Aus der Anatomischen Anstalt
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Die Rolle von β1-Integrin-Rezeptoren bei der Anti-Tumor-Wirkung von Resveratrol auf das kolorektale Karzinom in multizellulären 3D-Kulturmodellen *in vitro*

Dissertation

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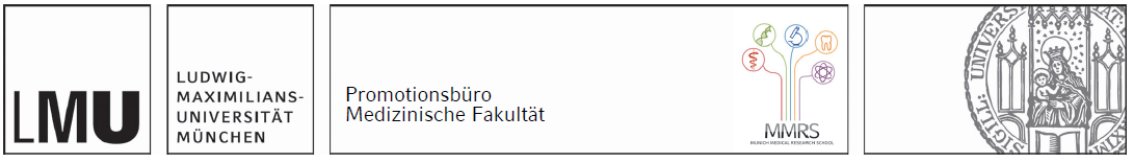
Geduld ist ein Baum,
dessen Wurzeln bitter sind,
dessen Früchte aber sehr süß sind.

Persisches Sprichwort

Inhalt

Affidavit



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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

Die Rolle von β1-Integrin-Rezeptoren bei der Anti-Tumor-Wirkung von Resveratrol auf das kolorektale Karzinom in multizellulären 3D-Kulturmodellen *in vitro*

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Paper I

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Paper II

Brockmueller A., A. Mueller, P. Shayan, M. Shakibaei: "β1-Integrin Plays a Major Role in Resveratrol-Mediated Anti-Invasion Effects in the CRC Microenvironment" Frontiers in Pharmacology, 2022, doi: 10.3389/fphar.2022.978625 ***IF 5.988***

Paper III / Anhang

Brockmueller A., S. Girisa, A. Kunnumakkara, M. Shakibaei: "Resveratrol Modulates Chemosensitisation to 5-FU via β1-Integrin/HIF-1α Axis in CRC Tumor Microenvironment" International Journal of Molecular Sciences, 2023, doi: 10.3390/ijms24054988 ***IF 6.208***

Vollständige Publikationsliste

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Abkürzungen

5-FU	– 5-Fluorouracil
Akt	– Serin/Threonin Proteinkinase B
Anti-β1	– Antikörper gegen β1-Integrin-Rezeptoren
Anti-β5	– Antikörper gegen β5-Integrin-Rezeptoren
β1-ASO	– β1-Integrin-Antisense-Oligonukleotide
β1-SO	– β1-Integrin-Sense-Oligonukleotide
CEA	– carcinoembryonales Antigen
COX	– Cyclooxygenase
CXCR4	– CXC-Motiv-Chemokinrezeptor 4
EMT	– epithelial-mesenchymale Transition
ERK	– extrazellulär Signal-regulierte Kinase
FAK	– fokale Adhäsionskinase
IL	– Interleukin
JAK	– Januskinase
JNK	– c-Jun N-terminale Kinase
MAPK	– mitogen-aktivierte Proteinkinase
MEK	– mitogen-aktivierte Proteinkinase-Kinase
MMP	– Matrix-Metalloproteinase
NF-κB	– nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NF-κB-ASO	– NF-κB-Antisense-Oligonukleotide
NF-κB-SO	– NF-κB-Sense-Oligonukleotide
PI3K	– Phosphoinositid-3-Kinase
RGD-Peptid	– Aminosäuresequenz Arginin, Glycin, Asparaginsäure
RGE-Peptid	– Aminosäuresequenz Arginin, Glycin, Glutaminsäure
Sirt-1	– Sirtuin-1
STAT3	– Signalüberträger und Transkriptionsaktivator 3
TGF-β	– Transforming growth factor-β
TNF	– Tumornekrosefaktor
TME	– multizelluläre, entzündungsfördernde Tumorumgebung

1. Einleitung

1.1. Krebserkrankungen

Tumore, die zu den fünf klassischen Entzündungszeichen nach Celsus und Galen zählen, werden in benigne und maligne Schwellungen unterteilt. Tumore benigner Genese stellen hierbei Raumforderungen dar, während es sich bei malignen Tumoren mit unkontrolliertem Zellwachstum sowie destruierender Tendenz um Krebserkrankungen handelt (1). Hierbei unterscheiden sich solide Primärtumore, die häufig abgrenzbaren Organen zuzuordnen sind und deren Zellen sich als Metastasen in weiteren Organen ansiedeln können, von hämatologischen Tumoren, welche bei Befall des lymphatischen Systems oder des Knochenmarks diffus im gesamten Körper auftreten (1). Maligne Neoplasien können somit alle Körperregionen sowie Gewebearten betreffen und bei Patienten jeder Altersgruppe auftreten. Die Summe aller Krebsarten führt weltweit jährlich zu über 19 Millionen Neudiagnosen sowie zu fast 10 Millionen krebsbedingten Sterbefällen (2), sodass es gesellschaftlich und medizinisch hochrelevant ist, deren komplexe Pathogenesen zu verstehen.

Krebserkrankungen sind vielstufige Prozesse und entstehen hauptsächlich auf der Basis von chronischen Entzündungsgeschehen. Um die Onkogenese systematisch greifbar und somit gezielt behandelbar zu machen, hat sich die Definition von „Cancer Hallmarks“ als Säulen der Krebserkrankung etabliert. Bereits 2011 schufen Hanahan & Weinberg mit der Festlegung von zehn „Cancer Hallmarks“ (3), bei denen Entzündungsgeschehen eine wichtige Rolle zugemessen wird, die Basis dieser Definition. Aufgrund wachsender Bedeutung von Metastasierung, welche bei kolorektalen Karzinomen besonders häufig beobachtet wird (4), erfolgte im Jahr 2019 durch Welch & Hurst eine Erweiterung um vier „Hallmarks of metastasis“ (5) mit dem Fokus auf invasiven Vorgängen. Um den Erkenntnissen der aktuellen Forschung gerecht zu werden, definierten Senga & Grose im Jahr 2021 schließlich weitere vier „Cancer Hallmarks“ (6), wodurch nun auch Faktoren des modernen Lebensstils wie ein verändertes Mikrobiom sowie epigenetische Dysregulationen Beachtung finden und diese sind bei der Risikoermittlung für kolorektale Karzinome von hoher Bedeutung.

Die weitere Systematisierung maligner Erkrankungen einschließlich deren Karzinogenese bleibt ständiger Gegenstand aktueller Forschungsfragen und ist entscheidend für die Weiterentwicklung von Krankheitsverständnis und Therapiemöglichkeiten.

1.2. Entzündungsprozesse der Karzinogenese

Akute, lokale Entzündungsreaktionen, deren Hauptsymptome als Schwellung (tumor), Rötung (rubor), Schmerz (dolor), Erwärmung (calor) sowie eingeschränkte Funktionsfähigkeit (functio laesa) definiert wurden, sind im physiologischen Maße überlebensnotwendig, da sie in gesundem Gewebe der Abwehr von Infektionen und Krankheitserregern dienen (7). Dauern akute Entzündungen länger als drei Wochen an, können diese chronisch werden (7), was die Dedifferenzierung von Zellen und somit die Krebsentstehung begünstigt.

Mit der Krebsausbreitung sind multiple Signalwege verbunden, wobei vor allem der entzündlichen Kaskade um nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) eine Schlüsselrolle zukommt (8). Dieser Haupt-Transkriptionsfaktor der Entzündung (9) reguliert über 500 Gene und deren Endprodukte (10), sodass eine Missregulation weitreichende Folgen haben kann, auch im Sinne einer expandierenden Inflammation sowie Tumorproliferation, Invasion und Metastasierung. Des Weiteren sind die Kaskaden verschiedener Kinasen oder Aktivatoren (11) wie beispielsweise die der mitogen-aktivierten Proteinkinase (MAPK) (12, 13), mitogen-aktivierten Proteinkinase-Kinase (MEK) (14), extrazellulär Signal-regulierten Kinase (ERK) (14), c-Jun N-terminalen Kinase (JNK) (12, 15), Phosphoinositid-3-Kinase (PI3K) (13), Serin/Threonin Proteinkinase B (Akt) (13, 15), Januskinasen (JAKs) (16) und des Signalüberträgers und Transkriptionsaktivators 3 (STAT3) (16) in inflammatorische Geschehen der Kanzerogenese involviert.

Gesamthaft unterstreicht diese Vielfalt an beteiligten Signalwegen und Botenstoffen, dass Entzündungsprozesse in Tumorzellen von zentraler Bedeutung sind und einen hohen Anteil an der Variabilität von Krebserkrankungen haben.

1.3. Kolorektales Karzinom

Maligne Neoplasien des Kolon- oder Rektumepithels, welche die Lamina muscularis mucosae durchdringen und damit lokal infiltrieren, werden als kolorektale Karzinome bezeichnet (17). Unter den Krebserkrankungen, die zu den häufigsten Krankheitsdiagnosen und Todesursachen zählen, nehmen kolorektale Karzinome mit einem Gesamtanteil von ca. 10% weltweit einen erheblichen Stellenwert ein: 2020 wurden in 185 Ländern insgesamt fast zwei Millionen Neudiagnosen des kolorektalen Karzinoms gestellt und es wurden über 935.000 damit assoziierte Todesfälle erfasst (2). Die höchsten Fallzahlen für Kolonkarzinome registrierten Nordamerika, Europa, Australien und Neuseeland,

die meisten Rektumkarzinome traten dagegen in ostasiatischen Ländern auf (2). Statistisch gesehen sind die verschiedenen Darmabschnitte in unterschiedlicher Häufigkeit betroffen, wobei Kolonkarzinome meist proximal (40%) oder distal (22%) gefunden werden und Rektumkarzinome rund ein Drittel (29%) der Erkrankungen ausmachen (18). Während kolorektale Malignome bis vor wenigen Jahren hauptsächlich Patienten über 65 Jahre betrafen, steigt derzeit vor allem die Rate der Erkrankten zwischen 50 und 64 Jahren (18), wobei Frauen sowie Männer gleichermaßen betroffen sind (2).

Beachtenswert ist hierbei, dass ein Viertel der Patienten bereits zum Zeitpunkt der Erstdiagnose Metastasen aufweisen. Im Verlauf der Erkrankung werden solche sogar von mehr als der Hälfte aller Patienten mit kolorektalem Karzinom entwickelt (4), was das hohe Aggressionspotential der Erkrankung unterstreicht. Obwohl die Zahl der vorsorglichen Koloskopien und weiterer Früherkennungsmöglichkeiten wächst, ist insgesamt in den nächsten Jahren mit weiter steigenden Patientenzahlen zu rechnen (18).

1.4. Risikofaktoren des kolorektalen Karzinoms

Die hohen Fallzahlen des kolorektalen Karzinoms kommen dadurch zustande, dass im Rahmen der Karzinogenese sowohl endogene als auch exogene Faktoren eine große Rolle spielen können. Zu den endogenen Risikofaktoren zählen genetische Veränderungen, die für 5-10% aller Krebserkrankungen (19), aber für bis zu 20% der kolorektalen Karzinome (20) verantwortlich gemacht werden und in diesen Fällen zu hereditärem nicht-polypösem Kolonkarzinom-Syndrom (21), familiärer adenomatösen Polyposis (21, 22), Aktivierung von Onkogenen (22) oder dem Verlust von Tumorsuppressorgenen (23) führen können. Außerdem entstehen 1-2% der malignen Neoplasien in Kolon oder Rektum aufgrund vorab bestehender chronisch-entzündlicher Darmerkrankungen wie Colitis Ulcerosa oder Morbus Crohn (24). Das sporadische Erkrankungsrisiko erhöht sich generell mit steigendem Lebensalter, besonders durch die Adenom-Karzinom-Theorie, bei der die Entstehung von malignen Karzinomen verstärkt aus vorhandenen benignen kolorektalen Adenomen beobachtet wird (25).

Insgesamt werden bei über 80% dieser Erkrankungen (19, 20) ein ungesunder Lebensstil mit fettfokussierter Ernährung, Bewegungsmangel, Adipositas (19, 26, 27), gesteigertem Alkoholkonsum oder Zigarettenrauchen (28) und weiteren exogenen Umweltfaktoren (19) als ursächlich angesehen. Diese Erkenntnis klingt erschreckend in Bezug auf den aktuellen Lebensstil weiter Teile der modernen Bevölkerung und bietet dennoch gleichzeitig eine große Chance zur präventiven

epigenetischen Regulation. So wird die Modulation der Alltagsgewohnheiten durch sportliche Betätigung, gesunde Ernährung und Vermeidung von Übergewicht in jedem Lebensalter empfohlen (27, 29, 30) und für die asymptomatische, über 50-jährige Bevölkerung sind darüber hinaus regelmäßige Früherkennungsmaßnahmen ratsam, möglichst in Form von Vorsorge-Koloskopien oder fäkalen immunchemischen Tests (30, 31).

1.5. Erkennung und Behandlung des kolorektalen Karzinoms

Zunehmende Verdauungsbeschwerden (20), ein positiver Stuhltest auf okkultes Blut (20) oder ein auffälliger Befund bei der digital-rektalen Untersuchung (30) können erste Hinweise auf eine maligne kolorektale Neoplasie liefern, woraufhin eine Koloskopie mit Probenentnahme erfolgen sollte. Wird der Befund eines kolorektalen Karzinoms histologisch bestätigt, wird ein umfassendes Staging mittels bildgebender Verfahren, kombiniert aus Abdomen-Sonographie, Thorax-Röntgen und Becken-Computertomographie, durchgeführt (30). Tumormarker wie das carcinoembryonale Antigen (CEA) haben sich bisher nicht als organspezifisch erwiesen und können dadurch nicht zur Früherkennung von kolorektalen Karzinomen eingesetzt werden. Während der Behandlung stellen sie allerdings einen wichtigen Parameter in der Verlaufskontrolle dar, da ein Anstieg des CEA-Wertes im Vergleich zum Zeitpunkt der Diagnosestellung auf eine Lebermetastasierung oder ein Rezidiv hinweisen kann (32).

Die Therapie eines kolorektalen Karzinoms besteht in Abhängigkeit des Allgemeinzustandes sowie des Erkrankungsstadiums und unter Einbeziehung psychoonkologischer Betreuung aus chirurgischer Resektion, Poly-Chemotherapie nach dem FOLFOX4-, FOLFOX6- oder CAPOX-/XELOX-Schema und/oder Radiochemotherapie (30). Besondere Herausforderungen stellen fortgeschrittene oder metastasierte Karzinome dar oder solche, deren Tumorzellen Resistenzen gegen klassische Chemotherapeutika, beispielsweise 5-Fluorouracil (5-FU) oder Oxaliplatin (30), gebildet haben.

1.6. Entzündungsprozesse der kolorektalen Karzinogenese

In der Pathogenese kolorektaler Malignome spielen entzündliche Prozesse eine zentrale Rolle. Bei einem Parametervergleich zwischen Gesunden und Patienten mit kolorektalem Karzinom wurde bei den Erkrankten nicht nur ein erniedrigter Plasmaspiegel des Immunglobulins A festgestellt, sondern es lag gleichzeitig eine erhöhte Lymphozytenzahl im Stroma des Karzinoms vor (33). In nekrotisierten Tumorbereichen wurde zudem eine erhöhte Anzahl an

Makrophagen und polymorphkernigen Zellen gefunden (33). Interessanterweise wurden zusätzlich die peritumoralen Zellinfiltrate untersucht, die durch Entzündungsprozesse entstanden und mit 47% Lymphozyten, 19% Plasmazellen, 15% Makrophagen/Monozyten und 5% granulierten Mastzellen zum Großteil aus Immunzellen bestanden, wobei auch hier bereits 15% der Immunzellen polymorphkernig verändert waren (33). Insgesamt sprach die Analyse für ein stark inflammatorisches Geschehen und die entsprechenden Signalwege wurden weiter untersucht.

Hierbei zeigte sich die besondere Bedeutung des immunmodulatorischen Transkriptionsfaktors NF-κB, dessen Phosphorylierung sowie Translokation in den Zellkern im Rahmen von Entzündungsprozessen durch Zytokine wie Tumornekrosefaktor (TNF)-α und Interleukin (IL)-1 induziert wird (34, 35), wodurch die Aktivierung von entzündungs- und damit tumorfördernden Endprodukten wie Matrix-Metalloproteinasen (MMPs), CXC-Motiv-Chemokinrezeptor 4 (CXCR4), Cyclooxygenase (COX)-2 und dadurch auch Prostaglandin E2 voranschreitet (11, 35-38). Bei der Proliferation und Metastasierung von Zellen kolorektaler Neoplasien kristallisierten sich MMP-9, CXCR4 und auch der involvierte Apoptosemarker Caspase-3 als hochrelevant heraus (8). Des Weiteren werden Proteine der Transforming growth factor-β (TGF-β) Familie in inflammatorischer Tumorumgebung fehlreguliert, sodass ihre ursprünglich tumorunterdrückende Wirkung zu einer tumorunterstützenden Komponente wird (39). Zur tierversuchsfreien Simulation dieser komplexen entzündlichen Vorgänge, hat sich mittlerweile der Einsatz von multizellulären 3D-Kulturen *in vitro* (40, 41) bewährt und etabliert, da durch die Zusammensetzung von Tumorzellen, Fibroblasten und T-Lymphozyten in einer Petrischale der interzelluläre Cross-Talk verschiedener Zellarten und somit auch der Austausch ihrer Botenstoffe *vivo*-nah nachgeahmt werden kann.

1.7. Plastizität während der kolorektalen Karzinogenese

Unter zellulärer Plastizität versteht man im Rahmen der Onkogenese die Fähigkeit von Zellen, mit einem folgenreichen phänotypischen Wandel auf pro-kanzerogene Stimuli zu reagieren. Im Falle des Intestinaltraktes führen inflammatorische Trigger zu Umprogrammierung des Stoffwechsels und epithelial-mesenchymaler Transition (EMT) von Darmepithelzellen (42) sowie der Bildung von Krebsstammzellen (43).

EMT meint die Fähigkeit von Zellen, sowohl ihre Polarität als auch ihren Phänotyp von epithelial zu mesenchymal zu verändern, was zu migratorischen Eigenschaften führt (44). Während dieser Prozess zu Zeiten der embryonalen

Entwicklung im Rahmen der Organogenese in gesunden Zellen physiologisch und sogar notwendig ist (44), ist er im Kontext einer Krebserkrankung als hochpathologisch anzusehen, weil er den Tumorzellen die Wanderung und damit die Metastasierung ermöglicht (45). Außerdem werden das Wachstum sowie die Aggressivität von kolorektalen Malignomen maßgeblich durch Tumorstammzellen beschleunigt (43). Daher besteht ein großes Forschungsinteresse, die Vorgänge der zellulären Plastizität in allen Stadien der kolorektalen Karzinogenese, die Initiation, Promotion sowie Progression umfassen (46), zu entschlüsseln.

Hierbei ist die Untersuchung markanter Parameter auf Ebene der Proteinexpression von besonderer Aussagekraft. Während erhöhte Bildung von Tumorstammzellen mit der Expression von ALDH1, CD44 sowie CD133 einhergeht (43), wird EMT durch den Transkriptionsfaktor Slug, den mesenchymalen Marker Vimentin sowie den epithelialen Stabilisator E-Cadherin repräsentiert (47, 48). Aufgrund des ausgeprägten Invasionspotentials wurden diese Parameter in verschiedenen Zelllinien des kolorektalen Karzinoms (Caco-2, HCT116, HT29, RKO, SW480, SW620) untersucht mit der Feststellung einer erhöhten Expression von ALDH1, CD44, CD133, Vimentin sowie Slug und der gleichzeitig eingeschränkten Bereitstellung von E-Cadherin (47, 48). Untersuchungen in multizellulären 3D-Kulturen mit tumorassoziierten Fibroblasten, T-Lymphozyten und Zellen des kolorektalen Karzinoms zeigten darüber hinaus, dass durch intensiven, entzündungsfördernden interzellulären Austausch von Botenstoffen alle Komponenten der Plastizität verstärkt werden (41, 47, 49), was insgesamt die Entstehung einer Chemoresistenz begünstigt (50).

Diese Erkenntnisse des tumorfördernden Cross-Talks sollen künftig als Angriffspunkte für Modulationen bei aggressiven kolorektalen Neoplasien dienen. So wurde bereits nachgewiesen, dass kolorektale Karzinomzellen mit geringem E-Cadherin-Anteil besonders gut auf die Chemotherapeutika Irinotecan und Oxaliplatin ansprechen (48). Gleichzeitig zeigen Phytopharmaka wie die Curcuminoide Calebin A und Curcumin sowie das Stilbenoid Resveratrol gute präventive Ansätze, um Plastizitätsprozessen in solchen Tumorzellen vorzubeugen oder die Chemosensibilität gegenüber klassischen Zytostatika zu erhöhen (47, 51, 52).

1.8. Resveratrol

Das Polyphenol Resveratrol wurde bereits 1939 als sekundärer Pflanzenstoff aus *Veratrum grandiflorum* extrahiert (53) und anschließend als Bestandteil vieler

Nahrungsmittel wie roten Trauben und dem daraus entstehenden Wein (54), Preiselbeeren, Heidelbeeren, Moosbeeren, Johannisbeeren, Erdbeeren (55) sowie Pistazien (56) und anderen Nüssen detektiert. In diesen Früchten übt Resveratrol eine Abwehrfunktion gegenüber Pathogenen mit besonderer Schutzfunktion vor Pilzinfektionen aus (57).

Mittlerweile sind auch die Auswirkungen dieses Phytopharmakons auf menschliche Zellen gut untersucht und der Naturstoff zeigt umfangreiche gesundheitsfördernde Wirkungen. So wurden beim Menschen ebenso infektionseindämmende (58) Effekte gezeigt, wobei sich Resveratrol aufgrund seiner anti-oxidativen Wirkung (59) zudem schützend auf ganze Organsysteme wie das Nervensystem (60), die Herz-Kreislauf-Organe (61) sowie die Verdauungsorgane (62) auswirkt und den Blutglukosespiegel (40) reguliert (Abbildung 1). In gesundem Gewebe, beispielsweise in Sehnen und Knorpel, kann der Naturstoff über die schützende Funktion hinaus sogar anabol wirken (63, 64), was unterstreicht, dass es sich bei Resveratrol nicht um ein einseitig, sondern um ein regulatorisch wirkendes Polyphenol handelt. Besonders hervorzuheben ist dessen modulatorische Fähigkeit in Bezug auf die Prävention und Bekämpfung von Entzündungen (36), welche die Chronifizierung von Krankheitsprozessen verhindern oder verzögern kann.

Als Multi-Target-Molekül kann Resveratrol somit an verschiedenen Stellen in die Entstehungs- und Ausbreitungskaskaden unterschiedlicher chronischer Erkrankungen einschließlich Krebserkrankungen (65-68) eingreifen und ist in der Lage, Tumorzellen durch ein breitgefächertes Wirkspektrum wie die Regulation des pH-Wertes und Warburg-Effektes (40) gezielt auszuschalten.

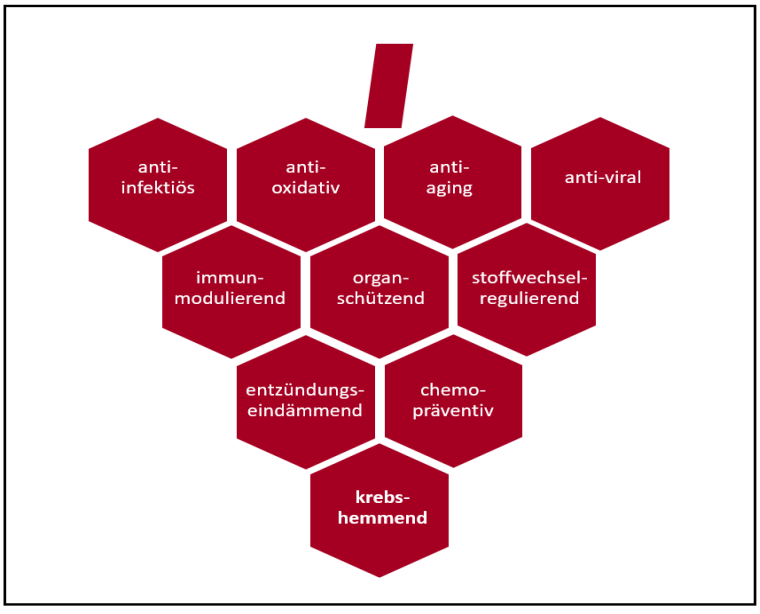

Abbildung 1: Übersicht über die gesundheitsfördernden Eigenschaften des Polyphenols Resveratrol.

1.9. Wirkung von Resveratrol auf das kolorektale Karzinom

An kolorektalen Karzinomzellen wurden bereits stark tumoreindämmende und metastasierungshemmende Effekte durch Resveratrolbehandlung gezeigt. So konnte das Phytopharmakon in mehreren Zelllinien kolorektaler Malignome (HCT116, RKO, SW480) die durch beispielsweise TNF-α oder TNF-β hervorgerufene EMT umkehren (69). Außerdem ist bekannt, dass verschiedene Zytokine und Enzyme Entzündungs- sowie Adhäsionskaskaden in kolorektalen Tumorzellen fördern. Eine Behandlung der Zellen mit Resveratrol hemmt diesbezüglich sowohl die Phosphorylierung des entzündungsfördernden Transkriptionsfaktors NF-κB als auch die Aktivierung der fokalen Adhäsionskinase (FAK), die als zentrales Enzym der Tumorausbreitung gilt (70). Darüber hinaus wirkt sich der in einer multizellulären 3D-Tumorkultur beobachtete Cross-Talk zwischen tumorassoziierten Fibroblasten, T-Lymphozyten und kolorektalen Karzinomzellen förderlich auf die Inflammationsexpansion, das Überleben, die Migrationsfähigkeit sowie die Krebsstammzellbildung aus und Resveratrol kann in diese vielschichtigen Prozesse durch die Hemmung des Cross-Talks lindernd eingreifen (49, 71). Hierbei stellte sich neben der NF-κB-Kaskade auch Sirtuin-1 (Sirt1), das Schlüsselenzym der intrazellulären Signaltransduktion, als Hauptangriffspunkt von Resveratrol heraus (72, 73).

Da die Entwicklung von Therapieresistenzen gegen klassische Zytostatika eine große medizinische Herausforderung darstellt, wurde die Wirkung von Resveratrol auch dahingehend untersucht. Eine Zugabe des natürlichen Polyphenols erhöht den Behandlungserfolg des Standard-Chemotherapeutikums 5-FU in kolorektalen Karzinomzellen der Linien HCT116, SW480 und SW620. Interessanterweise entfaltet Resveratrol seine anti-tumoröse Wirkung sogar in Zelllinien (HCT116R, SW480R), die bereits eine Resistenz gegenüber 5-FU zeigen, verbunden mit der Fähigkeit, die NF-κB-Aktivierung, EMT-Entstehung sowie Krebsstammzellbildung zu reduzieren und gleichzeitig die Apoptose dieser Tumorzellen zu fördern (66, 74, 75). Diese Erkenntnis liefert wertvolle Hinweise auf Resveratrols Modulationsmöglichkeiten bezüglich Plastizitätsprozessen in allen Stadien der Onkogenese des kolorektalen Karzinoms.

Die tumoreindämmenden Effekte bezüglich kolorektaler Malignome wurden außerdem bereits sowohl in Tiermodellen (76) als auch in klinischen Studien mit humanen Patienten (77) bestätigt, wobei keine relevanten Nebenwirkungen auftraten (78). Vor diesem Hintergrund scheint sich Resveratrol sowohl als Prophylaxe zur Gesunderhaltung des Intestinaltraktes als auch als Co-Therapeutikum für Patienten mit kolorektalen Neoplasien zu empfehlen.

1.10. β1-Integrin-Rezeptoren in kolorektalen Karzinomzellen

Bereits 1986 wurden Integrine als integrale Membrankomplexe definiert, die bei der transmembranen Verbindung zwischen Zytoskelett und extrazellulärer Matrix von Bedeutung sind (79). Es handelt sich hierbei um heterodimere Glykoproteine, die sich aus je einer α- und einer β-Kette zusammensetzen. Mittlerweile sind 18 α- sowie 8 β-Untereinheiten bekannt, aus deren Kombination sich im menschlichen Körper mindestens 24 Integrin-Typen ergeben (80). Während die α-Kette hierbei eine regulatorische Funktion übernimmt, ist überwiegend die β-Kette für den Empfang und die Weitergabe von Signalen zuständig (81). Die relevanten Untereinheiten wurden durch die Isolation mit spezifischen Antiseren nachgewiesen, so auch β1-Integrin-Rezeptoren im Jahr 1988 (82). Deren basale Bedeutung wird bereits während der embryonalen Entwicklung deutlich, wobei sie als Rezeptoren der extrazellulären Matrix an Vorgängen wie der myogenen, chondrogenen sowie epidermalen Differenzierung essenziell beteiligt sind (83-85). Als multifunktionelle Signalempfänger und Signalüberträger, die beispielsweise Fibronektin, Kollagene und Laminine erkennen, sind β1-Integrin-Rezeptoren für die Aufrechterhaltung von Morphologie, Stabilität sowie Funktionstüchtigkeit in gesundem Gewebe lebenslang unentbehrlich (86).

Diese existentiellen Eigenschaften machen sich allerdings auch maligne Neoplasien zunutze. So ist inzwischen bekannt, dass β1-Integrine wesentlich an der Metastasierung von Tumoren beteiligt sind. Hierbei gleiten Tumorzellen mit aktivierten, β1-Integrin-reichen Vorwölbungen am Endothel entlang, welche sich über Laminin mit der subendothelialen Matrix verbinden (87). Durch die anschließende Anreicherung des Strukturproteins F-Actin und die Translokation der Tumorzelle in das Nachbargewebe erfolgt somit eine β1-Integrin-vermittelte Invasion über vaskuläre Basalmembranen (87). Dies ist auch bei stark metastasierenden kolorektalen Tumoren von Bedeutung, denn in mehreren kolorektalen Karzinom-Zelltypen, beispielsweise HCT116 und SW480, wurde bereits eine erhöhte β1-Integrin Expression nachgewiesen (70), sodass β1-Integrin als Tumormarker, mindestens aber als Prognosemarker, diskutiert werden kann. Ob sich β1-Integrin-Rezeptoren darüber hinaus auch als Zielstruktur in die Behandlung kolorektaler Malignome involvieren lassen, wie es kürzlich für αvβ3-Integrin (88) vorgeschlagen wurde, ist noch nicht geklärt.

Die Frage nach zukünftigen Behandlungsoptionen des kolorektalen Karzinoms unter Einbezug von β1-Integrin-Rezeptoren bietet ein großes Forschungspotenzial und war ausschlaggebend für den thematischen Schwerpunkt dieser Dissertation.

2. Projektbeschreibung

2.1. Fragestellung dieser Dissertation

Da es sich beim kolorektalen Karzinom um eine der weltweit häufigsten sowie tödlichsten Krebserkrankungen handelt, bei der die konventionellen Therapiemöglichkeiten nicht ausreichend zu sein scheinen, fokussiert sich dieses Dissertationsprojekt auf die diesbezüglich präventiven und supportiven Möglichkeiten des Phytopharmakons Resveratrol. Dessen gesundheitsfördernde und anti-kanzerogene Wirkung (Abbildung 1 und 2) wird seit langem untersucht (40, 72, 89) und da hinsichtlich Zellen kolorektaler Malignome bereits vielversprechende entzündungs- und tumorhemmende Ergebnisse vorliegen (49, 69, 70, 72, 75, 90), besteht ein großes medizinisches Interesse daran, die exakten Wirkmechanismen zu entschlüsseln.

