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Resveratrol induces chemosensitization to 5-fluorouracil through up-regulation of intercellular junctions, Epithelial-to-mesenchymal transition and apoptosis in colorectal cancer

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1. Introduction

Colorectal cancer (CRC) ranks among the third most frequent cancer in the world, affecting both men and women [1]. There is a tremendous clinical interest in improving therapeutic management of primary CRC, as well as advanced, metastatic cancers after surgery or chemotherapy, since ~50% of cases develop chemoresistance to standard of care chemotherapeutic drugs such as 5-fluorouracil (5-FU) [1,2]. Metastasis of tumor cells presents a malignant event that leads to spreading of cancer cells to other tissues, and often results in increased mortality [3]. In addition, conventional chemotherapies associate with

Abbreviations: AT, ambient temperature; BSA, bovine serum albumin; CRC, colorectal cancer; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial-mesenchymal transition; FBS, fetal bovine serum; IKK, IκB kinase; 5-FU, 5-Fluorouracil; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol 2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; PARP, poly(ADP-Ribose) polymerase; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl-fluoride; Sirt1, Sirtuin-1; SDS–PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; TEM, transmission electron microscope; TGF, transforming growth factor.

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severe side effects, including abdominal pain mucositis, as well as high expense [4]. These limitations highlight the need for development of novel anti-tumor agents that may enhance chemosensitivity of tumor cells and suppression of metastasis.

It has been reported that intercellular communication through junctional complexes, such as desmosomes, tight and gap junctions increases intercellular adhesion, thereby stabilizing tissue homeostasis [5,6]. In contrast, loss of such intercellular communication closely correlates with increased metastatic potential, acts as a promotor for tumor cell proliferation, detachment and invasion [5,7]. Epithelial cells in the intestine form a barrier separating the external environment from the inside of the body. Adherence junctions and desmosomes play a critical role in cell–cell adhesion in enterocytes and provide an adhesive force to ensure the integrity of the cellular layer, whereas tight junctions seal off these compartments [8]. These functional structures have a similar organization, whereby transmembrane adhesive components are bound to cytosolic adapter proteins providing a link to the cytoskeleton [9]. Tight junctions are composed of proteins from the claudin, occludin and IgG-like family of junctional adhesion molecules [10,11]. However, claudins are the determining structures for barrier properties [12]. Claudins are transmembrane proteins that contain two extracellular domains and one intracellular domain with N- and C-termini facing the cytoplasm [13]. Expression of claudin −1, −2, −3, −4, −7, −8, −12 and −15 have been identified in the colon, whereby in human colorectal cancer, claudin-1, −2, −3, −4, −7 expression levels have been found to be significantly upregulated [12,14–16]. Moreover, the expression of claudins is regulated by many factors, including epithelial–mesenchymal transition (EMT)-related transcription factors such as Snail, hormones and various cytokines [17–19]. In general, most claudins are epithelial tight junction proteins that are down-regulated by EMT factors such as Snail and Slug [3]. Recently, it has been recognized that cytoskeletal reorganization, acts as a mediator of physiological and pathophysiological tight junction regulation [8].

A hallmark for EMT is the functional loss of E-cadherin, which is associated with loss of adherence between the epithelial cells converting them to become more motile, invasive and acquire more mesenchymal characteristics [20,21]. Loss of polarity during EMT is associated with loss of tight junctions, a process which is thought to be mediated by Snail and Slug independently of E-cadherin regulation [22,23]. Indeed, enhanced tight junction formation has been observed only in weakly metastatic tumor cells, whereas highly metastatic cancer cells have markedly poor expression of tight junctions [5].

It is well recognized that the majority of solid and hematopoietic cancer cells have constitutively active NF-κB [24]. NF-κB plays an important role in cell proliferation and malignant transformation in different cells, through its binding to DNA target sites as homo- or heterodimers and by influencing downstream gene expression [25,26]. Pro-inflammatory cytokines, chemotherapeutic agents and radiation therapy, which induce apoptosis, also activate NF-κB and thus may mediate chemoresistance and radioresistance of cancer cells [27].

The natural polyphenol Resveratrol (*trans*-3,5,4′-Trihydroxystilben) is found in the skin of grapes, various berries and nuts, and possesses a wide spectrum of pharmacological properties, including anti-tumor metastasis activities [28,29]. Resveratrol is a potent natural activator of Sirtuin-1 (SIRT1), a nucleus related histone deacetylase class III [30]. Resveratrol further inhibits IκB kinase (beta)-mediated NF-κB activation [31–33]. In addition, several studies have shown that in cancer, resveratrol induces cell cycle arrest and apoptosis [34–36], inhibits EMT-associated cancer cell migration and invasion through the inhibition of the PI-3K/Akt/NF-κB signaling pathway, inhibition of TGF-β1 and inhibition of hedgehog signaling pathway [29,37,38].

In the present study, we investigated the effect of resveratrol individually, and in combination with 5-FU on various growth regulatory parameters and extensive characterization of underlying mechanisms in a series of CRC cell lines. Our data firstly reveal that the up-regulation of intercellular junctions and MET by resveratrol, via down-regulation of NF-κB activation is one of the principle mechanisms of inhibition of tumor growth and invasion, thereby sensitizing CRC cells to 5-FU and potentiating apoptosis. These findings highlight the potential possibility of using such natural, safe and relatively inexpensive compounds as potential adjunctive treatments in improving the overall treatment response of patients with CRC in future.

2. Material and methods

2.1. Antibodies

Polyclonal anti-claudin-2 was obtained from Abcam PLC (Cambridge, UK). Polyclonal antibodies to active caspase-3 were from R&D Systems, Inc., (Heidelberg, Germany). Monoclonal anti-pan-IκBα, anti-E-cadherin, anti-vimentin and anti-Slug were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal poly(ADP-ribose) polymerase (PARP) antibodies were purchased from Becton Dickinson (Heidelberg, Germany). Monoclonal anti-β-actin antibody was purchased from Sigma-Aldrich Chemie (Munich, Germany). Anti-phospho-specific p65 (NF-κB) /(Ser536), anti-phospho-specific p50 (NF-κB) and anti-phospho-specific IκBα (Ser-32/36) were obtained from Cell Technology (Beverly, MA, USA). Anti-IKK-α and anti-IKK-β antibodies were obtained from Imgenex (Hamburg, Germany). Alkaline phosphatase–linked sheep anti-mouse and sheep anti-rabbit secondary antibodies for immunoblotting were purchased from EMD Millipore (Schwalbach, Germany). Secondary antibodies used for fluorescence labelling were purchased from Dianova (Hamburg, Germany). Gold particle-conjugated secondary antibodies were purchased from Amersham (Braunschweig, Germany).

2.2. Growth media, chemicals and cytokines

Cell culture growth medium consisting of Dulbecco's modified Eagle's medium/Ham's F-12 (1:1), 10% fetal bovine serum (FBS), 1% amphotericin B solution, 1% penicillin streptomycin solution (10,000 IU/10,000 IU), 75 μg/ml ascorbic acid, 1% essential amino acids and 1% glutamine was obtained from Seromed (Munich, Germany). Epon was obtained from Plano (Marburg, Germany). Alginate and 5-Fluorouracil (5-FU) were purchased from Sigma (Munich, Germany). Resveratrol with purity greater than 98% was purchased from Sigma. A 100 mM stock solution of resveratrol (molecular weight 228.2) was prepared in ethanol and further diluted in cell culture medium to prepare working concentrations. The maximum final content of ethanol in cultures was less than 0.1%. This concentration was also used as a control. A 100 mM stock of 5-FU was prepared in absolute DMSO and stored at −20 °C. For treatment, the 5-FU stock solution was diluted in DMEM/F12 and added to cultures to achieve the desired concentration. The final concentration of DMSO was less than 1% of drug treatment. For treatment, 5-FU was diluted in DMEM and added to cultures to give the desired final concentration.

2.3. Cell lines and cell culture

Human HCT116 CRC cells were obtained from the European Collection of Cell Cultures (Salisbury, UK). SW480 CRC cells were purchased from ATCC. We also generated 5-FU resistant derivatives

of these cell lines, referred to as HCT116R and SW480R respectively, which were established through repetitive treatment of the parental cell lines to increasing concentrations of 5-FU over a 10–12 month period, as described previously [39]. Both the parental and 5-FU resistant derivative cell lines were used to investigate the efficacy of individual and combined 5-FU and resveratrol treatments. The cells were maintained in tissue culture flasks in growth medium and in a humidified incubator at 37 °C in an atmosphere of 95% air and 5% CO_2. The medium was changed every three days, and cells were passaged using Trypsin/EDTA.

2.4. Alginate culture and Experimental design

A detailed description of the cell cultivation in alginate is given by Shakibaei and de Souza [40] and [41]. Briefly, the pellet of HCT116, HCT116R, SW480 and SW480R cells (1×10^6/ml) was resuspended in alginate (2% in 0.15 M NaCl) and drop wise added into a solution containing 100 mM $CaCl_2$ at ambient temperature (AT). The alginate beads polymerized in the presence of $CaCl_2$ after 10 min. Subsequently, the $CaCl_2$ solution was removed and the alginate beads washed three times with 0.15 M NaCl solution and twice with serum-starved medium (3% FBS). In this study, the experiments were performed on human CRC cells and were specifically designed to mimic cellular events that occur in the clinical condition of colorectal cancer. Alginate beads were left untreated, treated with various concentrations of resveratrol (0.1, 1, 5, 10, 20 μM), 5-FU (0.01, 0.1, 1, 10 nM) or the combinatorial treatment with resveratrol/5-FU (5 μM/0.01 nM or 5 μM/0.1 nM) in serum-starved medium.

Additionally, in another set of experiments, for investigation of claudin-2, E-cadherin, slug and vimentin expression, NF-κB translocation and IκBα phosphorylation, alginate cultures of HCT116 and HCT116R cells were either left untreated, treated with resveratrol (5 μM) or treated with 5-FU (0.01, 0.1, 1 nM) alone or co-treated with 5 μM resveratrol and with 5-FU (1 nM) for 10 days. Whole cell lysate and nuclear extracts were prepared. These experiments were performed in triplicate, and the results are provided as mean values from three independent experiments. The medium was changed every 3 days.

2.5. Cell proliferation and viability assay (MTT assay)

HCT116, HCT116R, SW480 and SW480R cell lines (1×10^6/ml) were cultured in alginate beads in petri dishes, treated with or without different concentrations of individual resveratrol or 5-FU to obtain IC_{50} value of each drug after 21 days culture. In a separate set of experiments, CRC cells were co-treated with 5 μM resveratrol and with different concentrations of 5-FU (0, 0.01, 0.1 nM) for 21 days. To examine the cell viability of colorectal cancer cells in alginate bead culture, cells were retrieved from alginate and measured using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). To release the CRC cells from the alginate, alginate beads were washed two times with PBS and dissolved in 55 mM sodium citrate solution. To remove excess alginate, cells were centrifuged, washed twice with sterile PBS and re-suspended in 320 μl modified cell culture medium (DMEM without phenol red, without ascorbic acid and only 3% FBS). Subsequently, in triplicate, 100 μl of cell suspension was distributed in each well of a 96-well-plate, and in each well 10 μl MTT solution (5 mg/ml) was immediately added and the plate was incubated for 4 h at 37 °C. Finally, 100 μl of the MTT solubilization solution (10% Triton × -100/acidic isopropanol) was added per well, and the cells incubated overnight at 37 °C. Metabolically active cells were evaluated by measuring the Optical Density at 550 nm (OD550) using revelation 96-well multiscanner plate ELISA reader (Bio-Rad Laboratories Inc. Munich, Germany). The results obtained were represented as surviving cells relative to controls. Effects of drugs combination used in this investigation were calculated using the combination index (CI) by Chou-Talalay equation: CI = (D) 1/(Dx) 1+(D) 2/(Dx) 2 [42,43] at 50% inhibitory concentration to show synergism between resveratrol and 5-FU. Based on the theory of Chou-Talalay, CI is defined additive effect as CI = 1, synergism as CI < 1, and antagonism as CI > 1 in two drugs combination. This experiment was repeated 3 times independently, and statistical analysis was done to obtain the final values.

