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Zebrafish embryo extracts enhance 5-FU anti-cancer effects upon breast cancer cells

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Abstract. – OBJECTIVE: The inhibition of the metastatic capability of cancer cells is a pivotal aim of current anticancer strategies. We investigated herein the anti-migrating and anti-invasive properties of Zebrafish embryo extracts (SL) – an integrative formula comprising morphogenetic factors extracted from zebrafish embryos – alone or in association with 5-Fluoro-Uracil (5-FU), when added to metastatic breast cancer cells (MDA-MB-231) and in normal epithelial breast cells (MCF10A) committed toward an inflammatory phenotype upon TGF-β1 stimulation.

MATERIALS AND METHODS: Invasiveness, migrating capability, cytoskeleton architecture and related molecular factors involved in the epithelial-mesenchymal transition were studied after treatment with 5-FU, with and without SL.

RESULTS: Remarkably, in both circumstances, embryo extracts amplify the migratory inhibition triggered by the anticancer drug 5-Fu. The fact that such an effect is noticed in normal as well as in cancerous cells suggests that the critical target of embryo extracts is specifically represented by the migrating/invasive phenotype. However, while 5-FU was unable in antagonizing the invasiveness of cancerous cells, the association with SL can significantly impair the invasive capability of tumor cells. These findings are noticeably associated with the reversion of the EMT phenotype in SL-treated cells, as documented by the contemporary downregulation of TCTP and some EMT-related molecular effectors, like α-SMA and Vimentin.

CONCLUSIONS: Embryo fish extracts significantly counteract the migrating and invasive phenotype of cancerous and inflammatory breast cells treated with the chemotherapeutic drug 5-FU. The availability of a compound able to amplify 5-Fu activity while significantly hampering the invasive phenotype of breast cancer should provide invaluable benefits, namely if we consider that this compound is substantially deprived of side-effects.

Key Words:
Tumor reversion, TCTP, Zebrafish embryo extracts, Breast cancer, 5-Fluorouracil, Migrastatics.

Introduction

Some studies[1,2] demonstrated that some low molecular weight factors expressed by early embryonic microenvironments remarkably modulate cancer behavior, by inducing apoptosis, growth arrest and phenotypic reversion through inhibition of the migrating/invasive phenotype of highly malignant cancer cells. Namely, several cancers have been shown to undergo partial or complete reversion when exposed to embryonic environments or treated with diffusible factors extracted from oocytes or embryonic cells (reviewed in[3]). This process entails mitigation of proliferation rate[4], apoptosis enhancement[5], as well as partial or complete reversion of the malignant phenotype, achieved through selective demethylation processes that ultimately promote the rewiring of the gene regulatory network and an overall system's change mediated by cytoskeleton remodeling[6-8]. Noticeably, embryo extracts show the capability to induce a phenotypic "repro-

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gramming" through which cancer cells dismiss their migrating/invasive character to revert toward a native non-malignant phenotype, deprived of significant metastatic potentiality. This process would significantly entail a partial/total transition from the mesenchymal toward an epithelial phenotype by enacting a reversion process that, as previously shown by Telerman's team[9], requires the downregulation of Translationally Controlled Tumor Protein (TCTP), a key protein that govern the "reprogramming" transition in cancer. We have already shown that stage developmental factors extracted from embryos of zebrafish can efficiently arrest cancer proliferation and induce apoptosis, both *in vitro*[10] and *in vitro*[11]. Moreover, sublingual administration of zebrafish embryo extracts showed to improve response to conventional chemotherapy while reducing the incidence of drug-resistance in a pilot study on human colon cancer patients[12], while a significant clinical improvement has been observed in patients with advanced hepatocellular carcinoma resistant to conventional chemotherapy and treated with zebrafish embryo extracts (SL)[13].

Therefore, we hypothesized that the association of zebrafish embryo extracts to conventional chemotherapy would significantly increase the drug activity in promoting the reversal of the pro-metastatic neoplastic phenotype into a less malignant phenotype, especially by inhibiting its migrating and invasive capabilities. Herewith, we addressed such issues by investigating both triple-negative breast cell line (MDA-MB-231), and normal immortalized breast cell line (MCF10A) – committed toward a migrating inflammatory phenotype under Transforming growth factor beta-1 (TGF-β1) stimulation – treated with 5-Fluorouracil (5-FU), SL or both compounds in association.

Materials and Methods

Experimental Cell Model

The human Caucasian breast adenocarcinoma cell line MDA-MB-231 (ECACC Cat# 92020424) was obtained from Sigma-Aldrich (St. Louis, MO, USA), the non-tumorigenic epithelial cell line MCF-10A (ATCCCRL-10317) was obtained from LGC Standards S.r.l, MI, Italy. Cells were seeded into 25 cm^2 flasks (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA). MDA-MB-231 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/mL, gentamycin 200 μg/mL; all from Euroclone Ltd., Cramlington, UK); MCF-10A were grown in Dulbecco's Modified Eagle's Medium/nutrient mixture F12 Ham (Sigma-Aldrich, Merck, Darmstadt, Germany) supplemented with 10% horse serum (Euroclone Ltd., Cramlington, UK) and EGF 500 μg/5 mL (Santa Cruz Biotechnologies, Dallas, TX, USA), Hydrocortisone (50 microM), cholera toxin (0.5 mg/mL), insulin (10 mg/mL) (all from Sigma Chemical Co, USA) and antibiotics (penicillin 100 IU/mL, streptomycin 100 μg/mL, gentamycin 200 μg/mL) all from Euroclone Ltd., Cramlington, UK. The cells were cultured at 37°C in an atmosphere of 5% CO_2 in air. The medium was changed every third day. At confluence, the cells were sub-cultured after removal with 0.05% trypsin–0.01% EDTA. In MDA-MB-231, 0.1 mg/mL 5-FU (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/mL 5-FU + 0.3 μg/mL SL and 0.3 μg/mL SL were added in DMEM supplemented with 0.1% FBS. MCF-10A cells were firstly treated with 10 nanog/mL TGF-β1 (PeproTech catalog#100-21) for five days and on fifth day 0.1 mg/mL 5-FU, 0.1 mg/mL 5-FU + 0.3 μg/mL SL and 0.3 μg/mL SL were added in F12 Ham supplemented with 0.1% horse serum.