Daher gelten die Bestrebungen dieser Dissertationsschrift dem Verständnis der funktionellen Interaktion zwischen Resveratrol und kolorektalen Karzinomzellen sowie der Art und Weise, wie die Signaltransduktion auf den Tumorzellen beginnt. Zum Zeitpunkt der Projektkonzeption wurde die erhöhte Expression von β1-Integrin-Rezeptoren in Tumoren tendenziell mit einer schlechten Prognose verknüpft (70, 91, 92). Es war allerdings nicht bekannt, ob diese Rezeptoren als Zielstrukturen und Überträgermoleküle für die Resveratrol-spezifischen anti-tumorösen Signale genutzt werden. Außerdem wurde bereits berichtet, dass andere Mitglieder der großen Integrin-Familie, wie αvβ3-Integrin, als Bindungsmolekül oder Rezeptor auf der Oberfläche von Tumorzellen eine wichtige Rolle bei der krebseindämmenden Wirkung von Resveratrol spielen (93, 94), die Rolle der β1-Integrine bei den krebshemmenden Effekten des Phytotherapeutikums war jedoch nicht geklärt. Diese Lücken in der Literatur führten zu dem vorliegenden Dissertationsthema, das die funktionelle Bedeutung von β1-Integrin-Rezeptoren für die Anti-Tumor-Wirkung des Phytopharmakons Resveratrol (Abbildung 2) in drei verschiedenen, etablierten Zelllinien des kolorektalen Karzinoms (HCT116, SW480, RKO) in An- und Abwesenheit mehrerer spezifischer β1-Integrin-Inhibitoren untersucht.

Zusammenfassend lautet die Hauptfragestellung dieser Arbeit, ob β1-Integrin-Rezeptoren an der Vermittlung der anti-proliferativen, anti-metastatischen, pro-apoptotischen und somit anti-kanzerogenen Signalwege des Multi-Target-Moleküls Resveratrol in kolorektalen Tumorzellen beteiligt sind. Die Ergebnisse der hierzu durchgeführten Studien wurden in zwei Publikationen, „Evidence that β1-integrin is required for the anti-viability and anti-proliferative effect of resveratrol in CRC cells“ (95) und „β1-integrin plays a major role in resveratrol-

mediated anti-invasion effects in the CRC microenvironment“ (96), zusammengefasst und veröffentlicht.

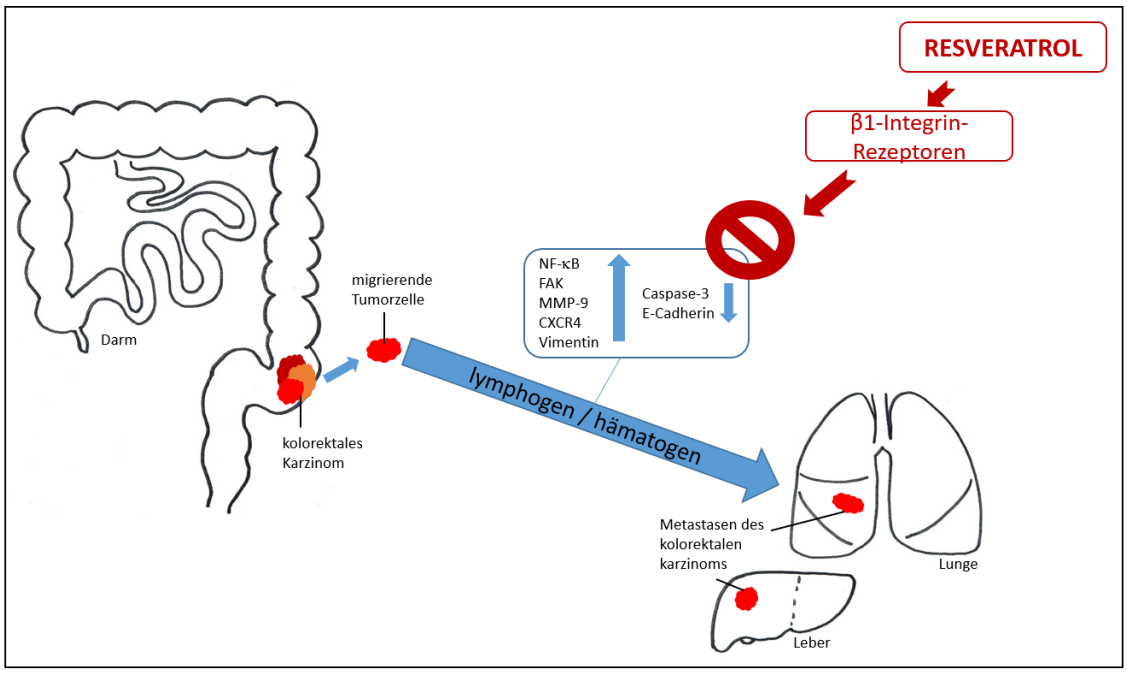

Abbildung 2: Projektübersicht. Tumorzellen eines kolorektalen Primärtumors metastasieren lymphogen oder hämatogen bevorzugt in Leber oder Lunge. Resveratrol nutzt β1-Integrin-Rezeptoren als eines von mehreren wichtigen Schlüsselmolekülen, um die tumorspezifischen Parameterveränderungen zu modulieren und seine krebshemmende Wirkung zu entfalten.

2.2. Methoden dieser Dissertation

2.2.1. Multizelluläre 3D-Tumor-Mikroumgebung

Die entzündungsfördernde multizelluläre 3D-Tumor-Mikroumgebung, welche in den englischsprachigen Publikationen als „tumor microenvironment (TME)“, bezeichnet wird, stellt das zentrale Kulturmodell und Methodenelement dieser Arbeit dar. Es wurde entwickelt (8, 49), um einen tumorbefallenen Körper mithilfe einer Komposition verschiedener Zellsorten *vivo*-ähnlich *in vitro* (Abbildung 3) zu simulieren, sodass die präklinischen Experimente ohne Tierversuche ablaufen können.

Zunächst wurden Zellen des kolorektalen Karzinoms der Linien HCT116, SW480 oder RKO (Abbildung 4) als Monolayer in Zellkulturflaschen gezüchtet und unter Standardbedingungen bei 37°C und 5% CO_2 kultiviert. Die jeweils zweite oder dritte Passage diente der Initiierung der Versuche, wozu die kolorektalen Karzinomzellen gezählt und in Alginat resuspendiert wurden. Die Alginat-Zell-Suspension wurde tropfenweise in $CaCl_2$ gegeben und nach einer Polymerisationszeit von 10 Minuten wurden die entstandenen Alginatkugeln dreimal mit Hanks-Lösung und zweimal mit Zellkulturmedium gereinigt (95).

Anschließend erfolgte die Umsetzung der 3D-Alginatkugeln in bereits vorbereitete 12-Wellplatten mit einem am Boden angewachsenen Fibroblasten-Monolayer aus MRC-5-Zellen (20.000 Zellen pro Well, Abbildungen 3 und 4) und im Zellkulturmedium schwimmenden T-Lymphozyten von Zelltyp Jurkat (20.000 Zellen pro Well, Abbildungen 3 und 4). Die Behandlungsdauer der Tumorzellen mit unterschiedlichen Stoffen und Konzentrationen betrug 10-14 Tage und jeden zweiten Tag erfolgte die Umsetzung der Alginatkugeln in frische 12-Wellplatten (95, 96).

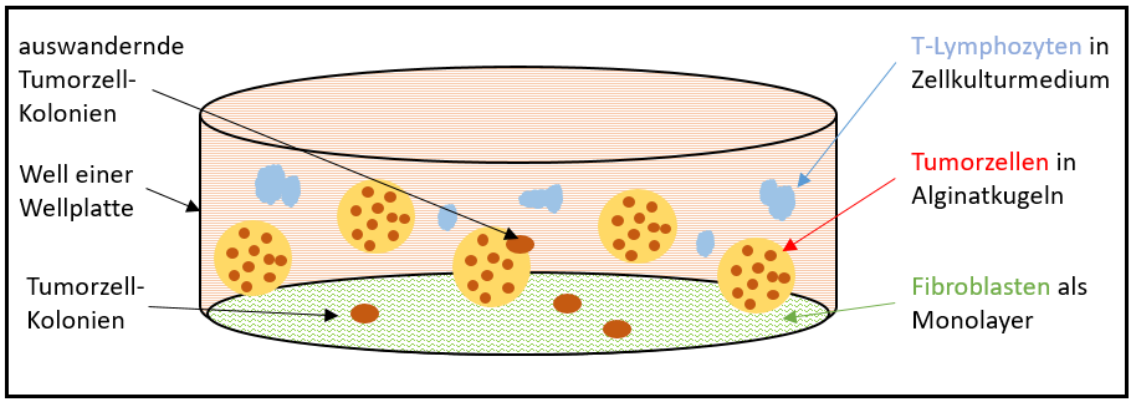

Abbildung 3: Aufbau des multizellulären TME. Tumorzellen wuchsen in Alginatkugeln, wanderten aus und setzten sich als Tumorzell-Kolonien am Wellplatten-Boden ab. Es kam zu einem interzellulären Cross-Talk der Tumorzellen mit am Boden wachsenden Fibroblasten und im Zellkulturmedium schwimmenden T-Lymphozyten.

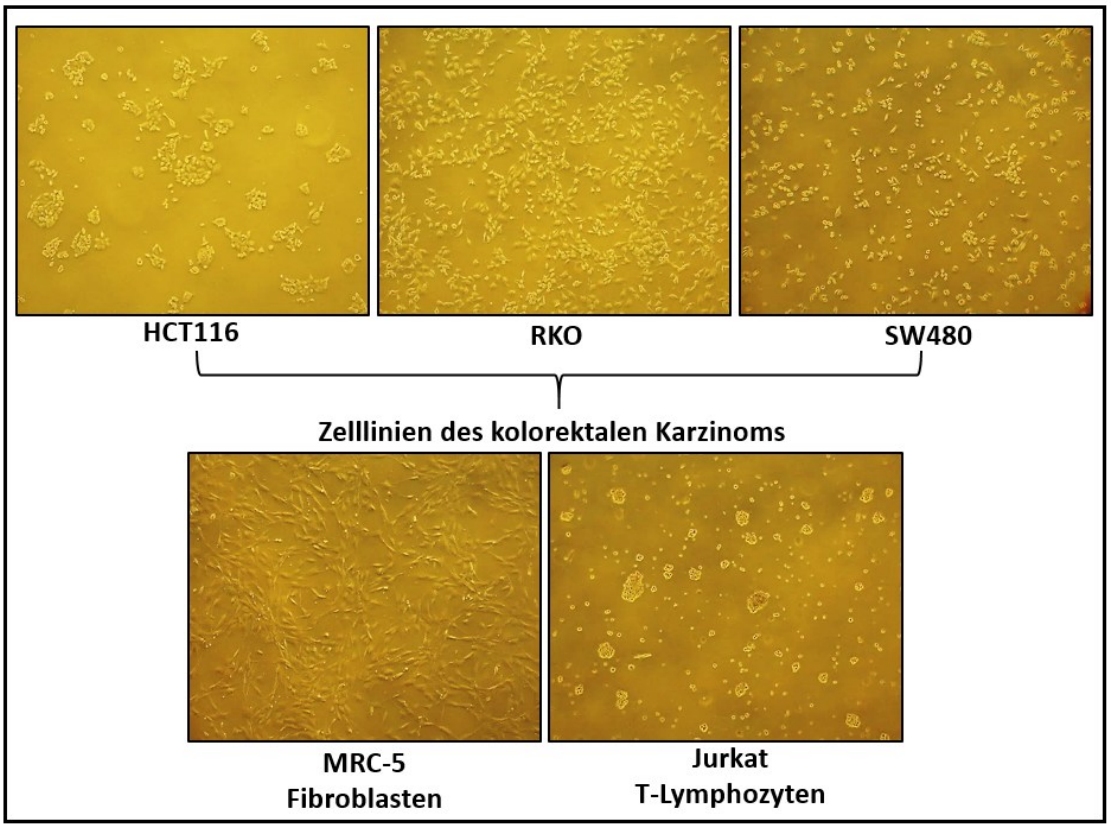

Abbildung 4: Übersicht über die in dieser Arbeit verwendeten Zelllinien. Die Zellen des kolorektalen Karzinoms (HCT116, RKO, SW480) sowie die Fibroblasten (MRC-5) adhärierten am Boden von T175-Zellkulturflaschen, während die T-Lymphozyten (Jurkat) traubenartig formiert im Zellkulturmedium schwammen.

2.2.2. Phasenkontrast-Mikroskopie

Mithilfe eines Zeiss Axiovert 40 CFL Mikroskops (Oberkochen, Deutschland) erfolgte die Beobachtung und Dokumentation der Versuche per Phasenkontrast. Hierbei wurden die unterschiedliche Entwicklung der Tumorzell-Kolonien in den Alginatkugeln (95) oder die Unterschiede in den aus den Alginatkugeln ausgewanderten und am Boden der Wellplatten angesiedelten Kolonien (96) dokumentiert (Abbildung 5).

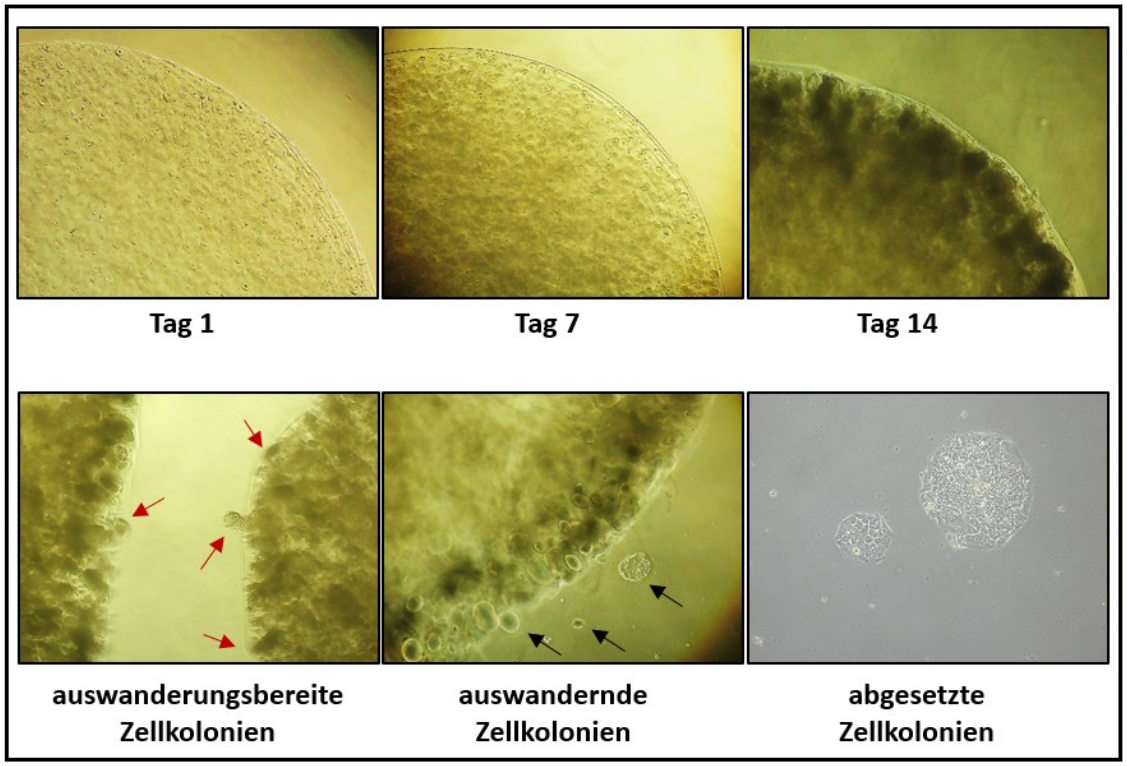

Abbildung 5: Entwicklung von HCT116-Zellen in Alginatkugeln. Im Zeitverlauf kam es zu Vermehrung, Wachstum und Ausbildung von Zellkolonien, welche zu auswanderungsbereiten Zellkolonien reiften (rote Pfeile), letztendlich auswanderten (schwarze Pfeile) und sich am Boden der Wellplatten absetzten.

2.2.3. Koloniefärbungen

Nach Beendigung der Versuchslaufzeit und nach Entnahme sowohl der Alginatkugeln als auch der T-Lymphozyten wurden die 12-Wellplatten mit Karnovsky-Lösung fixiert und anschließend mit Toluidinblau gefärbt (95), um die je nach Behandlung unterschiedliche Ansiedlung der Kolonien am Boden der

Wellplatten sichtbar zu machen (Abbildung 6). Diese waren anhand ihrer Größe und Morphologie klar von den Fibroblasten unterscheidbar.

In einer zusätzlichen Untersuchungsanordnung siedelten sich die ausgewanderten Tumorzell-Kolonien auf rechteckigen Objektträgern an und wurden mit der DAPI-Methode gefärbt (96), um den Anteil der vitalen Zellen an den Kolonien sichtbar zu machen.

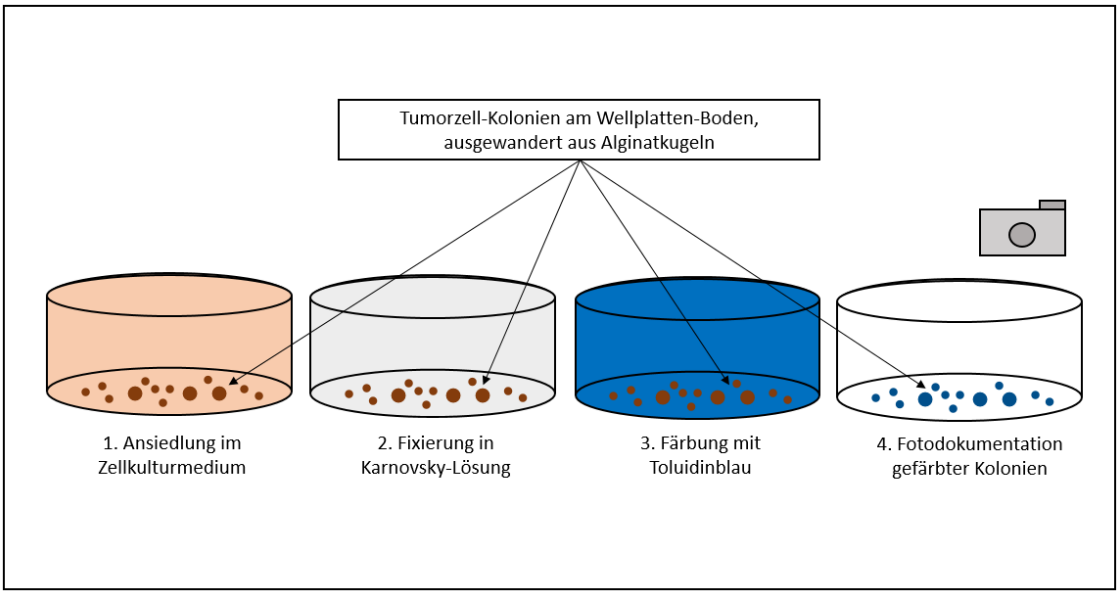

Abbildung 6: Färbung von Tumorzell-Kolonien. Tumorzellen haben sich in Alginatkugeln vermehrt, sind ausgewandert und haben sich als Kolonien am Boden der Wellplatten angesiedelt. Das Zellkulturmedium wurde entfernt, die Tumorzell-Kolonien in Karnovsky-Lösung fixiert, mit Toluidinblau gefärbt und anschließend fotografiert.

2.2.4. Western Blot

Zur Gewinnung der Western Blot-Proben wurden die tumorzellhaltigen Alginatkugeln nach Ablauf Versuchslaufzeit mithilfe einer gebogenen Pinzette aus den Wellplatten entnommen und in Natriumcitrat-Lösung aufgelöst. Nach Entfernung der Alginatreste mittels Zentrifugation, was die spätere ausschließliche Untersuchung von Tumorzellen sicherstellte, wurden die kolorektalen Karzinomzellen in einem Lysismix resuspendiert, erneut zentrifugiert und der Überstand wurde umgehend bei -80°C eingefroren (95). Anschließend erfolgte die Proteinbestimmung sowie die Reduktion mit Mercaptoethanol und die Lagerung der Proben bei -20°C. Die Proben wurden auf selbst hergestellte Gele aufgetragen, mit einem Apparat von Bio-Rad (München, Deutschland) geblottet und anschließend auf eine Nitrozellulosemembran übertragen. Diese wurde nach Blockierung der unspezifischen Bindungen über Nacht bei 4°C in Primär-Antikörper inkubiert, am folgenden Tag mit Sekundär-Antikörper prozessiert,

anschließend entwickelt und wiederum am Folgetag mit einem Densitometer von Bio-Rad (München, Deutschland) quantifiziert und ausgewertet (70).

2.2.5. MTT-Assay

Zur objektiven Messung der Proliferation und Vitalität der Tumorzellen unter unterschiedlichen Behandlungsbedingungen wurde der MTT-Assay ausgewählt. Auch hier wurden die Alginatkugeln aus den Versuchsplatten entnommen und in Natriumcitrat-Lösung aufgelöst und anschließend mittels Zentrifugation und Hanks-Lösung von Alginatresten gereinigt. Nach Resuspension in MTT-Medium erfolgte die Übertragung und Vermischung mit MTT-Reagenz in 96-Wellplatten. Die Reaktion wurde nach drei Stunden gestoppt und die optische Dichte bei 550nm mit einem Microplate-Reader von Bio-Rad (München, Deutschland) gemessen (70, 95).

2.2.6. Immunfluoreszenz-Mikroskopie

Zur Auswertung der Tumorzellen per Immunfluoreszenz-Mikroskopie wurde das multizelluläre 3D-Kulturmodell modifiziert (Abbildung 7). Diesmal wuchsen die Tumorzellen für 24 Stunden auf runden Glasplättchen, die anschließend auf kleine Stahlbrücken in 6-Wellplatten gelegt wurden. Am Boden der 6-Wellplatten befand sich ein Fibroblasten-Monolayer aus MRC-5-Zellen (50.000 Zellen pro Well) und das Zellkulturmedium war mit 10.000 T-Lymphozyten (Jurkat) pro Milliliter angereichert. Nun wurde die entsprechende Behandlungssubstanz für vier Stunden hinzugefügt. Nach Ablauf der Versuchszeit wurden die Glasplättchen entnommen und mit Methanol fixiert. Es folgte die Inkubation mit einem Primär-Antikörper über Nacht bei 4°C sowie die Inkubation in einem Sekundär-Antikörper und die Färbung mit DAPI am Folgetag. Ein Leica DM 2000 Mikroskop (Wetzlar, Deutschland) mit zugehöriger LAS V4.12 Software diente der Aufnahme sowie Evaluation der Immunfluoreszenz-Bilder (96).

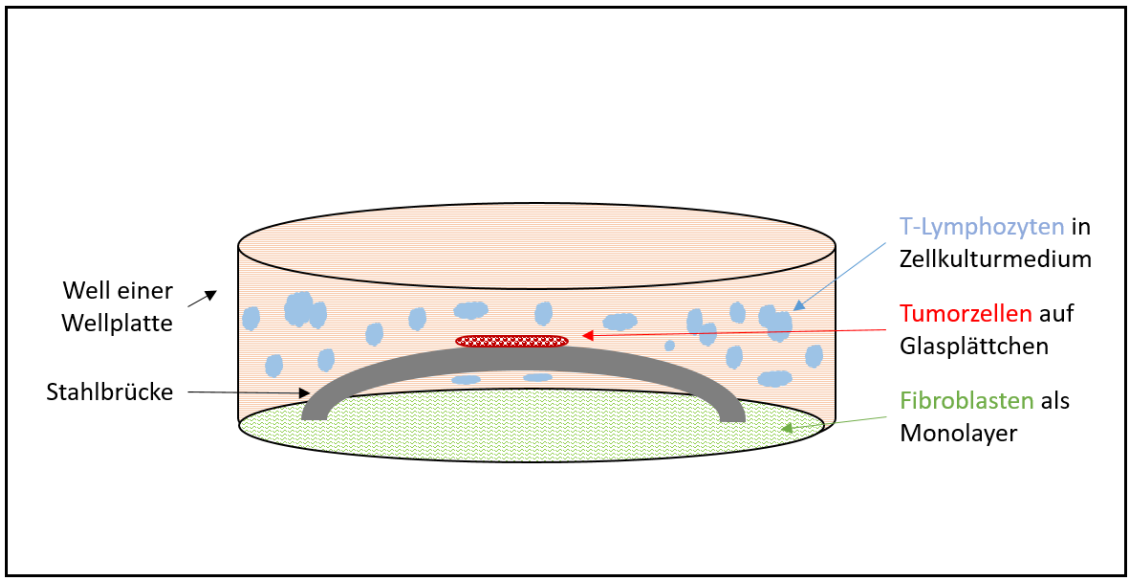

Abbildung 7: Modifiziertes multizelluläres TME. Tumorzellen wurden auf Glasplättchen gezüchtet und auf kleine Stahlbrücken in die Tumorumgebung (T-Lymphozyten, Fibroblasten) gelegt, dort behandelt und anschließend markierungsbreit fixiert.

2.2.7. Anmerkung

Alle eingesetzten Methoden sowie die Herkunft aller verwendeten Zellsorten, Antikörper und Reagenzien sind in den jeweiligen Publikationen detailliert ausgewiesen (95, 96).

3. Zusammenfassung

Um zu überprüfen, ob β1-Integrin-Rezeptoren auf der Oberfläche der Zellen des kolorektalen Karzinoms eine signifikante Rolle bei der proliferationshemmenden Wirkung von Resveratrol spielen, wurden im Rahmen der ersten Publikation "Evidence that β1-integrin is required for the anti-viability and anti-proliferative effect of resveratrol in CRC cells" (95) die β1-Integrin-assoziierten Signalwege in HCT116 und SW480 Zellen untersucht, indem diese mit Hemmstoffen wie a) dem Antikörper Anti-β1, b) dem selektiven β1-Integrin-Inhibitor Bacitracin, c) dem Integrin-bindenden RGD-Peptid oder d) den Kontrollsubstanzen Anti-β5, RGE-Peptid und/oder Resveratrol in multizellulären 3D-Tumorkulturen behandelt wurden.

Zunächst wurde die Erhöhung der Proliferationsrate sowie die verstärkte Expression von β1-Integrin-Rezeptoren auf der Oberfläche der HCT116 und SW480 Zellen durch das TME bestätigt. Dann zeigte sich überraschenderweise eine Veränderung des Expressionsmusters der β1-Integrin-Rezeptoren durch eine Behandlung mit Resveratrol von homogen in ungleichmäßig und punktförmig akkumuliert. Außerdem reduzierte das Phytopharmakon die Vitalität, Proliferation sowie Koloniebildung der Tumorzellen, wohingegen es die Anzahl der Apoptosen signifikant steigerte. Interessanterweise wurden diese Wirkungen des natürlichen Polyphenols durch die spezifischen β1-Integrin-Hemmstoffe größtenteils aufgehoben. Auf Ebene der Proteinexpression hemmte Resveratrol die Aktivierung des Entzündungsmarkers NF-κB, des Proliferationsmarkers Cyclin D1 sowie des Invasionsparameters FAK, wohingegen der Apoptoseparameter Caspase-3 gleichzeitig anstieg. Auch diese Wirkungen wurden durch die Hemmstoffe Anti-β1, Bacitracin oder RGD-Peptid, im Gegensatz zu den Kontroll-Antikörpern oder dem RGE-Peptid, überwiegend aufgehoben (95), sodass β1-Integrin-Rezeptoren eines der potenziellen Schlüsselmoleküle bei der proliferationshemmenden Resveratrol-Wirkung zu sein scheint.

Aufgrund der während der beschriebenen Untersuchungen bemerkten TME-induzierten, erhöhten Metastasierungsbereitschaft der Tumorzellen erfolgte eine Erweiterung der Fragestellung, sodass sich die Experimente im Rahmen der zweiten Publikation "β1-integrin plays a major role in resveratrol-mediated anti-invasion effects in the CRC microenvironment" (96) mit der Rolle der β1-Integrin-Rezeptoren bei der invasionshemmenden Wirkung von Resveratrol befassten. In einem Vergleich zweier Zelllinien des kolorektalen Karzinoms (HCT116 und RKO) wurden hierzu die Auswirkungen eines β1-Integrin Knockdowns mittels transienter Transfektion per Antisense-Oligonukleotide (β1-ASO) und den

entsprechenden Sense-Oligonukleotiden (β1-SO) als Kontrollsubstanz sowie zusätzlich die Effekte eines NF-κB Knockdowns durch Antisense-Oligonukleotide (NF-κB-ASO) und Sense-Oligonukleotiden (NF-κB-SO) als Kontrolle untersucht.

Hierbei ergab sich die Beobachtung der Bildung von lokalen Adhäsionsstellen an den zytoplasmatischen Membranen der Tumorzellen in Form von kleinen plaque-ähnlichen Anlagerungen, die konzentrationsabhängig von Resveratrol induziert wurden. Dies stand im Einklang mit einer ebenfalls von der Resveratrol-Konzentration abhängig festgestellten Erhöhung des Tumorsuppressorgens E-Cadherin, welches den epithelialen Phänotyp der Tumorzellen wahrte und damit einen invasionsfördernden Umschlag in den mesenchymalen Phänotyp im Rahmen der EMT verhinderte. Außerdem blockierte eine Resveratrol-Behandlung größtenteils die Expression des Adhäsionsadapterproteins Paxillin, das gleichzeitig als Tumormarker gilt. Ergänzend hierzu hemmte das Phytopharmakon in beiden Zellsorten die TME-geförderte Phosphorylierung sowie die nukleare Translokation von NF-κB und dadurch auch die Expression verschiedener Proteine, die mit dem NF-κB-Signalweg assoziiert sind. Während E-Cadherin sowie Caspase-3 durch eine Resveratrol-Behandlung anstiegen, sanken sowohl die mesenchymalen Parameter Vimentin und Slug als auch die Invasionsmarker FAK, MMP-9 sowie CXCR4. Interessanterweise hielt eine Knockdown-Behandlung mit β1-ASO, im Gegensatz zu β1-SO, alle genannten tumoreindämmenden Eigenschaften des natürlichen Polyphenols weitgehend auf (96), sodass die Untersuchungen einen deutlichen Hinweis auf eine Schlüsselfunktion von β1-Integrin-Rezeptoren bei der invasionshemmenden Wirkung von Resveratrol ergaben. Zusätzlich hatte ein spezifischer Knockdown von NF-κB mittels transienter Transfektion per NF-κB-ASO, wie auch NF-κB-SO, keinen hemmenden Einfluss auf die anti-tumoröse Wirkung von Resveratrol. Gegenteilig kam es in diesem Fall sogar zu einem synergistischen Effekt beider Substanzen (96), was die Bedeutung der entzündungs- und tumorhemmenden Resveratrol-Wirkung über die NF-κB Signalkaskade unterstreicht.