2.6. Cell invasion and migration assay

The cell invasion and migration ability was evaluated using alginate beads. For invasion assay, 1×10^6/ml cells were cultured in alginate beads in petri dishes, treated with or without different concentrations of individual resveratrol (5 μM) or 5-FU (0.01, 0.1, 1 nM) or combined (5 μM/0.01 nM) treatment. After incubation for 1, 8, 11, 15, 18 and 22 days, noninvasive cells and alginate beads were removed from the petri dishes, and migrated cells adhered and formed colonies on the bottom of the petri dish were stained with toluidine blue for 5 min and carefully washed two times with PBS and photographed under the light microscope (Zeiss, Germany). The number of migrated and positive stained adhered colonies were quantified and evaluated manually by counting all colonies.

2.7. Transmission Electron Microscopy

The alginate cultures were fixed for 1 h in Karnovsky's fixative followed by post-fixation in a 1% O_sO_4 solution in phosphate buffer, as previously described [40]. After rinsing and dehydration in ascending alcohol series, the samples were embedded in Epon and ultrathin sections prepared with a Reichert-Jung Ultracut E (Leica, Darmstadt, Germany). Ultrathin sections were contrasted with 2% uranyl acetate/lead citrate and examined under FEI Tecnai12 transmission electron microscope operated at 120 kV. Digital images were taken with an OSIS Megaview III CCD camera or Carl Zeiss Microscopy TEM 902, operated at 80 kV, images were digitized using a slow-scan charge-coupled-device camera (Pro Scan; Scheuring, Germany) or Jeol 1200 EXII, operated at 80 kV, Akishima Tokyo, Japan (Department of Anatomy and Cell Biology, Munich, Germany).

2.8. Immunoelectron microscopy (pre-embedding technique)

Detection of E-cadherin and claudin-2 expression on HCT116 and HCT116R cells by pre-embedding immunogold labeling was performed as previously described [44]. Cells were treated with anti-E-cadherin or -claudin-2 for 10 min at a concentration of 1:50 in cell culture medium, followed by treatment with 10 nanometer goat anti-rabbit/anti-mouse gold-conjugated secondary antibodies for additional 10 min at AT (diluted 1:50 in growth medium). Cells were fixed with 2% glutaraldehyde for 5 min and postfixed in 1% O_sO_4 solution for 5 min. After washing with PBS and dehydration in a series of ethanol, pellets were embedded in Epon and ultrathin sections were contrasted with a mixture of 2% uranyl acetate/lead citrate. Sections were examined under a Zeiss 10 transmission electron microscope (Institute of Pharmacology, Berlin, Germany).

2.9. Quantification of apoptotic cell death

Ultrathin sections of the alginate samples were prepared and evaluated with a TEM. To quantify the apoptotic CRC cells, the number of cells with typical and specific morphological features of

apoptotic cell death was determined by scoring 200 cells from 35 microscopic fields.

2.10. Western Blot Analysis

For Western blot analysis proteins were extracted from the alginate cultures with 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, 0.01% (v/v) aprotinin, 4 μg/ml of pepstatin A, 10 μg/ml of leupeptin, 1 mM phenyl methyl sulfonylfluoride, PMSF) on ice for 30 min as previously described [45,46]. Total protein concentration was measured with the bicinchonic acid assay system (Uptima, Monlucon, France) using bovine serum albumin as a standard. Samples were further reduced with 2-mercaptoethanol and equal quantities of protein (500ng/lane), separated under reducing conditions by SDS-PAGE and transferred onto nitrocellulose membranes using a transblot apparatus (Bio-Rad). After pre-incubation in blocking buffer (5% skimmed milk powder in PBS, 0.1% Tween 20) for 1 h, membranes were incubated with primary antibodies at 4 °C overnight, washed three times with blocking buffer, and then further incubated with alkaline phosphatase-conjugated secondary antibodies for 2 h at AT. After further washing in 0.1 M Tris, pH 9.5, containing 0.05 M $MgCl_2$ and 0.1 M NaCl, specific antigen-antibody complexes were detected using nitro blue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (*p*-toluidine salt; Pierce). Specific β-actin antibody was used for the internal control to normalize the sample amounts.

2.11. Isolation of CRC cytoplasmic and nuclear extracts

Cell nuclear extracts were isolated as previously described [47]. Briefly, cells were trypsinized and washed twice in 1 ml of ice-cold PBS. The supernatant was carefully removed. The cell pellet was re-suspended in hypotonic lysis buffer containing protease inhibitors and was incubated on ice for 15 min. Then, 12.5 μl of 10% Nonidet P-40 was added and the cell suspension was vigorously mixed. The extracts were centrifuged for 1.5 min. The supernatants (cytoplasmic extracts) were frozen at −70 °C. 25 μl of ice-cold nuclear extraction buffer were added to the pellets and incubated for 30 min with intermittent mixing. Extracts were centrifuged and the supernatant (nuclear extracts) was transferred to pre-chilled tubes for storage at −70 °C.

2.12. Immune complex kinase assay

To examine the effect of resveratrol on 5-FU-induced IKK activation, immune complex kinase assays were performed as previously described [48]. The IKK complex was immunoprecipitated from whole cell lysates with antibodies against IKK-α and IKK-β and subsequently incubated with protein A/G-agarose beads (Pierce, Germany). After 2 h incubation, the beads were washed with lysis buffer and resuspended in a kinase assay solution containing 50 mM HEPES (pH 7.4), 20 mM $MgCl_2$, 2 mM dithiothreitol, 10 mM unlabeled ATP and 2 mg substrate IκBα (amino acid 1–54), incubated at 30 °C for 30 min and boiled in SDS-PAGE sample buffer for 5 min. SDS-polyacrylamide gel electrophoresis was performed under reduced conditions as described above. Phosphorylation of IκBα was assessed using a specific antibody against phosphorylated IκBα (Ser 32/36). To demonstrate the total amounts of IKK-α and IKK-β in each sample, whole-cell lysates were transferred to a nitrocellulose membrane after SDS-PAGE electrophoresis was performed as described above. Detection of phosphorylated-IκBα, IKK-α and IKK-β was performed by immunoblotting.

2.13. Immunofluorescence microscopy analysis of alginate tumor microenvironment cultures

HCT116 and HCT116R cells in tumor microenvironment alginate cultures were either left untreated, treated with resveratrol (5 μM) or 5-FU (1 nM). Alginate beads of HCT116 and HCT116R cells were evaluated after 10 days. Cultures were embedded in Tissue-Tek (Sakura Finetek Europe, The Netherlands) and immediately frozen in liquid nitrogen. Glass plates bearing 10-μm-thick sections through alginate beads were fixed with acetone (10 min) and washed twice (5 min) in PBS/BSA at AT. The slides were incubated first with serum (diluted 1:20 in PBS/BSA), for 10 min at AT, and then with the primary antibodies (diluted 1:30 in PBS/BSA), in a humid chamber overnight at 4 °C. Cells were rinsed as described above, followed by incubation with rhodamine- or FITC-coupled secondary antibodies (diluted 1:80 in PBS/BSA) for 1 h at AT and finally washed again three times with aqua destilled. Slides were covered with fluormount and examined under a fluorescent microscope (Leica, Germany).

2.14. Statistical analysis

Each experiment was performed three times as individual experiments with three replicates. Parameters are expressed as the mean values (+/−SD). Results were analyzed by unpaired Student's *t*-test and by one-way ANOVA followed by a post-hoc test to compare the parameters of each group. Differences were considered to be statistically significant for $p < 0.05$.

3. Results

The aim of this study was to examine whether resveratrol might have a role in the treatment of CRC either alone or in combination with 5-FU, and what specific mechanisms are involved. We used

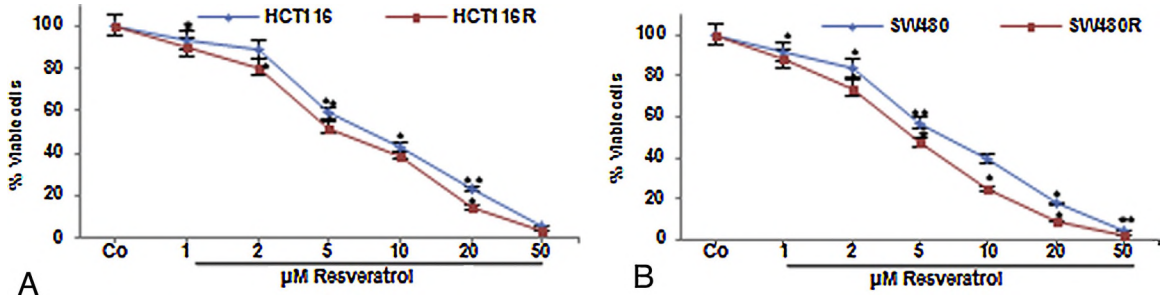

Fig. 1. A–B. Resveratrol blocks the proliferation of CRC cell lines. MTT assay showed inhibition of cell proliferation and viability in 4 CRC cell lines (HCT116, HCT116R, SW480, SW480R) treated with different concentrations of resveratrol (1, 2, 5, 10, 20 and 50 μM) cultured in alginate beads for 21 days. Data shown are representative of 3 independent sets of experiments. Values were compared with the control and statistically significant values with $p<0.05$ were designated by an asterisk (*) and $p < 0.01$ were designated by two asterisks (**).

4 different well-characterized CRC cell lines to address various questions. We evaluated the effects of resveratrol signaling on tight junction, adhesion molecules, EMT/MET, apoptosis and invasiveness in CRC cells in 3D alginate-based tumor microenvironment.

3.1. Resveratrol blocks proliferation of human colorectal cancer cells in alginate tumor microenvironment

To determine the 50% cell growth inhibitory concentrations (IC_{50}) and to understand the cytotoxic effect of resveratrol in 4 different cell lines (HCT116, HCT116R, SW480 and SW480R cells) in alginate culture, MTT cell viability assays were performed. Cells were treated with different concentrations of resveratrol (0, 1, 2, 5, 10, 20 and 50 μM) in serum-starved medium for 21 days and cell viability was measured using the MTT assay. Resveratrol blocked the proliferation of all 4 human CRC cell lines in a dose-dependent manner (Fig. 1A–B). The IC_{50} value of individual resveratrol was approximately 5 μM in all 4 CRC cell lines ($p < 0.05$).