Cell Migration Assay

The 2.5×10^4 cells non-stimulated (ctrl) and stimulated 0.1 mg/ml 5-FU, 0.1 mg/ml 5-FU + 0.3 μg/ml SL and 0.3 μg/mL SL respectively, were placed in 500 μL DMEM + 0.1% FBS medium (DMEM F12 + 0.1% horse serum + 10 nanog/ml TGF-β1 in case of MCF-10A cells) in the upper side of 8 mm filters (Falcon, BD Biosciences, San Jose, CA, USA) (upper chamber) and placed in wells of a 24-well plate (Falcon, BD Biosciences, Franklin Lakes, NJ, USA) (lower chamber), containing 0.8 ml of DMEM + 10% FBS medium (DMEM F12 + 10% horse serum in case of MCF-10A cells). After 24 h of incubation, the migratory cells on the lower surface of membranes were fixed, stained with Hemacolor® (HX54775574, Merck, Darmstadt, Germany) and examined microscopically. Cellular migration was determined by counting the number of cells on membranes in at least 4-5 randomly selected fields using a Zeiss Axiovert 10 optical microscope. For each data point, four independent experiments in duplicate were performed.

Cell Invasion Assay

The 2.5 × 10^4 cells non stimulated (CTRL) and 0.1 mg/mL 5-FU, 0.1 mg/mL 5-FU + 0.3 μg/mL SL and 0.3 μg/mL SL as single agent respectively, were placed in 500 ml DMEM + 0.1% FBS medium (DMEM F12 + 0.1% horse serum + 10 nanog/mL TGF-β1 in case of MCF-10A cells) in the upper side of 8-mm filters (BD Bio-CoatTM growth factor reduced MATRIGEL™ invasion chamber (BD Biosciences-Discovery Labware, Two Oak Park, Bedford, MA, USA) (upper chamber) and placed in wells of a 24-well plate (Falcon, BD Biosciences, USA) (lower chamber), containing 0.8 mL of DMEM 10% FBS medium (DMEM F12 + 10% horse serum in case of MCF-10A cells). After 24 h of incubation, the invasive cells on the lower surface of membranes were fixed, stained with Hemacolor® (HX54775574, Merck, Darmstadt, Germany) and examined microscopically. Cellular invasion was determined by counting the number of cells on membranes in at least 4–5 randomly selected fields using a Zeiss Axiovert 10 optical microscope. For each data point, four independent experiments in duplicate were performed.

Wound Healing Assay

To evaluate the migratory phenotype of treated or non-treated cells, we performed the wound-healing assay using special double well culture inserts (Ibidi GmbH, Martinsried, Germany). Each insert was placed in 8-well m-slides (Ibidi GmbH, Am Klopferspitz 19, D-82152 Martinsried, Germany) and 3.5 x 10^4 cells were placed into both wells of each insert with 70 ml of complete medium. When cells were confluent, the culture inserts were gently removed, and cells were fed with 10% FBS DMEM (CTRL), 0.1 mg/mL 5-FU, 0.1 mg/mL 5-FU + 0.3 μg/mL SL and 0.3 μg/mL SL for 18 h.

Confocal Microscopy

To evaluate the migratory phenotype of treated or non-treated cells, we perform the wound-healing assay using special double well culture inserts (Ibidi GmbH, Martinsried, Germany). Each insert was placed in 8-well μ-slides (Ibidi GmbH, Am Klopferspitz 19, D-82152 Martinsried, Germany) and 3.5 × 10^4 cells were placed into both wells of each insert with 70 μl of complete medium. When cells were confluent, the culture inserts were gently removed, and cells were fed with 10% FBS DMEM (CTRL), 0.1 mg/mL 5-FU, 0.1 mg/mL 5-FU + 0.3 μg/mL SL and 0.3 μg/mL SL for 24 h.

Cells were fixed with 4% paraformaldehyde for 10 min at 4°C and washed twice for 10 min with PBS. The cells were permeabilized for 30 min using PBS, 3% BSA, 0.1% Triton X-100 followed by anti-vinculin (7F9): sc-73614 (from Santa Cruz Biotechnology, Santa Cruz, CA, USA) staining in PBS, 3% BSA at 4°C overnight. The cells were washed with PBS and incubated for 1 h at room temperature with appropriate secondary antibody FITC conjugated (Invitrogen Molecular Probes Eugene, OR, USA).

For F-actin visualization, Rhodamine Phalloidin (Invitrogen Molecular Probes Eugene, OR, USA 1:40 dilution) was used. The cells were then washed in PBS and mounted in buffered glycerol (0.1 M, pH 9.5). Finally, analysis was conducted using a Leica confocal microscope TCS SP2 (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) equipped with Ar/ArKr and He/Ne lasers. Laser line were at 543 nm and 488 nm for TRITC and FITC excitation, respectively. The images were scanned under a 40X oil objective. To analyze the colocalization of actin and vinculin, optical spatial z series composed of about 8/10 optical section with a step size of 1 μm were performed. Color channels were merged and colocalization were analyzed with the Leica confocal software.