Insgesamt deuteten diese Daten darauf hin, dass das Phytopharmakon Resveratrol seine anti-proliferativen, anti-invasiven, pro-apoptotischen und damit tumorhemmenden Wirkungen in kolorektalen Karzinomzellen in relevantem Maß auf eine β1-Integrin-abhängige Weise entfaltet (Abbildung 8). Dies betont das große Potenzial der Möglichkeit, das Multi-Target-Molekül Resveratrol sowohl in die Prävention als auch Therapie von kolorektalen Karzinomen miteinzubeziehen.

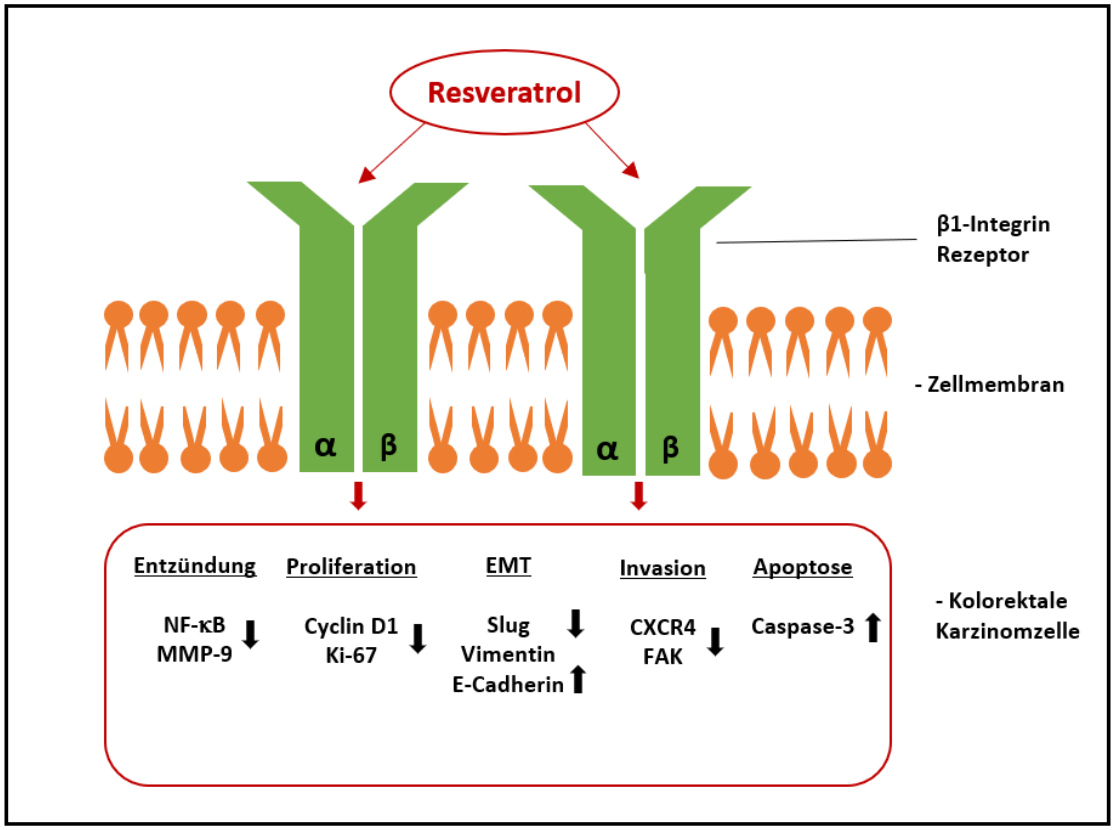

Abbildung 8: Modulierende Auswirkungen von Resveratrol über β1-Integrin Rezeptoren auf Entzündungs-, Proliferations-, EMT- und Apoptoseparameter in Zellen des kolorektalen Karzinoms.

4. Abstract/Summary

To verify whether β1-integrin receptors on the surface of colorectal cancer cells play a significant role in the proliferation-inhibitory effect of resveratrol, the first publication "Evidence that β1-integrin is required for the anti-viability and anti-proliferative effect of resveratrol in CRC cells" (95) investigated the β1-integrin-associated signaling pathways in HCT116 and SW480 cells by treating them with inhibitors such as a) the antibody anti-β1, b) the selective β1-integrin inhibitor bacitracin, c) the integrin-binding RGD peptide or d) the control substances anti-β5, RGE peptide and/or resveratrol in multicellular 3D-tumor-cultures.

First, the increased proliferation rate and the enhanced expression of β1-integrin receptors on the surface of HCT116 and SW480 cells by TME was confirmed. Then, surprisingly, a change in the expression pattern of β1-integrin receptors from homogeneous to non-uniform and punctate accumulation by resveratrol treatment was showed. In addition, the phytopharmaceutical reduced tumor cell viability, proliferation and colony formation, whereas it significantly boosted the number of apoptosis. Interestingly, these effects of the natural polyphenol were largely reversed by the specific β1-integrin inhibitors. At the protein expression level, resveratrol inhibited the activation of the inflammatory marker NF-κB, the proliferation marker cyclin D1 and the invasion parameter FAK, whereas the apoptosis parameter caspase-3 increased simultaneously. These effects were also predominantly reversed by the inhibitors anti-β1, bacitracin or RGD peptide, in contrast to the control antibodies or RGE peptide (95), so that β1-integrin receptors seem to be one of the potential key molecules in resveratrol‘s proliferation-inhibiting effect.

Due to the TME-induced elevated metastasis propensity of the tumor cells during the described investigations, the research hypothesis was expanded, so that the experiments in the framework of the second publication "β1-integrin plays a major role in resveratrol-mediated anti-invasion effects in the CRC microenvironment" (96) dealt with the role of the β1-integrin receptors in the invasion-inhibiting effect of resveratrol. In a comparison of two colorectal carcinoma cell lines (HCT116 and RKO), the effects of β1-integrin knockdown by transient transfection with antisense oligonucleotides (β1-ASO) and the corresponding sense oligonucleotides (β1-SO) as control as well as the effects of NF-κB knockdown with antisense oligonucleotides (NF-κB-ASO) and sense oligonucleotides (NF-κB-SO) as control were investigated.

In this relation, the formation of local adhesion sites on the cytoplasmic membranes of the tumor cells in the form of small plaque-like deposits was observed, which were induced by resveratrol in a concentration-dependent manner. This was consistent with a resveratrol-concentration-dependent increase in the tumor suppressor gene E-cadherin, which preserved the epithelial phenotype of the tumor cells and thus prevented an invasion-promoting switch to the mesenchymal phenotype during EMT. Moreover, a resveratrol treatment largely blocked the expression of the adhesion adaptor protein paxillin, which represents also a tumor marker. In addition, the phytopharmaceutical inhibited TME-promoted phosphorylation and nuclear translocation of NF-κB in both cell types, thereby reducing the expression of different proteins associated with the NF-κB signaling pathway. While E-cadherin and caspase-3 increased with resveratrol treatment, the mesenchymal parameters vimentin and slug as well as the invasion markers FAK, MMP-9 and CXCR4 decreased. Interestingly, knockdown treatment with β1-ASO, in contrast to β1-SO, largely halted all of the aforementioned tumor-repressing properties of the natural polyphenol (96), so that the studies provided clear evidence for a key function of β1-integrin receptors in the invasion-inhibiting effect of resveratrol. Aside from that, the specific knockdown of NF-κB by transient transfection via NF-κB-ASO, as well as NF-κB-SO, had no inhibitory effect on the anti-tumor effect of resveratrol. In contrast, there was even a synergistic effect of the two substances (96), which underlines the importance of the anti-inflammatory and anti-tumor effect of resveratrol via the NF-κB signaling cascade.

Overall, these data indicated that the phytopharmaceutical resveratrol exerts its anti-proliferative, anti-invasive, pro-apoptotic and thus tumor-inhibitory effects in colorectal cancer cells at least in part in a β1-integrin-dependent manner (Abbildung/Figure 8). This emphasizes the great potential of the possibility of including the multi-targeting molecule resveratrol in both the prevention and therapy of colorectal carcinomas.

5. Beitrag zu den Veröffentlichungen

5.1. Beitrag zu Paper I

Evidence that β1-integrin is required for the anti-viability and anti-proliferative effect of resveratrol in CRC cells

Der Artikel „Evidence that β1-integrin is required for the anti-viability and anti-proliferative effect of resveratrol in CRC cells“ wurde am 25.04.2022 im International Journal of Molecular Sciences publiziert (95). Diese Arbeit ist unter der Leitung meines Hauptbetreuers Herrn Prof. Dr. Mehdi Shakibaei (Universität München) und unter Mitwirkung von Herrn Prof. Dr. Parviz Shayan (Universität Teheran) entstanden.

An diesem Projekt war ich von Beginn an und durchgehend in allen Bereichen beteiligt. Aufbauend auf vorangehende Arbeiten der Forschungsgruppe von Herrn Prof. Dr. Mehdi Shakibaei (AG Shakibaei) entwarfen wir ein Projektkonzept, welches die Themen kolorektales Karzinom und Resveratrol enthalten sollte. Nach gründlicher Recherche konnte ich mich hierbei vor allem bei der Auswahl des Schwerpunktes β1-Integrin, den geeigneten Hemmstoffen (Anti-β1, RGD, Bacitracin) sowie der Gestaltung des Zeitkonzeptes einbringen. Die beschriebenen Untersuchungen habe ich selbständig durchgeführt, da ich durch meine Tätigkeit als Technische Assistentin der AG Shakibaei bereits in die notwendigen Labormethoden eingearbeitet war. So führte ich alle vorbereitenden Labormaßnahmen, Zellkulturarbeiten, Versuchsabläufe, Probenentnahmen sowie Versuchsauswertungen durch.

Die in diesem Paper präsentierten Labormethoden beinhalten die Züchtung von kolorektalen Krebszellen als Monolayer und im 3D-Alginat-Kulturmodell, lichtmikroskopische Proliferationsdokumentation, MTT-Assay, Western Blot und Immunfluoreszenz-Mikroskopie. Zur Sicherstellung der Ergebnis-Reproduzierbarkeit wurden die Experimente in unserem Labor (Universität München) dreimal durchgeführt und zusätzlich von Herrn Prof. Dr. Parviz Shayan (Universität Teheran) wiederholt. Die Interpretation und Zusammenfassung der Ergebnisse wurden von Herrn Prof. Dr. Mehdi Shakibaei koordiniert. Zum Schreibprozess konnte ich in allen Bereichen beitragen. Die Ergebnisinterpretation, Zusammenstellung der Bilder, finale Überarbeitung sowie das Reviewing und Proofreading fand gemeinsam mit Herrn Prof. Dr. Mehdi Shakibaei und Herrn Prof. Dr. Parviz Shayan statt.

5.2. Beitrag zu Paper II

β1-integrin plays a major role in resveratrol-mediated anti-invasion effects in the CRC microenvironment

Der Artikel „β1-integrin plays a major role in resveratrol-mediated anti-invasion effects in the CRC microenvironment“ wurde am 02.09.2022 im Journal Frontiers of Pharmacology publiziert (96). Diese Arbeit ist unter der Leitung meines Hauptbetreuers Herrn Prof. Dr. Mehdi Shakibaei (Universität München) und unter Mitwirkung von Herrn Prof. Dr. Parviz Shayan (Universität Teheran) als Fortsetzung zur erstgenannten Publikation entstanden.

Auch an diesem Projekt war ich von Beginn an und ohne Unterbrechung beteiligt. Unter der Leitung meines Hauptbetreuers Herrn Prof. Dr. Mehdi Shakibaei konnte ich bei der Recherchearbeit und Paper-Konzeption mitwirken. Sämtliche Versuchsvorbereitungen, Versuchsdurchführungen und Versuchsauswertungen habe ich selbständig und in dreifachem Ansatz im Labor der AG Shakibaei (Universität München) durchgeführt. Die Hauptversuche wurden zum Zwecke der Ergebnis-Reproduktion zusätzlich durch Herrn Prof. Dr. Parviz Shayan (Universität Teheran) wiederholt.

Die hier eingesetzten Labormethoden waren Zellzüchtung, Versuchsaufbau als Monolayer und 3D-Alginatkultur, Transfektion, Invasionsdokumentation, Western Blot, Immunfluoreszenz-Mikroskopie, Toluidinblau-Färbung von Invasionsplatten sowie die DAPI-Färbung ausgewanderter Tumorzellkolonien. An der Zusammentragung und Interpretation der Ergebnisse war ich zu jeder Zeit beteiligt. Alle Abschnitte des Manuskriptes wurden von mir geschrieben, durch Herrn Prof. Dr. Mehdi Shakibaei wissenschaftlich ergänzt und durch Frau M.Sc. Anna-Lena Müller kritisch und sprachlich überprüft. Die endgültige Überarbeitung, das Reviewing und Proofreading führte ich zusammen mit Herrn Prof. Dr. Mehdi Shakibaei und Herrn Prof. Dr. Parviz Shayan durch.

6. Bestätigung der Co-Autoren

6.1. Paper I

Aus Datenschutzgründen entfernt.

6.2. Paper II

Aus Datenschutzgründen entfernt.

7. Paper I

International Journal of *Molecular Sciences*

MDPI

Article

Evidence That β1-Integrin Is Required for the Anti-Viability and Anti-Proliferative Effect of Resveratrol in CRC Cells

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Abstract: The β1-integrin receptor is broadly expressed on tumor and other cells in the tumor microenvironment (TME), and is an unfavorable prognostic factor for cancers. Nature-derived resveratrol has preventive and apoptotic effects on tumors, but whether resveratrol can exert its suppressive actions on TME-induced tumorigenesis through β1-integrin on the surface of CRC cells is still unknown. HCT116 or SW480 cells were exposed to inhibitory antibodies against β1-integrin, bacitracin (selective β1-integrin inhibitor), integrin-binding RGD (Arg-Gly-Asp) peptide, and/or resveratrol. We evaluated the anti-tumor actions and signaling impacts of resveratrol in colorectal cancer (CRC)-TME. We found that resveratrol completely altered the β1-integrin distribution pattern and expression on the surface of CRC cells in TME. Moreover, resveratrol down-regulated CRC cell proliferation, colony formation, viability, and up-regulated apoptosis in a concentration-dependent way. These actions of resveratrol were antagonized mainly by inhibitory antibodies against β1-integrin but not β5-integrin, and by an integrin-binding RGD peptide but not by RGE peptide, and by bacitracin in TME. Similarly, resveratrol-blocked TME-induced p65-NF-kB and its promoted gene markers linked to proliferation (cyclin D1), invasion (focal adhesion kinase, FAK), or apoptosis (caspase-3), were largely abrogated by anti-β1-integrin or RGD peptide, suggesting that β1-integrin is a potential transmission pathway for resveratrol/integrin down-stream signaling in CRC cells. The current results highlight, for the first time, the important gateway role of β1-integrins as signal carriers for resveratrol on the surfaces of HCT116 and SW480 cells, and their functional cooperation for the modulatory effects of resveratrol on TME-promoted tumorigenesis.

Keywords: tumor microenvironment; β1-integrin; resveratrol; RGD peptides; proliferation; NF-kB; bacitracin

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1. Introduction

Colorectal cancer (CRC), mainly due to epigenetic alterations, has become widely recognized as being among the most common aggressive adenocarcinomas causing cancer deaths worldwide, and unfortunately, the incidence of CRC is increasing [1–3]. In current statistics, CRC ranks third in the number of new cancer incidents as well as in the total of patients who have died from cancer [4]. This high value shows the importance of developing complementary and adequate treatment strategies. The different stages of cancer development have been known for years and are of great relevance because each stage offers the opportunity to intervene in the tumor process. Tumors also have the ability to recruit seemingly normal cells to help them establish a specific tumor microenvironment (TME) [5], underscoring cancer as a complex, multi-stage disease that requires multi-targeting therapy.

In recent years, several papers have highlighted the relevance of the TME in CRC progression, proliferation, and metastasis. However, the extracellular matrix (ECM) com-

pounds take a central role in this context. Among the components of the ECM responsible for adhesion, collagens, and proteoglycans are some of the most important matters controlling cancer-related processes at each stage of tumorigenesis [6–8]. Therefore, the TME plays a central role in tumor research and also in the generation of novel drugs for the therapeutic intervention of tumors. A multicellular TME consists of a variety of active cells (immune cells, tumor-associated fibroblasts, endothelial cells, pericytes, adipocytes, and other stromal cells) as well as many ECM proteins and enzymes produced by tumor cells and stromal cells [5,9,10]. This complex network of ECM in the TME plays a fundamental role in tumor progression and proliferation, and is partially discussed as a specific biomarker for tumor malignancy [11–13].

An intense interaction exists between cells and ECM in TME that is critical for tumor cell progression. One of the most important cell surface receptor families that plays a major role in TME in cell-matrix interaction and simultaneously functions as signaling molecules is the integrin family [8,9], thus representing a very important and attractive therapeutic target in TME. It is known that integrins are transmembrane glycoproteins, composed of non-covalent, heterodimeric complexes of an α- and a β-chain, that can signal bidirectionally upon binding to their ECM compounds, and thus regulate a number of important biological activities [14–17]. Association of integrins with the ECM component causes an active interaction between integrins and cytoskeleton, and signaling complexes that induce the assembly of cytoskeletal filaments. Modulation of cytoskeletal filaments further activates integrins to bind more matrix, resulting in an enhanced feedback system. Furthermore, this interaction induces specific adaptor molecules to form focal adhesion aggregates. Thus, integrins as functional integrators, enable binding and also signal transduction between ECM and cytoskeletal proteins, and these very highly orchestrated complexes are designated as focal adhesion sites [18], highlighting their role in angiogenesis, growth, migration, and invasion of tumors. Consequently, preclinical and clinical works displayed interventions in tumor progression by integrin antagonists [19,20]. Notably, distinct integrins on tumor cells have been found to play essential functional roles in the progression of diverse tumors, such as αvβ3, αvβ5, α5β1, α6β4, and α4β1, which serve as a prognostic marker for early-stage overall survival in TME, suggesting that integrin receptors act as regulators of tumor survival and progression [21–26].

Natural plant compounds such as resveratrol can promote health, and support both disease prophylaxis and co-treatment of many diseases. This is possible because they are multi-targeting agents having the ability to modulate various signaling pathways. Resveratrol (3,5,4′trihydroxy-trans-stilbene) has been identified as a plant polyphenol in berries, grapes, red wine, soy, and peanuts [27]. However, with respect to cancer cells, resveratrol showed anti-tumor, anti-mutagenic, chemopreventive, anti-oxidative, and anti-inflammatory activities. In fact, resveratrol is known to exert these properties in CRC through modulation of various signaling pathways such as CamKKB/AMPK, talin-FAK, Sirt1, NF-kB, and also glycolysis, and pentose phosphate pathway [28]. Moreover, the cysteine-rich domain of integrin is assumed to hold the binding site for resveratrol. Indeed, resveratrol has previously been reported to bind to the integrin αvβ3 receptor in breast cancer cells, thereby activating ERK1/2 and AMPK to induce COX-2 accumulation, and subsequently, p53-dependent apoptosis [29]. In SW480 CRC cells, the αvβ3-integrin receptors have been shown to be even involved in both resveratrol-uptake by the CRC cells as well as resveratrol-induced apoptosis [26].

Although the anti-tumor activity of some active components via αvβ3-, αvβ5-, and β1-integrins in many different tumors has been reported more frequently in the past, the anti-tumor activity of resveratrol in association with β1-integrin receptors in CRC cells is still unknown. In our previous study, we found that resveratrol attenuated TME-induced CRC cell growth and migration [30]. Now, we addressed whether resveratrol can interact with β1-integrin on the surfaces of HCT116 or SW480 cells and exert its anti-proliferative, anti-viable, and pro-apoptotic effects in an in vitro TME model.

2. Results

In our previous research, we have demonstrated that resveratrol attenuates pro-inflammatory TME-induced CRC cell growth and migration [30], and what we want to further evaluate is regarding the specificity and details of relevant pathways of these interactions. In this work, we assessed the potential of resveratrol to act on β1-integrin receptors at the surface of CRC cells to drive its anti-tumor-related cellular actions in an in vitro 3D-TME model.

2.1. Resveratrol Alters the Expression and Distribution Pattern of β1-Integrin Receptors on the Surface of HCT116 and SW480 Cells in the TME

To analyze potential functional targets of the anti-tumorigenesis effect of resveratrol in the TME microarchitecture in CRC cells, we initially left the HCT116 and SW480 cells in the TME untreated or treated with resveratrol (5 μM), and with or without anti-β1-integrin antibody (2 μg/mL). Cells were immunolabeled with β1-integrin antibody, and DAPI counterstaining was carried out to reveal the cell nuclei.

Immunolabeling study by immunofluorescence microscopy in TME control cultures showed that HCT116 and SW480 cells had strong uniform and homogeneous cell membrane labeling for β1-integrin (Figure 1B,F), compared with HCT116 and SW480 cells in the basal control (Figure 1A,E) without TME. Interestingly and surprisingly, we found that treatment of TME cultures with resveratrol caused a remarkable change in the expression and distribution pattern of β1-integrin receptors in HCT116 and SW480 cells (Figure 1C,G, yellow arrows). Indeed, the expression of integrin receptors became punctate rather than homogeneous on the surface of the cells. In contrast, simultaneous treatment of HCT116 and SW480 cells in TME with resveratrol and a specific anti-β1-integrin blocked the resveratrol-induced punctate distribution pattern of β1-integrin receptors again, i.e., the expression and distribution pattern of β1-integrin receptors on the surface of tumor cells remained homogeneous (Figure 1D,H), as in the TME control (Figure 1B,F). These findings are in accordance with other reports demonstrating that integrins are overexpressed in the cell membranes of mitotic-active and various tumor cells [31]. Taken together, these results underline the critical role of β1-integrin receptors as one of the first transmission molecules of resveratrol signaling pathway in the TME, and this modulatory effect of resveratrol on the distribution of β1-integrin receptors on the CRC cell membrane was not cell line-specific.

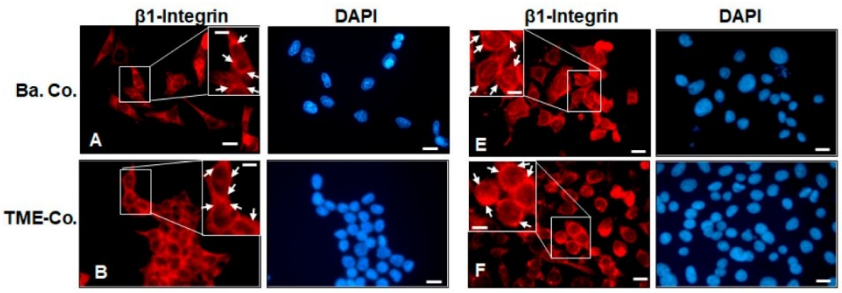


Figure 1. *Cont.*

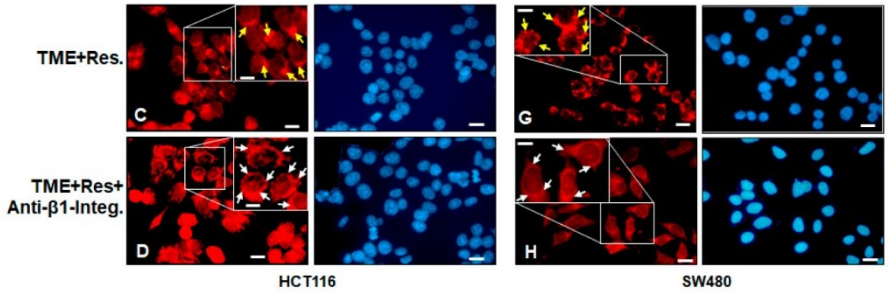


Figure 1. Immunofluorescence microscopy visualization of β1-integrin receptors under resveratrol challenge on the surface of HCT116 and SW480 cells in the TME. β1-integrin immunolabeled (white arrows) and DAPI-stained nuclei from untreated basal control (**A**,**E**); TME-grown (**B**,**F**); resveratrol-treated (5 μM) (**C**,**G**), and further addition of anti-β1-integrin (2 μg/mL) (**D**,**H**) HCT116 and SW480 cells. Yellow arrows = change in distribution pattern of β1-integrin receptors. Microscope: Leica DM 2000. Magnification ×600; scale bar = 30 μm. Insets: magnification: ×1200; scale bar = 15 μm.

2.2. Resveratrol Blocks TME-Stimulated Expression of β1-Integrin, but Not β5-Integrin in CRC Cells

It has been previously reported that integrin receptors in the TME are overexpressed and play an essential role in tumor cell progression, survival, and as markers for tumor prognosis [23]. We wanted to investigate the action of resveratrol on up-regulated and activated integrin expression in the TME. For this purpose, HCT116 cells were cultured in 3D-alginate by themselves (basal control) or co-cultured as 3D-alginate culture in the TME treated with various doses of resveratrol (0, 1, 5, 10, and 20 μM) for 10–14 days, as outlined in the Materials and Methods. Western blotting results showed that the expression of β1-integrin was markedly elevated in TME-control cells compared to basal control. However, treatment of HCT116 cells in the TME with resveratrol clearly showed a dose-dependent down-regulation of β1-integrin but not β5-integrin compared with TME-control (Figure 2). Resveratrol exerts a profound dose-dependent action on TME-activated β1-integrin expression in HCT116 cells. To note, at a concentration of 10 μM resveratrol, β1-integrin expression was markedly reduced, and at a concentration of 20 μM of resveratrol, β1-integrin was largely blocked compared with TME-control (Figure 2). Importantly, the concentration of resveratrol (5 μM) used as a working dose and the timing of treatment had marginal effects on β1-integrin expression. This was confirmed by quantitative densitometry. Indeed, there was no apparent effect of added resveratrol on the expression of β5-integrin, underscoring the role of β1-integrin in resveratrol's specific signal transmission. Taken together, these data indicate a critical role of β1-integrin for TME-inducing tumorigenic effects in CRC cells, and further highlight that resveratrol suppresses, at least in part, TME-stimulated progression of HCT116 cells primarily via gateway β1-integrin receptor and down-stream β1-integrin signaling pathway.

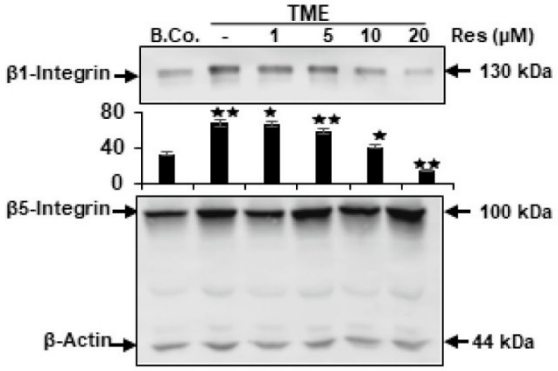


Figure 2. The impact of resveratrol on TME-triggered expression of β1- and β5-integrin. HCT116 cells in alginate matrix in basal control (B. Co.) or in TME were untreated or TME treated with diverse dosages of resveratrol (1, 5, 10, 20 μM) for 10–14 days as outlined in Materials and Methods. Immunolabeling of cell lysates was conducted by Western blotting with antibodies against β1- and β5-integrin. Data are originated from three separate assays, and β-actin was used as a reference. *Y*-axis: densitometric units confirming Western blot results. * $p < 0.05$, ** $p < 0.01$ compared with TME control.

2.3. β1-Integrin Serves as a Signal Transmission Receptor of Resveratrol in HCT116 and SW480 Cells

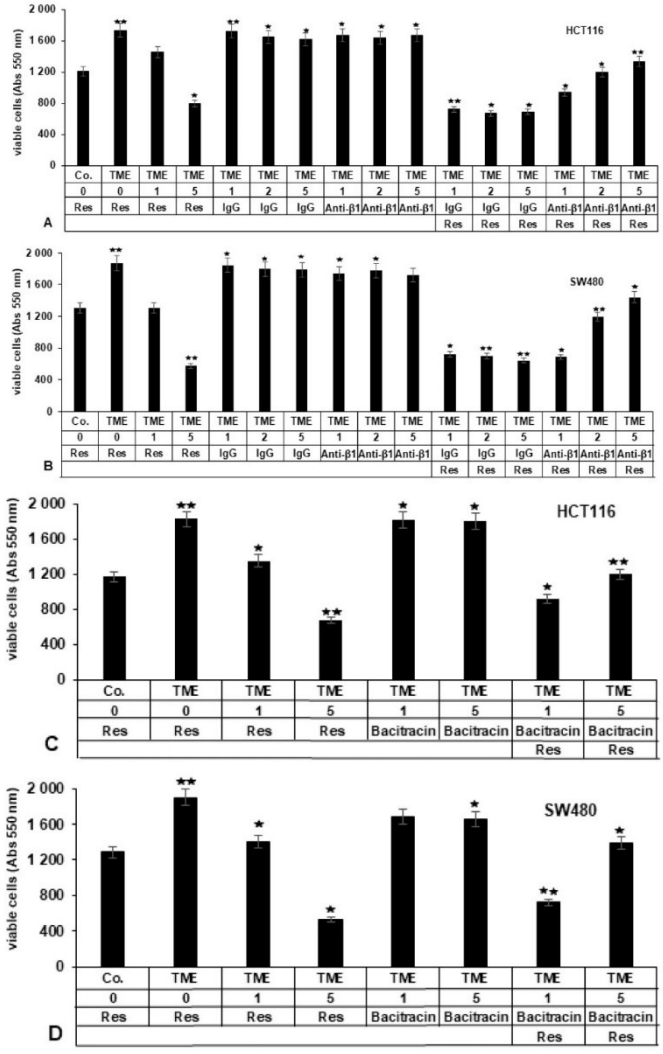
HCT116 or SW480 cultures were treated with resveratrol (0, 1, 5 μM) in the presence or absence of IgG (1, 2, 5 μg/mL), anti-β1-integrin (1, 2, 5 μg/mL), anti-β5-integrin (5 μg/mL), RGD peptide (1, 2, 5 μM), RGE peptide (5 μM), or bacitracin (1, 5 μM) for 10–14 days.

2.3.1. Repression of β1-Integrin by Antibody Inhibits the Blocking Effect of Resveratrol on TME-Promoted Viability of CRC Cells

To investigate whether β1-integrin signaling is involved in resveratrol-induced anti-proliferative activity in HCT116 or SW480 cells in TME, the CRC cells were grown in 3D-alginate as basal control (Co.) or in TME control, or TME was treated with resveratrol (1, 5 μM), or anti-mouse IgG (served as the negative control) (1, 2, 5 μg/mL), or inhibitory anti-β1-integrin antibody (1, 2, 5 μg/mL) by itself, or were co-treated with 5 μM resveratrol and anti-mouse IgG (1, 2, 5 μg/mL), or anti-β1-integrin (1, 2, 5 μg/mL). Cell proliferation was examined by MTT assay as detailed in Materials and Methods.