3.2. Resveratrol synergizes the effect of 5-FU on colorectal cancer cells in alginate tumor microenvironment

We next examined the effect on cellular proliferation following incubation with resveratrol or 5-FU alone or in combination with increasing concentrations of 5-FU in 2 CRC cell lines (parental HCT116 and chemoresistant HCT116R) using phase contrast microscopy and MTT assay. Whether resveratrol can potentiate the anti-proliferation effect of 5-FU against these 2 cell lines were also examined by phase contrast microscopy. As shown in Fig. 2A, the individual dose of resveratrol (5 μM) or 5-FU (0.01, 0.1 nM) themselves had a minimum inhibitory effect on cell proliferation, but showed a strong synergistic, anti-proliferative effect when used in combination in both cell lines. To confirm these morphological results, we performed MTT assay in long-term cultures. Our results revealed that after 21 day treatment resveratrol (5 μM) caused growth inhibition of parental HCT116 by 56%, but much more effectively in HCT116R cells (up to 34%). In contrast, 5-FU treatment resulted in 27–62% proliferation inhibition of parental HCT116 in a concentration-dependent manner, but no growth inhibition was observed in HCT116R cells (Fig. 2B–C). Interestingly, combined treatment with resveratrol (5 μM) and 5-FU (0.01, 0.1 nM) resulted in a much greater inhibition in cellular growth of parental HCT116 cells (73.5%, 85.3%) and of 5-FU-chemoresistant cells HCT116R (57.6%, 74.5%) (Fig. 2B–C). Chou-Talalay CI values indicate that the combined effect of resveratrol (5 μM) with 5-FU (0.1 nM) was found to be significant synergistic in cytotoxicity in both cell lines (CI < 1) with a CI value of 0.507 (parental HCT116 cells), 0.302 (HCT116R cells). Doses that show synergistic effect were used for further studies. These findings indicate that a very low amount of 5-FU, when combined with a moderate dose of resveratrol, can produce

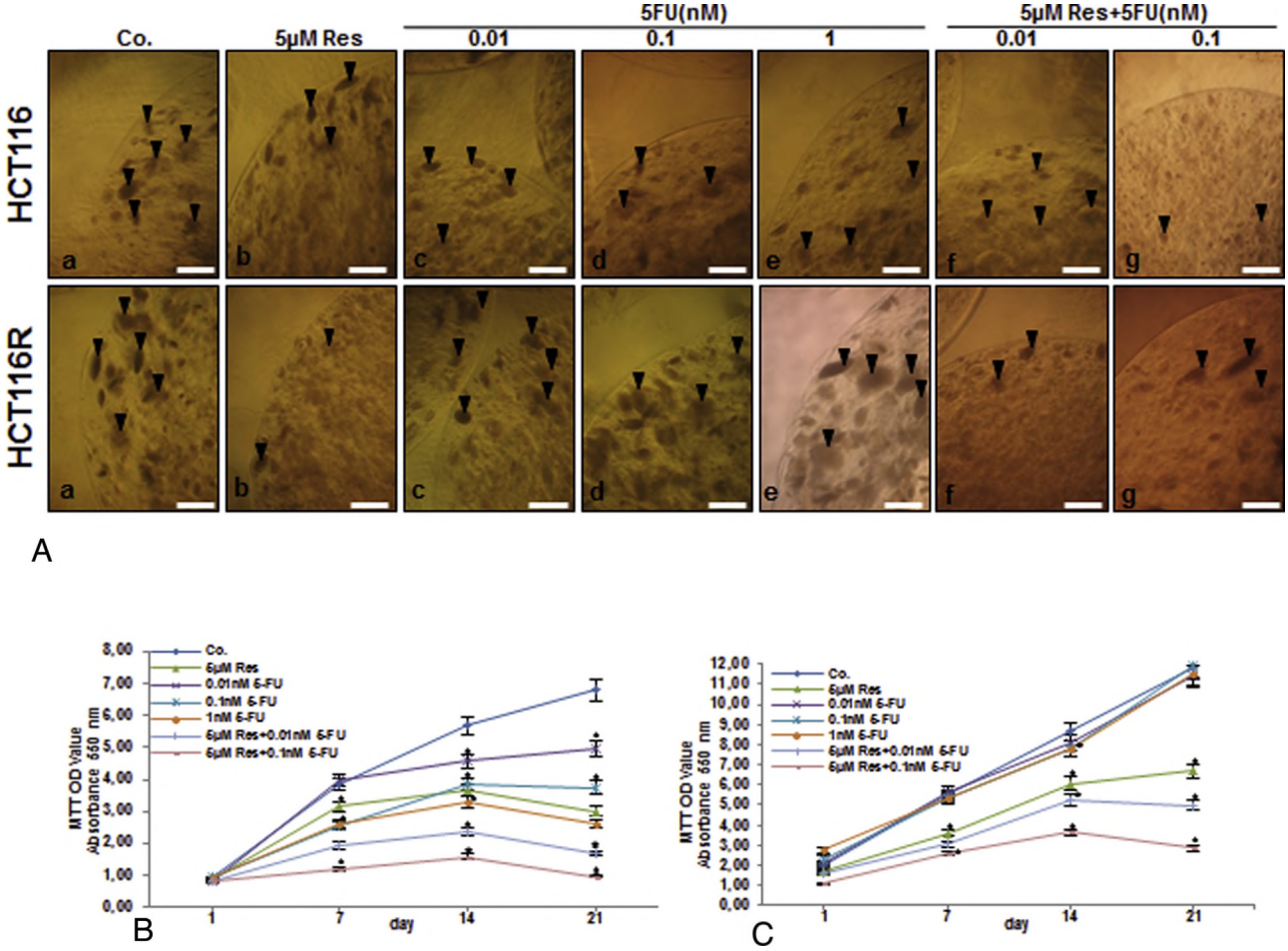

Fig. 2. A–C. Resveratrol synergizes the anti-tumor effects of 5-FU on CRC cells in alginate tumor microenvironment. (A) Serum-starved HCT116 and HCT116R cell lines in alginate culture (1×10^6/ml) were either left untreated (a) or treated with resveratrol alone (5 μM) (b), 5-FU alone (0.01, 0.1, 1 nM) (c, d, e) or were treated with resveratrol (5 μM) followed by treatment with 5-FU (0.01, 0.1 nM) (f, g). After 21 days of culture, phase-contrast microscopic observations of HCT116 cells and HCT116R cells in alginate showed the formation or inhibition of spheroids (arrowheads) and viability of cells by resveratrol, 5-FU alone and in combination of them. B–C: Cell viability was determined in alginate beads by MTT assay in HCT116 (B) and HCT116R (C) over a period of 21 days. Values were compared with the control and statistically significant values with $p < 0.05$ were designated by an asterisk (*). Samples from 3 experiments were analyzed and representative data are shown. Scale bar = 0.2 mm in all cases.

synergistic cytotoxicity and chemosensitizes HCT116R cells to 5-FU-based chemotherapy.

3.3. Resveratrol potentiates the 5-FU-mediated anti-invasion effect against CRC cells in alginate tumor microenvironment

To examine the combined effect of resveratrol and 5-FU on cell motility and invasion through 3D alginate-based tumor microenvironment, HCT116 and HCT116R cells (1×10^6/ml) were cultured in alginate beads, treated as described above and the capacity of migration and invasion was determined with toluidine blue staining as described in detail in material and methods. As shown in Fig. 3, treatment of the CRC cells with resveratrol (5 μM) alone significantly blocked ($p < 0.05$) the migration rate of HCT116 and HCT116R cells through the alginate-based matrix after an incubation time of 22 days by 77% and 61%, respectively compared to untreated cells. Treatment of the cells with 5-FU (0.01, 0.1, 1 nM) alone inhibited migration of HCT116 cells through the alginate-based matrix by 19%, 33%, 61.5%, respectively (Fig. 3B). Interestingly, it should also be stated here that there was little or no effect of 5-FU on HCT116R cells, even after treatment with 1 nM dose (Fig. 3C), suggesting that HCT116R cells are resistant to 5-FU. Furthermore, the combined treatment of resveratrol and 5-FU was much more effective in comparison to the individual compound. Treatment of the cells with resveratrol (5 μM) and co-treatment with 5-FU (0.01 nM) synergistically enhanced inhibition of the invasion ability of HCT116 cells by 90% (CI values = 0.643) and of HCT116R by 89% (CI values = 0.492) (Fig. 3B–C).

3.4. Distribution profile of cell–cell-contacts in CRC cells in tumor microenvironment alginate in the presence of 5-FU or/and resveratrol revealed by transmission electron microscopy

To investigate whether the growth-inhibitory effect of resveratrol and 5-FU in HCT116 and HCT116R colonosphere formation in 3D alginate culture microenvironment correlates with the changes in ultrastructural cell morphology, cell–cell behavior and the induction of apoptosis, transmission electron microscopic evaluations were performed. After 10 days culture period, untreated alginate cultures of HCT116 and HCT116R (not shown) showed typical morphological and ultrastructural features of viable cell proliferation and aggregation formation embedded in alginate structure. Cells were mainly round to oval, contained a well-developed rough endoplasmic reticulum, a large golgi apparatus and other organelles, such as mitochondria, small vacuoles and granules, all of which indicate maintenance of metabolic functions (Fig. 4A: a). CRC cells contained numerous abundant microvilli and cell surface processes and showed numerous requisite intercellular junctions on the planar surface. Contrariwise, in the presence of 5-FU, cells exhibited a mesenchymal-like morphology associated with increased abundant

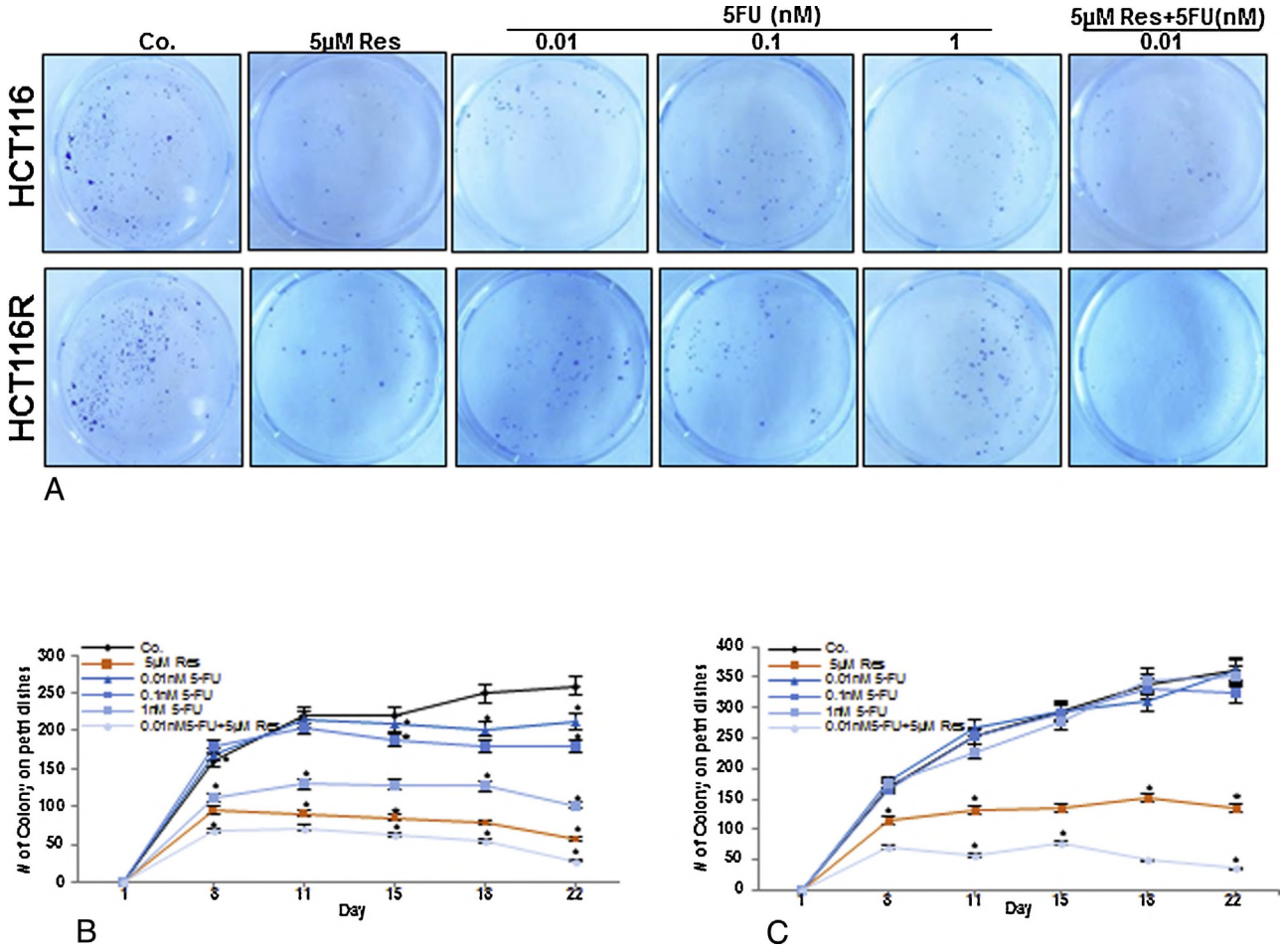

Fig. 3. A–C. Resveratrol inhibits the invasive phenotype of CRC cells in alginate. (A) Serum-starved HCT116 and HCT116R cell lines in alginate culture (1×10^6/ml) were either left untreated or treated with resveratrol alone (5 μM), 5-FU alone (0.01, 0.1, 1 nM) or were treated with resveratrol (5 μM) followed by treatment with 5-FU (0.01 nM). The invasive properties of HCT116 and HCT116R cells were analyzed after an incubation time of 22 days. (B–C) Resveratrol suppressed colony formation in both CRC cell lines. The values given are the means ± standard errors of the mean of three replicates. One of three independent experiments is shown. Values were compared with the control and statistically significant values with $p<0.05$ were designated by an asterisk (*).

