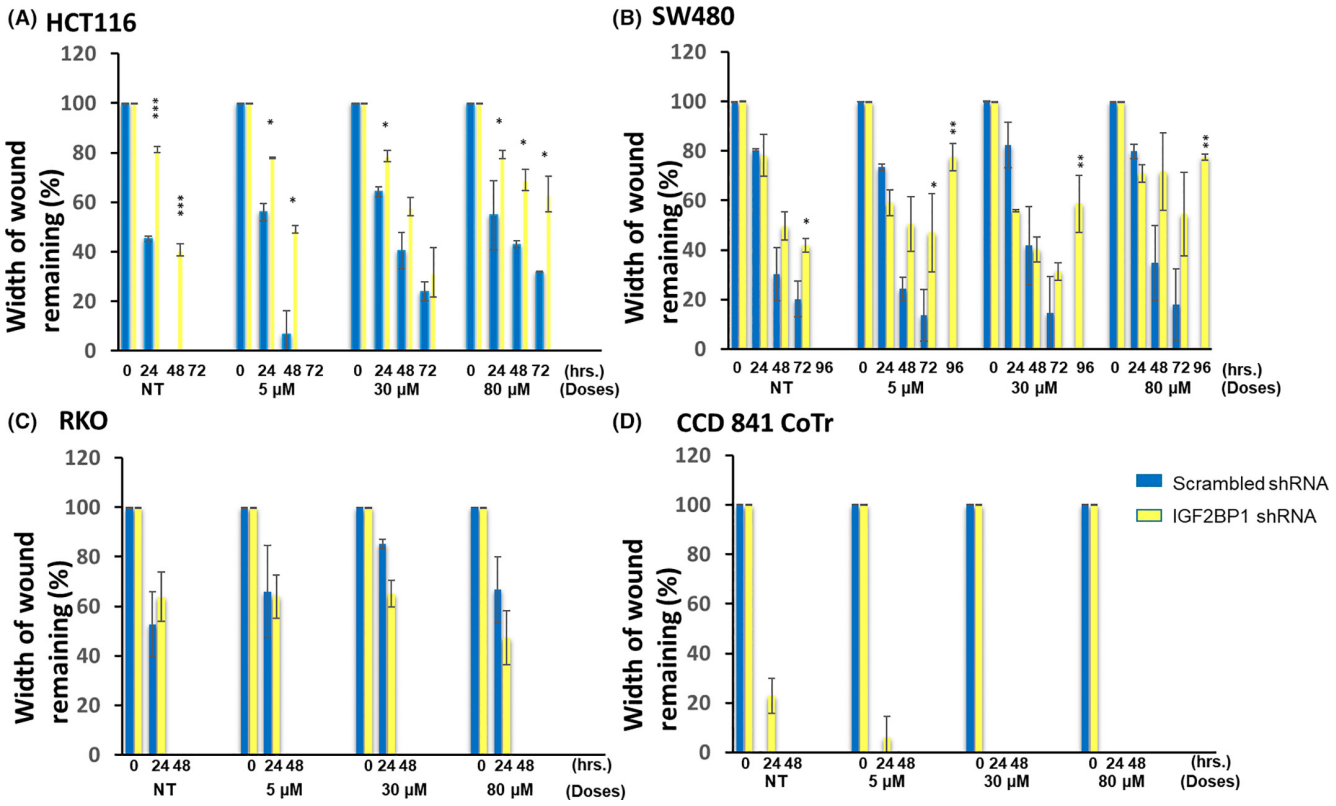

FIGURE 9 Wound healing assay. Inhibition of IGF2BP1 significantly reduces migration in cells with activated Wnt signaling pathway and treated with 5-FU. Stable clones of each cell line expressing scrambled shRNA or IGF2BP1 shRNA plasmids were seeded in Culture-Insert 3 Well from Ibidi at the density of 5–8.5104 cells. 24–48 h after seeding, the inserts from the dish were removed and the cells were treated with 5-FU at the indicated concentrations. The migration of the cells was recorded every 24 h, and the size of the wounds was measured using ImageJ. (A) Migration of HCT116 cells. (B) Migration of SW480 cells. (C) Migration of RKO cells. (D) Migration of CCD-841 CoTr cells. Each experiment was performed at least three times and in duplicates. The *Y*-axis represents the remaining width of the wound relative to the initial width (t_0) and the *X*-axis represents the concentrations of the drugs and time points. Each value represents Mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