TME significantly increased the viability and thus indirectly, the proliferation of HCT116 and SW480 cells, as evidenced by the fact that the measurement rate of viable cells for both cell lines was more than one-third higher in TME than in the basal control (Figure 3A,B). However, resveratrol significantly and concentration-dependently suppressed TME-enhanced viability in both CRC cell lines by around 19% and 55% in HCT116 as well as 32% and 69% in SW480 cells at 1 or 5 μM resveratrol compared with TME control. Treatment with IgG or anti-β1-integrin (Anti-β1) by itself did not significantly suppress cell proliferation in HCT116 or SW480 cells compared to untreated TME control cultures (Figure 3A,B). Of note, in opposite to control co-treatment with IgG and resveratrol, which reduced CRC cell viability by around 58% in HCT116 and 64% in SW480, co-treatment of CRC cells with inhibitory anti-β1-integrin antibody and resveratrol significantly abrogated resveratrol-induced inhibition of proliferation in tumor cells in a concentration-dependent way, so that at a concentration of 5 μg/mL anti-β1 and 5 μM resveratrol, only around 20% (HCT116) and 25% (SW480) respectively, less viable cells were measured than in the TME control (Figure 3A,B). These results are consistent with other findings stating that other integrins such as integrin αvβ3 are able to bind resveratrol on the surface of breast tumor

cells [32]. Collectively, these data underline that the β1-integrin receptor is actively involved in the anti-viability and anti-proliferative effect of resveratrol on the surface of CRC cells (HCT116 and SW480). Furthermore, the involvement of β1-integrin in the suppressive effect of resveratrol on CRC cell viability and proliferation was not cell line-specific.



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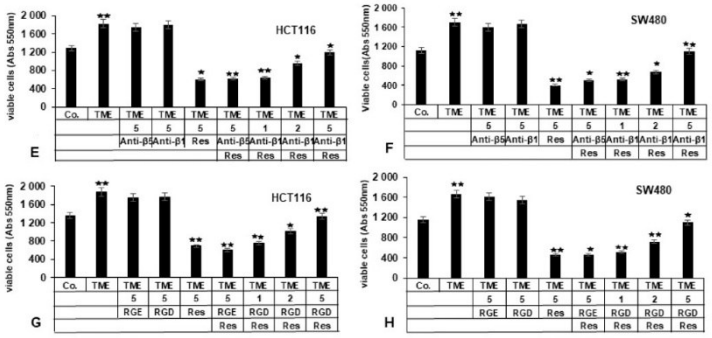

Figure 3. The importance of β1-integrin for resveratrol-mediated down-modulation of tumor cell viability: (**A**,**B**) CRC cells (HCT116, SW480) from basal control (Co.) or TME control were not treated or treated with resveratrol (1, 5 μM) in the presence or absence of IgG (1, 2, 5 μg/mL), the blocking anti-β1-integrin antibody (1, 2, 5 μg/mL); (**C**,**D**) bacitracin (1, 5 μM); (**E**,**F**) the blocking anti-β1-integrin antibody (1, 2, 5 μg/mL), anti-β5-integrin (5 μg/mL); (**G**,**H**) RGD peptide (1, 2, 5 μM), RGE peptide (5 μM) for 10–14 days. Tumor cell survival and, indirectly, proliferation were assessed by MTT assay. Relative to TME control, * $p < 0.05$ and ** $p < 0.01$.

2.3.2. Bacitracin (β1-Integrin Inhibitor) Suppresses the Inhibitory Impact of Resveratrol on TME-Induced Viability in CRC Cells

To confirm that β1-integrins are actively involved as surface receptors for the resveratrol signaling pathway on TME-promoted viability and thus indirect proliferation of CRC cells, HCT116 or SW480 cells were treated with resveratrol (0, 1, 5 μM) in the presence or absence of bacitracin (1, 5 μM), a selective pharmacological inhibitor of β1-integrin and β7-integrin-mediated cell adhesion [33], and evaluated by MTT assay. As demonstrated in Figure 3C,D, TME indirectly stimulated the proliferation of CRC cells in alginate beads, with over a third more viable CRC cells (HCT116, SW480) in the TME control than in the basal control. However, resveratrol inhibited TME-induced viability of cells in a concentration-dependent fashion by decreasing cell viability around 23% and 67% in HCT116 as well as 27% and 59% in SW480 at 1 or 5 μM resveratrol, compared to TME. However, there was no significant difference between the bacitracin treatment by itself and TME control group. Notably, bacitracin suppressed resveratrol-induced inhibition of HCT116or SW480 cell viability in a concentration-dependent way (Figure 3C,D), visible by a reduction of viable cells by 32% (HCT116) and 27% (SW480) respectively, compared to TME, at a co-treatment with 5 μM bacitracin and 5 μM resveratrol. These data confirm the results from Figure 3A,B and underline that resveratrol-promoted inhibition of CRC cell viability is, at least in part, dependent on β1-integrin signaling pathway, and this was not cell line specific.

2.3.3. RGD-Peptide, Similar to Anti-β1-Integrin, in Opposite to RGE Peptide or Anti-β5-Integrin, Inhibits Resveratrol-Suppressed TME-Induced Viability in CRC Cells

To further support the specific role of β1-integrin in signal transmission for resveratrol in HCT116 or SW480 cells, they were alternatively treated with resveratrol (5 μM) by itself or co-treated with anti-β5-integrin (5 μg/mL), or anti β1-integrin (1, 2, 5 μg/mL) (Figure 3E,F) or with an integrin inhibitor RGD peptide (1, 2, 5 μM), or control RGE-peptide (5 μM) (Figure 3G,H). Cell viability and thus indirectly, proliferation was investigated by MTT test as outlined in Materials and Methods. TME-induced viability of HCT116 as well as SW480 cells (over one third more viable CRC cells in TME control than in basal control) was clearly suppressed at a concentration of 5 μM resveratrol by around 67% (3E) and 64% (3G) respectively in HCT116, and 77% (3F) and 76% (3H) respectively in SW480, compared

to TME as demonstrated before. To note, the RGD peptide reduced cell viability by around 27% in HCT116 and 32% in SW480 when co-treated with 5 μM RGD and 5 μM resveratrol, similar to the blocking antibody to β1-integrin (by around 26% in HCT116 and 33% in SW480 when co-treated with 5 μg/mL anti-β1-integrin and 5 μM resveratrol), in opposite to RGE peptide or anti-β5-integrin, and thus significantly blocked resveratrol-inhibited viability of CRC cells in a concentration-dependent way (Figure 3E–H), compared to TME control. Taken together, these data clearly demonstrate that β1-integrin and the β1-integrin signaling pathway, at least in part, is a potential receptor transfer pathway for the anti-tumor effect of resveratrol on the tumor cell membrane, and this was not cell line-specific, due to reproducibility in different CRC cell lines (HCT116, SW480).

2.4. β1-Integrin Signaling Pathway Is Involved in Resveratrol-Modulated TME-Induced Colony Formation in HCT116 Cells

To evaluate further effects of β1-integrin receptor on resveratrol's anti-tumor mechanisms, colony formation [34] of HCT116 cells, an essential and prominent feature of tumor cells, was performed in 3D-alginate TME cultures with resveratrol in the absence or presence of IgG or anti-β1-integrin, as outlined in Materials and Methods. Resveratrol by itself suppressed the colonosphere development of HCT116 (Figure 4A,B) in a dose-dependent mode. To note, TME initiated the quantity of colonosphere formations in CRC cells compared to that in basal control cultures of HCT116 cells (Figure 4A,B), indicating the pro-tumor role of TME in initiating the aggressiveness of CRC cells. In contrast, it was seen that there was no effect of IgG and anti-β1-integrin alone on colonosphere formation in CRC cells. Interestingly, only anti-β1-integrin, but not IgG, abolished the anti-colonosphere formation effects of resveratrol, so that the number of colonospheres formed in the 3D-alginate TME culture was similar to that in the control TME (Figure 4A,B). Thus, resveratrol has promising anti-CRC efficacy by inhibiting cell colony formation via β1-integrin.

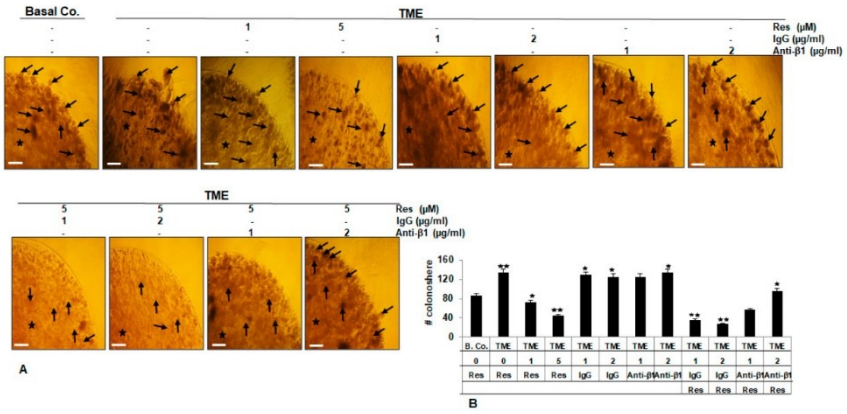

Figure 4. The implication of β1-integrin in resveratrol-mediated down-modulation of tumor cell colony assembly in TME: (**A**) HCT116 cells from basal control (Basal Co.) or untreated TME or TME were treated with different concentrations of resveratrol (1, 5 μM) in the presence or absence of IgG (1, 2 μg/mL) or blocking anti-β1-integrin antibody (1, 2 μg/mL) for 10–14 days as indicated in Materials and Methods; (**B**) Colonies (black arrows) were quantified by scoring 20 different microscopic patches. Microscope: Zeiss Axiovert 40 CFL. Magnification (A): ×24, bar = 0.2 mm. "star" = Alginate. * $p < 0.05$, ** $p < 0.01$ compared with TME control.

2.5. β1-Integrin Is Required for Anti-Tumor Effects of Resveratrol in CRC Cells

To more specify and confirm one of the primary functional proteins that recruits resveratrol to the surface membranes of HCT116 cells in TME, which has not been fully elucidated yet, the following studies were performed. HCT116 cells in 3D-alginate TME were provoked with or without an appropriate neutralizing antibody against β1-integrin (1, 2, 5 μg/mL), or antibody against β5-integrin (5 μg/mL) (Figure 5A), with or without an RGD peptide (1, 2, 5 μM), or RGE peptide (5 μM) (Figure 5B) for 10–14 days, as detailed in the Materials and Methods. Samples were tested by Western blotting with antibodies against FAK, p-FAK, (downstream of β1-integrin), pro-inflammatory transcription factor p65-NF-kB, cyclin D1 (proliferation), and activated caspase-3 (apoptosis). Immunoblotting of TME control cultures, similar to anti-β5- or β1-integrin, or to RGD-, or RGE-peptides by themselves, showed high expression of p65-NF-kB and p-FAK, cyclin D1 from HCT116 cells, and very low expression of activated caspase-3 (Figure 5A,B). As demonstrated in Figure 5A,B, treatment of HCT116 cells in TME with resveratrol resulted in down-regulation of p-FAK, p65-NF-kB, and cyclin D1 but a significant up-regulation of activated caspase-3 (Figure 5A,B). In contrast, we found that in combination with resveratrol and neutralizing antibodies against β1-integrin or resveratrol and RGD peptides, the effect of resveratrol on the above-stated proteins was revised in a concentration-dependent way (Figure 5A,B). However, the β5-integrin antibody or an inactive RGE peptide had no inhibitory effect on this process. Collectively, because of this, the down-regulatory actions of resveratrol on HCT116 cell proliferation are linked with the regulation of β1-integrin dependent FAK-, NF-kB-, and cyclin D1-signaling.

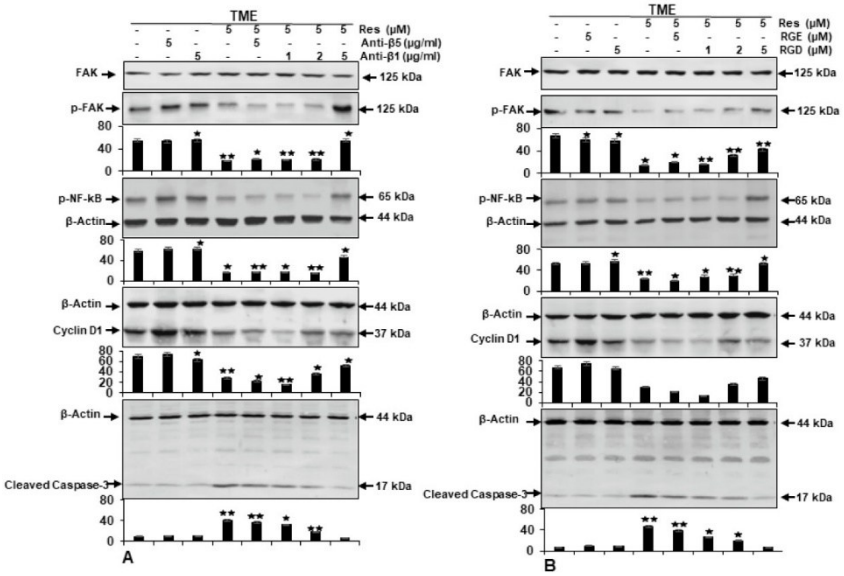

Figure 5. β1-integrin is required for anti-tumor effects of resveratrol. TME-HCT116 cells were treated with resveratrol (5 μM) in the presence or absence of anti-β1- (1, 2, 5 μg/mL), or anti-β5- integrin (5 μg/mL) (**A**) or of RGD (1, 2, 5 μM), or RGE (5 μM) peptide (**B**) as mentioned in Materials and Methods. Western blot samples were probed with antibodies against FAK, p-FAK, p-NF-kB, cyclin D1, and activated caspase-3. In addition, anti-β-actin was used as a loading control. *Y*-axis: densitometric units confirming Western blot results. * $p < 0.05$, ** $p < 0.01$ compared with TME control.

3. Discussion

Recently, accumulated evidence showed that tumor proliferation, malignancy, and growth may be motivated by ECM components and a variety of active paracrine interactions between stromal cells and tumor cells within the pro-inflammatory TME [30,35]. Consequently, a combination of anti-tumor and anti-stromal therapies has become an important research focus to develop new therapies for tumor treatment [35]. Therefore, in this research, we tested the possible contribution of the specific ECM adhesion receptor β1-integrin on the surface of HCT116 and SW480 cells as an initial transmitter for resveratrol-mediated anti-tumorigenesis signaling in TME.

It is widely accepted and, like others we have previously shown, that resveratrol, as a naturally occurring multi-targeting agent with biological activities, is extensively investigated with beneficial properties (anti-oxidative, anti-inflammatory, anti-microbial) in several biomolecular systems. Moreover, it is associated with a range of anti-cancer activities by directing multiple signaling pathways linked with cancer initiation, promotion, angiogenesis, and metastasis, as well as induction of apoptosis in various tumors [28,30,36–39]. However, the initial cellular-level interactions of resveratrol in CRC cells are incompletely understood, and a cellular receptor site of resveratrol signaling initiation has not been fully described yet.

Integrins are heterodimeric transmembrane proteins that have the ability to functionally connect cells to their specific microenvironment. Furthermore, they have been described as a family of cell membrane receptors involved in almost all functions and properties of many different tumors [14,18]. β1-integrin is capable of forming most of the junctions of multiple receptor complexes and thereby functionally associates with other subcellular signaling pathways, stimulating a number of genes and kinases such as FAK, which then leads to stimulation of cell adhesion, proliferation, and invasion [19,40]. Therefore, attempts have been made to develop targeting drugs directed against integrins. However, the results of treatment with these drugs in cancer therapy are very limited so far, as the specific integrin inhibitors are not approved for clinical use [19,41] yet.

We wondered whether our pro-inflammatory CRC-TME model induces β1-integrin expression in the HCT116 or SW480 cell line and, if so, whether this phenomenon is specifically modulated by resveratrol, which would be important for a therapeutic molecular experiment of tumor fate. At first, our immunofluorescence microscopy results showed that the expression of β1-integrin was significantly up-regulated in the HCT116- or SW480-TME compared to HCT116 or SW480 cells from the basal control environment. More interestingly, treatment of CRC cells with resveratrol changed the distribution pattern of β1-integrin receptors on the cell surface from homogeneous and uniform to a punctate. Indeed, it must be emphasized here that the alteration of the β1-integrin expression pattern by resveratrol observed is not an activation but rather a modulation of the β1-integrin receptors, which may facilitate the uptake or transduction capacity of resveratrol's signals by the β1-integrin receptors. Moreover, it should be noted that co-treatment of HCT116 or SW480 cells with resveratrol and an anti-β1-integrin (neutralizing antibody) in the TME system abolished the resveratrol-induced change in the distribution pattern of β1-integrin receptors on the surface of both CRC cells. This shows that A) resveratrol modulates β1-integrin receptors for its signaling on the surface of tumor cells and B) resveratrol needs functional β1-integrin receptors, at least in part, to fully exert its anti-carcinogenic effect in CRC cells.

We further demonstrated that resveratrol significantly suppressed the viability and thus indirectly, proliferation of HCT116 and SW480 cells, and this suppression could be prevented by function-blocking antibodies to β1-integrin or treatment with RGD, or β1-integrin inhibition by specific inhibitor (bacitracin). Moreover, resveratrol was shown to inhibit TME-stimulated colony formation of HCT116 cells, and this inhibition was reversed by anti-β1-integrin. These results clearly suggest a critical, mediating, and important role of β1-integrin as an adhesion receptor and signaling molecule in TME-promoted proliferation and colony formation of CRC cells, and for the resveratrol signaling pathway in cellular changes, too. These results further suggest that pro-inflammatory TME-dependent up-

regulation of β1-integrin is more likely a feature of CRC cells. Furthermore, this is consistent with other studies having shown that other types of integrin, such as β6-integrin, are sparsely expressed in normal intestinal epithelium but are highly up-regulated in the TME or by exogenous pro-inflammatory cytokines and act as tumor promoters [42,43]. More interestingly, these overall data are also concordant with earlier work by Lin et al. outlining that antibody against integrin αvβ3, but not αvβ5, or RGD peptide specifically impede the initiation of ERK1/2- and p53-dependent resveratrol-induced apoptosis in human breast cancer cells MCF-7 or MDA-MB231, thereby supporting the notion that integrin αvβ3 carries a receptor site for resveratrol [24]. In addition, αvβ3-integrin receptors have already shown an important role in resveratrol-uptake in SW480 cells [26], which we also used in the present work.

NF-kB has already been reported to be an active player in integrin expression, tumor survival, and malignant proliferation [44,45]. In addition, it has been repeatedly published that pro-inflammatory TME simultaneously induces phosphorylation of the transcription factor NF-kB and tumor-promoting NF-kB-governed proteins implicated in proliferation, metastasis, growth, and apoptosis of CRC cells [46,47]. Moreover, FAK has been shown to be a receptor tyrosine kinase that has an essential function in the intracellular signaling of integrins [18,19,40]. Therefore, to obtain more information about the underlying mechanism, we screened for NF-kB signaling in this work and found that p65-NF-kB, FAK (downstream of β1-integrin), and cyclin D1 (protein regulating cell cycle progression), strongly and at the same time, caspase-3 (apoptosis) was poorly expressed in TME. In numerous previous publications by our group, we compared the expression of these proteins in the TME of the cell lines also used in this work with a basal control without TME, confirming the notion that the described protein expressions are significantly induced in the TME [30,46,48–50]. In parallel, blocking the β1-integrin receptor by the antibody against β1-integrin but not against other integrin chains, the β5-integrin antibody, or by treatment with the integrin epitope suppressor RGD, but not with RGE peptides, led to inhibition of resveratrol-depressed p65-NF-kB, p-FAK, and cyclin D1 expression, and resveratrol-activated caspase-3. This finding suggests that this so-called central signaling cascade is engaged in the action of TME and β1-integrin-linked signaling is participating in the anti-tumorigenic effects of resveratrol in CRC cells. Furthermore, these outcomes are quite in line with past findings which have shown that FAK is an important regulating signaling molecule throughout the process of integrin-mediated signaling in various types of tumor cells, and plays a fundamental role in survival and invasion [51,52]. Indeed, integrins have been repeatedly reported to be a negative prognostic factor that also promotes cancer cell migration/invasion and metastasis [53–55], suggesting that they are possible cancer targets [25].

Collectively, these observations illuminate that the resveratrol/β1-integrin/FAK/p65-NF-kB pathway may be an important underlying mechanism for resveratrol-promoted inhibition of CRC cell viability, growth, and proliferation in the TME. However, because our in vitro pro-inflammatory TME model is not sufficient to elucidate the precise physiological or pathological role of β1-integrin in vivo, further studies, possibly using transgenic mice with manipulated β1-integrin expression, are required (Figure 6).

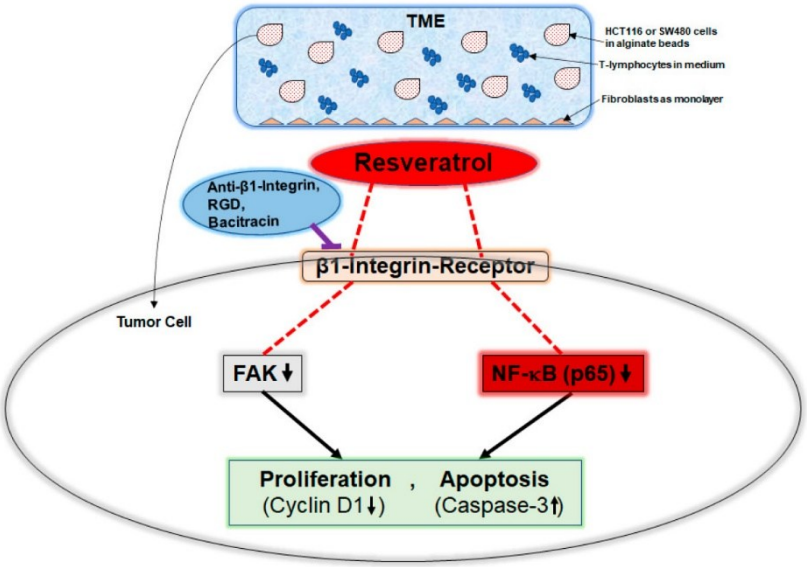

Figure 6. Working model demonstrating resveratrol-mediated anti-proliferative and anti-viability activity through modulation of the β1-integrin receptor in CRC cells in the pro-inflammatory TME.

4. Materials and Methods

4.1. Antibodies and Chemicals

Anti-phospho p65-NF-kB (#MAB7226), anti-caspase-3 (#AF835), and anti-cyclin D1 (#MAB4314) were purchased from R&D Systems (Heidelberg, Germany). Anti-phospho-FAK (#558540) and anti-FAK (#610088) antibodies were from Becton Dickinson (Heidelberg, Germany). Anti-β-actin (#A4700) antibodies, MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DAPI, resveratrol, bacitracin, and alginate were from Sigma-Aldrich (Taufkirchen, Germany). Monoclonal antibodies to β1-integrin (specifically recognizing the active conformation) are from BD Biosciences (#610468) (Monoclonal, 18/CD29) (San Diego, CA, USA). Neutralizing monoclonal antibodies against β1-integrin (#sc-374429), β5-integrin (#sc-374429), and normal mouse IgGs (#sc-2025) were from Santa Cruz (#sc-398214) (CA, USA). RGD (Arg-Gly-Asp) and RGE (Arg-Gly-Glu) peptides were obtained from Bachem (Torrance, CA, USA). Secondary rhodamine-coupled antibodies were from Dianova (Hamburg, Germany). Sheep anti-mouse and sheep anti-rabbit alkaline phosphatase-linked secondary antibodies were from Millipore (Schwalbach, Germany). RGD/RGE peptides were prepared by dilution in serum-free medium to minimize the effects of serum proteins. Resveratrol was prepared as a 100 mM stock in ethanol and further diluted in cell culture medium for experimental investigations.

Cell culture medium from Seromed (Munich, Germany) consisting of Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) were completed with 3% FBS (fetal bovine serum, serum-starved) or 10% FBS, 1% glutamine, 1% penicillin/streptomycin solution (10,000 IU/10,000 IU), 75 µg/mL ascorbic acid, 1% essential amino acids, and 0.5% amphotericin B solution.

4.2. Cancer Cells, T-Lymphocyte, and Fibroblast Cell Growth Culture

HCT116 and SW480 are human colorectal cancer cell strains that differ in their KRAS mutation, and HCT116, among others, showed faster growth than SW480 [56]. Both CRC

cell lines and MRC-5, a human fibroblast cell strain, were acquired from the European Collection of Cell Cultures (Salisbury, UK). T-cell leukemia cells Jurkat (ACC 282), THP-1 (ATCC® TIB-202™), a human T-lymphocyte cell strain, were purchased from the Leibniz Institute (DSMZ-German Collection of Microorganisms and Cell Cultures). All cell strains were cultured under routine culture conditions at 37 °C and 5% CO_2 in T175 flasks until a confluence of 70% was reached. HCT116, SW480, and MRC-5 cells grow as monolayers, whereas Jurkat cells freely float in suspension. Before starting an experiment, cells underwent three rinses with medium containing serum (3% FBS) and were pre-incubated in the same medium for 30 min.

4.3. Tumor Microenvironment and Study Design

The purpose of this research was to evaluate the anti-cancer value of resveratrol via β1-integrin and to test different β1-integrin inhibitors in a pro-inflammatory TME in vitro, simulating a pro-inflammatory tumor situation in vivo. For this purpose, a model was set up with 3D-alginate beads as reported in our previous work [30,46].

As "basal control", HCT116 or SW480 cells encapsulated in alginate matrix, were cultivated by itself in whole-cell culture medium. To accomplish the pro-inflammatory multicellular TME, fibroblasts were seeded as monolayers (20,000/well) and incubated in whole-cell culture medium containing 10% FBS. Subsequently, HCT116 or SW480 cells in 3D-alginate beads were added with 20,000 Jurkat cells/well (T-lymphocytes) to petri dishes containing the fibroblasts in serum-starved medium (3% FBS), as described before [30,46,50]. This composition of CRC-alginate beads, Jurkat cells, and fibroblasts served as "TME control". TME cultures were either not treated, or treated with resveratrol (1, 5 µM) in the presence or absence of IgG (1, 2, 5 µg/mL), the functional blocking anti-β1-integrin antibody (1, 2, 5 µg/mL), anti-β5-integrin antibody (5 µg/mL), RGD peptide (1, 2, 5 µM), RGE peptide (5 µM), and bacitracin (1, 5 µM) for 10–14 days.

4.4. Alginate Bead Culture

Human colon cancer cells (HCT116 or SW480) were embedded as beads in a sterile alginate suspension (2% in 0.15 M NaCl) as reported in our previous works [30,46,48,50]. For this purpose, CRC cells were counted, resuspended in alginate (1 Mio. CRC cells/1 mL alginate), and added dropwise to $CaCl_2$ (100 mM) solution. After 10 min of polymerization, the beads were washed three times with NaCl (0.15 M) solution, then two times with cell culture medium (10% FBS), and before transfer to the investigation well-plates, beads were incubated with serum-starved medium (3%) for 30 min.

4.5. MTT Assay

To estimate the vitality and thus indirectly, the proliferation potential of HCT116 and SW480 cells in TME, the cells were detached from alginate, and an MTT assay was carried out as explained in detail previously [57,58]. To ensure that only the properties of the CRC cells were measured, each individual CRC alginate bead (size: ca. 0.5 cm) was first removed from the original experimental plates using bent tweezers, transferred to new plates containing fresh buffer (Hanks salt solution), and carefully washed on a shaker with gentle waving. This procedure was repeated at least three times (5 min each time) to ensure that no Jurkat cells (size: ca. 12 µM) adhered to the alginate beads. Each time, the CRC-alginate beads were observed under a phase-contrast microscope to check whether Jurkat cells adhered to the alginate beads. When it was 100% certain that no Jurkat cells were visible, the pure CRC-alginate beads were prepared for further processing. The clean CRC-alginate beads were then dissolved in sodium citrate (55 mM) for 30 min. After centrifugation and removal of sodium citrate and alginate residues, cells were washed with Hanks salt solution, resuspended in modified cell culture medium (3% FBS, without phenol red, without vitamin C) and distributed to a 96-well-plate with cell suspension (100 µL) and MTT solution (10 µL) to each well. The reaction was stopped by adding 100 µL of MTT solubilization solution (10% Triton x-100/acidic isopropanol) to each well after 3 h.

The Optical Density (OD) was measured at 550 nm (OD550) using a 96-well-plate multiscanner ELISA reader from Bio-Rad (Munich, Germany). At no time during the entire experiment, collection, and evaluation was there a risk of contamination with other cell types, as there was no direct contact.

4.6. Proliferation and Colony Formation

As described in the study design section, HCT116 were cultured in alginate beads for 10–14 days. Treatment-dependent proliferation differences of CRC cells were observed, and their colonosphere formation in the alginate beads was quantified by counting 20 microscopic fields using a Zeiss Axiovert 40 CFL microscope (Oberkochen, Germany) as explained in detail previously [58]. Images from all different treatments were stored digitally and statistically evaluated.

4.7. Immunofluorescence Study

The TME was modified to perform immunofluorescence; 6-well-plates with fibroblast monolayers on the bottom and Jurkat cells in suspension were used. CRC cells were seeded on glass coverslips as a monolayer with 6000 cells/cover glass. After 24 h of incubation in 10% FBS cell culture medium, the glass plates were placed on small steel mesh bridges in the 6-well-plates as a basal control, or as TME with fibroblasts on the bottom, and 10,000 T-lymphocytes per mL of cell culture medium. Cells were incubated in basal control or TME for 24 h before treatment (5 µM resveratrol and/or 2 µg anti-β1-integrin) began. Immunofluorescence experiments were carried out as detailed in our earlier reports [30], where the dilution was 1:80 for the primary antibody and 1:100 for the secondary antibody. This experimental setup ensured exclusive labeling of HCT116 and SW480 CRC cells after removal of glass coverslips from the TME, as the steel bridges provided a distance to the fibroblast monolayer and the T-lymphocytes, which were floating in the cell culture medium and remained in the well-plates.

4.8. Western Blot Analysis

After 10–14 days of 3D-cultivation, the HCT116-alginate beads were separated from the TME, ensuring that the subsequent Western blot results were exclusively related to CRC cells. Therefore, the alginate beads, which contained only HCT116 cells, as described before, were removed from the experimental well-plates, transferred to a new well-plate, and carefully washed. Then, HCT116 were dissolved from alginate in sodium citrate solution and resuspended in lysis buffer (50 mM Tris-HCl, pH 7.2; 150 mM NaCl; 1% (*v*/*v*) Triton x-100; 1 mM sodium orthovanadate; 50 mM sodium pyrophosphate; 100 mM sodium fluoride; 4 µg/mL pepstatin A; 1 mM PMSF). After 30 min of centrifugation, the liquid supernatant was frozen at −80 °C. Standard Western blot assay was carried out as detailed in our earlier paper [46,48,58]. Briefly, proteins were separated by SDS-PAGE with a transblot apparatus from Bio-Rad (Munich, Germany). After pre-incubation in milk-powder-based blocking buffer (PBS, 5% milk-powder, 0.1% Tween 20), nitrocellulose membranes were incubated with primary antibodies (dilution 1:10,000) overnight and with secondary antibodies (dilution 1:10,000) for 90 min. The antibodies used are described in detail in "Antibodies and Chemicals" section and β-actin served as loading control. Bindings were quantified by densitometry using the program Quantity One from Bio-Rad (Munich, Germany).