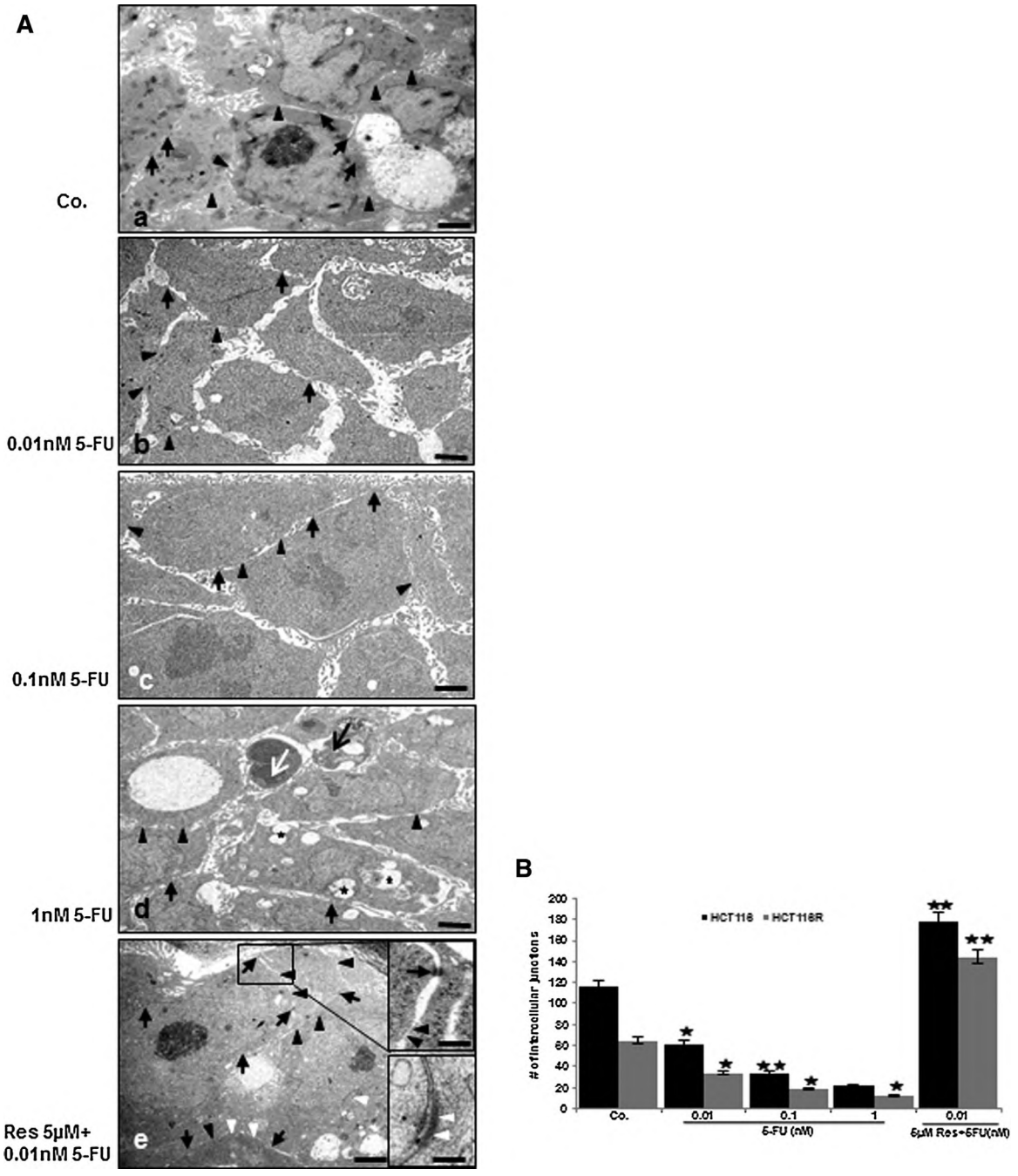

Fig. 4. A–D. Transmission electron microscopic evaluation of cell–cell-contacts in CRC cells in alginate beads after treatment with resveratrol or/and 5-FU. (A) Serum-starved HCT116 cells in alginate culture (1×10^6/ml) were either left untreated (a) or treated with 5-FU alone (0.01, 0.1, 1 nM) (b, c, d) or were treated with resveratrol (5 μM) and with 5-FU (0.01 nM) (e). After 10 days of culture, transmission electron microscopic observations of HCT116 cells in alginate showed in control cultures, tumor cells with planar surface and microvilli on their surface (a). 5-FU-treated tumor cells contained abundant microvilli on their surface, multiple cytoplasmic vacuolization (stars) and apoptosis (white arrows) (d). Co-treated tumor cells showed only few microvilli but they had contact planar surface with numerous desmosomes (black arrows) and tight- and adherence junctions (black arrowheads) (a–e) and gap junctions (white arrowheads)(e) (insets, arrows and black and white arrowheads). Magnification: Scale bar = 1 μm, Insets: Scale bar = 0.075 μm. (B) To quantify degenerative changes, alginate cultures of *A* were examined for apoptosis by counting 200 HCT116 and HCT116R cells from 35 microscopic fields. The examination was performed in triplicate, and the results are provided as the mean values with S.D. from three independent experiments. Values were compared with the control and statistically significant values with $p<0.05$ were designated by an asterisk and $p<0.01$ were designated by two asterisks. (C) Resveratrol-induced tight-, adherence junctions and desmosomes between tumor cells. Serum-starved CRC cell lines in alginate culture (1×10^6/ml) were either left untreated (a) or treated with different concentrations (1, 5, 10 μM) of resveratrol (Res) for 10 days (b,c,d). After 10 days of culture, ultrathin sections were prepared and evaluated by transmission electron microscopy. Micrographs shown are representative of all the cultures evaluated. In the presence of resveratrol, the tumor cells showed abundant planar cell surface and this was associated with increase of tight- and adherence junctions (black arrowheads), gap junctions (white arrowheads), desmosomes (black arrows), multiple cytoplasmic vacuolization (stars) and apoptosis (white arrows). These morphological changes were dose dependent. Scale bar = 1 μm, Insets: Scale bar = 0.1 μm. (D) Serum-starved CRC cell lines in alginate culture (1×10^6/ml) were either left untreated or treated with different concentrations (1, 5, 10, 20 μM) of resveratrol (Res), or with 5-FU alone (0.01, 0.1, 1 nM) or were co-treated with resveratrol (5 μM) and 5-FU (0.01, 0.1, 1 nM). After 10 days of culture, apoptosis was quantified by counting 200 cells with morphological features of apoptotic cell death from 35 different microscopic fields and results presented are mean values with standard deviations from three independent experiments. Values were compared with the control and statistically significant values with $p<0.05$ were designated by an (*) and $p<0.01$ were designated by two (**).

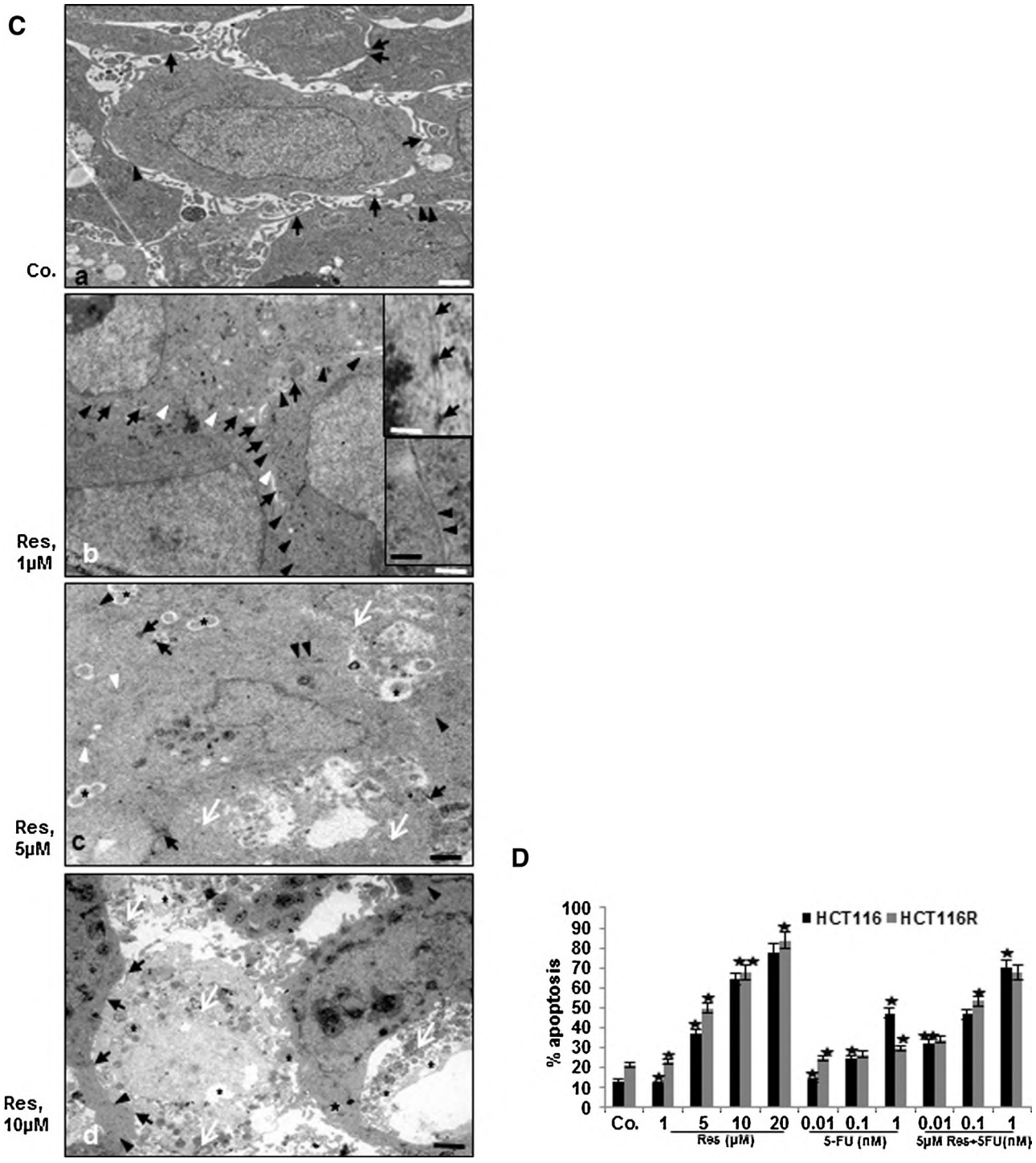

Fig. 4. (Continued)

membrane extensions, microvilli and long filaments resembling filopodia (Fig. 4A: b–d). These ultrastructural alterations occurred in a 5-FU dose-dependent manner. When treated with higher 5-FU concentrations (1 nM), apoptosis was observed (Fig. 4A: d). Interestingly, through co-treatment with 5-FU and resveratrol, a dramatic conversion to an epithelial ultrastructural morphology was observed. CRC cells were observed to grow in close clusters expressing numerous intercellular junctions including desmosomes, tight- gap- and adherence junctions (Fig. 4A: e). By quantification, we confirmed that the number of intercellular junctions (desmosomes, tight- gap- and adherence junctions) decreased in the presence of 5-FU in a dose-dependent manner, but significantly increased when given combined treatment with resveratrol, particularly on the cell surface of HCT116 and HCT116R cells compared to control tumor cultures (Fig. 4B).