Western Blots

Control and stimulated cells were washed twice with ice-cold PBS and scraped in RIPA lysis buffer (Sigma Aldrich, St. Louis, MO, USA). A mix of protease inhibitors (Complete-Mini Protease Inhibitor Cocktail Tablets, Roche, Mannheim, Germany) and phosphatase inhibitors (PhosStop; Roche, Mannheim, Germany) was added just before use. Cellular extracts were then centrifuged at 8000 × g for 10 min. The Bradford assay was used to determine protein contents. For western blot analysis, cellular extracts were separated on SDS-polyacrylamide gels and proteins were blotted onto nitrocellulose membranes (BIO-RAD, Bio-Rad Laboratories, Hercules, CA, USA). The following antibodies were analyzed: anti-vimentin (sc-6260 from Santa Cruz Biotechnology, Santa Cruz, CA, USA), alpha-sma (ab5694 from Abcam), anti-TPT1 (TCTP) (E-AB-31729) from Elabscience. Antigens were detected with an enhanced chemiluminescence kit (Western Bright ECL HRP Substrate, Advansta Inc., Menlo Park, CA, USA), according to the manufacturer's instructions.

Densitometry

All Western blot images were acquired and analyzed through Imaging Fluor S densitometer (Biorad-Hercules, CA, USA). Optical density (OD) of each condition was normalized *vs.* the signal of internal control GAPDH (anti-GAPDH #2118 from Cell Signaling Technology, Danvers, MA, USA).

Results

Zebrafish Embryo Extracts Antagonizes Cancer Cell Invasiveness and Migrating Capability

To ascertain to what extent zebrafish embryo extracts (SL) can significantly reverse the malignant phenotype, we plan to investigate the invasive and the migratory capability of the malignant breast cancer cell line MDA-MB-231 and of the immortalized normal breast cell line MCF10A, the latter committed toward a pro-inflammatory, invasive phenotype when stimulated by TGF-β1, a cytokine able in promoting the epithelial-mesenchymal transition (EMT)[14].

MCF10A Cells

The immortalized, normal breast cell line MCF10A, at five days after the stimulation with TGF-β1 10 nanog/ml, shows a dramatic increase of invasiveness (up to eight-fold) and migrating capability (up to three-fold), *vs.* control (Figure 1A-B). Both 5-FU and SL significantly inhibits migration and invasiveness, albeit SL seems to exert a more pronounced effect. Noticeably, the association 5-FU+SL leads to an additive result, besides statistical meaning is consistent only for invasiveness.

MDA-MB-231 Cells

Highly metastatic breast cancer cells exhibit a marked invasive and migrating phenotype, as estimated at 24 hours. Invasiveness is poorly affected by 5-FU and SL when added individually; however, a significant decrease (-45%) is observed when both are simultaneously added to breast cancer cells (Figure 2A). On the contrary, cell motility is remarkably reduced by 5-FU and SL (-30% and 50%, respectively), nonetheless the association of both does not lead to any significant additive effect (Figure 2B).

Wound Healing Assay

These findings are mirrored by results obtained with the Wound Healing Assay. MCF10A cells stimulated with TGF-β1 display a remarkable increase in the speed at which the open area is replenished, leading to an almost complete wound recovery after only 18 hours (Figure 3A). Noteworthy, while 5-FU only slows down the reparative process, SL, and the association 5-Fu+SL dramatically inhibits the healing process. Namely, the association 5-FU+SL eventually deepens the wound as the open area increases significantly (+26%) when compared to control value (Figure 3B). Unfortunately, such findings are only partially confirmed when 5-FU and SL are added to MDA-MB-231 cells. While SL still display an appreciable, albeit marginal inhibition upon the wound healing process, neither 5-Fu nor the association 5-FU+SL seem to exert a valuable effect on the repair process (Figure 4A-B).

Overall, these data suggest that the association of SL to conventional chemotherapy can successfully hamper cancer invasiveness, while the anticancer drug by itself is deprived of such remarkable capability. Nonetheless, motility of cancer cells is only marginally influenced by the association involving 5-FU and SL.

Epithelial-Mesenchymal Transition

Our findings support the idea that embryo factors, as those represented in the SL formula, can be even more effective than conventional chemotherapy in reversing prominent malignant features like invasiveness and migratory behavior. To ascertain if this effect could be traced back to the epithelial-mesenchymal (EMT) features harbored by invasive cancer cells, we investigated if the treatment with 5-FU and SL – as singularly or in association – could hamper and eventually revert the pivotal phenotypic traits associated with EMT.

Cytoskeleton Remodeling

Changes in the migratory/invasive phenotype are associated with recognizable cytoskeleton rearrangement. Conversely, treatment aiming at inhibiting the invasive/migrating phenotype are likely to modify the cytoskeleton architecture. Indeed, control MDA-MB-231 cells were found to harbor a dense texture of stress fibers, with F-actin filaments distributed all along the cytosol (Figure