4.9. Statistical Evaluation

Our studies were conducted in three separate assays with three different control samples. Results were analyzed by an unpaired Student's t-test and by one-way ANOVA followed by a post hoc test to compare the parameters of each group. A p value of <0.05 indicates statistically significant differences.

5. Conclusions

Our results show for the first time that β1-integrins are overexpressed in TME-stimulated CRC cells, and resveratrol has a modifying impact on its expression and distribution on the tumor cell surface. It was shown here that resveratrol-promoted suppression of CRC cell proliferation, viability, and survival in TME occurs through down-regulation of FAK, p65-NF-kB, cyclin D1 activation, and up-regulation of caspase-3. As these effects can be reversed by anti-β1-integrin or RGD peptides, we conclude that resveratrol uses β1-integrin receptors as one of its major transmission molecules in its modulatory signal transduction in tumor cells (Figure 6). These results suggest a possible therapeutic implication of β1-integrins being a target for the development of anti-tumor drugs containing resveratrol or resveratrol-like natural components with the potential to inhibit viability as well as proliferation and induce apoptosis of CRC tumor cells. Further investigation of the role of β1-integrin in tumorigenesis in vivo will help to understand the potential therapeutic value of β1-integrin in the future treatment of colorectal cancer.

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8. Paper II

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β1-Integrin plays a major role in resveratrol-mediated anti-invasion effects in the CRC microenvironment

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Background: Tumor microenvironment (TME) is one of the most important factors in tumor aggressiveness, with an active exchange between tumor and other TME-associated cells that promotes metastasis. The tumor-inhibitory effect of resveratrol on colorectal cancer (CRC) cells has been frequently reported. However, whether resveratrol can specifically suppress TME-induced CRC invasion *via* β1-integrin receptors has not been fully elucidated yet.

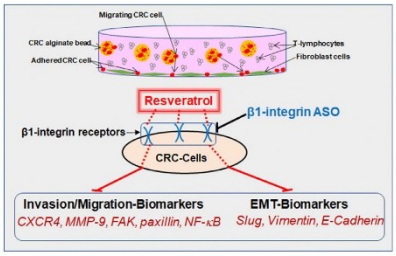
Methods: Two CRC cell lines (HCT116, RKO) were cultured in multicellular, pro-inflammatory 3D-alginate TME cultures (containing fibroblasts, T-lymphocytes) to investigate the role of β1-integrin receptors in the anti-invasive and anti-metastatic effect of resveratrol by antisense oligonucleotides (ASO).

Results: Our results show that resveratrol dose-dependently suppressed the migration-promoting adhesion adapter protein paxillin and simultaneously enhanced the expression of E-cadherin associated with the phenotype change of CRC cells, and their invasion. Moreover, resveratrol blocked TME-induced phosphorylation and nuclear translocation of p65-NF-κB, which was associated with changes in the expression pattern of epithelial-mesenchymal-transition-related biomarkers (slug, vimentin, E-cadherin), metastasis-related factors (CXCR4, MMP-9, FAK), and apoptosis (caspase-3). Finally, transient transfection of β1-integrin, in contrast to knockdown of NF-κB, abrogated most anti-invasive, anti-metastatic effects as well as downstream signaling of resveratrol, resulting in a concomitant increase in CRC cell invasion, indicating a central role of β1-integrin receptors in the anti-invasive function of resveratrol.

Conclusion: These results demonstrate for the first time that silencing β1-integrins may suppress, at least in part the inhibitory effects of resveratrol on invasion and migration of CRC cells, underscoring the crucial homeostatic role of β1-integrin receptors.

KEYWORDS

β1-integrin, CRC, inflammation, invasion, metastasis, NF-κB, tumor microenvironment, resveratrol



GRAPHICAL ABSTRACT

Introduction

Cancer is one of the most commonly diagnosed diseases that causes many deaths among populations worldwide. According to global cancer statistics, there were over 19 million new cancer cases and 10 million cancer deaths globally in 2020 (Sung et al., 2021). Herein, colorectal cancer (CRC) represented 10% and the total number is expected to further increase within the next 20 years (Sung et al., 2021). In fact, CRC is the second leading cause of cancer deaths in the United States (Siegel et al., 2020). It is known that not only patients of advanced age are at risk, but also unhealthy lifestyle, sedentary behavior, excessive obesity or smoking play a decisive role in the development of cancer disease (Siegel et al., 2020). Moreover, despite the rising prevalence of screening and the growing number of colonoscopies (Siegel et al., 2020), more investment is needed to detect CRC at an early stage. Indeed, it has been reported that when a CRC is first diagnosed, up to 25% of patients already have been developing metastases, and as the disease progresses, as many as half of all affected individuals develop metastases (Vatandoust et al., 2015).

In that regard, metastatic tumors are cells that have spread outside of the colorectal tract *via* the blood or lymphatic system and are then located predominantly in the liver or lungs. The metastatic process, which is one of the six hallmarks of cancer, is usually a sign of a high-progressive disease and explains why patients die more often from secondary than from primary tumors (Koklesova et al., 2020). Furthermore, these lethal complications can also be triggered by metastases damaging affected organs, leading to dysfunction, organ failure, and ultimately to death in patients.

As inflammatory processes are one of the major underlying mechanisms of cancer diseases, colorectal tumors also contain large numbers of cells that can trigger excessive immune responses and cytokine production, leading to increased activation of pro-inflammatory and oncogenic transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) (Rasool et al., 2021). In the advanced cascade of tumor invasion and metastasis, epithelial–mesenchymal transition (EMT) plays an essential role, which describes the process of cells changing their polarity from epithelial to mesenchymal characteristics, thus are able to migrate. While this transformation is a physiological process during the embryonic phase, it is considered highly pathological in the context of cancer development and migration (Koklesova et al., 2020). In addition, there are numerous signs of EMT, such as reduced E-cadherin expression acting as an epithelial marker with simultaneously increased vimentin expression as a mesenchymal marker (Koklesova et al., 2020). Moreover, it has been reported that the inflamed multicellular microenvironment of CRC significantly promotes expression of EMT proteins and transcription factors such as slug (Buhrmann et al., 2015). Indeed, this complex disease process requires therapy that attacks various levels in a multi-faceted manner, thus it is advisable to incorporate the power of nature-derived substances into treatment, such as the polyphenol and phytoalexin resveratrol. Resveratrol was found as a stilbenoid in several plants such as grapevines and peanuts, where it is synthesized in order to protect plants against pathogens (Fritzemeier et al., 1983; Hain et al., 1993).

Based on preclinical studies in CRC, resveratrol has already been shown promising effects in the subject of anti-inflammatory, anti-proliferative and anti-invasive properties in tumor cells (Brockmueller et al., 2022), and its clinical application

is currently under intense investigation (Brockmueller et al., 2021). Thus, previous studies by our group among others have shown that resveratrol is able to suppress the dangerous and tumor-promoting cross-talk between CRC cells and stromal cells in a pro-inflammatory tumor microenvironment (TME) *in vitro*. Furthermore, resveratrol, as a multi-target molecule has been reported to interfere and negatively affect NF-κB signaling pathways, which is one of the major inflammatory transcription factors and markers promoted by the TME, that was shown to affect invasion behavior matrix metallopeptidase 9 (MMP-9) and metastasis process chemokine receptor type 4 (CXCR4) in CRC cells (Bergman et al., 2013; Buhrmann et al., 2016; Suh et al., 2018; Buhrmann et al., 2020).

Moreover, the prevention of EMT mechanisms in tumor cells is one of the most important goals in cancer research and therapy and has been increasingly attributed to be of fundamental importance. In this context, resveratrol has been frequently reported to possess potent and targeted subcellular modulatory effects on EMT, particularly the inhibition of EMT-promoting proteins (vimentin, slug) and simultaneous promotion of the epithelial protein, E-cadherin (Buhrmann et al., 2015), thus showing an inhibitory effect on the highly metastatic tumors.

Resveratrol has also been announced to make use of integrins as both, surface receptors and signaling molecules, for exerting its anti-tumor effects (Lin et al., 2006; Belleri et al., 2008; Varoni et al., 2016). Integrin families are heterodimeric cell adhesion and signaling molecules that play a central role as signal transducers in the interaction between cells and extracellular matrix (Shakibaei et al., 1997; Shakibaei, 1998; Shakibaei and Merker, 1999; Mueller et al., 2022), thus display a crucial role in the differentiation and function of cells in healthy tissues (Carter et al., 1990; Chen, 1990; Shakibaei, 1995). Moreover, these specific compounds are remarkably up-regulated in cancer cells, what is associated with increased risk for tumor migration and metastasis (Huang et al., 2021). Researchers aim to take advantage of this property and thus are investigating the chance of using integrins as tumor markers (Jones et al., 1992). Specifically for colorectal carcinoma, α1-integrin has already been shown to be associated with increased tumorigenesis, CRC cell migration and invasion (Li et al., 2020), and β6-integrin provided even more accurate prognosis than the already established tumor marker carcinoembryonic antigen (CEA) for tumor surveillance of patients with advanced CRC stage (Bengs et al., 2019). Furthermore, there are indications that inhibition of αvβ6-integrin could reduce the risk of liver metastasis in diabetic CRC patients (Wang et al., 2021) and that resveratrol-binding αvβ3-integrin inhibits tumor growth and metastasis as promising target for cancer therapy (Cheng et al., 2021; Chen et al., 2022). Moreover, down-regulation of focal adhesion kinase (FAK), a specific subcellular target protein of integrins (Lipfert et al., 1992) that is also of great importance in cell migration and invasion (Buhrmann et al., 2017), represents an important mechanism here (Cheng et al., 2021) and resveratrol modulates FAK phosphorylation (Buhrmann et al., 2017).

Recently, we have demonstrated that resveratrol shows one of its important anti-proliferative and -viable properties *via* modulation of the β1-integrin pathway by rearrangement of β1-integrin receptors to use them for signal transduction as well as for exertion of resveratrol's proliferation- and viability-inhibitory capabilities (Brockmueller et al., 2022) in CRC cells.

In the present work, we address the question of whether and how resveratrol can affect the invasion and metastasis potential and thus the EMT of CRC cells *via* β1-integrin axis. Within this study, implications of β1-integrin-SO/ASO (β1-SO/ASO) and NF-κB-SO/ASO in HCT116 and RKO CRC cells were compared in a multicellular, pro-inflammatory TME *in vitro*. This 3D-tumor cultures, composed of cancer cells, T-lymphocytes and fibroblasts have been established throughout our previous studies (Buhrmann et al., 2020; Brockmueller et al., 2021) as well as in other research groups (Gao et al., 2021).

Material and methods

Antibodies and reagents

Monoclonal antibodies to NF-κB (#MAB5078), and phospho-specific p65-NF-κB (#MAB7226), MMP-9 (#MAB911), polyclonal caspase-3 (#AF835) were purchased from R&D Systems (Heidelberg, Germany). Monoclonal antibodies to anti-FAK (#610088), anti-phospho-FAK (#558540) were from Becton Dickinson (Heidelberg, Germany). Monoclonal antibodies to β-actin (#A4700), resveratrol, alginate, DAPI, Fluoromount were from Sigma-Aldrich (Taufkirchen, Germany). Monoclonal anti-E-cadherin (#sc-21791), anti-vimentin (#sc-53464), anti-slug (#sc-166476), anti-paxillin (#sc-365059) normal mouse IgG (#sc-2025) were from Santa Cruz Biotechnology (Dallas, Texas, United States). Monoclonal anti-β1-integrin (#14-0299-82) and anti-CXCR4 (#35-8800) were from Thermo Fisher Scientific (Langenselbold, Germany). Secondary rhodamine-coupled antibodies for immunofluorescence were from Dianova (Hamburg, Germany), and alkaline phosphatase-linked antibodies for Western blotting were from EMD Millipore (Schwalbach, Germany). Resveratrol was prepared in 100 mM stocks with ethanol and directly diluted in the cell culture medium for CRC cell treatment, without exceeding an ethanol concentration of 0.1% during the investigations.

Cells and preparation

For the presented studies, two different human CRC cell lines [HCT116 from European Collection of Cell Cultures (Salisbury, United Kingdom) and RKO from American Type Culture Collection (Manassas, Virginia, United States)], human

T-lymphocytes [Jurkat from DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany)] and human fibroblasts [MRC-5 from European Collection of Cell Cultures (Salisbury, United Kingdom)] were used. The cell culture setting and preparation corresponds to the one already described earlier (Buhrmann et al., 2020; Brockmueller et al., 2022). 3% fetal bovine serum (FBS) or 10% FBS, 1% glutamine, 1% penicillin/streptomycin solution (10.000 IU/10.000 IU), 75 µg/ml ascorbic acid, 1% essential amino acids and 0.5% amphotericin B solution were added to Dulbecco's modified Eagle's medium/F-12 from Sigma-Aldrich (Taufkirchen, Germany) and used as cell culture medium.

Transfection

To study the effects of transfection, 0.5 µM antisense oligonucleotides (ASO) or control sense oligonucleotides (SO) was incubated in Lipofectin transfection reagent (Invitrogen, Karlsruhe, Germany) and added into the experimental well-plate. Oligonucleotides from Eurofins MWG Operon (Ebersberg, Germany) were modified with phosphonothioate to preserve the oligonucleotides from cell nucleases, the exact procedure has already been described (Buhrmann et al., 2016; Buhrmann et al., 2020), and used for transient transfection with antisense/sense oligonucleotides (ASO/SO) based on β1-integrin or p65-NF-κB:

a) β1-integrin-ASO (5′-TAGTTGGGGTTGCACTCACAC A-3′),
b) β1-integrin-SO (5′-TGTGTGAGTGCAACCCCAACTA-3′),
c) NF-κB/p65-subunit-ASO (5′-gGAGATGCGCACTGTCCC TGGTC-3′),
d) NF-κB/p65-subunit-SO (5′-gACCAGGGACAGTGCGCA TCTC-3′).

3D-alginate tumor culture *in vitro*

In the current investigations, the *in vivo* condition of a cancerous patient was simulated by a 3D-alginate culture model *in vitro*. This enables an animal-free investigation of resveratrol's anti-tumor effect in a pro-inflammatory tumor microenvironment. The present study focuses on the exploration of resveratrol's β1-integrin-mediated effects on the migration and invasion of CRC cells using β1-integrin-ASO/SO as well as NF-κB-ASO/SO for comparison.

The experiments were carried out with CRC-alginate balls comprising an average size of 4 mm which were produced as described in numerous publications of our research group (Buhrmann et al., 2020; Brockmueller et al., 2022). Then they were assembled in a composition of MRC-5 fibroblasts as monolayer on the bottom of 12-well-plates, and Jurkat T-lymphocytes floating in the cell culture medium, which has been established as a suitable TME simulation for other cancer cells as well (Gao et al., 2021). In the present work, the alginate balls were made from HCT116 or RKO cells in 12-well-plates and the alginate coating ensures that the CRC cells do not mix with the other cell types during the experiments. A control without TME (Ba. Co.) and a TME control (TME Co.) without treatment additives were established. The treated cells received resveratrol (1, 5 µM), β1-integrin-ASO/SO (0.5 µM), NF-κB-ASO/SO (0.5 µM) or a combination thereof as supplements to 3% FBS (serum-starved) cell culture medium with a running time of 10–14 days for all samples.

Three invasion attempts

To observe the invasion and to draw conclusions about CRC's metastatic properties, HCT116 and RKO were treated as explained before (Ba. Co., TME Co., resveratrol, β1-integrin-SO/ASO, NF-κB-SO/ASO) for 10–14 days and evaluated using three different invasion assays:

A) Firstly, the bottom of 12-well-plates was photographed in phase contrast with a Zeiss Axiovert 40 CFL microscope (Oberkochen, Germany) in order to compare the CRC cell colonies that had settled. To determine the average size of settled colonies, 25 colonies of each treatment were measured.
B) Secondly, the 12-well-plates were fixated with Karnovsky solution for 30 min, stained with toluidine blue. To determine the average colony, count of stained colonies, labelled as invasion, colonies were counted from three wells at each treatment.
C) Thirdly, the experiment was set up with identical treatments in 6-well-plates. As a special feature, a square cover glass was placed in each well on which the migrating CRC cell colonies settled. The glass slides were fixated with methanol for 30 min, then rinsed with Hank's solution (3 times), incubated for 15 min in the dark with DAPI and covered in Fluoromount for photographic analysis using a Leica DM 2000 microscope (Wetzlar, Germany).

Immunofluorescence investigation

Leica (Wetzlar, Germany) DM 2000 microscope with LAS V4.12 software was used for the generation of immunofluorescence images. For preparation, 6000 CRC cells (HCT116 or RKO) were sown on a small round glass plate. After 24 h, the TME was recreated in a modified manner by placing the small glass plates on a meshed bridge in a 6-well-plate containing fibroblasts as monolayer, floating T-lymphocytes and reagents according to the treatment pattern described above. After 1 day,

the glass slides were removed from well-plates and washed with Hank's salt solution three times, ensuring that only CRC cells were left for immunolabelling. Then, CRC-glass plates were fixated in methanol for 30 min and prepared for immunofluorescence microscopy as described in detail before (Brockmueller et al., 2022; Mueller et al., 2022). In the present work, CRC cells were immunolabeled with E-cadherin-, NF-κB-paxillin- or slug-antibodies. The primary antibody dilution was 1: 80 and the secondary antibody dilution was 1:100 each. To reveal the cell nuclei, each slide was stained with DAPI for 15 min and then fixated with Fluoromount.

Immunoblotting

For Western blot analysis, HCT116 or RKO 3D-alginate balls were treated with the aforementioned substances. After 10–14 days, the alginate balls were removed from 12-well-plates with bent tweezers, transferred to 12-well-plates containing Hank's salt solution, and washed on a gentle waving shaker. This procedure was repeated at least three times, ensuring that no T-lymphocytes adhered to the CRC-alginate balls which was verified by observation with a phase contrast microscope. Then, the clean CRC-alginate balls were dissolved in sodium citrate (55 mM) for 30 min to isolate CRC cells which were resuspended in lysis buffer as described before (Brockmueller et al., 2022). After 30 min of centrifugation, the liquid supernatant was kept frozen (−80°C) and samples were further processed with a Bio-Rad (Munich, Germany) transblot apparatus for densitometric evaluation with Bio-Rad Quantity One analysis software as explained in detail in our earlier work (Brockmueller et al., 2022; Mueller et al., 2022). In the current study, the beforementioned antibodies were used in 1: 10.000 dilution and β-actin served as loading control.

Statistical analysis

We performed all assays as three independent repetitions and used an unpaired student's t-test for statistical analysis. Results matched by one-way ANOVA followed by post hoc test to compare parameters within the groups. At the outcomes, a *p*-value less than 0.05 was considered as statistically significant.

Results

The aim of our study was to determine the role of β1-integrin receptors in the anti-invasive, anti-metastatic and anti-inflammatory effects of resveratrol in two CRC cell lines (HCT116, RKO) in a pro-inflammatory, multicellular, *in vivo*-like tumor microenvironment *in vitro* by β1-integrin knockdown and NF-κB knockdown *via* antisense oligonucleotides (ASO).

Resveratrol targets the β1-integrin receptors to promote TME-down-regulated E-cadherin expression and suppress up-regulated paxillin expression in CRC cells

Investigations on CRC primary tumors showed that they were prone to have a dedifferentiated, mesenchymal phenotype, a noticeably reduced E-cadherin expression, an increased paxillin expression, and a high tendency to metastasize (Kaihara et al., 2003; Buhrmann et al., 2015; Wen et al., 2020). Therefore, we examined HCT116 and RKO cells, grown on glass slides, in more detail using the phase contrast microscope, and observed that the HCT116 and RKO control cultures exhibited evenly distributed cell colonies with a slightly epithelial morphology, smooth surface, and close cell to cell contacts (Figures 1A,B), similar to treatment with only β1-integrin-SO or -ASO (β1-SO or -ASO). In contrast, the RKO control cells showed a more fibroblast-like to mesenchymal morphology, less colony formation, and fewer cell to cell contacts. Interestingly, treatment of both CRC cells with resveratrol resulted in 1) increased cell to cell contact (epithelial shape) and a rounder shape of CRC cells though and 2) the development of small conspicuous plaque-like deposits of the cell membrane on HCT116 (Figure 1A, top row, black arrows) and RKO cells (Figure 1B, top row, black arrows) in a dose-dependent (1, 2, 5 μM resveratrol) manner, similar to the combined treatment with resveratrol (5 μM) and/or β1-SO (0.5 μM). Even more interestingly, treatment with resveratrol and β1-ASO significantly decreased or resolved the development of small conspicuous plaque-like deposits of the cell membrane on HCT116 (Figure 1A, top row) and RKO cells (Figure 1B, top row) in a dose-dependent (0.1, 0.2, 0.5 μM β1-ASO) manner in both cell lines. Together, these findings indicate that the detected resveratrol-induced plaque formation in CRC cells by targeting β1-integrin receptors was related to an increased E-cadherin expression, leading to a more stable local adhesion and therefore rather epithelially shape. We wondered whether this resveratrol-induced plasma membrane plaques contain the biomarker for epithelial phenotype, E-cadherin, and therefore examined its expression by immunofluorescence analysis (Figures 1A,B; middle row each). The untreated CRC cells in TME showed minimal E-cadherin expression on the surface, similar to treatment with β1-SO (0.5 μM) or β1-ASO (0.5 μM) alone. On the other hand, the expression of E-cadherin on the surface of CRC cells treated with resveratrol (1, 2, 5 μM) alone showed a significant increase in E-cadherin expression in a concentration-dependent manner, which was regulated similar

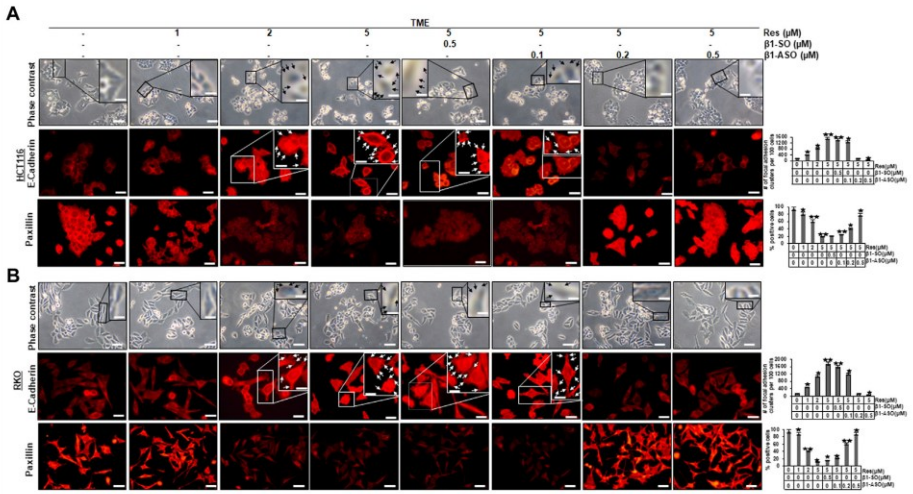


FIGURE 1
Impact of resveratrol or/and ASO against β1-integrin on TME-mediated cell morphology and expression of E-cadherin and paxillin in CRC cells. HCT116 **(A)** and RKO **(B)** cells were grown on coverslips in TME, left untreated or treated with increasing doses of resveratrol (1, 2, 5 μM) or were transfected with β1-SO or β1-ASO as outlined in Material and Methods. After rinsing with phosphate-buffered water, the cell morphology of CRC cells was examined by phase contrast **(A,B)**, (top row each). Immunolabeling (red) with anti-E-cadherin **(A,B)**, (middle row each) or paxillin **(A,B)**, (bottom row each) was observed by immunofluorescence microscopy. Special attention is paid to the discovered resveratrol-induced plaque-like deposits, highlighted with black **(A,B)**, (top row each) and white **(A,B)**, (middle row each) arrows. Statistical analysis: Plaque-like deposits were quantified by couting in 20 different microscopiy fields. Related to TME control, $*p < 0.05$ and $**p < 0.01$. Microscope: Leica DM 2000. Magnification ×600; scale bar = 30 μm. Inset magnification: ×1200; scale bar = 15 μm.

to cells treated with β1-SO and resveratrol in combination (Figures 1A,B; middle row each, white arrows). In contrast, knockdown of β1-integrin with β1-ASO in a concentration-dependent (0.1, 0.2, 0.5 μM) manner abolished the blocking effect of resveratrol on the expression of the above-mentioned epithelial biomarkers. To further investigate the impact of the β1-integrin-mediated effect of resveratrol on EMT processes in CRC cells, we also immunolabelled HCT116 as well as RKO cells with an antibody against the cell migration marker paxillin (Figures 1A,B; bottom row each). Confirming previous results, both CRC cell lines showed high paxillin expression in the untreated TME control, which decreased significantly with increasing resveratrol (1, 2, 5 μM) concentration. The largely suppressed expression of paxillin by resveratrol (5 μM) in CRC cells remained low even with combined treatment of the CRC cells with 0.5 μM β1-SO. However when β1-ASO (0.1, 0.2, 0.5 μM) was added to resveratrol-treated HCT116 or RKO cells, paxillin expression became increasingly intense. In summary, these results indicate a strong migration inhibitory effect of resveratrol on CRC cells (Figures 1A,B) and the important role of β1-integrin receptors in resveratrol-enhancing anti-tumor effect on CRC cells in TME, whereby these effects were not cell line specific.

Resveratrol targets β1-integrin receptors and suppresses TME-up-regulated phosphorylation and nuclear NF-κB translocation in CRC cells

As resveratrol is known to be a compound that inhibits inflammation in CRC cells (Buhrmann et al., 2016), we wanted to examine its influence on NF-κB activation and furthermore the role of β1-integrin receptors in this context. For this purpose, CRC cells (HCT116, RKO) were cultured on round glass coverslips and examined by immunofluorescence microscopy as described in the Material and Methods section. Here, HCT116 cells in untreated TME (TME control) showed a distinct, luminescent immunolabeling (Figure 2A, exemplary marked with white arrows) as a sign of high, inflammation-induced NF-κB phosphorylation and nuclear translocation in the majority of CRC cells (Figures

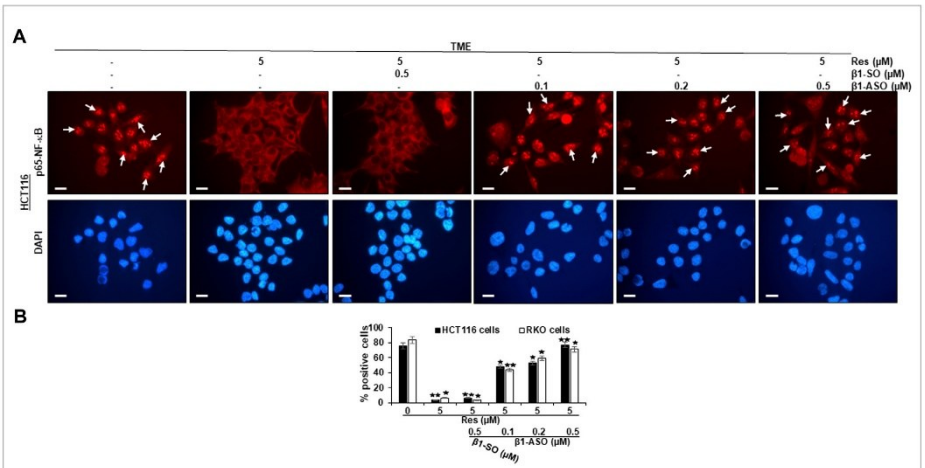


FIGURE 2
Impact of resveratrol or/and ASO against β1-integrin on TME-mediated activation and nuclear translocation of p65-NF-κB in CRC cells. HCT116 **(A,B)** and RKO **(B)** cells in TME on cover glasses without treatment or only treated with resveratrol (5 μM) or resveratrol-treated (5 μM) with addition of β1-SO (0.5 μM) or knocked down with β1-ASO (0.1, 0.2, 0.5 μM). Anti-phosho-NF-κB immunolabeled (red) and DAPI-stained (blue). White arrows = p65-NF-κB positive CRC cells. Microscope: Leica DM 2000. Magnification ×600; scale bar = 30 μm. Statistical evaluation: $*p < 0.05$ and $**p < 0.01$, compared to TME control.

2A,B) and confirmatory, this expression was similar to TME-HCT116 cells treated with β1-SO (0.5 μM) or β1-ASO (0.5 μM) alone. Moreover, treatment with resveratrol (5 μM) led to impressive changes in TME grown CRC cells with a significant down-regulation of NF-κB phosphorylation and nuclear translocation compared to the TME control, resulting in barely labeled, pale cell nuclei (Figure 2A). This effect remained the same with β1-SO (0.5 μM) addition to resveratrol-treated HCT116 cells in TME, validating β1-SO as suitable control substance (Figures 2A,B). However, when β1-ASO (0.1, 0.2, 0.5 μM) was added to resveratrol-treated TME cultures for the purpose of β1-integrin knockdown, a concentration-dependent increase of phosphorylation and nuclear translocation of NF-κB was found in CRC cell nuclei, due to the gradual removal of resveratrol's effect (Figures 2A,B). Interestingly, the statistical analysis of RKO CRC cells undergoing same treatments (Figure 2B) confirmed our observations made on HCT116 (Figures 2A,B), leading to the assumption that these are transferable to other CRC cell lines, whereas DAPI staining verified the vitality of the photographed CRC cells by blue DNA labeling. Altogether, these results demonstrate a strong, non-cell line specific anti-inflammatory effect of resveratrol and furthermore suggest a significant limitation of resveratrol's anti-inflammatory impact by β1-integrin knockdown.