3.5. Resveratrol induces an epithelial conversion in CRC cells in alginate tumor microenvironment culture

To extend our studies and gain further insight into the role of resveratrol in ultrastructural cell morphology and cell–cell behavior and its involvement in MET (mesenchymal-to-epithelial transition)

in alginate microenvironment, CRC cells were treated with different concentrations of resveratrol (1, 5, 10 μM) and cultured in alginate beads for 10 days. In control cultures, within the colonosphere, cells contain microvilli and cell membrane processes and partial planar intercellular junctions (Fig. 4C: a). In the presence of 1 μM resveratrol, we observed that the CRC cells gained planar cell–cell contacts, retained typical epithelial morphology and continued to grow as groups of colonospheres. Furthermore, the cells became more round and lost their cell surface processes, microvilli and the surface became planar with intensive cell–cell contact. Surprisingly, these ultrastructural morphological changes induced by resveratrol were accompanied by high expression of junctional intercellular communication, suggesting evidence of a EMT (epithelial-to-mesenchymal transition) (Fig. 4C: b). Similar morphological changes on the ultrastructural level were observed in CRC cells in the presence of 5 or 10 μM resveratrol, but were much more pronounced. In these cells additional degeneration of tumor cells, including areas of condensed heterochromatin within the nuclei, chromatin aggregation and multiple cytoplasmic vacuolization, signs of apoptosis were noticed (Fig. 4C: c–d). These ultrastructural morphological results were not quantitative in nature, but they qualitatively supported the observations that CRC cells and their morphology and behavior were relatively well-maintained in this tumor microenvironment.

3.6. Resveratrol enhances 5-FU-mediated apoptosis of HCT116 cells and their 5-FU-chemoresistant cells in alginate cultures

Treatment of HCT116 and HCT116R cells with 5-FU or resveratrol alone resulted in degeneration of cell organelles, mitochondrial swelling and appearance of multiple vacuoles, with prominent signs of apoptosis. In contrast, such effects were not evident in 5-FU treated HCT116R cells. However, a combinatorial treatment with 5-FU and resveratrol for 10 days markedly enhanced degeneration of all tumor cells. Statistical quantification of the ultrastructural sections highlighted the prominent effects of combined 5-FU (0.01, 0.1, 1 nM) and resveratrol (5 μM) treatment in inducing and significantly synergistic enhancing apoptosis in a dose-dependent manner in both HCT116 (CI values = 0.850, 0.574, 0.576) and HCT116R (CI values = 0.352, 0.295, 0.462) cells compared to control tumor cultures (Fig. 4D), indicating that resveratrol may sensitize 5-FU in the chemoresistant cell line. However, it was noted that the above mentioned degenerative changes were significantly more in 5-FU resistant cells compared with the parental HCT116 cells (Fig. 4D). Thus, the findings indicate that 5-FU chemoresistant cells are sensitive to chemotherapeutic agents, such as resveratrol and the resveratrol and 5-FU combination represents a potential treatment option for 5-FU resistant colon cancer cells.

3.7. Resveratrol induces the expression of E-cadherin and claudin-2 in CRC cells in alginate beads: E-cadherin co-localizes with claudin-2

It has been suggested that down-regulation of intercellular adhesive molecules leads to disruption of adherent cell–cell junctions leading to ultrastructural morphological changes related to EMT [49]. Moreover, loss of cell–cell adhesion is a central process that reduces tumor cell adhesion leading to tumor cell growth, invasion and metastasis [50]. We therefore, investigated the status of known tight junction and EMT markers in CRC cells by immunofluorescence analysis. The alginate cultures of HCT116 (Fig. 5A) and HCT116R (Fig. 5B) cells were either left untreated or treated with 1 nM 5-FU or 5 μM resveratrol for 10 days. The alginate cultures were subjected to immunofluorescence labeling with primary antibodies for claudin-2 and E-cadherin followed by incubation with rhodamine- or FITC-coupled secondary antibodies. As shown in Fig. 5, in untreated cultures the expression of claudin-2 and E-cadherin was found diffusely distributed on the round HCT116 and HCT116R cells cultured in basal control alginate cultures (Fig. 5A, B). Interestingly, in contrast, treatment of CRC cells with 1 nM 5-FU led to down-regulation of E-cadherin and claudin-2 proteins, concomitant with the apparent loss of cell–cell junctions in both cells (Fig. 5A, B). Cells showed a weak punctuate staining of the two proteins on the cell membrane. However, treatment of CRC cells with 5 μM resveratrol over 10 days markedly enhanced the expression of both tight junction and adhesion molecules (Fig. 5A, B). More interestingly, immunolabeling of both molecules showed that they were co-localized on the cell surface. Our results demonstrate that resveratrol induces the central hallmarks of tight junctions and a MET phenotype in CRC cells in alginate tumor microenvironment.

To confirm electron microscopic and immune-cytochemical observations, we performed western blot analysis. As shown in Fig. 6A, when the HCT116 cells (I–II) and their respective 5-FU resistant cells (III-IV) were treated with resveratrol (0.1, 1, 5, 10 μM) in alginate cultures for 10 days, the expression of claudin-2 and E-cadherin was significantly increased in a concentration-dependent manner. In contrast, 5-FU (0.01, 0.1, 1 nM) treatment caused a decrease of the claudin-2 and E-cadherin expression in a concentration-dependent manner (Fig. 6B). Interestingly, co-treatment of HCT116 cells (I-II) and their respective 5-FU resistant cells (III-IV) with resveratrol (5 μM) and 5-FU (0.01, 0.1 nM) clearly caused an up-regulation in claudin-2 and E-cadherin expression in both cell types (Fig. 6B: I-IV). Quantitative western-blot analyses by densitometric evaluation of the representative experiment performed in triplicate confirmed the transmission electron microcopy and immunofluorescence results (Fig. 6A–B).

3.8. Resveratrol-induced up-regulation of E-cadherin and claudin-2 protein expression in CRC cells revealed by immunoelectron microscopy

To further visualize the precise localization of E-cadherin and claudin-2 proteins on an ultrastructural level during treatment with resveratrol, we employed immunoelectron microscopy to study cell surface expression of E-cadherin (7A) and claudin-2 (7B) proteins. HCT116 and HCT116R (not shown) were either left untreated (Fig. 7:A, B: a) or treated with 1 μM (Fig. 7A, B: b), 5 μM (Fig. 7A, B: c) or 10 μM (Fig. 7A, B: d) resveratrol for 10 days in alginate tumor microenvironment. Immunoelectron microscopic images show low basal expression of E-cadherin and claudin-2 diffusely distributed on the round HCT116 and HCT116R cells cultured in control alginate cultures (Fig. 7A, B: a). Surprisingly, the treatment with resveratrol induced a round shape with high expression of E-cadherin, claudin-2 and this was localized predominantly on the planar cell body between the cells (Fig. 7A, B: b–d). Taken together, our result showed that resveratrol significantly stimulated E-cadherin and claudin-2 expression in a dose-dependent manner. Moreover, immunoelectron microscopic results confirmed the observations in Figs. 5 and 6 that resveratrol induces expression of adhesion and tight junction molecules in HCT116 colon cancer cells in the microenvironment and stimulation of these interactions by resveratrol induces biochemical and functional changes towards MET. Indeed, E-cadherin and claudin-2 are important components of adherence junctions, tight junctions and regulate cancer cell progression, invasion and metastasis through maintenance of cell–cell contacts.

3.9. Resveratrol blocks and synergizes 5-FU-induced EMT in CRC cells in alginate tumor microenvironment cultures

To gain further insights into the functional inhibiting roles of resveratrol and its synergistic effect with 5-FU during

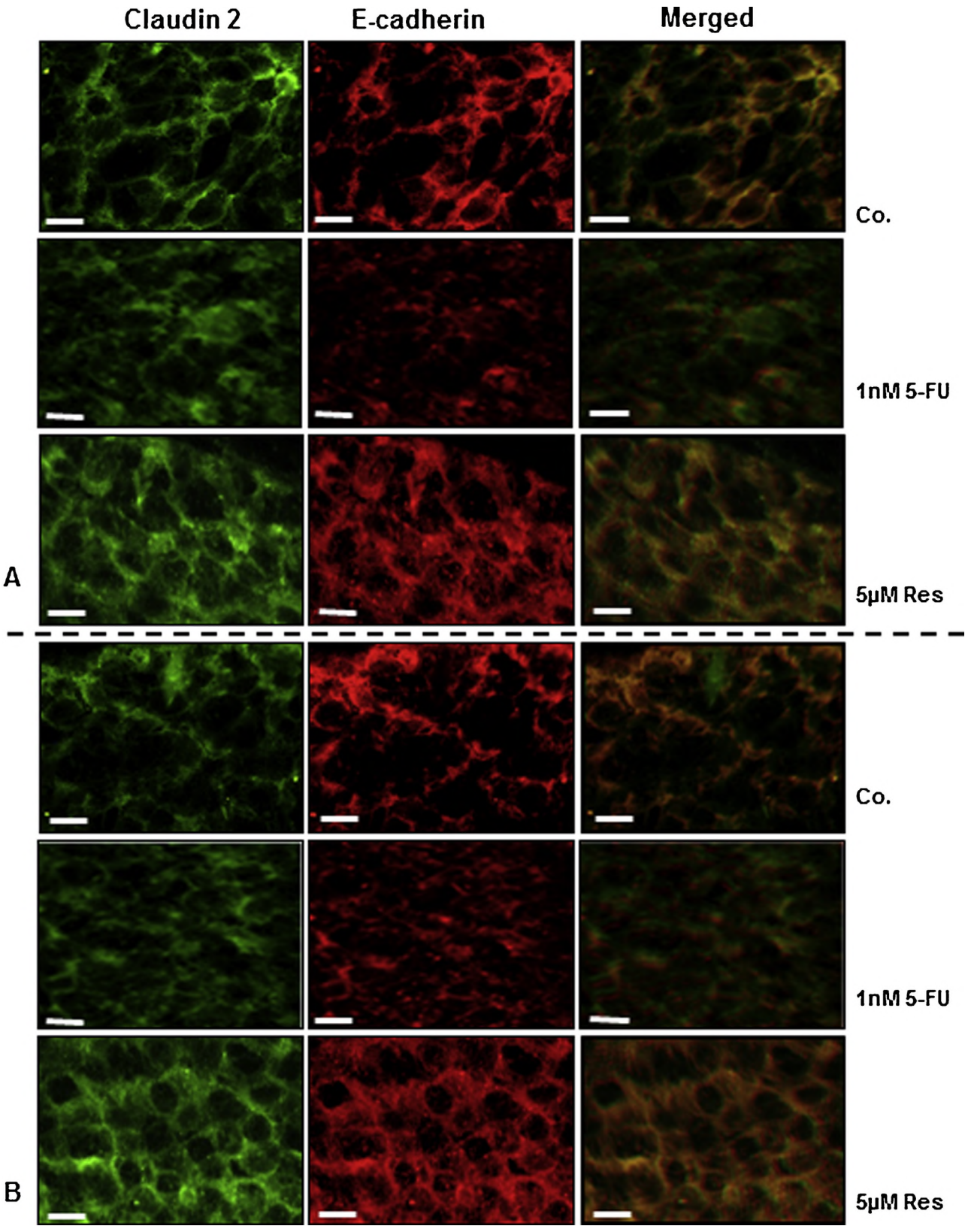

Fig. 5. A–B. Resveratrol induces and 5-FU suppresses claudin-2 and E-cadherin expression in HCT116 and HCT116R cells in alginate tumor microenvironment. Alginate cultures of HCT116 (A) and HCT116R (B) cells were left untreated, treated with 5-FU (1 nM) or treated with resveratrol (5 μM) for 10 days. The cultures were subjected to immunofluorescence labeling with primary antibodies for claudin-2 (green) and E-cadherin (red) followed by incubation with rhodamine- or FITC-coupled secondary antibodies. Images shown are representative of three different experiments. Scale bar = 12 μM.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