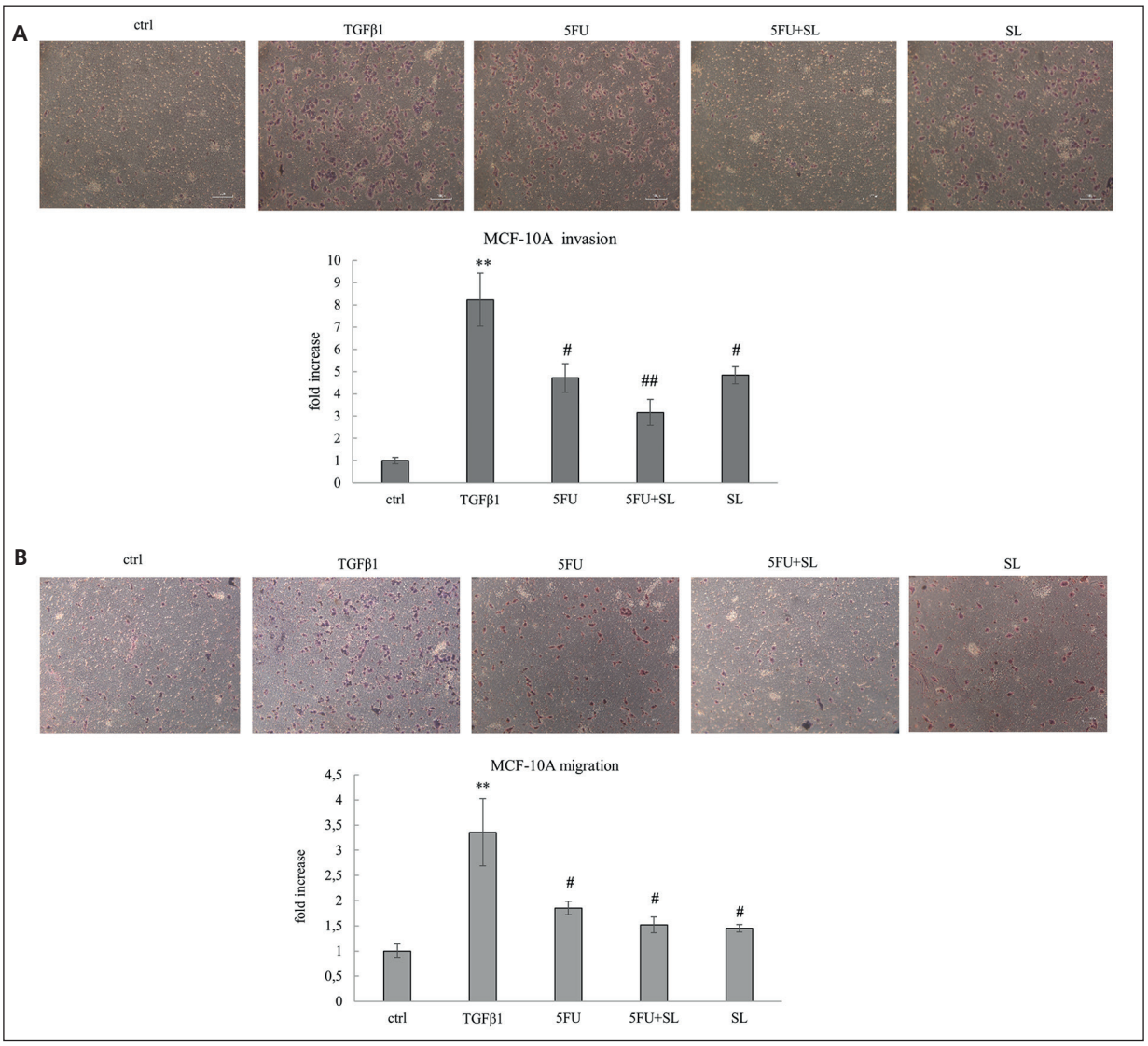

Figure 1. Effects of 5FU, 5FU+SL and SL on invasion (**A**) and migration (**B**) in MCF-10A cells. Transwell assays were performed in MCF-10A cells untreated (ctrl) and pre-treated with TGF-β1 for 5 days. TGF-β1 stimulated MCF-10A cells were then treated with 5FU, 5FU+SL and SL for 24 h. Values, expressed as fold increase of control value considered as 1, are means of three independent experiments performed in duplicate, with SD represented by vertical bars. *$p<0.05$, **$p<0.01$ *vs.* ctrl; $^{\#}p<0.05$; $^{\#\#}p<0.01$ *vs.* TGF-β1 by ANOVA followed by Bonferroni post-test. Images were obtained by optical microscopy, with ×100 magnification.

5A), a pattern that is usually associated with the migratory/invasive phenotype, and that can bestow cells with the required capability to sustain motility and invasion[15]. Noticeably, 5-FU-treated cells do not show appreciable changes in F-actin pattern of distribution, whereas stress fibers are clearly identifiable and strongly expressed (Figure 5B). On the contrary, SL-treated cells show a F-actin distribution predominantly concentrated along the membrane border (Figure 5C), and an almost complete disappearance of stress fibers. In breast cancer cells treated with the association 5-FU+SL mixed results were found, with appreciable reduction in the density of stress fibers and recovery of a normal F-actin distribution (Figure 5D). Moreover, SL-treated MDA-MB-231 cells almost completely lost pseudopodia and lamellipodia, two prominent structures required by migrating/invasive cells, as evidenced in control and 5-Fu-treated MDA-MB-231 cells, in which polarized lamellipodia with treadmilling filaments, as well as filopodia were easily recognizable.