Resveratrol targets β1-integrin receptors and suppresses TME-induced activation of EMT-related transcription factor (slug) in CRC cells

Since we have already found out that a pro-inflammatory, multicellular TME promotes EMT and resveratrol is able to intervene this conversion (Buhrmann et al., 2015), we were then interested in the extend of major EMT transcription factor slug expression, the influence of resveratrol and especially the role of β1-integrin receptors in this process. In Figure 3, RKO (Figure 3A) and HCT116 are shown (Figure 3B), which were grown on small cover glasses in TME, subsequently immune-marked with anti-slug antibody and analyzed by immunofluorescence microscopy as described in the Material and Methods section. We observed same significant expression tendencies in both cell lines, RKO and HCT116: In the TME (TME control), the majority of CRC cells showed cell nuclei with bright, slug-positive labeling, exemplary highlighted with white arrows (Figures 3A,B), whereas β1-SO (0.5 μM) or β1-ASO (0.5 μM) addition had no visible influence. However, resveratrol-treatment (5 μM), remarkably suppressed slug expression in the TME compared to TME control, leading to pale labelled inconspicuous cell nuclei. Interestingly, addition of 5 μM resveratrol to β1-SO (0.5 μM) treated CRC cells did not change this expression pattern, leading to confirmation of β1-SO as suitable

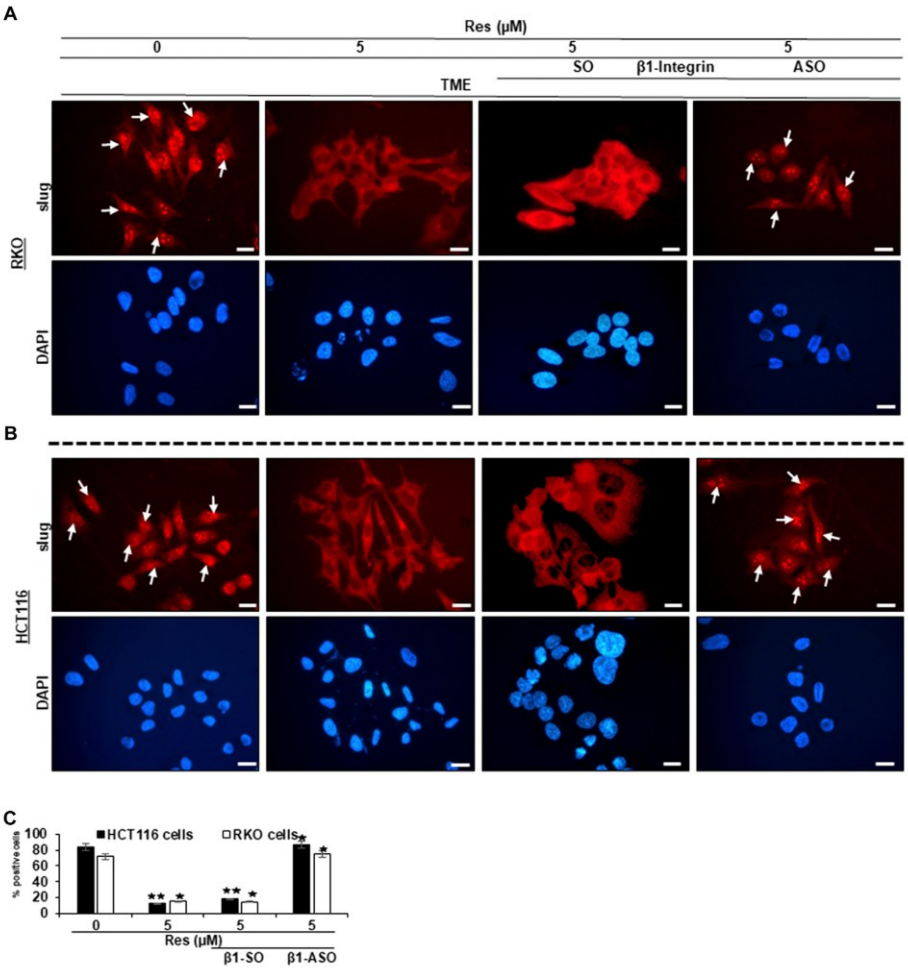


FIGURE 3
Impact of resveratrol or/and ASO against β1-integrin on TME-mediated EMT-linked transcription factor slug activation in CRC cells. RKO **(A,C)** and HCT116 **(B,C)** cells in TME on coverslips, left untreated or treated with resveratrol (5 μM) or resveratrol-treated (5 μM) with addition of β1-SO (0.5 μM) or knocked down with β1-ASO (0.5 μM). CRC cells were immunolabeled with anti-slug (red) and DAPI-stained (blue). White arrows = slug positive CRC cells. Microscope: Leica DM 2000. Magnification ×600; scale bar = 30 μm. Statistics: $*p < 0.05$ and $**p < 0.01$, in relation to TME control.

control substance (Figures 3A,B). In contrast, in the combined treatment consisting of 5 μM resveratrol and 0.5 μM β1-ASO, there were almost as many slug-positive labeled CRC cells found as in the TME control (Figures 3A,B), underscoring that resveratrol was not able to fully exert its EMT-inhibitory effect when β1-integrin was knocked down. The direct statistical comparison of RKO and

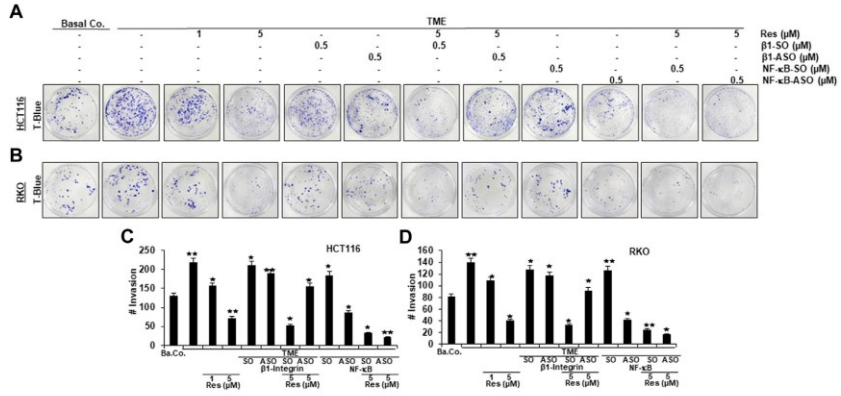


FIGURE 4
Impact of resveratrol or/and ASO against β1-integrin or against NF-κB on TME-promoted CRC cell invasion in alginate cultures. Serum-starved HCT116 **(A)** and RKO **(B)** cells, cultured in 3D-alginate, emigrated under following treatments: untreated (Basal Co.), TME control, resveratrol (1, 5 μM), β1-SO/ASO (0.5 μM), NF-κB-SO/ASO (0.5 μM) or co-treatment of sense or antisense oligonucleotides with resveratrol (5 μM). CRC cells were stained with toluidine blue (T-Blue) after settling on the bottom of 12-well-plates. Statistical evaluation: Compared to TME control, $*p < 0.05$ and $**p < 0.01$ for HCT116 **(C)** and RKO **(D)**.

HCT116 (Figure 3C) proved the described non-cell line specific effects. To ensure the viability of evaluated CRC cells, supplementary DAPI staining (blue) was performed (Figures 3A,B). In summary, these results highlight the significant EMT-inhibitory and reproducible effect of resveratrol in the two CRC cell lines RKO and HCT116, and point out the major role of β1-integrin receptors in mediating these effects.

Resveratrol-promoted repression of TME-induced migration and invasion, similarly to knockdown of NF-κB, is blocked by knockdown of β1-integrin in CRC cells

In order to further investigate the role of β1-integrin receptors in mediating the anti-metastatic and anti-invasive effects of resveratrol, we carried out three invasion assays of HCT116 (Figures 4A,C, Figures 5A–C) and RKO (Figures 4B,D, Figures 5D–F) as described in the Material and Methods section. For this purpose, β1-integrin was knocked down *via* β1-ASO and because of its important role in inflammation and tumor progression (Rasool et al., 2021), NF-κB was knocked down as well using NF-κB-ASO.

The focus of our invasion assays was the observation of spheres of CRC cells that have been migrated from 3D-alginate beads, which represent an *in vivo*-like simulation of metastasis and invasion in an *in vitro* culture model. The emigrated CRC-colonies that had attached to the bottom of well-plates were stained with toluidine blue (Figure 4), whereas colonies that had settled on square cover glasses were analyzed by phase contrast (Figures 5A,D) or additionally stained with the before mentioned DAPI-method (Figures 5B,E), in order to demonstrate the vitality of CRC cells. Cross-methodology, based on invasion assays and statistical analysis, showed that the pro-inflammatory, multicellular TME increased both, the number of invaded colonies and the average diameter of colonies compared to the baseline control (Ba.Co.) without fibroblasts and T-lymphocytes (Figures 4A,B Figures 5A,B,D,E). The administration of β1-SO (0.5 μM) or β1-ASO (0.5 μM) alone had no significant modifying effects on the CRC-colonies in TME. Apart from that, a concentration-dependent reduction of invaded colonies and colony size by resveratrol (1, 5 μM) treatment in the TME was clearly visible, and could also be observed in concomitant treatment of resveratrol (5 μM) together with β1-SO (0.5 μM), validating β1-SO as an appropriate control agent. However, the knockdown of β1-integrin *via* β1-ASO strongly reduced the inhibitory effect of resveratrol, barely affecting colony size and number of migrated CRC cells. Even TME-treatment using a lower concentration of resveratrol (1 μM) and without β1-integrin knockdown, did show greater effects (Figures 4A,B, Figures 5A,B,D,E).

Simultaneously, resveratrol (5 μM), comparable to the knockdown of NF-κB (0.5 μM NF-κB-ASO), significantly reduced the number of CRC cell migration and their average size, highlighting resveratrol's anti-invasive effect and its ability to even intensify the inhibitory effect of NF-κB-ASO (0.5 μM). Moreover, it was found

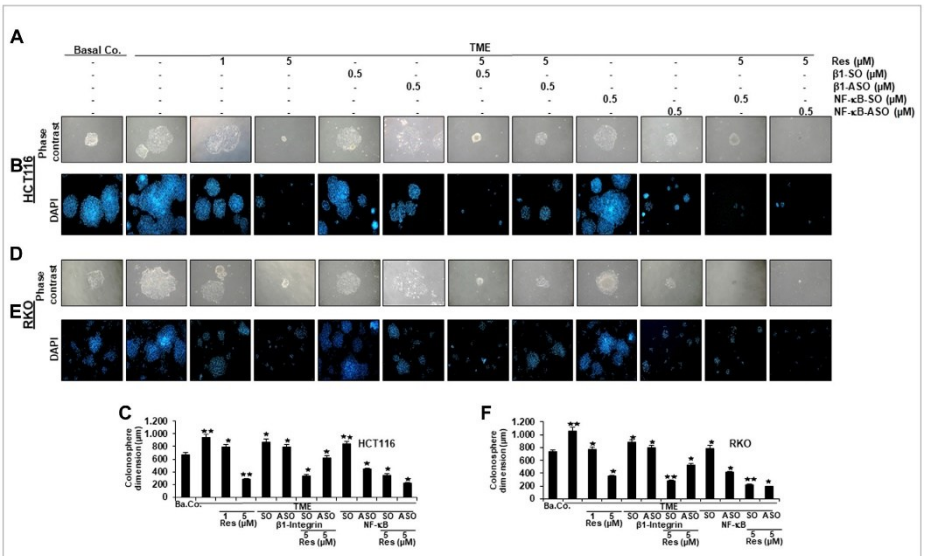


FIGURE 5
Impact of resveratrol or/and ASO against β1-integrin or against NF-κB on TME-promoted CRC cell metastasis and formation of colonospheres in alginate cultures. Serum-starved HCT116 **(A,B)** and RKO **(D,E)** cells from 3D-alginate, settled on square cover glasses as colonies under different treatment conditions: untreated (Basal Co.), TME control, resveratrol (1, 5 μM), β1-SO/ASO (0.5 μM), NF-κB-SO/ASO (0.5 μM) or combination of SO/ASO (0.5 μM) and resveratrol (5 μM). CRC cells were photographed using phase contrast (magnification ×100, Zeiss Axiovert 40 CFL microscope) and DAPI-staining (magnification ×50, Leica DM 2000 microscope). Statistical analysis for HCT116 **(C)** and RKO **(F)**: Relative to TME control, $*p < 0.05$ and $**p < 0.01$.

that there was no significant difference between co-treatment of resveratrol (5 μM) and NF-κB-SO (0.5 μM) or resveratrol (5 μM) alone on reduced inhibition of migration and invasion and their size on CRC cells (Figures 4A,B, Figures 5A,B,D,E). These findings were further supported by the quantification of colonosphere formation, migrated CRC cells and their size. All of the described results were reproducible in both cell lines, HCT116 and RKO, indicating a non-cell line specific effect. Altogether, these findings further support the idea that resveratrol uses β1-integrin receptors to repress the pro-inflammatory TME-induced migration, invasion and signaling pathway of CRC cells.

Resveratrol targets β1-integrin receptors to block TME-induced expression of metastasis-related factors and p65-NF-κB phosphorylation in CRC cells

To screen changes in protein expressions and associated modulation of various signaling pathways, we carried out extensive Western blot analyses. Samples were obtained from CRC cells (HCT116 or RKO) grown as alginate bead cultures and treated as described in Material and Methods for 10–14 days.

At first, we found that the metastasis parameters CXCR4 and phosphorylated FAK as well as inflammation parameter phosphorylated NF-κB were highly expressed in HCT116 or RKO cells grown in TME without any treatment. Interestingly, compared to TME control, same biomarkers were significantly down-regulated by resveratrol (5 μM) in TME-cultures, similar to β1-SO-cultures (0.5 μM) co-treated with 5 μM resveratrol, (Figure 6). Contrarily, in CRC cultures with β1-integrin knockdown (0.5 μM β1-ASO), no suppression of CXCR4, phosphorylated FAK and phosphorylated NF-κB could be observed by resveratrol co-treatment (5 μM) so that their expression pattern resembled the TME control (Figure 6). In addition, cultures solely treated with resveratrol (5 μM) or with resveratrol (5 μM) in combination with β1-SO (0.5 μM) clearly showed increased apoptosis, manifested by a high caspase-3 level in the TME compared to a low apoptosis rate in the untreated TME control. In β1-integrin knockdown (0.5 μM β1-ASO) cultures, however, resveratrol's anti-apoptotic effect could not be detected and a low apoptosis rate was observed again

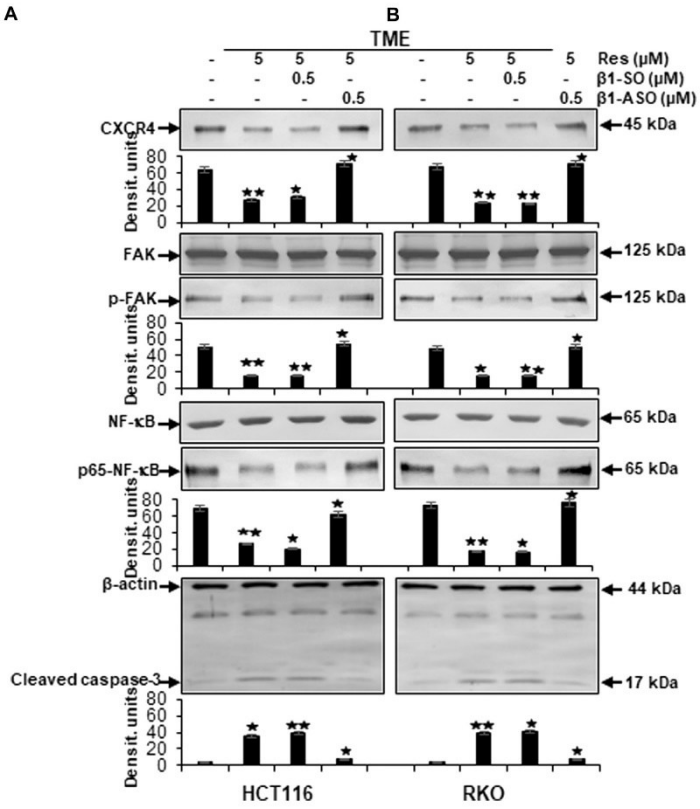


FIGURE 6
Impact of resveratrol or/and ASO against β1-integrin on TME-promoted activation of metastasis and apoptosis parameters in CRC cells. HCT116 **(A)** and RKO **(B)** derived from 3D-alginate cultures were grown untreated or treated with 5 μM resveratrol alone or in combination with 0.5 μM β1-SO or 0.5 μM β1-ASO (*x*-axis) and probed with antibodies against CXCR4, FAK, p-FAK, NF-κB, p65-NF-κB and cleaved caspase-3. Loading control: β-actin. Densitometric units complementing Western blot results (*y*-axis). For densitometric analysis, data were compared to TME control: $*p < 0.05$ and $**p < 0.01$.

(Figure 6). Our results were supported by the consistent expression of non-phosphorylated FAK and NF-κB in all of the treatments, serving as reference control, and consistent expression of β-actin serving as loading control (Figure 6).

Furthermore, to reassure the effectiveness of β1-integrin knockdown (β1-ASO) and control substance (β1-SO), or NF-κB knockdown (NF-κB-ASO) and the control substance (NF-κB-SO), we also performed immunoblots with anti-β1-integrin or with anti-p65-NF-κB that confirmed the chosen dosages (0.5 μM β1- and NF-κB-SO/ASO) in both cell lines (HCT116 and RKO), by demonstrating significant β1-integrin or NF-κB down-regulation of β1-ASO or NF-κB-ASO treated CRC cells, respectively, whereas β1-integrin and NF-κB were up-regulated in untreated TME or TME-CRC cells treated with β1-SO or NF-κB-SO (Figures 7A,B). These results, supporting our assumption that resveratrol uses β1-integrin receptors and NF-κB transcription factor to inhibit metastasis in CRC cells, encouraged us to further investigate the level of EMT-protein expression.

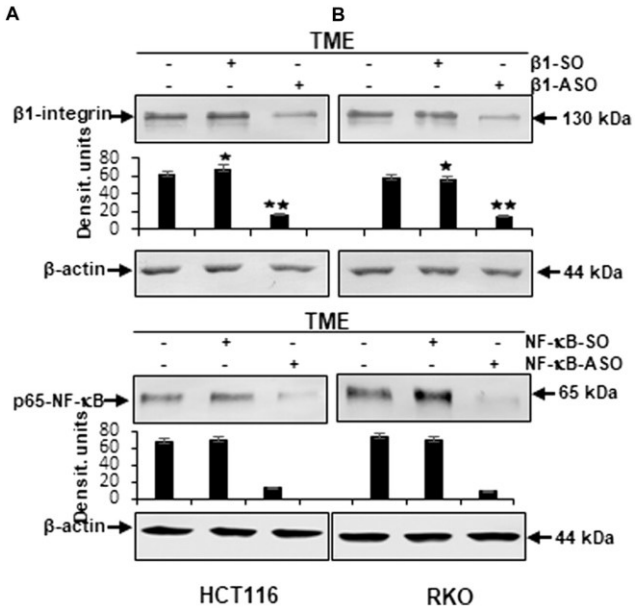


FIGURE 7
Western blot investigation on the efficacy of β1-integrin and NF-κB knockdown by antisense oligonucleotides in CRC cells. *X*-axis: HCT116 **(A)** and RKO **(B)** samples from 3D-alginate TME were untreated or treated with β1-SO/NF-κB-SO or β1-ASO/NF-κB-ASO (0.5 μM). Immunoblotting with anti-β1-integrin or with anti-p-65-NF-κB and β-actin as a loading control. *Y*-axis: Densitometric units (*y*-axis) complementing Western blot results. Statistical analysis: $*p < 0.05$ and $**p < 0.01$, comparison to TME control.

Resveratrol modulates TME-triggered inflammation, EMT, invasion, and TME-suppressed apoptosis and acts synergistically with NF-κB-ASO in CRC cells but not by knockdown of β1-integrin in CRC cells

Whether and by which pathway resveratrol can modulate TME-induced EMT, invasion, migration as well as upregulation of NF-κB, NF-κB-dependent inflammation and apoptosis, was investigated by using HCT116 and RKO CRC cells. Analogous to previously explained invasion assays (Figure 4), cells were left untreated in alginate beads as a baseline control (Ba.Co.) or in TME (TME control) or treated with different concentrations of resveratrol (1, 5 μM) or with NF-κB-SO/-ASO (0.5 μM) or with β1- SO/-ASO (0.5 μM) or with a combination of resveratrol (5 μM) and NF-κB-SO/-ASO (0.5 μM) or β1-SO/-ASO (0.5 μM) for 10–14 days as described in Methods.

Resveratrol targets β1-integrin and blocks TME-induced expression of EMT-related biomarkers in the same way as NF-κB-ASO in CRC cells

Initially, with Western blot studies of previously described cell samples we aimed to visualize EMT-reflective parameters (E-cadherin, vimentin, slug) to further elucidate the importance of resveratrol as well as its effect *via* β1-integrin receptors and the NF-κB signaling pathway.

The expression of E-cadherin, representing epithelial phenotype, was low in untreated baseline control (Ba.Co.) and untreated TME control as well as in TME culture treated with β1-SO (0.5 μM) or β1-ASO (0.5 μM) alone (Figures 8A,B). However, resveratrol when added developed a concentration-dependent (1, 5 μM), strong enhancing effect of E-cadherin expression in both, RKO (Figure 8A) and HCT116 (Figure 8B) cells, compared to cells of the TME control. As this increase was also distinct with

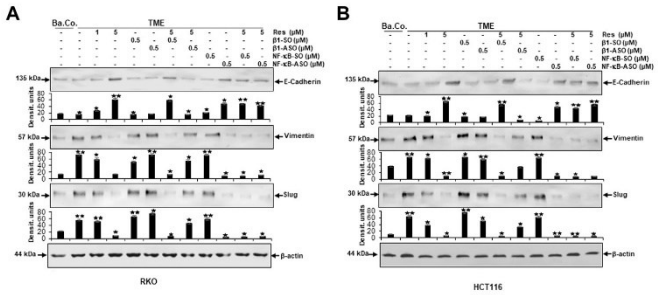


FIGURE 8
Impact of resveratrol or/and ASO against β1-integrin or against NF-κB on TME-promoted activation of EMT-linked biomarkers in CRC cells. 3D-alginate RKO (**A**) and HCT116 (**B**) CRC cells were detected against E-cadherin, vimentin, slug and loading controlled with β-actin after 10–14 days of treatment. *X*-axis: untreated (Ba.Co.), TME control, resveratrol (1, 5 μM), β1-SO/ASO (0.5 μM), NF-κB-SO/ASO (0.5 μM) or combination of 0.5 μM SO/ASO and resveratrol (5 μM). *Y*-axis: Densitometric units (*y*-axis) complementing Western blot results and for analysis, data were compared to TME control: $*p < 0.05$ and $**p < 0.01$ were considered statistically significant.

combined administration of resveratrol (5 μM) and β1-SO (0.5 μM) to the TME, β1-SO proved to be a reliable control reagent. When TME was treated with resveratrol (5 μM) and β1-ASO (0.5 μM), though, E-cadherin expression remained down-regulated because resveratrol could not exert its full epithelial-stabilizing effect in both CRC cell lines (Figures 8A,B). It was noted that treatment with NF-κB-SO (0.5 μM) did not alter the low E-cadherin level in TME. In contrast, addition of NF-κB-ASO (0.5 μM) to the TME, resulted in down-regulation of inflammatory spread and, concurrently, increased E-cadherin expression. Furthermore, resveratrol-treatment (0.5 μM) of NF-κB-SO/-ASO-TME (0.5 μM) also reduced inflammation and markedly promoted epithelial features of RKO or HCT116 cells (Figures 8A,B). The mesenchymal markers vimentin and slug showed opposite results. Both biomarkers were significantly increased in TME compared to baseline control (Ba.Co.) and not significantly affected by β1-SO/-ASO addition (0.5 μM each) in both CRC cell lines (Figures 8A,B). Impressively, resveratrol treatment led to a significant down-regulation of these parameters, which also persisted with combined treatment of resveratrol and β1-SO (0.5 μM). With β1-integrin knockdown by β1-ASO (0.5 μM), however, resveratrol (5 μM) could no longer fully exert its anti-EMT effects, what resulted in a strong increase of vimentin and slug (Figures 8A,B). Whereas the high expression of both biomarkers in TME was unaffected by treatment with NF-κB-SO (0.5 μM), both, NF-κB knockdown (0.5 μM NF-κB-ASO) and the combination treatment of resveratrol and NF-κB-SO or NF-κB-ASO lead to strong down-regulation in HCT116 and RKO, compared with TME control (Figures 8A,B). For all examinations, β-actin served as an internal control. In summary, resveratrol showed a considerable anti-EMT effect (Figures 8A,B) and, supported by the immunofluorescence results (Figures 1, 3), these findings suggest the use of β1-integrin receptors by resveratrol to exert its anti-invasion impact in CRC cells which was not cell line specific.

Resveratrol targets β1-integrin receptors, modulates TME-induced NF-κB phosphorylation and NF-κB-associated migration, metastasis and apoptosis proteins in the same manner as NF-κB-ASO in CRC cells

In the next step, same Western blot samples as used before were examined for the influence of resveratrol's unfolding effects *via* β1-integrin receptors on metastasis and apoptosis markers. In addition, the effects of NF-κB knockdown (with NF-κB/p65-subunit-ASO as outlined in Material and Methods) were also taken into account, whereby RKO (Figure 9A) and HCT116 (Figure 9B) presented similar results:

Compared to untreated baseline control (Ba.Co.), TME significantly promoted the expression of phosphorylated NF-κB (p-NF-κB), phosphorylated FAK (p-FAK), MMP-9 as well as CXCR4 in both CRC cell lines, which are all known as inflammation- and metastasis-related factors. This effect was confirmed in both, the untreated TME and the control treatments with 0.5 μM β1-SO or 0.5 μM β1-ASO alone (Figures 9A,B). Resveratrol's concentration-dependent (1, 5 μM) down-regulation of all these parameters was surprising and equally observed in the presence of the control substance β1-SO (0.5 μM). In β1-ASO (0.5 μM) treated CRC cells, resveratrol was unable to exert its effect properly though, so that the

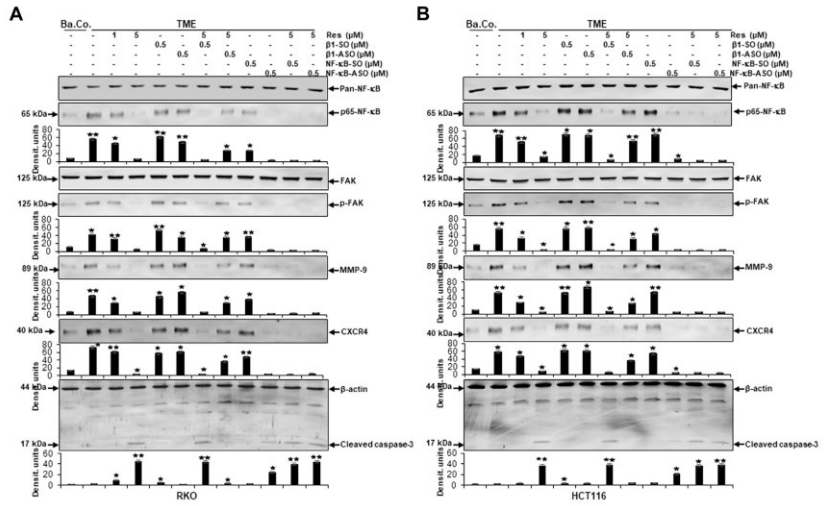


FIGURE 9
Impact of resveratrol or/and ASO against β1-integrin or against NF-κB on TME-promoted activation of metastasis- and apoptosis-linked biomarkers in CRC cells. Serum-starved RKO **(A)** and HCT116 **(B)** CRC cells, grown in 3D-alginate and differently treated for 10–14 days [untreated (Ba.Co.), TME control, resveratrol (1, 5 μM), β1-SO/ASO (0.5 μM), NF-κB-SO/ASO (0.5 μM) or combination of 0.5 μM SO/ASO and resveratrol (5 μM); *x*-axis] were investigated with antibodies against pan-NF-κB, p65-NF-κB, FAK, p-FAK, MMP-9, CXCR4 and cleaved caspase-3. β-actin served as loading control and statistical significance is shown by: $*p < 0.05$ and $**p < 0.01$ compared with the TME control. *Y*-axis: Densitometric units (*y*-axis) complementing Western blot results.

expression of metastasis-related and inflammatory biomarkers was up-regulated instead of suppressed. Furthermore, NF-κB-SO (0.5 μM) addition barely affected TME, whereas NF-κB knockdown with 0.5 μM NF-κB-ASO or combined treatment with resveratrol and NF-κB-SO or NF-κB-ASO suppressed indicators of inflammation and invasion in RKO and HCT116 (Figures 9A,B). In line with these observations, the complementary study of apoptosis presented inverse results with low caspase-3 detection in all control samples, meaning baseline control (Ba.Co.), TME control as well as TME-CRC cells treated with 0.5 μM β1-SO or 0.5 μM β1-ASO (Figures 9A,B). Also in this regard, resveratrol showed a major modulatory effect by significantly increasing caspase-3 levels in a concentration-dependent (1, 5 μM) manner, both in TME-CRC cells and in β1-SO-treated (0.5 μM) TME-CRC cells, which was inhibited by β1-integrin knockdown (0.5 μM β1-ASO), visible by very low caspase-3 expression. Although the apoptosis rate was low in TME control treated with 0.5 μM NF-κB-SO, caspase-3 expression increased by NF-κB knockdown (0.5 μM NF-κB-ASO) and was maintained at a high level by resveratrol (5 μM) in both, NF-κB-SO- and NF-κB-ASO-treated RKO and HCT116 cells (Figures 9A,B).

The results described were supported by the consistent expression of non-phosphorylated FAK and NF-κB (pan-NF-κB) in all treatments, serving as a reference control while the uniform β-actin expression functions as a loading control. Overall, these findings indicate the utilization of β1-integrin receptors by resveratrol to unfold its strong anti-metastasis effects in CRC cells. Furthermore, it is noticeable that resveratrol developed its strongly anti-apoptotic effect even beyond NF-κB knockdown, suggesting resveratrol to powerfully complement its action. In summary, all Western blot results presented (Figures 6–9) were reproducible in both cell lines, HCT116 and RKO.

Discussion

After revealing the significance of β1-integrin in the context of resveratrol's anti-viability and anti-proliferative impact on CRC cells *in vitro* in our latest work (Brockmueller et al., 2022), the present study was devoted to the role of β1-integrin in association with anti-invasive and anti-metastatic resveratrol treatment, focusing on metastasis formation to detect a

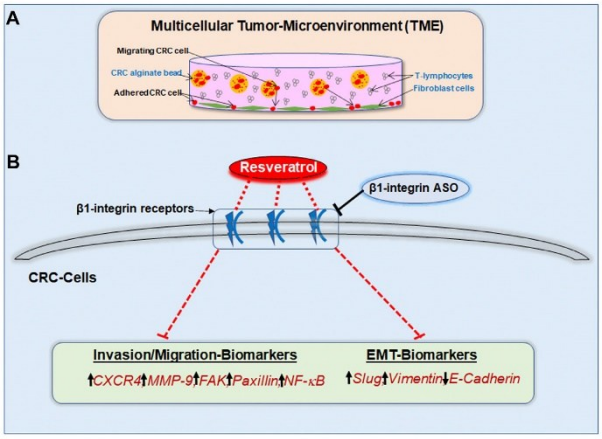


FIGURE 10
(A) Study model of the pro-inflammatory 3D-tumor microenvironment consisting of a fibroblast monolayer, floating T-lymphocytes in cell culture medium and CRC cells encapsulated in alginate. **(B)** Graphical outcome of resveratrol's anti-invasive, anti-metastasis and anti-EMT effects by using β1-integrin receptors for signal transmission. The black arrows illustrate the biomarker situation within unaffected CRC cells. The red dashed lines show the modulatory effect of resveratrol on invasion-, migration- as well as EMT-parameters *via* β1-integrin receptors. β1-integrin-ASO can weaken this effect of resveratrol through β1-integrin receptor inhibition.

potential association. The central new, and in two CRC cell lines reproducible, insights gained within our study were: 1) resveratrol uses β1-integrin receptors to shift the balance from mesenchymal to epithelial morphology in CRC cells; 2) resveratrol uses β1-integrin receptors to down-regulate invasion as well as metastasis of CRC cells; 3) furthermore, resveratrol uses β1-integrin receptors to suppress inflammation in CRC cells; 4) and finally, resveratrol synergistically amplifies NF-κB knockdown, thereby increasing the anti-inflammatory effect on CRC cells.