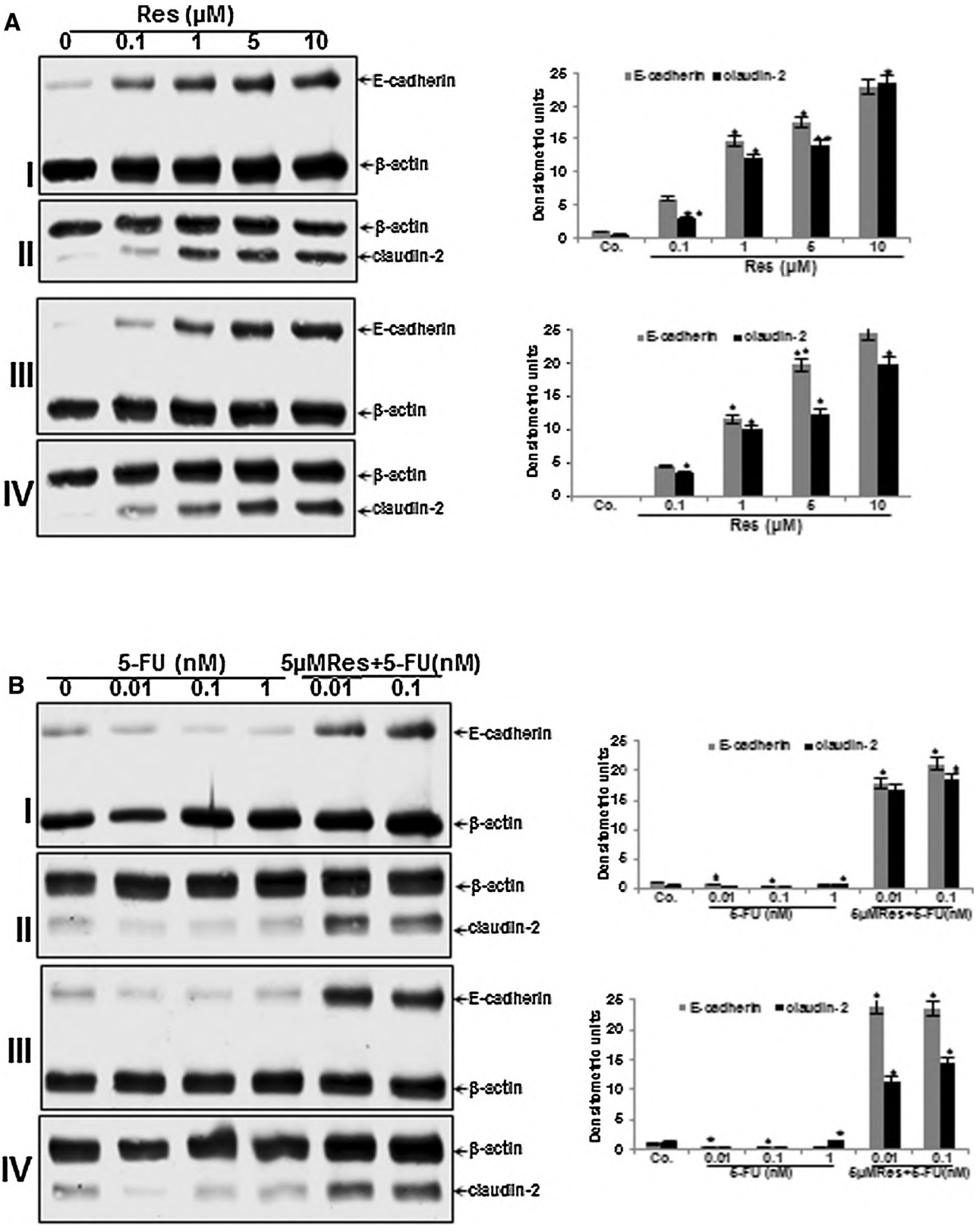

Fig. 6. A–B. Effect of resveratrol or/and 5-FU on the expression of E-cadherin and claudin-2 in CRC cells in alginate tumor microenvironment. (A) Serum-starved HCT116 (I-II) and HCT116R (III–IV) cell lines in alginate culture (1×10^6/ml) were either left untreated or treated with different concentrations (0.1, 1, 5, 10 μM) of resveratrol (Res) for 10 days. After 10 days of culture, whole cell lysates (500ng/lane) were fractionated and subjected to western blotting with antibodies against E-cadherin and claudin-2. (B) Serum-starved HCT116 (I-II) and HCT116R (III-IV) cell lines in alginate culture (1×10^6/ml) were either left untreated or treated with 5-FU alone (0.01, 0.1, 1 nM) or co-treated with resveratrol (5 μM) and 5-FU (0.01, 0.1 nM). After 10 days of culture, total cell lysates of HCT116 and HCT116R cultures were prepared and immunoblotting performed for E-cadherin (I–III) or claudin-2 (II–IV). Housekeeping protein β-actin served as a loading control in all experiments. Densitometric evaluation was performed for E-cadherin and claudin-2 expression. Each experiment was performed in triplicate and mean values and standard deviation are indicated. Values were compared with the control and statistically significant values with $p < 0.05$ were designated by an asterisk and $p < 0.01$ were designated by two asterisks.

carcinogenesis in alginate cultures in relation to EMT [51], we have analyzed the expression of EMT-associated signaling molecules, such as vimentin, E-cadherin and the transcription factor Slug. The alginate cultures of HCT116 (Fig. 8A) and HCT116R (Fig. 8B) were either left untreated or treated with resveratrol (5 μM), 5-FU (0.01, 0.1, 1 nM) alone or co-treated with resveratrol (5 μM) and 5-FU (1 nM) for 10 days. Immunoblotting of cell lysates of the HCT116 and HCT116R alginate tumor microenvironment cultures showed basal expression of vimentin and E-cadherin (Fig. 8A-B:I-II). Treatment of HCT116 or HCT116R tumor microenvironment cultures with resveratrol down-regulated the expression of mesenchymal marker vimentin, whereas it increased epithelial marker E-cadherin expression (Fig. 8A–B:I–II). In contrast, immunoblotting analysis of CRC tumor microenvironment cultures

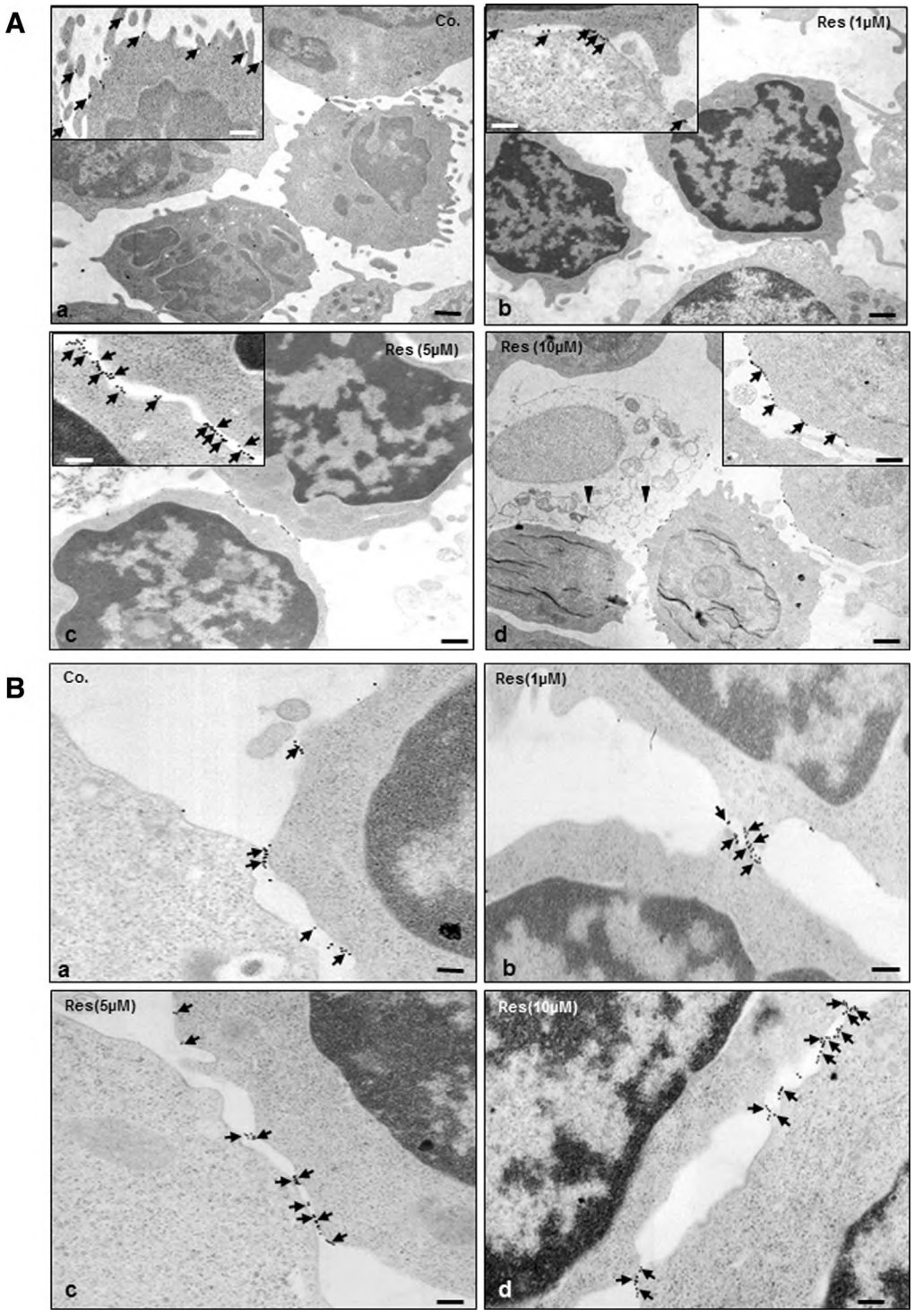

Fig. 7. A–B: A–B Immunoelectron microscopic images showing E-cadherin (A) and claudin-2 (B) expression by immunogold labeling. Serum-starved HCT116 cells in alginate culture (1×10^6/ml) were either left untreated or treated with different concentrations (1, 5, 10 μM) of resveratrol (Res) for 10 days. After 10 days of culture, HCT116 cells were either left untreated without primary antibody (A, B: a) or with primary antibody (A, B: b, c, d). Immunogold labeling (arrows) is detected at the plasma membrane of tumor cells with antibodies against E-cadherin (A) and claudin-2 (B). Images shown are representative of three different experiments. A: Scale bar = 0.2 μm; Insets: Scale bar = 0.09 μm. B: Scale bar = 0.08 μm.

treated with 5-FU showed marked dose dependent up-regulation of vimentin, but down-regulation of E-cadherin (Fig. 8A–B). Taken together, our data support the paradigm that EMT is linked with down-regulation of tight junctions, and the development of increased resistance to chemotherapeutic agents [3,21]. To investigate this issue, we examined whether resveratrol can modulate the effect of 5-FU-induced vimentin or E-cadherin expression. Co-treatment of cultures with resveratrol and 5-FU, showed to be most effective in down-regulation of vimentin or up-regulation of E-cadherin in HCT116 (IC value=0.743) and HCT116R (IC value=0.363) cells (Fig. 8A–B:I–II).