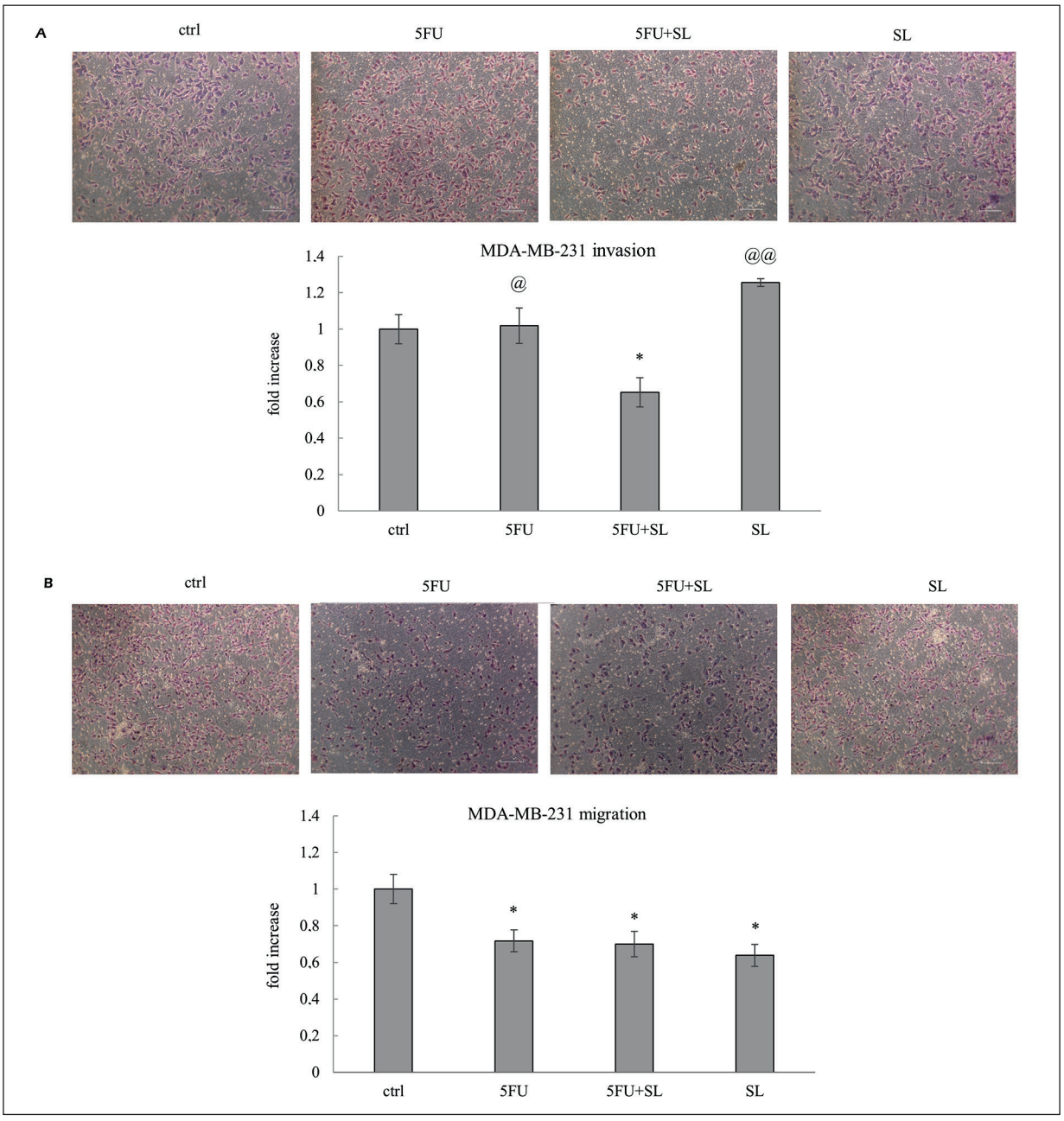

Figure 2. Effects of 5FU, 5FU+SL and SL on invasion (**A**) and migration (**B**) in MDA-MB 231 cells. Transwell assay were performed in MDA-MB 231 cells untreated (ctrl) and treated with 5FU, 5FU+SL and SL for 24 h. Values, expressed as fold increase of control value considered as 1, are means of three independent experiments performed in duplicate, with SD represented by vertical bars. *$p< 0.05$ *vs.* ctrl; $^{@}p<0.05$; $^{@@}p<0.01$ *vs.* 5FU+SL by ANOVA followed by Bonferroni post-test. Images were obtained by optical microscopy, with ×100 magnification.

Moreover, in MDA-MB-231 cells vinculin is mostly internalized and significantly reduced at the adhesion sites (Figure 5A). Vinculin stabilizes focal adhesions, and negatively drives cell spreading, lamellipodia formation and locomotion, thereby suppressing cell migration. Noticeably, both 5-FU and SL increase vinculin redistribution behind the cell membrane, with the most remarkable effect recorded with the association 5-FU+SL (Figure 5D).

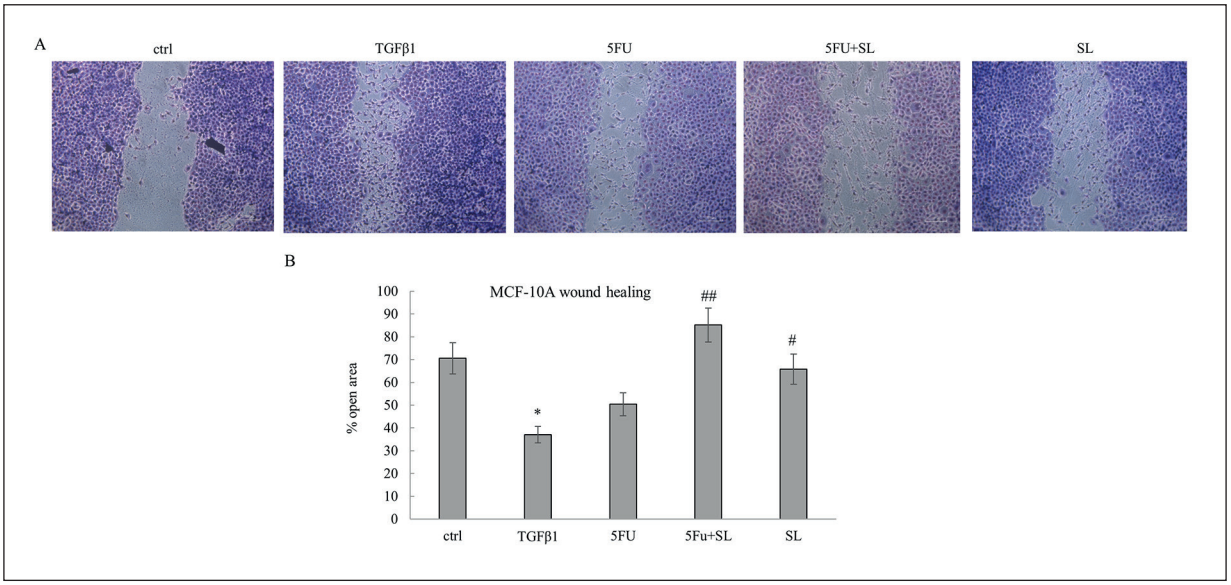

Figure 3. Wound-healing assay in MCF-10A cells. (**A**) Representative phase-contrast images of wound-healing assay taken 18 h after insert removal. Images were obtained by optical microscopy, with ×100 magnification (**B**) Quantitative analysis of wound closure after 18 h from insert removal. Values, expressed as the mean percentage of residual open area compared with the respective cell-free gap at T0, are means of three experiments performed in duplicate. *$p<0.05$ *vs.* ctrl; $^{\#}p<0.05$; $^{\#\#}p<0.01$ *vs.* TGF-β1 by ANOVA followed by Bonferroni post-test.