EMT together with its associated phenotype alteration pave the way for tumor cells to metastasize and invade peripheral tissue, serving as an indicator for severe disease progression. The EMT process, when tumor cells lose E-cadherin as epithelial organizer and instead take on a mesenchymal character, symbolized by slug overexpression, plays a strong role in CRC cells. It is known that EMT-promoting factors, such as cytokines and growth factors, are mainly generated by the cross-talk between tumor and immune cells (Buhrmann et al., 2020; Vuletić et al., 2021). Therefore, the ability of resveratrol to interrupt this exchange in our 3D-alginate model (Figure 10) represents a very important finding and simultaneously serves as encouragement for further investigation. The present results confirmed that resveratrol is able to prevent EMT-driven changes in the TME mainly by up-regulating the expression of E-cadherin and the down-regulation of the expression of multifunctional regulatory adhesion protein paxillin, intermediate filament vimentin and EMT-associated master transcription factor slug. These results confirm that paxillin as known downstream intracellular target protein of FAK, controls the interaction of integrin and extracellular ligands (Deakin et al., 2012). Interestingly, stimulation of paxillin has also been reported to alter the functional composition of focal adhesions, thereby significantly increasing cell motility (Devreotes and Horwitz, 2015). As these EMT-inhibitory properties are rendered impossible by β1-integrin knockdown, it can be assumed that resveratrol uses β1-integrin receptors in order to shift the balance from mesenchymal to epithelial phenotype in CRC cells, strongly highlighting the potential of resveratrol as an effective agent for cancer and metastasis prophylaxis *via* the utilization of β1-integrin receptors. The significance becomes even clearer in light of the fact that paxillin is considered a migration marker in CRC cells due to its direct connection to EMT (Wen et al., 2020).

Moreover, further evidence that previously described the relationship between integrin family members and EMT is provided by the finding of β4-integrin being responsible for the organization of vimentin filaments in lung cancer cells (Colburn and Jones, 2018). In addition, αvβ3-integrin is known to be a necessary essential condition for slug activation

in breast cancer cells (Desgrosellier et al., 2014). To concretely link molecular events with the clinically diagnosable course of a cancer disease, ten factors of tumorigenesis, that have become known as hallmarks of cancer (Hanahan and Weinberg, 2011), were noted by Hanahan and Weinberg further highlighting the importance of inflammation and invasion. Recently, Welch and Hurst complemented these factors by adding four hallmarks of metastasis including motility/invasion, modulated microenvironment, plasticity and colonization (Welch and Hurst, 2019), underlining their high relevance in the search of alternative treatment strategies for targeting metastatic tumors. Invasion and metastasis involve cell adhesion, cell growth, and degradation of tissue barriers and are inseparable in tumor progress (Gupta et al., 2010). Our invasion assays confirmed the already demonstrated increasement of the invasion capacity of CRC cells in TME and highlighted how this process was down-regulated by resveratrol treatment. Impressively, the metastasis-inhibiting impact of resveratrol was significantly abrogated by β1-integrin knockdown, what was further reinforced by Western blot results. Moreover, resveratrol treatment alone was able to down-regulate the metastasis-related factors including FAK, of which cascade activation is known to be integrin-dependent (Lipfert et al., 1992; Cheng et al., 2021) as well as CXCR4, shown to up-regulate αvβ6-integrin in CRC cells, thus promoting metastasis (Wang et al., 2014). Furthermore, resveratrol was able to up-regulate apoptosis-marker caspase-3, but all these effects were not detectable in β1-integrin knockdown CRC cells.

Interestingly, the down-regulation of caspase-3 by integrin knockdown in *Helicobacter pylori* infected gastric epithelial cells has been previously reported too (Li et al., 2021). Overall, it is apparent that resveratrol reduced CRC cell invasion and migration *via* β1-integrin receptors, what leads to the assumption that its signaling pathway might be a suitable co-treatment for CRC that is urgently needed, since at least 50% of CRC cases are associated with metastases (Vatandoust et al., 2015). The aforementioned hallmarks of CRC progression, EMT and metastasis, are promoted by chronic inflammatory processes (Vuletić et al., 2021) indicating the great importance of NF-κB, which is considered a major inflammatory and tumorigenesis marker (Ko et al., 2017) in its phosphorylated, thereby activated form. Our results clearly demonstrated that resveratrol suppresses NF-κB activation in pro-inflammatory TME, what is consistent with previous results (Buhrmann et al., 2020). However, a new observation found is that both, immunofluorescence and Western blot analysis, reproducibly displayed an abolition of resveratrol's anti-inflammatory effect by β1-integrin knockdown, making it obvious that resveratrol uses β1-integrin receptors to exert its inflammation-suppressing effect in CRC cells, in turn, leading to lower metastatic potential. On this background, the β1-integrin pathway represents a promising target in the fight against inflammation-based cancer.

Moreover, due to the strong association between phosphorylated NF-κB and cancer progression, we finally investigated on resveratrol's impact on CRC cells in an inflammatory environment when NF-κB was knocked down. Here, we found that NF-κB knockdown led to a decreased inflammatory spread and invasion capacity in both CRC cell lines HCT116 and RKO, whereby resveratrol treatment of NF-κB down-regulated CRC cells significantly enhanced their ability to act in a synergistic, powerful anti-inflammatory way. Based on this finding, it is worth considering the potential of resveratrol in the future with regard to the treatment of cancer and other chronic inflammatory diseases such as rheumatoid arthritis, where NF-κB activation also plays a crucial role or Crohn's disease, where dysregulation of NF-κB, physiologically necessary for intestinal homeostasis, triggers an inflammatory cascade (Nissim-Eliraz et al., 2021; Mueller et al., 2022). The fact that integrin receptors physiologically occur and are necessary in the embryonic development is also exploited by malignant cancer cells in the event of disease, thus for treatment of hepatocellular carcinoma, reduction of α2-, β1-, and β3-integrin expression has been proposed as a possible option (Relja et al., 2011). Also in neuroblastoma cells, up-regulated α2-, α3- and β1-integrin expression was shown and invasion and migration could be inhibited mainly by silencing β1-integrin (Lee et al., 2013). Moreover, the correlation between β1-integrin upregulation and the migration of triple negative breast cancer cells has been demonstrated in previous research (Schlienger et al., 2015).

Altogether, β1-integrin has already been shown to act as a fundamentally important receptor in various types of cancer in the digestive tract and other organ systems. Extremely high CRC case numbers underline the urgency to explore the exact mechanisms underlying colorectal cancer, whereby already existing studies of other cancer types support the potential of our observations made in HCT116 and RKO cells. Our findings show evidence that high β1-integrin expression in cancer cells can be considered as a promising target to be used in CRC therapy. For the first time we suggest to rather use β1-integrin receptors as promising gateways for CRC co-treatment with the bio-active phytopharmaceutical resveratrol, especially due to its anti-invasive potential.

Conclusion

The presented results shed light on β1-integrin's role in resveratrol-mediated increased E-cadherin, caspase-3 expression and decreased phosphorylated NF-κB, phosphorylated FAK, vimentin, slug, paxillin, CXCR4, MMP-9 expression (Figure 10) in HCT116 and RKO cells leading to a noticeable reduction in their invasion and metastasis activity. Resveratrol has been shown to stabilize epithelial balance and to prevent metastasis of CRC cells, whereby these effects were significantly weakened by the knockdown of β1-integrin. Overall, we conclude that resveratrol exerts its anti-

inflammatory, anti-invasive and thus cancer-inhibiting effect, to a relevant extent, *via* β1-integrin receptors. Therefore, we are convinced of the helpfulness of clinical studies to determine whether β1-integrin is suitable as a tumor marker for CRC and to emphasize the great opportunity of innovative, resveratrol-based drugs against highly metastatic CRC *via* the β1-integrin action mechanism.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

Conceptualization AB and MS; investigation AB and PS; methodology, validation and formal analysis AB and PS; writing—original draft preparation AB and MS; writing—review and editing AB, A-LM and MS; project administration MS. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Anhang: Paper III

International Journal of *Molecular Sciences*

MDPI

Article

Resveratrol Modulates Chemosensitisation to 5-FU via β1-Integrin/HIF-1α Axis in CRC Tumor Microenvironment

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Abstract: Frequent development of resistance to chemotherapeutic agents such as 5-flourouracil (5-FU) complicates the treatment of advanced colorectal cancer (CRC). Resveratrol is able to utilize β1-integrin receptors, strongly expressed in CRC cells, to transmit and exert anti-carcinogenic signals, but whether it can also utilize these receptors to overcome 5-FU chemoresistance in CRC cells has not yet been investigated. Effects of β1-integrin knockdown on anti-cancer capabilities of resveratrol and 5-FU were investigated in HCT-116 and 5-FU-resistant HCT-116R CRC tumor microenvironment (TME) with 3D-alginate as well as monolayer cultures. Resveratrol increased CRC cell sensitivity to 5-FU by reducing TME-promoted vitality, proliferation, colony formation, invasion tendency and mesenchymal phenotype including pro-migration pseudopodia. Furthermore, resveratrol impaired CRC cells in favor of more effective utilization of 5-FU by down-regulating TME-induced inflammation (NF-kB), vascularisation (VEGF, HIF-1α) and cancer stem cell production (CD44, CD133, ALDH1), while up-regulating apoptosis (caspase-3) that was previously inhibited by TME. These anti-cancer mechanisms of resveratrol were largely abolished by antisense oligonucleotides against β1-integrin (β1-ASO) in both CRC cell lines, indicating the particular importance of β1-integrin receptors for the 5-FU-chemosensitising effect of resveratrol. Lastly, co-immunoprecipitation tests showed that resveratrol targets and modulates the TME-associated β1-integrin/HIF-1α signaling axis in CRC cells. Our results suggest for the first time the utility of the β1-integrin/HIF-1α signaling axis related to chemosensitization and overcoming chemoresistance to 5-FU in CRC cells by resveratrol, underlining its potential supportive applications in CRC treatment.

Keywords: CRC; resveratrol; chemosensitisation; 5-FU resistance; β1-integrin; HIF-1α; cancer stem cells; 3D tumor microenvironment

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1. Introduction

Colorectal cancer (CRC) is defined as a malignant neoplasm of the colon or rectal epithelium, the treatment of which represents a major medical challenge worldwide [1]. Currently, in the United States of America, approximately 150,000 new cases of CRC are diagnosed annually [1]. In Germany, the number is more than 60,000, and despite numerous early detection measures and techniques, the relative 5-year survival rate in Germany is 63% for men and 65% for women [2]. After diagnosis, most patients receive chemotherapy containing first-line chemotherapeutic agents such as oxaliplatin and 5-flourouracil (5-FU) within the framework of the FOLFOX4 or FOLFOX6 therapy scheme [3–6].

Oxaliplatin is a platinum derivative chemotherapeutic agent, while 5-FU is one of the main components of the FOLFOX therapy schemes, a synthetic pyrimidine analogue that can be administered intravenously as a prodrug and exerts its effect via fluorinated

nucleotides that are incorporated into the patient's deoxyribonucleic acid (DNA) instead of the pyrimidine nucleoside thymidine [7,8]. It thus inhibits DNA replication and induces cell death in cancer cells [9]. However, in addition to the pronounced aggressiveness of CRC, recognizable by severe metastatic properties of the cells [10], it develops high resistance via activating multiple survival signaling pathways to the mono-target chemotherapeutic agents leading to complicated treatment process with lesser therapeutic outcome or success [6,7,9,11,12]. Moreover, due to the heterogeneity of this disease, it represents a major treatment hurdle, and thus, efforts are being made to explore the exact mechanism or pathways involved in the development of resistance, including dihydropyrimidine dehydrogenase, thymidylate synthase or thymidine phosphorylase signaling pathways [13–19]. At the same time, biomarkers are being sought that could indicate an unfavorable, chemoresistance-promoting course of FOLFOX therapy at an early stage, with complement compound 3 gene being suggested as an example [12]. Overcoming chemoresistance to mono-target therapies in CRC continues to be intensively explored, and an increasing amount of research is looking at combination treatment of standard anti-cancer drugs and herbal polyphenols [20]. This exciting research strategy is based on a broad, health-promoting spectrum of natural agents such as the blood-glucose-regulative action of chlorella [21] or vitamin C [22] and the anti-oxidative experience of miswak [23] or turmeric-components curcumin and calebin A [24].

Especially for the reduction of CRC-chemoresistance, potential is offered by the secondary plant compound, by the secondary plant compound resveratrol, is a well-known natural polyphenol which can be extracted from various grapes, berries and nuts [25]. Resveratrol has already been shown significant anti-inflammatory [26] and anti-tumor effects [27], especially by modulating the signaling pathways of the important pro-inflammatory nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-kB) and NF-kB-related gene cascades [28]. Consistent with the increased number of β1-integrins in CRC cells [29], which function as both cell-survival-protective adhesion and active signaling molecules, resveratrol specifically exploits these β1-integrin receptors in tumor cells by altering their expression pattern and using them as a gateway to transfer its anti-cancer effects and signals into the tumor cells [30]. Interestingly, it was previously described that resveratrol was even able to re-sensitise HCT-116R as well as SW480R CRC cells, which were already resistant to 5-FU by inducing the uptake of this chemotherapeutic agent, thereby overcoming 5-FU-resistance and inducing apoptosis in cancer cells, that would not have been targeted if treated with 5-FU alone [20]. Indeed, the development of chemoresistance is significantly influenced by cancer stem cells (CSC), which have a high renewal and differentiation potential [31].

Moreover, several biomarkers have already been used to detect CSC in HCT116 cells, and it is also known that CSC parameters can be down-regulated by resveratrol treatment in CRC cells [27]. Furthermore, the progression of CRC is also accompanied by an increase in angiogenesis factors, namely hypoxia-induced factors (HIF), as a reaction to hypoxic conditions in the tumor cells, which are responsible for the formation of a new vascular epithelium through the activation of vascular endothelial growth factor (VEGF) cascade [32]. In metastatic CRC, the intratumoral expression of HIF-1α is high as a particularly active subunit, and enables the invasive properties of CRC cells [33,34]. While there is initial evidence of resveratrol-binding HIF-1α restriction in HT-29 [35] and LoVo [36] colon carcinoma cells, thus exploring of the effects of resveratrol on HIF-1α signaling in HCT-116 and 5-FU-resistant HCT-116R CRC cells offers particular scientific interest.

In previous investigations, we have shown the role of β1-integrin receptors in the anti-viability, anti-proliferative and anti-invasive effects of resveratrol [10,30]. However, since it is not yet known whether β1-integrins are also involved in 5-FU-chemosensitisation by resveratrol, we have aimed to address this topic. In addition, we also wanted to shed light on the resveratrol/HIF-1α interaction in CRC cells and to find out whether this connection is influenced by the presence or absence of β1-integrins. Therefore, we chose a pre-clinical,

animal-free 3D-alginate tumor microenvironment (TME) culture in vitro and compared the tumor-inhibitory property of resveratrol in two CRC cell lines, HCT-116 and HCT-116R, the later represents a 5-FU-resistant variant of HCT-116 CRC cells.

2. Results

2.1. Resveratrol Increases 5-FU Sensitivity and Acts Anti-Carcinogenic via β1-Integrin Receptors in CRC Cells

The ability of resveratrol and its related analogs to increase the susceptibility to the chemotherapeutic agent 5-FU as well as to enhance its effect in CRC cells has already been demonstrated by several research groups worldwide [20,37]. Based on our previous findings that resveratrol employs β1-integrin receptors to transfer its anti-tumor signals into CRC cells [10,30], we hypothesized a connection of resveratrol's chemosensitisation via these β1-integrin receptors and cells were treated as follows:

First, basal control (CRC cells without additives), TME control (CRC cells in multicellular milieu, without additives), CRC-TME treated with 2 nM 5-FU or 5 µM resveratrol, CRC-TME treated with 2 nM 5-FU and 5 µM resveratrol, CRC-TME treated with 0.5 µM β1-SO (control compound) or 0.5 µM β1-ASO (knockdown compound). Further, CRC-TME treated with 0.5 µM β1-SO or 0.5 µM β1-ASO was supplemented with 2 nM 5-FU or 5 µM resveratrol or with a combination of both substances. Therefore, four different evaluation methods were chosen and their results are described in more detail below.

2.1.1. Reduction of CRC Cell Vitality

Compared to the basal control (Ba.Co., without fibroblasts and T-lymphocytes), HCT-116 cells were significantly more viable in the pro-inflammatory TME containing fibroblasts and T-lymphocytes. This remained when β1-SO or β1-ASO were added to the TME, confirming that these substances alone had no relevant effect on HCT-116 cells in the TME. When HCT-116 cells were treated with resveratrol (5 µM), 5-FU (2 nM) or a combination thereof (2 nM 5-FU with 5 µM resveratrol), their survival-capacity decreased markedly and it also decreased when 5-FU was added to HCT-116 cells in the β1-SO-TME or β1-ASO-TME. Resveratrol, however, was able to exert its inhibitory effect in the β1-SO-TME, but not in the β1-ASO-TME (Figure 1A), suggesting that a knockdown of β1-integrin receptors does not affect 5-FU's effect, but does predominantly restrict the anti-carcinogenic ability of resveratrol in HCT-116 CRC cells.

Then, HCT-116R cells, resistant to chemotherapeutic agent 5-FU, were investigated with the same treatments as HCT-116 cells. Besides the observation that more vital HCT-116R cells than non-resistant HCT-116 cells were measured in general, the main difference was the treatment failure of 5-FU in a TME containing HCT-116R cells and also in β1-SO-TME or β1-ASO-TME with HCT-116R cells. Surprisingly, an addition of resveratrol to TME or β1-SO-TME reduced the viability of HCT-116R cells (Figure 1B), while a knockdown with β1-ASO eliminated the anti-cancer potential and chemosensitising effect of resveratrol in these particularly combative CRC cells.

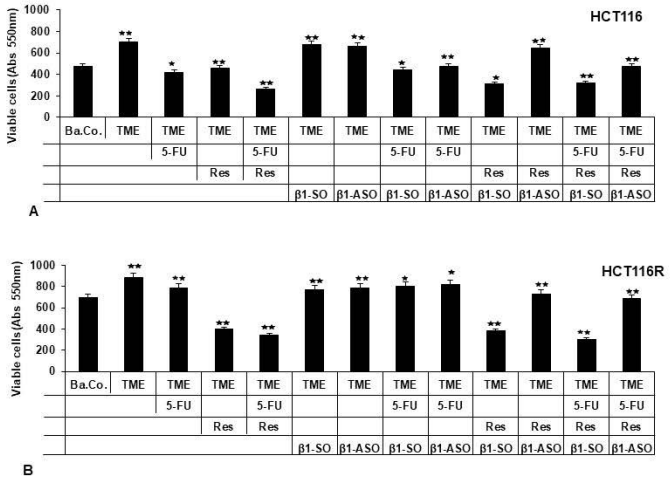

Figure 1. Resveratrol's viability inhibition and chemosensitisation to 5-FU via β1-integrin receptors in HCT-116/HCT-116R cells shown by MTT assay. *X*-axis: HCT-116 (**A**) and HCT-116R (B) cells, grown in alginate, were left untreated as basal control (Ba.Co.), as TME control (TME) or CRC cells in TME were treated with 5-FU (2 nM), resveratrol (5 μM) or β1-SO/β1-ASO (0.5 μM), alone or combined. *Y*-axis: viable CRC cells at 550 nm. Compared to TME control: $p < 0.05$ (*) and $p < 0.01$ (**).

2.1.2. Reduction of CRC Cell Colony Formation

In TME, HCT-116 cells formed considerably more colonies (black arrows) than in the basal control (Ba.Co.), which was observed to be uninfluenced by an addition of β1-SO or β1-ASO. However, the treatment of these cells with resveratrol, 5-FU, combination of 5-FU and resveratrol, 5-FU and β1-SO or 5-FU and β1-ASO visibly limited the colony formation. Furthermore, resveratrol inhibited proliferative formation of CRC cell colonies in β1-SO-TME but not in β1-ASO-TME (Figure 2A(a); "alginate"). However, when HCT-116R cells were treated with the same agents or additives, the formation of more colonies (black arrows) was noticed compared to HCT-116 cells, while the ineffectiveness of 5-FU in inhibiting colonies was also observed in TME, β1-SO-TME as well as β1-ASO-TME treatments. Interestingly, resveratrol strongly reduced the colony-forming ability of HCT-116R cells in TME as well as in β1-SO, but not in combination with β1-ASO (Figure 2B(a); "alginate"). Overall, this proliferation investigation confirmed the preceding vitality evaluation.

2.1.3. Reduction of CRC Cell Invasion

By comparison with CRC cells in the basal control (Ba.Co.), the number of migrated HCT-116 colonies was higher in TME and also in the β1-SO-TME and β1-ASO-TME. When TME was treated with 5-FU, resveratrol, 5-FU and resveratrol, the invasion capacity of HCT-116 cells was distinctly reduced, similar to an addition of 5-FU to β1-SO-TME or β1-ASO-TME. Moreover, resveratrol-treatment weakened CRC colony settling in TME with β1-SO but not in TME with β1-ASO (Figure 2A(b); "T-blue"). Further extended to 5-FU-resistant CRC cells, smaller but much more migrated colonies settled from HCT-116R cells compared to HCT-116 cells. The same treatments showed the inefficacy of 5-FU to contain HCT-116R invasional property in TME and TME with β1-SO or β1-ASO. Noteworthy, addition of resveratrol to TME or β1-SO-TME averted migration as well as formation of HCT-116R cell colonies, however this effect of resveratrol was not observed in β1-ASO-TME

treatment (Figure 2B(b); "T-blue"). These behavioural observations of CRC cell invasion confirmed the previously described MTT and colony formation assays that the β1-integrin receptor might play an important role in the resveratrol-sensitizing effect of 5-FU on CRC cell migration.

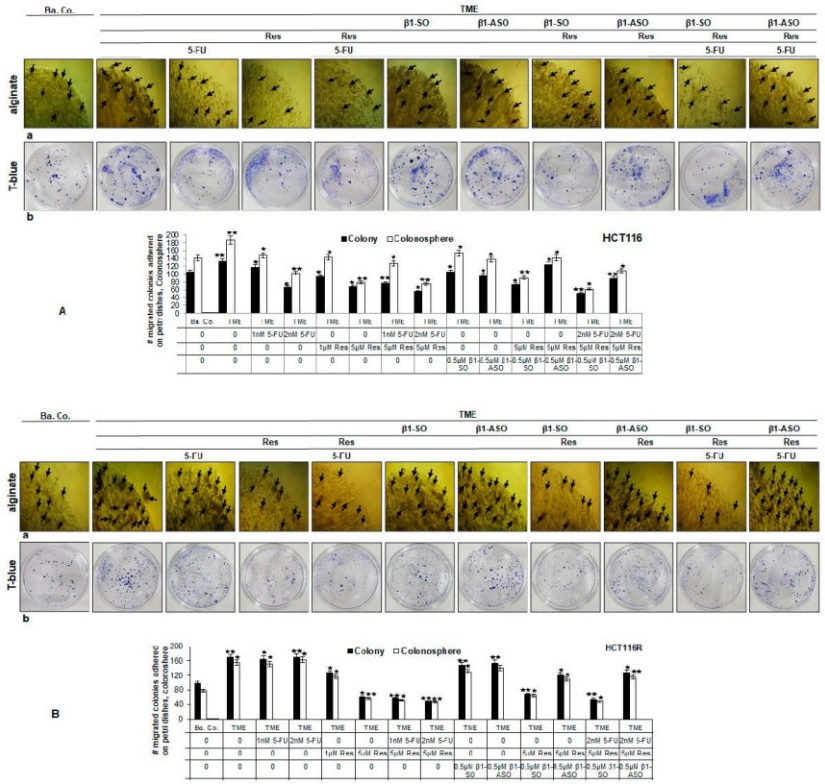

Figure 2. (**A**,**B**) Resveratrol's colony formation and migration inhibition as well as chemosensitisation to 5-FU via β1-integrin receptors in HCT-116/HCT-116R cells. HCT-116 (**A**) and HCT-116R (**B**) cells in alginate matrix maintained untreated as basal control (Ba.Co.), as TME control or TME-CRC cells were treated with 5-FU (1, 2 nM), resveratrol (1, 5 µM), β1-SO (0.5 µM), β1-ASO (0.5 µM) alone or in combination. CRC-alginate drops were photographed ((**A**,**B**), upper row each) and formed colonies were marked with black arrows. Migrated and settled CRC cell colonies were stained with toluidine blue ((**A**,**B**), lower row each). Statistical evaluation: $p < 0.05$ (⋆) and $p < 0.01$ (⋆⋆), related to TME control. White bars show the colonosphere formed in alginate drops and black bars show the bottom-settled CRC cell colonies.

2.1.4. Reduction of CRC Cell's Mesenchymal Phenotype

Both cell types, HCT-116 and HCT-116R, presented in the basal control with elongated cell bodies, planar surface with small pseudopodia on the surface and moderate cell-cell contact, representing a more epithelial morphology (Figure 3A(a,e)). However, in TME, where CRC cells have been influenced pro-inflammatory, HCT-116 as well as HCT-116R cells showed a distinctly mesenchymal shape, in that both the cell bodies and their extensions

appeared rounded, developed many thick pseudopodia on the surface with active nucleus and inclined to emigrate (Figure 3A(b,f)). A treatment with 5-FU reduced this TME-induced change in HCT-116 cells without completely restoring the appearance of the basal control (Figure 3A(c)) and occasionally led to the generation of apoptosis, (Figure 3A(c)). In HCT-116R cells, an addition of 5-FU to the TME showed only a slight effect, so that the CRC cells remained morphologically similar to the TME control (Figure 3A(g)). Contrary to this, TME-fueled CRC cells clearly responded to a treatment with resveratrol, as single additive or combined with 5-FU. In both CRC cell lines, HCT-116 and HCT-116R, the cell bodies remained rather roundish to oval, but the round cell extensions needed for migration regressed (Figure 3A(d,h)) and partially re-extended and they had an epithelial shape without pseudopodia in the slightly less aggressive HCT-116 cells in resemblance to the basal control (Figure 3A(d)). Furthermore, the observation of numerous mitochondrial changes and apoptotic bodies, which were even more visible in HCT-116R cells than in HCT-116 cells (Figure 3A(d,h)), was remarkable when CRC cells were treated with resveratrol in combination with 5-FU. To explore the role of β1-integrin in resveratrol's chemosensitising signaling, HCT-116 and HCT-116R cells in the TME were further subjected to treatment with the knockdown substance β1-ASO or the control substance β1-SO and along with resveratrol alone or combination of resveratrol and 5-FU (Figure 3B). While in β1-SO-TME resveratrol or resveratrol with 5-FU had an almost unrestricted effect of the CRC cells resulting in a smooth epithelial surface (Figure 3B(a,c,e,g)) and initiation of mitochondrial changes as well as apoptosis (Figure 3B(a,c,e,g)), where this effect was severely limited in β1-ASO treatment (Figure 3B(b,d,f,h)). Numerous TME-induced cell extensions remained, especially at combined treatment of β1-ASO and resveratrol (Figure 3B(b,f)), which was very similar to the TME control.

In the combination treatment of β1-ASO with resveratrol and 5-FU, comparatively, strong but somewhat less round cell extensions were observed (Figure 3B(d,h)). As a whole, resveratrol promotes the tendency to an epithelial-like phenotype in CRC cells, making them more susceptible to treatment with the chemotherapeutic 5-FU, and increases apoptosis initiation in HCT-116 and HCT-116R cells, which coincides with outlined results of vitality, proliferation and invasion assays. Summarising this assay, it remains to be noted that (a) HCT-116R cells were indeed predominantly resistant to treatment with 5-FU, (b) resveratrol was effective in both CRC cell types, alone or synergistically in combination with 5-FU and (c) the anti-viable, anti-proliferative, anti-invasive as well as anti-mesenchymal effect of resveratrol was largely cancelled out by the addition of β1-ASO. All in all, the results suggested an anti-carcinogenic and 5-FU-chemosensitising property of resveratrol in CRC cells at least in part via β1-integrin receptors.

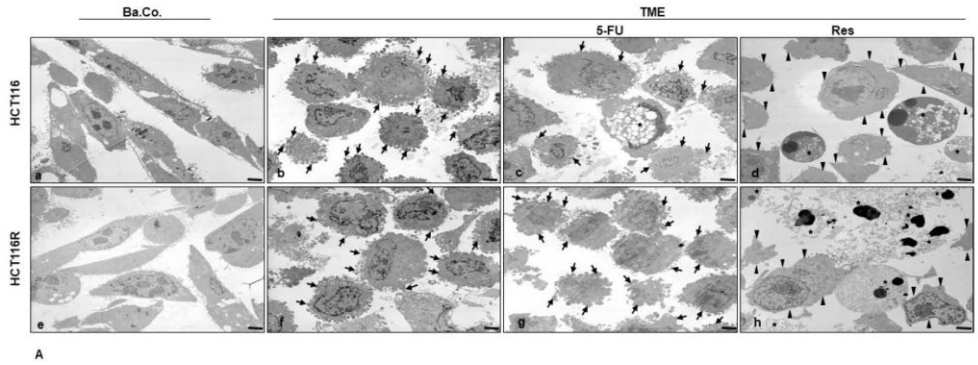

Figure 3. *Cont*

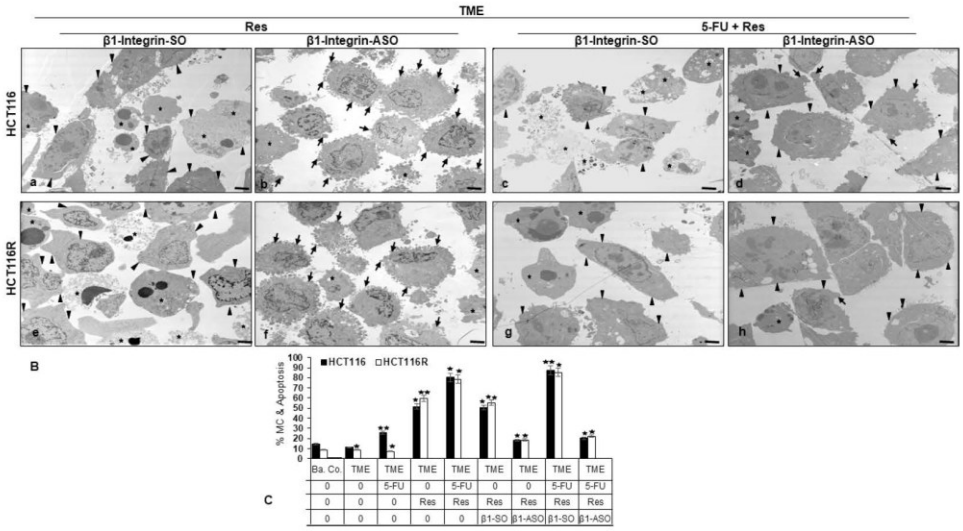

Figure 3. (**A**,**B**) Resveratrol's phenotype change, apoptosis induction and chemosensitisation to 5-FU via β1-integrin receptors in HCT-116/HCT-116R cells shown by transmission electron microscopy. HCT-116 (**A**(a–d),**B**(a–d)) and HCT-116R (**A**(e–h),**B**(e–h)) in monolayer cultures were cultivated as untreated basal control (Ba.Co.), untreated TME control (TME) or CRC cells in TME were treated with 2 nM 5-FU or 5 μM resveratrol alone or combined with 0.5 μM β1-SO or 0.5 μM β1-ASO and ultrastructural were investigated. Star: apoptosis, arrow: mesenchymal cell extensions, arrowhead: smooth epithelial cell surface. Scale bar 1 μm. (**C**) The statistic diagram illustrates mitochondrial changes (MC) and apoptosis (%) in HCT-116 (black bars) and HCT-116R (white bars) CRC cells with $p < 0.05$ (⋆) and $p < 0.01$ (⋆⋆), compared to TME control.