To date, the transcription factor Slug, a member of developmental transcription factors, has been linked to cancer metastasis. Furthermore, Slug is associated with transcriptional activation of vimentin and suppression of E-cadherin expression in the molecular EMT program [52–54]. As shown in Fig. 8A–B, the basal expression of Slug was inhibited by resveratrol, however the expression of Slug and above mentioned proteins was significantly more in 5-FU resistant cells compared with the parental HCT-116 cells (Fig. 8A–B:III). In

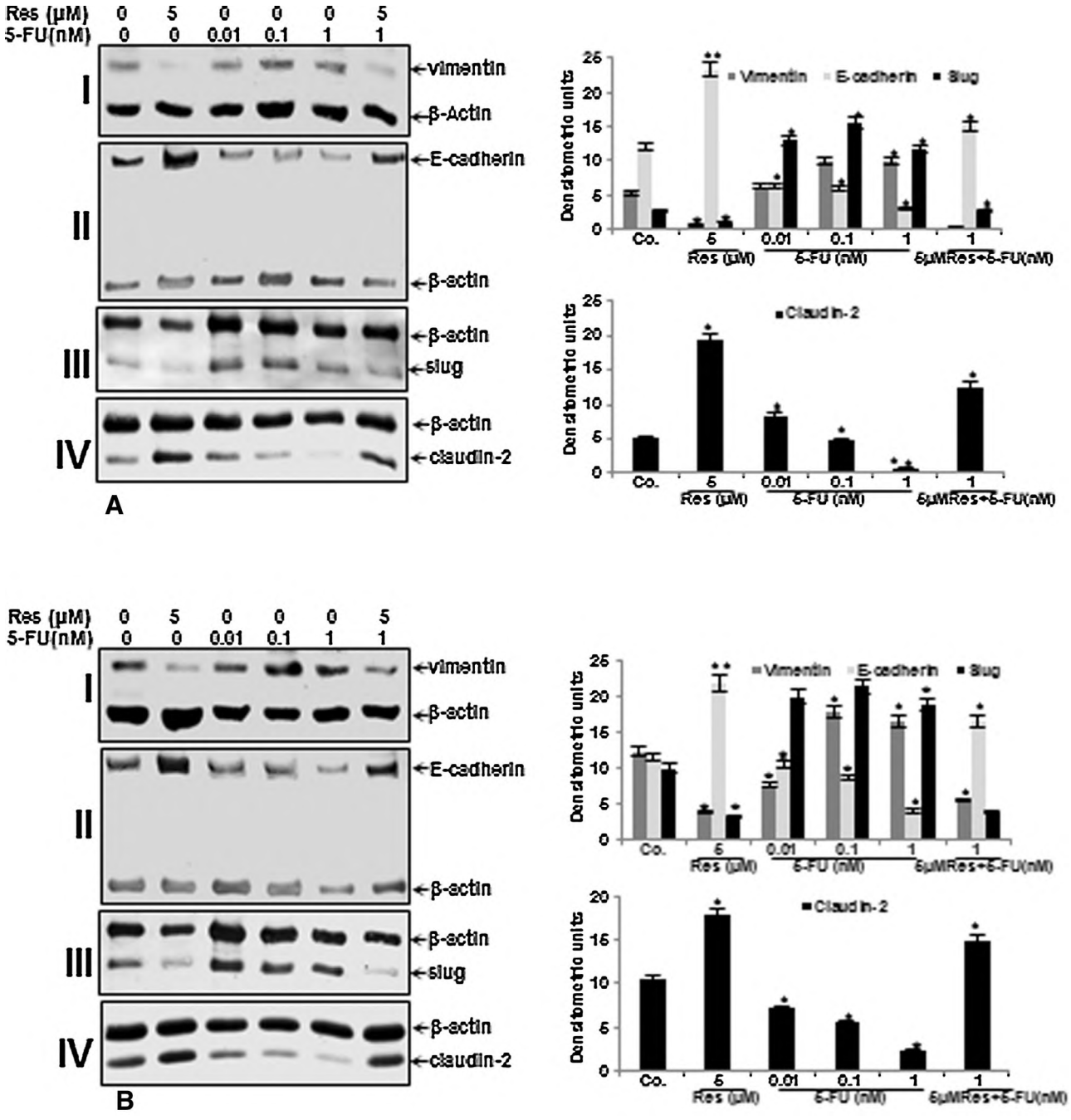

Fig. 8. A–B. Resveratrol induces MET with loss of mesenchymal markers and increase of epithelial markers. Serum-starved HCT116 (A) and HCT116R (B) cell lines in alginate culture (1×10^6/ml) were either left untreated or treated with resveratrol alone (5 μM), 5-FU alone (0.01, 0.1, 1 nM) or were treated with resveratrol (5 μM) and 5-FU (1 nM). After 10 days of culture, total cell lysates of cells were prepared and immunoblotting performed for vimentin (I) or E-cadherin (II), Slug (III) or claudin-2 (IV). Housekeeping protein β-actin served as a loading control in all experiments. Densitometric evaluation was performed for E-cadherin and claudin-2 expression. Each experiment was performed in triplicate and mean values and standard deviation are indicated. Values were compared with the control and statistically significant values with $p<0.05$ were designated by an asterisk and $p < 0.01$ were designated by two asterisks.

contrast, treatment of HCT116 or HCT116R with 5-FU induced a dose dependent up-regulation of Slug expression in both cell lines and this response was considerably blocked by resveratrol (Fig. 8A–B:III). It was noted that 5-FU resistant cells were more sensitive to resveratrol compared to the parental HCT116 cells. Moreover, we also examined in the same experimental model whether resveratrol up-regulates the expression of claudin-2 and inhibits 5-FU-induced down-regulation of claudin-2. The results (8A-B:IV) confirmed the qualitative changes we observed in another set of experiments in Figs. 6 and 7. Taken together, these results suggest that the alginate cultures might provide an ideal microenvironment to study 5-FU-induced malignancy and EMT in CRC cells, and inhibition of this effect by resveratrol induces biochemical and functional changes towards MET, thereby sensitizing CRC cells to 5-FU treatment.

3.10. Resveratrol blocks and synergizes the effect of 5-FU by down-regulation of NF-κB activation and NF-κB-regulated gene end-products in CRC cells

To elucidate the underlying mechanism, how resveratrol potentiates the effects of 5-FU in CRC cells, we analyzed whether NF-κB signaling pathway was involved. Indeed, it has been reported that NF-κB is a transcription factor that is expressed in tumor cells, and causes resistance to apoptosis and acts as the key mediator of tumorigenesis [55,56].

3.10.1. Resveratrol inhibits 5-FU-induced NF-κB activation in CRC cells in alginate tumor microenvironment

Based on the observed data, we speculated that resveratrol might inhibit the NF-κB pathway in CRC cells. The phosphorylation of NF-κB subunits (p50/p65) play an important role in IκBα-mediated activation of NF-κB transcriptional activity [57]. Therefore we evaluated, nuclear protein extracts from CRC cells for the phosphorylated form of NF-κB after treatment with resveratrol and/or 5-FU, as described above (Fig. 8). The western blot analysis showed (Fig. 9A–B: I), the basal expression of NF-κB subunits (p50/p65) was down-regulated by resveratrol, however, treatment with 5-FU alone markedly induced a dose dependent up-regulation of the NF-κB subunits (p50/p65) phosphorylation in HCT116 and HCT116R cells. In contrast, co-treatment with resveratrol blocked NF-κB phosphorylation in alginate tumor microenvironment. Taken together, these results support a functional role for the NF-κB pathway in 5-FU-induced signaling pathway in HCT116 and HCT116R cells.

3.10.2. Resveratrol inhibits 5-FU-dependent IκBα degradation in CRC cells

It was noted that the phosphorylation and degradation of IκBα, the natural inhibitor of NF-κB is required for the activation of NF-κB [58]. To investigate whether inhibition of 5-FU-induced NF-κB activation occurs through inhibition of IκBα degradation, HCT116 and HCT116R cells were either left untreated or treated as described above (Fig. 8) and examined for IκBa activation in the cytoplasm by western blot analysis. Treatment of HCT116 and HCT116R cells with resveratrol (5 μM) completely suppressed IκBα degradation. Interestingly, treatment with 5-FU alone markedly up-regulated IκBα subunit (p50/p65) phosphorylation and this was blocked by co-treatment with resveratrol (Fig. 9A–B: II–III). These results indicated that resveratrol inhibits both 5-FU-induced NF-κB activation and IκBα degradation.

3.10.3. Resveratrol suppresses IκBα Kinase activation induced by 5-FU in CRC cells

Next, we examined the effect of 5-FU on IκBα kinase (IKK) activation, which is required for phosphorylation of IκBα and subsequently NF-κB signaling pathway activation. HCT116 and

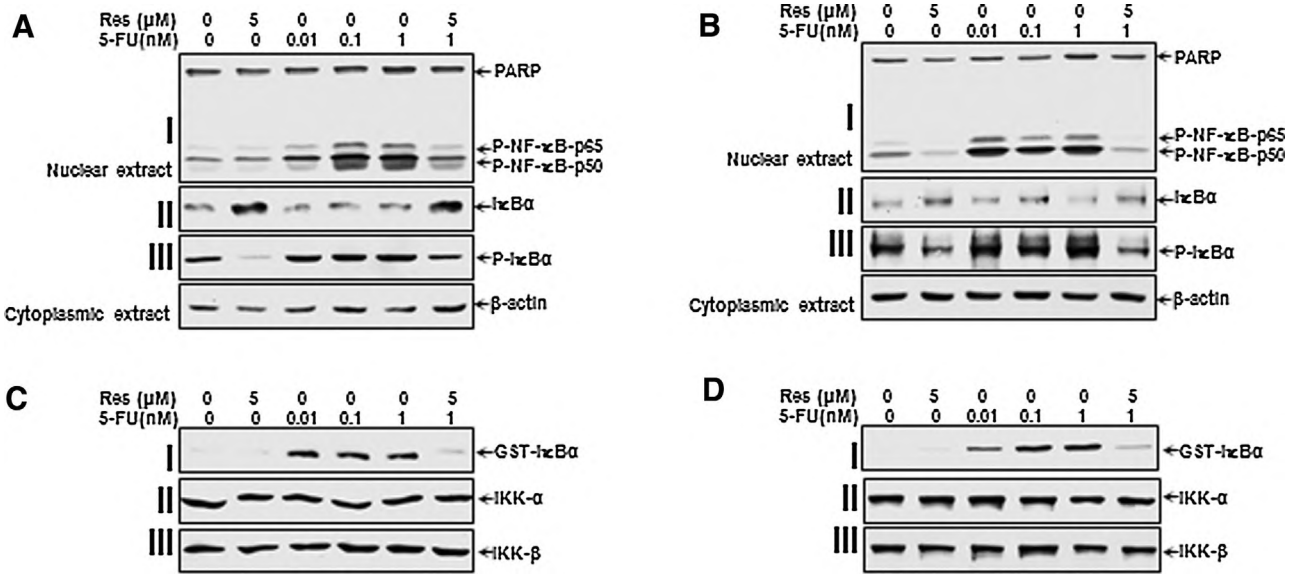

Fig. 9. A–D. Resveratrol inhibits 5-FU-induced phosphorylation and degradation of IκBα and phosphorylation and nuclear translocation of NF-κB In in CRC cells in alginate tumor microenvironment. A–B: The effect of resveratrol on 5-FU-induced IκBα phosphorylation and degradation of NF-κB phosphorylation and nuclear translocalization were investigated in HCT116 (A) and HCT116R (B) cell lines in alginate culture. Serum-starved HCT116 (A) and HCT116R (B) cell lines in alginate culture (1×10^6/ml) were either left untreated or treated with resveratrol alone (5 μM), 5-FU alone (0.01, 0.1, 1 nM) or were treated with resveratrol (5 μM) and 5-FU (1 nM). After 10 days of culture, nuclear extracts and cytoplasmic extracts were prepared, fractionated on 10% SDS–PAGE, and electrotransferred onto a nitrocellulose membrane. Western blot analysis was performed with anti-phospho-specific NF-κB (I), anti-IκBα (II) and anti-phospho-specific-IκBα (III) antibodies. The results shown are representative of two or three independent experiments. Housekeeping protein β-actin served as a loading control in all experiments. C-D: The effect of resveratrol on the activation of IKK by 5-FU was investigated in HCT116 (C) and HCT116R (D) cell lines in alginate culture. Cell extracts were immunoprecipitated with an antibody against IKK-α and immune complex kinase assays were performed as described in Materials and Methods. Equal amounts of total protein (500 ng protein/lane) were separated by SDS-PAGE under reducing conditions and then analyzed by immunoblotting using antibodies against phosphor-specific IκBα (I), IKK-α (II), and IKK-β (III). The results shown are representative of two independent experiments.