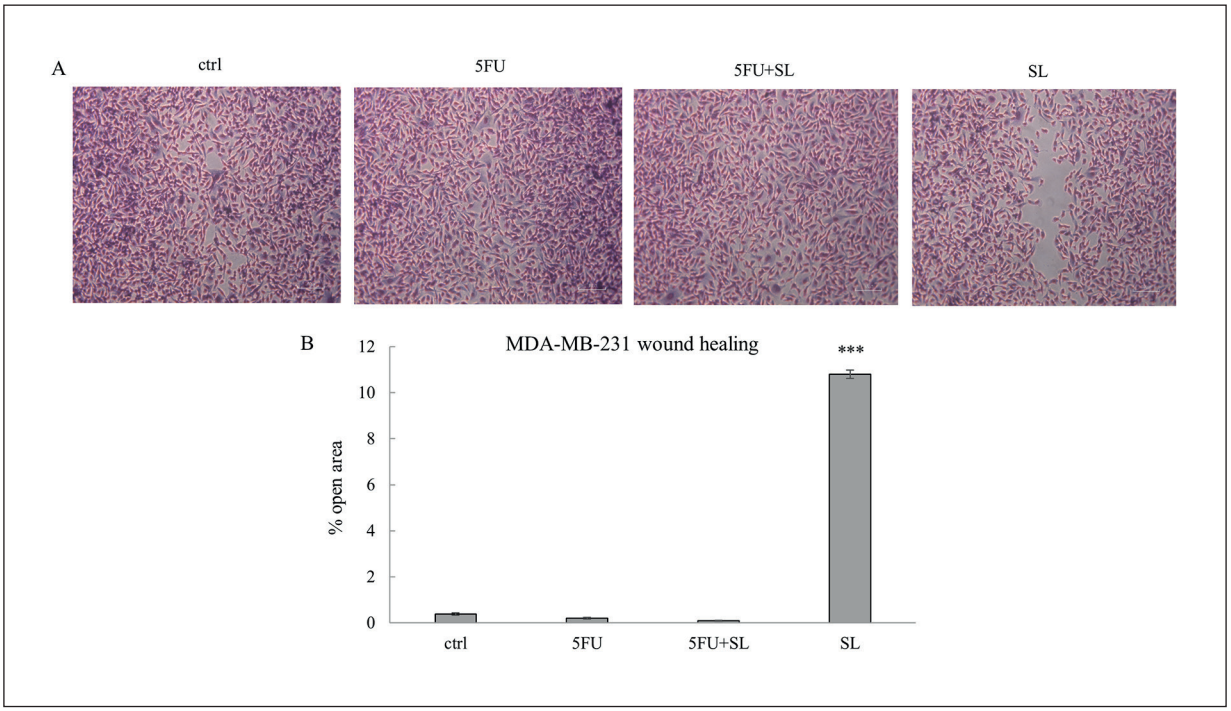

Figure 4. Wound-healing assay in MDA-MB-231 cells. (**A**) Representative phase-contrast images of wound-healing assay taken 18 h after insert removal. Images were obtained by optical microscopy, with ×100 magnification (**B**) Quantitative analysis of wound closure after 18 h from insert removal. Values, expressed as the mean percentage of residual open area compared with the respective cell-free gap at T0, are means of three experiments performed in duplicate. ***$p<0.001$ *vs.* ctrl by ANOVA followed by Bonferroni post-test.