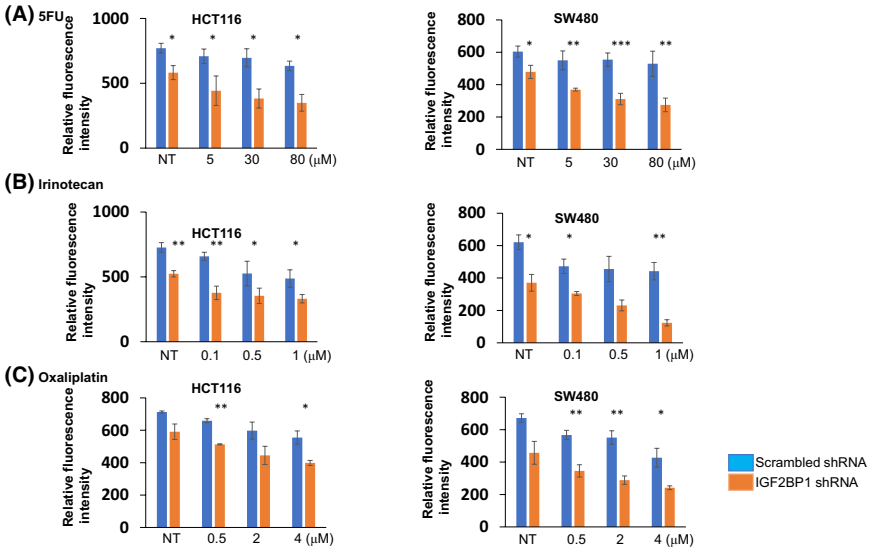

FIGURE 10 Transwell migration assay. Inhibition of IGF2BP1 significantly reduces migration of HCT116 and SW480 cells treated with 5FU, Irinotecan, or Oxaliplatin. 300,000 cells from each stable clone were suspended in low serum media (1% FBS) containing 5FU (A), Irinotecan (B), or Oxaliplatin (C) were seeded in the insert of the 24-well cell migration plate and incubated for 24 h in the 5% CO2 humidified incubator. 10% FBS in the culture media in the lower chamber served as a chemo-attractant. 24 h after seeding, the migrating cells were dislodged, lysed, and stained with the CyQuant GR dye according to the manufacturer's recommendations. 200 μl of each sample was transferred into a 96-well white plate with a clear bottom, and the fluorescence intensity was read using a plate reader (Omega). Experiments were performed at least three times and in duplicates. Each value represents Mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

of chemotherapy-resistant cancer cells with activated Wnt/β-catenin signaling pathway. Inhibition of IGF2BP1 also reduced the expression of the ATP-binding cassette transporters ABCB1 and ABCG2 mRNA in cancer cells.

This study proposes a novel target in the treatment of CRC. Currently, there are no approved drugs that target RNA-binding proteins involved in both cell proliferation and induction of multidrug resistance membrane transporters. IGF2BP1 is involved in both processes. These unique characteristics of IGF2BP1 make it an appealing candidate to be assessed as a potential target for the treatment of CRC. Additional supporting studies on the role of IGF2BP1 in CRC drug resistance may potentially lead to the design of the agents capable of affecting cell proliferation, migration, apoptosis, and drug efflux from the cells. In support, a recent study identified a small molecule inhibitor of IGF2BP that could prevent its interaction with its target Kras, therefore reducing Kras expression and a pro-oncogenic phenotype in cancer cells.[39] This would be more effective in the treatment of CRC or other conditions associated with high levels of IGF2BP1 expression.

AUTHOR CONTRIBUTIONS

Nicole Betson and Mohammed Hajahmed performed experimental work and prepared figures; Tsige Gebretsadek performed experimental work; Kenneth Ndebele performed experimental work and reviewed the manuscript; H. Anwar Ahmad helped with the statistical analyses and reviewed the manuscript; Paul B. Tchounwou reviewed the experimental design and the manuscript. Vladimir S. Spiegelman designed experiments and reviewed the manuscript; Felicite K. Noubissi designed and performed experimental work, analyzed data, prepared figures, and wrote the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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