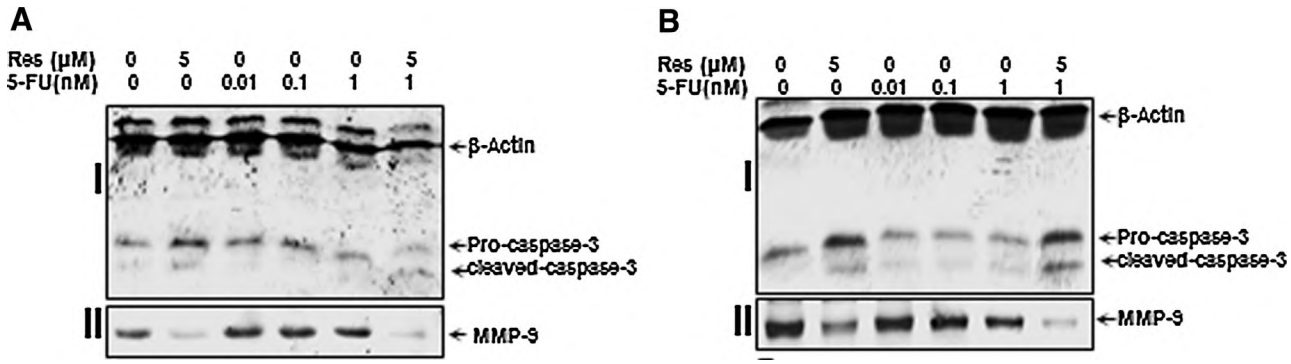

Fig. 10. A–B. Resveratrol blocks 5-FU-induced activation of NF-κB-regulated metastasis gene products (MMP-9) and enhances caspase-3-mediated apoptosis in CRC tumor cells. Serum-starved HCT116 (A) and HCT116R (B) cell lines in alginate culture (1×10^6/ml) were either left untreated or treated with resveratrol alone (5 μM), 5-FU alone (0.01, 0.1, 1 nM) or were treated with resveratrol (5 μM) followed by treatment with 5-FU (1 nM). After 10 days of culture, whole cell lysates (500 ng of protein/lane) were prepared and analyzed by western blotting for cleavage of caspase-3 (I) and expression of MMP-9 (II). The results shown are representative of two or three independent experiments. Housekeeping protein β-actin served as a loading control in all experiments.

HCT116R cells were either left untreated or treated as described above (Fig. 8) and whole cell extracts were immunoprecipitated with an antibody against IκBα kinase (IKK-α and IKK-β) and then analyzed by an immune complex kinase assay for phosphorylation of IκBα. No phosphorylated IκBα was found in basal control or resveratrol-treated cultures. As shown in Fig. 9C–D:I, the immune complex kinase assay showed that 5-FU induced the activation of IKK in a time dependent manner. In contrast, co-treatment of CRC cells with 5-FU followed by resveratrol resulted in an inhibition of 5-FU-induced effects on the activation of IKK (Fig. 9C–D:I). 5-FU and resveratrol had no direct effect on the expression of IKK-α and IKK-β proteins (Fig. 9C–D:II–III). Taken together, these results suggest that resveratrol suppressed IκBα phosphorylation/NF-κB signaling pathway and highlights the important functional role of resveratrol in inhibiting NF-κB pathway.

3.11. Resveratrol modulates 5-FU-induced NF-κB-dependent gene end-products involved in apoptotic and metastasis of CRC tumor cells

We examined further, whether resveratrol might modulate NF-κB-regulated gene products involved in apoptosis (cleavage of caspase-3) and metastasis (MMP-9) of CRC cells in alginate tumor microenvironment. HCT116 and HCT116R cells were either left untreated or treated as described above (Fig. 8) and whole cell extracts were examined by western blot analysis using specific antibodies. As shown in Fig. 10A–B, 5-FU induced expression of MMP-9 and blocked caspase-3 cleavage in a dose-dependent manner, and resveratrol blocked substantially MMP and induced caspase-3 cleavage in HCT116 (Fig. 10A:I–II) and HCT116R (Fig. 10B: I–II). The combined treatment with resveratrol and 5-FU resulted in significant synergistic enhancement in inducing caspase-3 cleavage in HCT116 (CI values = 0.742) and HCT116R (CI values = 0.276) cells compared to control tumor cultures (Fig. 10A–B: I–II), indicating that resveratrol enhances 5-FU-induced caspase-dependent cell death in CRC cells, rather than modulating 5-FU-induced NF-κB-regulated apoptotic genes. Taken together, these results further strengthen the role of resveratrol in modulating 5-FU-induced NF-κB-regulated gene products.

4. Discussion

Human colorectal cancer is one of the most commonly diagnosed cancers and one of the major causes of cancer-related morbidity and mortality worldwide. 5-FU is routinely employed in the management of colorectal cancer, but is toxic and ineffective in a large majority of patients, as they frequently develop metastasis. Furthermore, more than 50% of patients treated with 5-FU demonstrate resistance to 5-FU in the clinical setting. Therefore, there is a clear need for the development of novel therapeutic agents to treat these resistant tumors. The aim of this study was to determine whether resveratrol can individually, or in combination with 5-FU, has enhanced anti-cancer activity in the treatment of CRC cell lines in 3D-alginate tumor microenvironment.

The results of our study revealed that the proliferation of all 4 CRC cell lines (HCT116, HCT116R, SW480 and SW480R) was suppressed by resveratrol and this finding is in agreement with other studies showing that resveratrol can inhibit the growth of diverse tumor cells [59,60]. We could also successfully demonstrate that resveratrol in combination with 5-FU had significant synergistic effect (CI values lower than 1), in increasing the anti-cancer properties of 5-FU including suppression of proliferation, invasion and enhancing apoptosis in CRC cells confirming that resveratrol chemosensitizes to 5-FU-based drug regimens. Indeed, it has been previously reported that suppression of proliferation on cancer cells in mice by resveratrol is enhanced by 5-FU [61].

It is known that resveratrol inhibits tumor cell proliferation, invasion, metastasis and stimulates apoptosis via modulation of a wide range of signal transduction pathways [29,34–38]. However, the molecular mechanism(s) of resveratrol on adhesion molecules and cell–cell adhesion components has not been explored. Moreover, direct cell–cell communications are mediated by intercellular junction complexes, including adhesion molecules, desmosomes, tight- and gap junctions, which enables adhesion and communication between individual cells, suppress tumor metastasis and retain the integrity of tissues [5,7]. Here, we provide a comprehensive evidence with our ultrastructural electron microscopic evaluations that in the presence of 5-FU the CRC cells undergo ultrastructural changes that typify highly metastatic cells e.g. the development of microvilli on nearly the entire surface of the cells, microfilaments with high amount of endoplasmic reticulum and golgi apparatus. More interestingly, contrariwise, CRC cells treated with resveratrol and/or 5-FU revealed ultrastructural changes that mirrored weakly metastatic cells e.g. almost exclusively planar cell surface with tight cell–cell contacts with desmosomes, adhesion molecules and tight- and gap junctions, very small amount of microvilli. These effects manifested in a dose-dependent manner, with higher concentration of resveratrol accompanied with induction of apoptosis in CRC cells. Moreover, following resveratrol treatment we observed a dose-dependent increase of intracellular vesicles at or near intercellular junctions, highlighting that resveratrol enhances specific, target-orientated endo-/exocytosis controlled by intercellular junctions. Indeed, it has been previously acknowledged that intercellular junctions not only provide the cell with structural integrity, but

also function as landmarks, spatially confining signaling molecules and polarity cues as well as serving as docking sites for vesicles [62]. These results suggest that ultrastructural changes of CRC tumor cells are required for the rapid growth and metastasis of tumor cells. Indeed, tight junctions have been shown to be highly dynamic, multimolecular and multifunctional complexes [63]. These findings are further in agreement with those, which have shown that tumor cell surface morphological changes are closely associated with aggressive growth and proliferation of tumors [5,64]. Furthermore, it seems that the intercellular junction interactions may directly manage on the quality and malignity originating from tumor cells. Indeed, several lines of evidence have shown that cell–cell junction complexes, such as desmosomes, tight-, gap junctions and adherence molecules, may increase intercellular adhesion in tumor, leading to cell aggregation and stabilization of the tumor cells [5,6,65].

We also found a basal expression of claudin-2 and E-cadherin on CRC cells and this expression was significantly increased in the presence of resveratrol and decreased in the presence of 5-FU and interestingly this was accompanied with a conversion of epithelial-to-mesenchymal transition (EMT). Moreover, it has been reported that claudin-2 and E-cadherin are expressed in CRC cells [66,67]. Our immunfluorescence, western blotting and immunoelectron microscopy results showed that resveratrol, in opposite to 5-FU up-regulates significantly the expression of claudin-2 and E-cadherin. E-cadherin is an important component of cell surface adherence junction and regulates tumor cell progression, migration and metastasis through maintenance of cell–cell contacts [50]. It has been reported that EMT causes higher invasive properties and loss of cell–cell junctions is linked with highly metastatic spread in aggressive tumor cells [7]. Furthermore, claudins are epithelial tight junction components that are suppressed by EMT transcriptions factors such as Snail and Slug [3]. We found further in this study that 5-FU dose dependently increased mesenchymal cell morphology and EMT markers, such as vimentin, slug and decreased E-cadherin in CRC cells and in opposite to this, resveratrol induced an epithelial conversion in CRC cells in alginate tumor microenvironment with decreasing of vimentin and slug and increasing of E-cadherin. Consistent with these results, several studies have reported that in tumor cells an increase of mesenchymal features (N-cadherin and vimentin) and loss of epithelial features (E-cadherin) via EMT is proportional with cancer proliferation, motility, drug resistance and metastasis [68–71]. Recently, we have shown that up-regulation of TGF-β in the tumor microenvironment by CRC cells and fibroblasts play an active role in inducing EMT [67]. Interestingly, it has been previously reported that TGF-β-signaling induces disassembly of tight junctions during epithelial to mesenchymal transition [72].

These synergistic effects between resveratrol and 5-FU illustrate that different drugs mediate their ability to modulate different signaling molecules in cancer cells. It has been reported that resveratrol mediates its chemosensitization effects on cancer cells by targeting proteins involved in cell proliferation, cell survival, target transport and apoptosis [60,73]. In term of 5-FU, as a substitute, has a related structure to uracil and incorporates in DNA and RNA. This process inhibits cell proliferation by suppressing DNA production [74]. Indeed, based on the CI values, synergy between resveratrol and 5-FU by measuring CI values of different drug combinations according to the Chou-Talalay equation (CI values lower than 1), suggesting that the combination of these drugs has a synergistic effect in decreasing cell proliferation and invasion and resveratrol chemosensitizes to 5-FU-based drug regimens.

To study the molecular mechanism, by which resveratrol or/and 5-FU execute their effects against HCT116 and its FU-resistant cells, we found that resveratrol alone or in combination with 5-FU showed suppression of NF-κB and NF-κB-regulated gene end-products (caspase-3, MMP-9) that are involved in growth, proliferation, invasion and apoptosis in CRC cells. Furthermore, we could show that resveratrol down-regulates NF-κB signaling pathway through suppression of IκBα kinase activation and IκBα phosphorylation in both CRC cell lines. Indeed, it has been further reported that resveratrol blocks malignancy of tumor cells via modulation, at least in part of transcription factor NF-κB. In our earlier reports, we demonstrated clearly that resveratrol is a potent inhibitor of the NF-κB signaling pathway [75–77], which has been closely linked to inflammation and cancer [24] and was found to promote EMT and metastasis [78]. Our findings are further in agreement with studies that resveratrol down-regulates proliferation, invasion and metastasis in glioma, lung carcinoma and breast tumors [79–82]. These results suggest that the synergistic effects of resveratrol and 5-FU and chemosensitizing effect of resveratrol on 5-FU resistant cells is, at least in part, through suppressing NF-κB signaling in tumor cells.

In conclusion, for the first time, we describe herein that resveratrol chemosensitizes CRC cells to chemotherapeutic agent (5-FU), which has chemopreventive potential by affecting multiple cell adhesion molecules, desmosomes, tight-, gap junctions and signaling molecules involved in cell proliferation, invasion and metastasis and potentiated the anti-cancer effects of 5-FU. Based on these findings, further investigations are required to explore in detail the potential of resveratrol as an anti-cancer agent in CRC.

Competing interests

The authors declare that they have no competing interests.

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