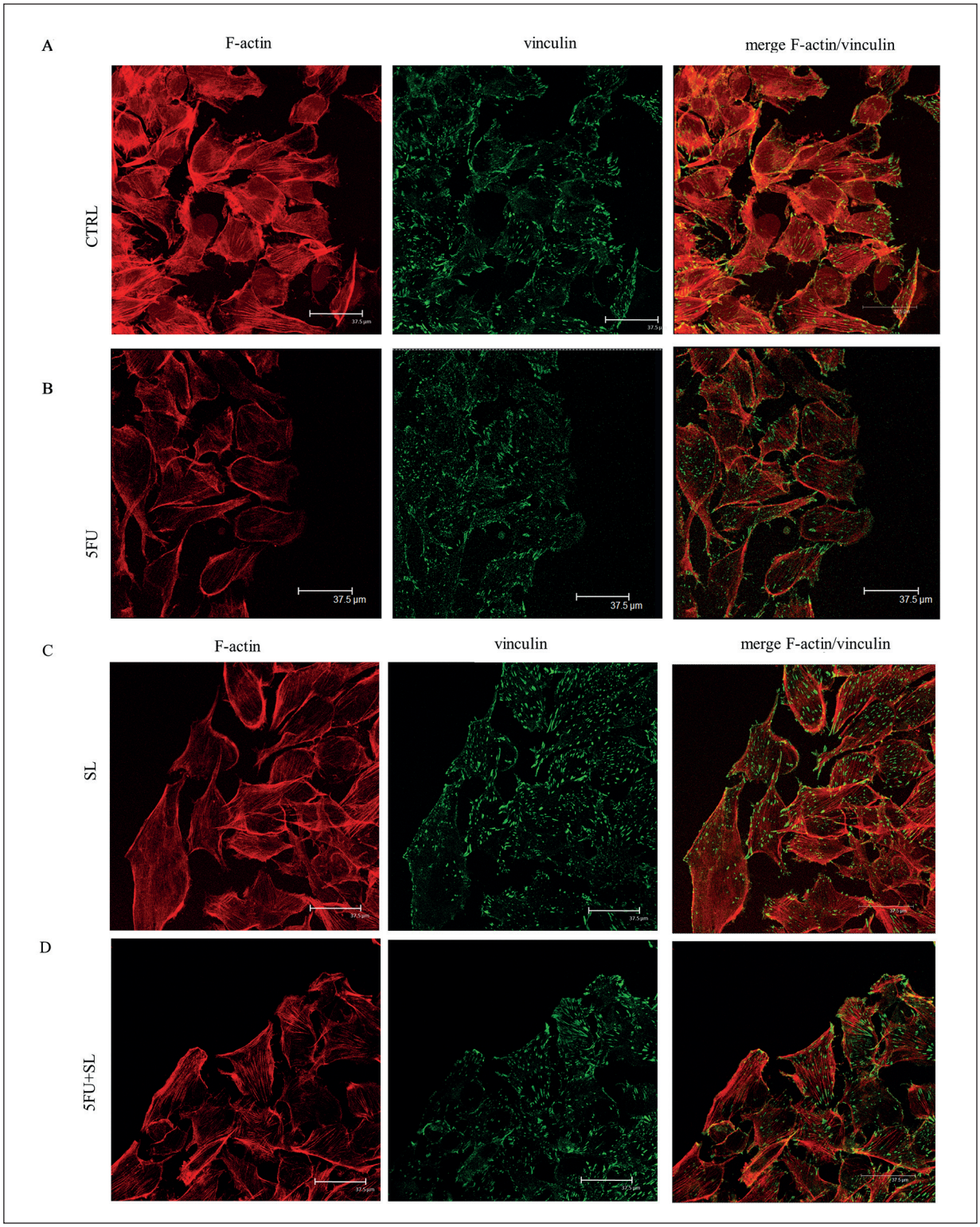

Figure 5. Distribution pattern of vinculin and F-actin in wound-healing assay performed on MDA cells cultured in control condition **(A)** or exposed to 5FU **(B),** SL **(C),** and 5FU+SL **(D)**. Confocal microscopy analysis of F-actin staining with rhodamine-phalloidin (red signal) merged with anti-vinculin immunofluorescence (FITC/green signal) on MDA-MB-231 cells subjected to wound healing assay, and cultured with or without 5FU, SL, 5FU+SL.

Molecular Parameters: Zebrafish Embryo Extracts Downregulate TCTP Expression in MDA-MB-231 Cells

TCTP has emerged as a critical regulator of cell fate determination. This protein regulates distinct biological processes, many of whom converge to a limited set of key events that participate in determining tumor reversion, i.e., the process through which cancerous cells recover a non-malignant phenotype[16]. Increased expression of TCTP has been noticed in several cancer cells and, conversely, TCTP silencing has been shown to promote cancer de-differentiation/regression in some instances, including breast cancer[17]. Downregulation of TCTP is also associated with reduced expression of mesenchymal molecular markers, like α-SMA and Vimentin, which play a critical role in sustaining EMT[18,19]. In our model, a significant down-regulation of TCTP was induced by SL treatment in MDA-MB-231 cells; the association in between 5-FU and SL does not provide any additive benefit, besides TCTP were still below the values measured in control cells (Figure 6A), while in 5FU-treated cells only a slightly, not significant decrease in TCTP levels was found. As expected, Vimentin was significantly reduced (Figure 6B) by SL treatment, whereas the combination of 5-FU+SL does not add further efficacy. On the contrary, only SL-treated cells showed an appreciable reduction in α-SMA levels (Figure 6C). Overall, such findings suggest that, upon treatment with SL alone or in association with anticancer drugs, cytoskeleton changes and reversion of the migrating/invasive phenotype are followed by concomitant modulation of key molecular factors – like TCTP – that play a pivotal role in enacting tumor differentiation.

Discussion

In the present study, we report that extracts obtained from fish embryonic tissues – a complex formula comprising diffusible low-molecular weight factors extracted from zebrafish embryos – can inhibit the invasive/migrating cell phenotype, in both metastatic breast cancer cells as well as in normal epithelial breast cells committed toward an inflammatory phenotype upon TGF-β1 stimulation. Remarkably, in both circumstances, SL greatly amplifies the inhibition of migration capability triggered by the anticancer drug 5-FU. The fact that such an effect is noticed upon normal as well as in cancerous cells, suggest that the critical target of SL is specifically represented by the migrating/invasive phenotype. Indeed, treatment with SL is associated with the reversion of the EMT phenotype, as documented by the contemporary downregulation of TCTP and some EMT-related molecular effectors, like α-SMA and Vimentin. However, it is worth noting that while 5-FU does not significantly impair cancer cells invasiveness, when SL was added a significant reduction in cancer cell invasive capability was observed. That finding suggest that morphogenetic embryo factors can efficiently enhance drug efficiency, namely by counteracting the invasive phenotype that frequently can be accidentally triggered by conventional treatments[20].

Downregulation of TCTP is a key event during tumor and EMT reversion, leading in both cases to a loss of the invasive/migrating traits of transformed cells. In turn, TCTP reduction entails a complex cascade of structural and biochemical events, including cytoskeleton remodeling,