2.2. HIF-1α Is Involved in Resveratrol-Promoted Chemosensitising CRC Cells to 5-FU

First of all, a slightly different morphology of the two cell lines should be noted, because while HCT-116R cells proliferated more and presented as many small roundish cells with a migratory mesenchymal character, the HCT-116 were in comparison somewhat larger and more epithelially spread out. Both, HCT-116 and HCT-116R were clearly HIF-1α-marked in the basal control (Ba.Co.) containing cell culture medium only. A resveratrol addition down-regulated the HIF-1α expression unambiguously (Figure 4A). This observation was reproduced also in TME composed of floating T-lymphocytes in the cell culture medium, fibroblast monolayers on the well-plate-bottom and CRC cells on glass coverslips. While the HIF-1α expression was strong in the TME control (TME), resveratrol treated HCT-116 and HCT-116R cells showed barely HIF-1α immunolabeling. Noteworthy, a treatment of the CRC cells with the chemotherapeutic agent 5-FU was ineffective in preventing HIF-1α expression, but the combined administration of 5-FU and resveratrol led to a down-regulation of HIF-1α, thus appearing HIF-1α to be a target of resveratrol but not of 5-FU (Figure 4A).

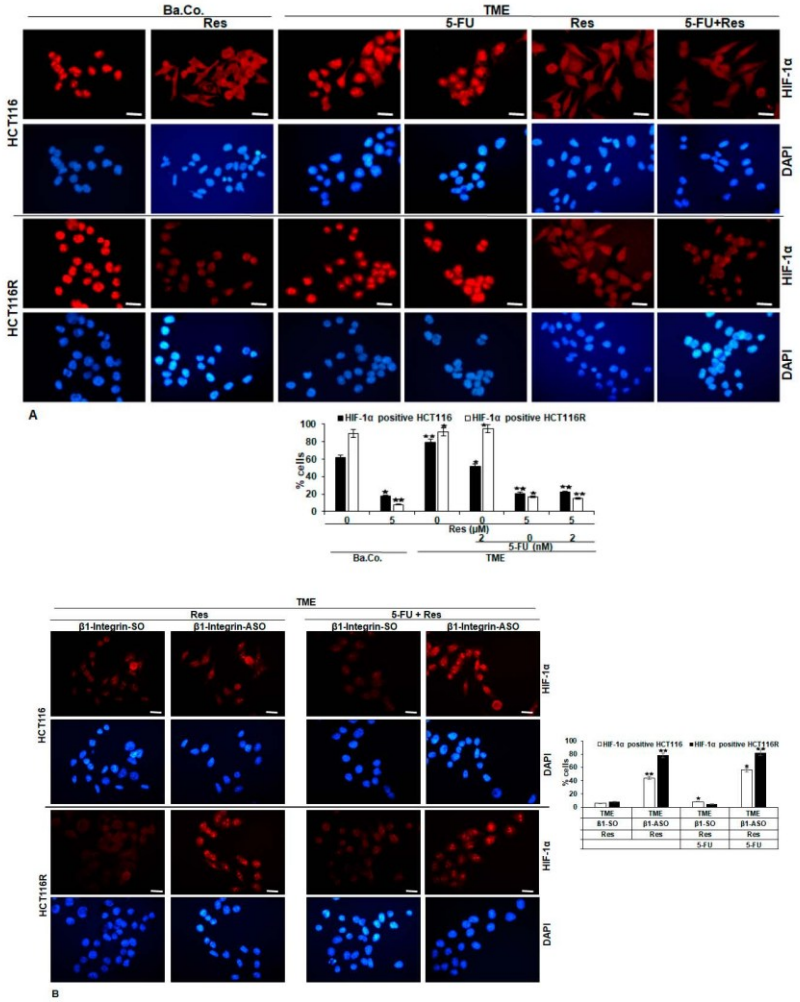

Figure 4. (**A**,**B**) Resveratrol's enhancement of chemosensitivity to 5-FU by targeting HIF-1α via β1-integrin receptors in HCT-116/HCT-116R cells shown by immunofluorescence microscopy. HCT-116 and HCT-116R were grown on glass coverslips. A basal control was left untreated or treated with 5 μM resveratrol. CRC cells in TME were left untreated or treated with 2 nM 5-FU, 5 μM resveratrol or a combination thereof (**A**). Further, CRC cells in TME were incubated with 0.5 μM β1-SO or 0.5 μM β1-ASO and treated with 5 μM resveratrol alone or 5 μM resveratrol and 2 nM 5-FU (**B**). Thereafter, HCT-116 and HCT-116R were immunolabeled with anti-HIF-1α (red) and DAPI-stained (blue). Magnification ×600, scale bar 30 μm. Statistics: $\star p < 0.05$ and $\star\star p < 0.01$.

Furthermore, investigations of the mechanistic action of resveratrol in these cell lines confirmed the anti-HIF-1α efficacy of resveratrol even in the presence of the control substance β1-SO alone as well as in the presence of β1-SO and 5-FU in HCT-116 and HCT-116R

cells (Figure 4B). Collectively, these results proposed an increased likelihood of HCT-116 and HCT-116R cells responding to 5-FU through resveratrol's ability to make the CRC cells vulnerable through the β1-integrin/HIF-1α axis.

2.3. *β1-Integrin Participated in Resveratrol-Mediated Down-Regulation of NF-kB Activation and Related Gene End Products*

Compatible with previous findings of elevated integrin values in CRC cells [38], a high β1-integrin level has been found in TME in the presence or absence of control β1-SO. But if β1-ASO was added to TME, the level of β1-integrin was down-regulated (Figure 5). All told, β1-integrin knockdown was successfully performed as transient transfection by oligonucleotides listed in Material and Methods.

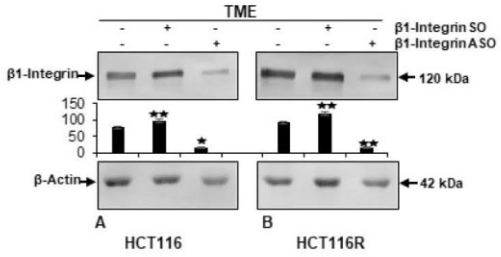

Figure 5. Effectiveness of β1-integrin knockdown by antisense oligonucleotides in HCT-116/HCT-116R cells shown by Western blot analysis. *X*-axis: HCT-116 (**A**) and HCT-116R (**B**) cells in TME-alginate were maintained without treatment or treated with 0.5 µM β1-SO (control) or 0.5 µM β1-ASO (β1-integrin knockdown). Samples were immunoblotted with anti-β1-integrin and further anti-β-actin as loading control. *Y*-axis: densitometric units. Compared to TME control, values were $p < 0.05$ (⋆) and $p < 0.01$ (⋆⋆).

For the extended examination of protein expression level, HCT-116 (Figure 6A) or HCT-116R (Figure 6B) were separated from alginate drops and immunoblotted by SDS-PAGE (Figure 6).

In both CRC cell lines, a sample verification was carried out by the uniform display of β-actin as loading control and the pan-NF-kB was equally represented as a vitality sign in all rehearsals (Figure 6A,B). Following a known, comprehensible signaling chain, the expression of phosphorylated NF-kB (p-NF-kB) as main inflammation parameter, vascularisation factor VEGF as well as CD44, CD133 and ALDH1 as cancer stem cell marker were comparable within each CRC cell line. In HCT-116 cells (Figure 6A), the expression of these markers was higher in TME-cultivated CRC cells than in the basal control and remained high when β1-SO or β1-ASO were added to the TME. With an addition of β1-SO or β1-ASO to 5-FU-treated HCT-116 cells in TME, the expression of parameters mentioned were comparative with the basal control level. Furthermore, a treatment of TME-HCT-116 cells with 5-FU, resveratrol or both agents in combination, significantly down-regulated inflammation (p-NF-kB), vascularisation (VEGF) and cancer stem cell (CD44, CD133, ALDH1) expression. However, remarkably, these anti-CRC effects of resveratrol were reversed by β1-integrin knockdown using β1-ASO, regardless of the presence or absence of 5-FU (Figure 6A). As another representative of vascularisation, HIF-1α level was investigated whereby a decisive difference became apparent. In contrast to resveratrol, 5-FU could not down-regulate HIF-1α and thus could not prevent the initiation of vascularisation. But interestingly, a dual treatment of resveratrol and 5-FU inhibited HIF-1α expression. Resveratrol's significantly suppressed HIF-1α expression which was also observed remarkably in β1-SO-TME, but not in β1-ASO-TME, regardless of 5-FU's presence or absence. In a further Western blot analysis on cleaved-caspase-3, this apoptosis marker was up-regulated in all HCT-116 cells in which resveratrol was able to unfold its

effect freely, alone or combined with 5-FU. Accordingly, an increased caspase-3 level was noticed in HCT-116 and HCT-116R cells, treated with resveratrol alone or a resveratrol-5-FU combination in TME or β1-SO-TME, but not in a β1-integrin knockdown via β1-ASO in TME (Figure 6A). The key difference in the dynamic observation of 5-FU-resistant HCT-116R cells showed that 5-FU alone or in combination with β1-SO or β1-ASO had no significant anti-inflammatory, anti-vascularising as well as anti-stemness effect (Figure 6B). This was confirmed by the observation where the expression level of all these parameters were comparatively similar to the expression in the TME control of HCT-116R cells (Figure 6B). In contrast, resveratrol alone as well as in combined treatment with 5-FU induced a strong anti-tumor effect against these parameters, both in TME and β1-SO-TME, but not in β1-ASO-TME. In summary, resveratrol reduces inflammation, vascularisation, particularly by inhibiting HIF-1α, and suppresses cancer stem cell formation and increases apoptosis in both HCT-116 and HCT-116R cells, acting chemosensitising and synergistic agent in combination with chemotherapeutic drug, 5-FU at least proportionally via β1-integrin receptors.

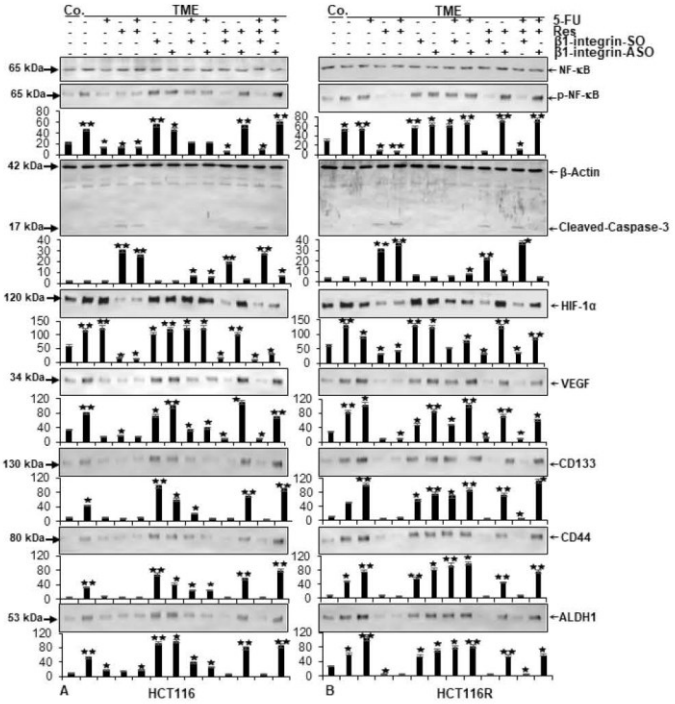

Figure 6. Resveratrol's reduction of inflammation, vascularisation as well as cancer stemness and elevation of apoptosis via β1-integrin receptors in HCT-116/HCT-116R cells shown by Western blot analysis. *X*-axis: HCT-116 (**A**) and HCT-116R (**B**) cells in alginate drops were left untreated alone (Co.) or in TME, where they were left untreated or were treated with 2 nM 5-FU, 5 μM resveratrol, 0.5 μM β1-SO, 0.5 μM β1-ASO or combinations thereof. Samples were immunoblotted with antibodies against NF-kB (unphosphorylated NF-kB), p-NF-kB (phosphorylated NF-kB), cleaved-caspase-3, HIF-1α, VEGF, CD44, CD133, ALDH1 and β-actin (loading control). *Y*-axis shows densitometric units. Relative to TME control, values were $p < 0.05$ (⋆) and $p < 0.01$ (⋆⋆).

2.4. Resveratrol Inhibits β1-Integrin/HIF-1a Axis in CRC Cells

The previous results suggested a functional molecular connection between the pathways of β1-integrin and master transcriptional regulator HIF-1α and to investigate this specifically, immunoprecipitation assay was chosen. For this purpose, HCT-116 (Figure 7A) and HCT-116R (Figure 7B) cells were cultured in alginate drops. After 10 days, CRC-samples were obtained, immunoprecipitated with anti-β1-integrin antibody and immunoblotted against HIF-1α to demonstrate the concatenation of both signaling pathways. Consistent with the known β1-integrin expression in the basal control [39], an expression of HIF-1α was analysed by densitometry. In comparison, the expression of HIF-1α in the TME control was markedly increased, consistent with the already shown high β1-integrin expression in Figure 5 and in agreement with the high HIF-1α expression in Figure 4A in both CRC cell lines. Resveratrol impressively suppressed the β1-integrin coupled HIF-1α expression in HCT-116 as well as HCT-116R cells. The uniform β-actin detection served as loading control. Overall, these results suggested for the first time an attenuation of TME-promoted β1-integrin/HIF-1α axis by resveratrol treatment, indicating the intracellular mode of action of resveratrol in inducing anti-tumor effect in CRC cells.

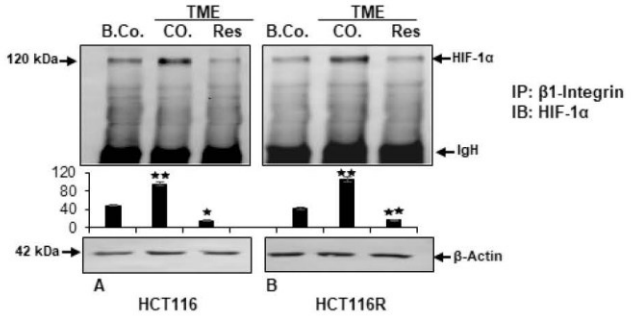

Figure 7. Resveratrol's attenuation of TME-promoted β1-integrin/HIF-1α axis in HCT-116/HCT-116R cells shown by immunoprecipitation assay. *X*-axis: HCT-116 (**A**) and HCT-116R (**B**) cells in alginate matrix alone (B.Co.) or in TME, were maintained without treatment (CO.) or treated with 5 μM resveratrol (Res). Proteins were immunoprecipitated (IP) with anti-β1-integrin. Each immunoprecipitates was fragmented by SDS-PAGE and immunoblotted (IB) with anti-HIF-1α. Initial samples were provided with anti-β-actin as loading control. *Y*-axis: densitometric units. Values were comparable to reference control, $p < 0.05$ (⋆) and $p < 0.01$ (⋆⋆). IgH means immunoglobulin heavy chain.

3. Discussion

Colorectal cancer management has benefited significantly over the past decade from the development of both target-based therapies and conventional chemotherapeutic substance, such as 5-FU, which have decisively increased the quality of patients' lives and their life expectancies [40]. However, the performance of these drugs is seriously compromised by the emergence of resistance and recurrence mechanisms which are observed in more than 50% of patients, in routine clinical practice [11]. Therefore, it is the need of the hour to design novel therapeutic compounds for the effective management of such resistant malignancies.

In the past, we demonstrated the importance of β1-integrin receptors in the anti-viability and anti-invasive action of resveratrol, as a natural chemopreventive compound on various CRC cell lines [30]. Pursuing this, the question was whether resveratrol would have a chemosensitising effect to 5-FU via β1-integrin receptors and related signaling pathways on CRC cell lines in a 3D alginate tumor microenvironment. The following core statements could be derived from the results which suggests that resveratrol enhances,

at least in part through the use of β1-integrin receptors, (I) intensifies the effectiveness of 5-FU in CRC cells, (II) paves the way for 5-FU efficacy in therapy-resistant CRC cells, (III) triggers the epithelial phenotype, (IV) targets the vascularisation marker HIF-1α and (V) specifically inhibits the association of β1-integrin with HIF-1α in 5-FU-resistant and non-resistant CRC cells.

The results of our study showed that in all the methods used, a significant containment of CRC cells in vitro was observed by treatment with resveratrol in the presence of β1-integrin. Moreover, resveratrol treatment in CRC wells also significantly enhanced the effect of 5-FU when both agents are used in combination. This finding confirms earlier scientific suspicions and hypotheses of a chemosensitising potential of resveratrol in CRC to 5-FU treatment [20,37,41] and it can be assumed that a combination application could also lead to an optimisation of patient's clinical therapy, for example through accelerated recovery or later during the process of development of resistance. Tumor cells, including CRC cells, are exposed to more severe environmental conditions such as cytokine storms related to inflammatory processes, increased oxidative and endoplasmic reticulum stress as well as hypoxia and changes in the local pH-value [42–44]. Such a complex situation was reproduced in our multicellular 3D model, simulating an advanced carcinogenic body situation without animal testing and, as in vivo, by habituation to this environment, the CRC cells become insensitive to environmental influences, including drugs.

A mesenchymal phenotype resulting from epithelial-mesenchymal transition (EMT) as consequence of a pole reversal is a hallmark of aggressive and therapy-resistant tumor cells. During the present study, we observed resveratrol's ability to change the migratory, mesenchymal and pseudopodia-rich phenotype of CRC cells to a more localised epithelial phenotype thus lowering migratory as well as invasive attendance. In contrast to 5-FU, resveratrol also operated in chemoresistant cells via β1-integrin receptors, thus repressing EMT and paving the way for 5-FU to exert its effect in HCT-116R cells. This could be a potential key component to develop complementary treatment options to control invasive stages of cancer, what is particularly important as more than 50% of patients endure metastases during the course of CRC disease [45] and these often lead to death from organ failure. These findings are in accordance with other studies that have shown that morphological changes and upregulation of intercellular junctions on the surface of cancer cells by resveratrol as a multitargeting component are strongly associated with its anti-malignant and anti-proliferation behaviour in tumor cells [20,46].

Rapidly growing cancerous mass and metastatic processes require a pronounced vascularisation and the angiogenic factor HIF-1α plays a crucial role in this process [47]. As a sensitive parameter, HIF-1α indicates milieu changes in CRC cells, generated in the event of oxygen deficiency, and induces new vessel formation [34] to increase an oxygen supply. In addition, up-regulated αvβ5-integrin or β1-integrin expression as well as NF-kB phosphorylation are associated with a strong HIF-1α increase in CRC cells [48,49]. Plant-derived polyphenols such as curcumin and its analogs could down-regulate HIF-1α expression by interrupting NF-kB phosphorylation in HCT-116 cells [50]. Moreover, HIF-1α has already been suggested as a potential target for resveratrol in various tumor types such as prostate and pancreatic cancers [51,52] or colon carcinoma [35,36], but to the best of our knowledge, the present work is the first study to demonstrate the potential of resveratrol to target HIF-1α in inducing cancer cell chemosensitisation in CRC. Thus, resveratrol could intervene the cancer cascades when the time for local intervention has already passed and the treatment possibilities in patients are limited, thereby increasing the possibilities of general healing chances for patients with advanced CRC.

Each decoding of cancer mechanisms leads to more precise therapy options, reaching the pathologically changed cells more directly and sparing the healthy cells. Thus, it is becoming increasingly interesting to use natural effects of plant ingredients as a co-treatment for various proliferative diseases. For example, inhibition of liver cancer cells by means of oxidative stress and DNA damage induced by safranel, an active component of the spice saffron, has been demonstrated [53]. Furthermore, the flavone quercetin supported

significantly the anti-cancer competence of sorafenib (a standard drug against liver cancer) in vitro as well as in vivo by down-regulation of inflammation and up-regulation of apoptosis [54]. Moreover, photosensitive compounds of the plant *Cichorium Pumilum* reduced oxidative stress as well as the activation of estrogen receptors in female Sprague-Dawley rats' breast tumors [55]. A crucial factor in chronic progressive diseases, including cancer, is the halting of inflammatory processes. This property has been demonstrated in numerous natural products such as hawthorn from *Crataegus oxyacantha* [56], dandelion from *Taraxacum officinale* [57] and also resveratrol from *Vitis vinifera* [10], that is the focus of this work, in whose detailed effect there is great interest. In establishing the mechanism of action of resveratrol, its effect on tumor suppressor gene p53 was already ruled out in previous study [58]. But interestingly, an in vivo investigation emphasised resveratrol's significant anti-carcinogenic effect through suppression of oncogenic Kras expression. In this context, the authors describe a 60% inhibition of CRC incidence in mice with activated Kras mutation by supplementing 150 or 300 ppm resveratrol (which in humans would correspond to a dose of 105 and 210 mg) for nine weeks [59]. And now, through this study, we have identified the effect of resveratrol through the β1-integrin signaling pathway as the fulcrum of numerous anti-CRC mechanisms such as proliferation and invasion, and appropriately, showed the importance of β1-integrin receptors in angiogenesis via targeting HIF-1α, and also in chemosensitisation to 5-FU. Especially in view of the fact that β1-integrin is abundant in CRC cells [29], this finding may be helpful in the future for the therapeutical or concomitant use of resveratrol and conventional chemotherapeutics in CRC management.

4. Materials and Methods

4.1. Trial Substances

The monoclonal antibodies against phospho-p65-NF-kB (#MAB7226), p65-NF-kB (#MAB5078) as well as polyclonal anti-cleaved-caspase-3 (#AF835) were acquired from R&D Systems (Heidelberg, Germany), while monoclonal antibody against β1-integrin (#14-0299-82) was from Thermo Fisher Scientific (Langenselbold, Germany). Monoclonal antibody to β-actin (#A4700), resveratrol, 5-FU, alginate, DAPI, MTT reagent and Fluoromount were purchased at Sigma-Aldrich (Taufkirchen, Germany). Monoclonal anti-HIF-1α (sc-13515), anti-VEGF (sc-7269) and normal mouse IgG were from Santa Cruz (Dallas, TX, USA). Anti-CD44 (ab243894) and anti-CD133 (ab278053), both monoclonal, were from Abcam PLC (Cambridge, UK) and anti-ALDH1 (A248522) was bought as monoclonal antibody from Acris Antibodies GmbH (Herold, Germany). Alkaline phosphatase-linked Western blot antibodies were from EMD Millipore (Schwalbach, Germany) and rhodamine-coupled secondary immunofluorescence antibodies were obtained from Dianova (Hamburg, Germany), while Epon was purchased from Plano (Marburg, Germany). Resveratrol (100 mM in ethanol) and 5-FU (1000 µM in ethanol) were prepared as stock solutions. Both were finally diluted in cell culture medium without exceeding an ethanol concentration of 0.1% during the CRC cell treatment.

4.2. Cell Types and Conditioning

HCT-116 (human CRC cells) were obtained from European Collection of Cell Cultures (Salisbury, UK). Chemoresistant tumor cells were produced from these cells by repeated and long-term treatment with 5-FU, and are hereafter referred to as HCT-116R [60]. MRC-5 (human fibroblasts) were from the same institute and additionally, Jurkat cells (human T-lymphocytes) were acquired from Leibniz Institute (Braunschweig, Germany). The preparatory cell culture has already been described in detail [30], as well as the composition of the Dulbecco's Modified Eagle medium/F-12 cell culture medium from Sigma-Aldrich (Taufkirchen, Germany), which was used with 10% fetal bovine serum (FBS) as growth medium or with 3% FBS as experimental medium [10].

4.3. Knockdown of β1-Integrin

Transient transfection was performed, as described earlier [10], with phosphorothioate-specific oligonucleotides from Eurofins MWG Operon (Ebersberg, Germany), incubated in Lipofectin transfection reagent from Invitrogen (Karlsruhe, Germany). The sequences used were β1-integrin-ASO (5′TAGTTGGGGTTGCACTCACACA3′) as antisense oligonucleotide (ASO) and β1-integrin-SO (5′TGTGTGAGTGCAACCCCAACTA3′) as sense oligonucleotide (SO).

4.4. Alginate Drop Preparation

Alginate drops were formed, following a method, shown in numerous previous work [30,61]. HCT-116 or HCT-116R were passaged, resuspended in sterile alginate (2% in 0.15M NaCl) and dripped into CaCl2 (100 mM) for polymerization. Afterwards, the resulting CRC-alginate drops were washed with 0.15M NaCl threefold and twofold with cell culture medium (10% FBS). Then, they were incubated in serum-starved cell culture medium containing 3% FBS for 30 min and to start the trial, CRC-alginate drops were transferred with bent tweezers to prepared 12-well-plates.

4.5. Carcinogenic Tumor Microenvironment

To illuminate β1-integrin's role in overcoming chemoresistance through resveratrol, a pro-inflammatory, vivo-near TME was constructed in vitro and treated differently. Therefore, CRC-alginate drops were prepared as described before and placed in experimental 12-well-plates with serum starved cell culture medium (3% FBS), which were changed every second day. The treatments for 10–14 days were as follows: Firstly, a basal control (alginate drops without TME) and a TME control for self-check. Then, concentration-dependent treatments with resveratrol (1, 5 µM) or 5-FU (1, 2 nM) or transfection with β1-ASO/SO (0.5 µM) and finally combinations thereof. Here, TME represents a multicellular, pro-inflammatory composition with a fibroblast-monolayer (MRC-5) on the bottom of the well, floating T-lymphocytes (Jurkat) suspended in the cell culture medium and added CRC-alginate drops. This 3D tumor study model has already been used by our group [10,30,61] as well as, in a similar way, by other research teams [62].

4.6. Vitality Assay

Vitality of CRC cells and thus indirect proliferation was detected by detaching the CRC cells from alginate and performing a MTT assay as previously described [30]. Briefly, the CRC-alginate drops were removed from 12-well-plates with bent tweezers and washed in Hank's salt solution, ensuring that only CRC were examined. Then, CRC cells were extracted from alginate drops by dissolving with sodium citrate. Afterwards, they were washed in Hank's solution and resuspended in MTT culture medium consisting 3% FBS, but without vitamin C and phenol red. After adding MTT solution and stopping the reaction after 3 h, the Optical Density of samples was measured by Bio-Rad ELISA reader (Munich, Germany) at 550 nm (OD 550).

4.7. Proliferation Assay

In order to document the proliferation of HCT-116 and HCT-116R, developed CRC cell colonospheres in alginate drops were photographed with a Zeiss Axiovert 40 CFL (Oberkochen, Germany) phase contrast microscope after 10–14 days of treatment. The images were stored digitally as already done earlier [10,30].

4.8. Invasion Assay

CRC cell colonospheres, that had formed in the alginate drops, emigrated from alginate and settled as colonies to the bottom of the 12-well-plates. These colonies were stained with toluidine blue after fixation with Karnovsky solution and stained colonies were manually counted (three independent trials of each treatment), whereas HCT-116 or

HCT-116R colonies were certainly distinguishable from fibroblast monolayer, as already published [10].

4.9. Immunofluorescence Microscopy

HCT-116 or HCT-116R, grown as monolayer on glass coverslips, were treated 4 h in a modified TME, the detailed procedures were described in our last papers [10,30,61,63], consisting of MRC-5 monolayer on the bottom, floating Jurkat cells in cell culture medium (3% FBS) and small mesh bridges for placing the glass coverslips in 6-well-plates. Afterwards, they were fixed in methanol and prepared for immunofluorescence microscopy as also already been described in detail [30,63,64]. The slides were immunolabeled with a primary antibody against HIF-1α (dilution 1:80), processed with a described secondary antibody (dilution 1:100), DAPI-stained for the assurance of CRC cell vitality and fixed in Fluoromount. Immunofluorescence images were taken with a Leica (Wetzlar, Germany) DM 2000 microscope and related LAS V4.12 software.

4.10. Electron Microscopic Evaluation

To investigate ultrastructural changes of CRC cell lines, HCT-116 and HCT-116R were cultivated according to the same experimental set-up as described in the Section 4.9. Afterward, the glass coverslips covered with CRC cells were fixed in Karnovsky solution for 1 h, transferred into a tube with a cell scraper and fixed in osmium tetroxide (OsO4) for 2 h. Then, the dehydration with an ascending series of alcohols and embedding of the cells with Epon were followed as described earlier [31,65]. With a Reichert-Jung Ultracut E (Darmstadt, Germany), samples were prepared, then contrasted with 2% uranyl acetate/lead citrate solution and evaluated with a transmission electron microscope (TEM) 10 from Zeiss (Jena, Germany).

4.11. Western Blot Analysis

For immunoblotting, alginate drops with HCT-116 or HCT-116R cells were removed from 12-well-plates with bent tweezers after 10–14 days of treatment. To ensure that Western blot samples contained only CRC cells, CRC-alginate drops were washed as described in the Section 4.6. After dissolving in sodium citrate (55 mM) and freeing from alginate residues, CRC cells were resuspended in lysis mix, centrifuged and the supernatant was frozen at −80 °C. The specimens were processed as described earlier [31,61,63]. We used mentioned primary antibodies and secondary antibodies in dilution 1:10.000 [30]. Samples were blotted with a transblot apparatus from Bio-Rad (Munich, Germany) and densitometric values were evaluated with related "Quantity One" analysis software. β-actin was used as a loading control.

4.12. Immunoprecipitation Assay

To investigate the functional relationship between β1-integrin and HIF-1α signaling pathways, CRC cell samples were obtained as described in Section 4.11. Afterwards, they were incubated with 25 µL of normal mouse or rabbit IgG serum and Staphylococcus aureus to preclear, treated with primary antibody against β1-integrin at 4 °C for 2 h and incubated with Staphylococcus aureus at 4 °C for 1 h again according to a proven method [64]. Samples were separated by SDS-PAGE with the explained Western blot technique and apparatus, and anti-HIF-1α antibody was used for this experiment.

4.13. Statistical Evaluation

Three independent repetitions were performed from all assays and analyzed by unpaired student's *t*-test. The results matched by ANOVA (one-way) followed by a post hoc test to compare group parameters. At all outcomes, a p-value < 0.05 was considered as statistically significant.

5. Conclusions

The current results demonstrate an inhibition of growth, viability and pathological morphological changes and thus both a chemosensitisation of non-5-FU-resistant HCT-116 cells and an overcoming of chemoresistance in 5-FU-resistant HCT-116R cells by treatment with resveratrol along with β1-integrin receptor. For this purpose, resveratrol not only acts as pro-apoptotic (caspase-3) but also induces its effect against inflammation (NF-kB), vascularisation (VEGF), and cancer stem cells (CD44, CD133, ALDH1), and also targets the β1-integrin/HIF-1α axis that is highly pronounced in CRC cells. In summary, resveratrol represents a multifunctional polyphenol that could complement the therapy options of advanced, metastatic or 5-FU-resistant CRC in the future.

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