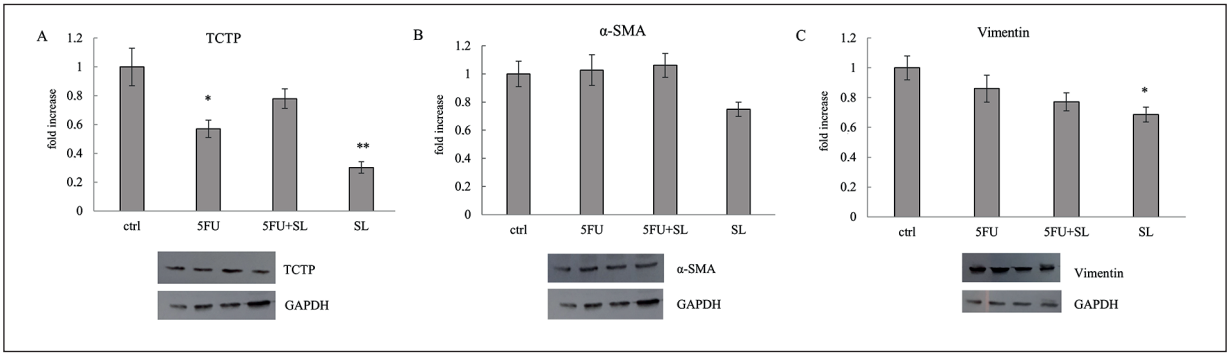

Figure 6. Effect of 5FU, 5FU+SL and SL on expression of TCTP **(A)** and α-SMA **(B)** and vimentin **(C)** in MDA-MB 231 cells. Columns represent densitometric quantification of optical density (OD) of specific protein signal normalized with the OD values of GAPDH served as a loading control and they are expressed as fold increase of control value considered as 1. Each column represents the mean value ± SD of three independent experiments. *$p<0.05$; **$p<0.01$ *vs.* ctrl by ANOVA followed by Bonferroni post-test. Representative western blot analysis relating to TCTP, α-SMA and vimentin expression in MDA-MB 231 cells untreated (ctrl) and treated with 5FU, 5FU+SL and SL for 24 h.

pathways modulation and even reframing of the gene regulatory network[21].

It is noticeably that, in parallel to TCTP inhibition, EMT is chiefly antagonized through cytoskeleton remodeling and re-establishment of the E-cadherin/β-catenin junctions. Those changes are key steps in promoting the reversal of the epithelial-mesenchymal transition[22]. It is remarkable that SL triggers additive effects when associated to chemotherapeutic drugs, especially when we are looking at the invasive properties of MDA-MB-231 cells. This is a critical issue, as the emergence of neoplastic cellular clusters displaying a pro-metastatic, highly invasive phenotype during the first chemotherapy settings represents a relevant threat and dramatically reduces the chance of clinical response[23]. It is noteworthy that the association of 5-FU+SL specifically increases the redistribution of vinculin at the membrane border, thus enhancing cell adhesion and inhibition of cell motility and invasiveness, as previously noticed in other cell models[24]. Indeed, an unwarranted complication of chemotherapy-based treatments is represented by the selection and promotion of selected, more-resistant, and invasive cancer cells, that ultimately compromise the effectiveness of therapy. Indeed, chemotherapy can provide tumor cells with enhanced disseminating properties, stem-cells features and chemoresistance[25]. In this context EMT represents the link between chemoresistance and metastatic potential although this connection is more complex than originally imagined and still need to be thoroughly investigated[26].

The availability of a compound able to amplify 5-FU activity while significantly hampering the invasive phenotype of breast cancer[5] should provide invaluable benefits, namely if we consider that – as reported by previous clinical studies – this compound is substantially deprived of side-effects[27]. Noticeably, 5-FU exerts only minimal if any effects on tumor reversion, especially when invasiveness and migrating capability are considered. Instead, the addition of SL to chemotherapy-treated cells, enhances the reversion of the invasive phenotype. This additive activity could be exploited in improving the clinical response to conventional drugs, as previously reported in colon cancer patients treated with chemotherapy and SL12.

Conclusions

Breast cells acquire a migrating/invasive phenotype upon inflammatory and/or cancerous transformation. Herewith, we extend previous our studies[28] on the anticancer effects displayed by zebrafish embryo extract by showing that while 5-FU was unable in antagonizing the migrating phenotype, the association with SL halves the invasiveness of breast cancer. Moreover, a further additive effect upon cell motility when the two compounds are simultaneously administered was observed. It is worth noting that such an effect is noticed upon normal as well as in cancerous cells, suggesting that the critical target of SL is specifically represented by the migrating/invasive phenotype. Overall, those changes are associated with the reversion of the EMT phenotype, as documented by the contemporary downregulation of TCTP and some EMT-related molecular effectors, like α-SMA and Vimentin. These results disclose entirely new opportunities for better fine-tuning chemotherapeutic protocols, while significant increasing their efficiency in fostering tumor reversion. Indeed, SL may fall in the class of "anti-migrastatics" drugs, which are neither cytotoxic nor antiproliferative on their own, but are directed towards inhibition of cancer cell motility[29]. This perspective deserves to be investigated thoroughly, and studies to address such issues are currently ongoing in our laboratory.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Joel M, Sandberg CJ, Boulland JL, Vik-Mo EO, Langmoen IA, Glover JC. Inhibition of tumor formation and redirected differentiation of glioblastoma cells in a xenotypic embryonic environment. Dev Dyn 2013; 242: 1078-1093.
2) Hendrix MJ, Seftor EA, Seftor RE, Kasemeier-Kulesa J, Kulesa PM, Postovit LM. Reprogramming metastatic tumour cells with embryonic microenvironments. Nat Rev Cancer 2007; 7: 246-255.
3) Proietti S, Cucina A, Pensotti A, Fuso A, Marchese C, Nicolini A, Bizzarri M. Tumor reversion and embryo morphogenetic factors. Semin Cancer Biol 2020; 10: S1044-579X(20)30194-2.

4) Giuffrida D, Rogers IM, Nagy A, Calogero AE, Brown TJ, Casper RF. Human embryonic stem cells secrete soluble factors that inhibit cancer cell growth. Cell Prolif 2009; 42: 788-798.

5) Cucina A, Biava P, D'Anselmi F, Coluccia P, Conti F, di Clemente R, Miccheli A, Frati L, Gulino A, Bizzarri M. Zebrafish embryo proteins induce apoptosis in human colon cancer cells. Apoptosis 2006; 11: 1617-1628.

6) Ferranti F, D'Anselmi F, Caruso M, Lei V, Dinicola S, Pasqualato A, Cucina A, Palombo A, Ricci G, Catizone A, Bizzarri M. TCam-2 seminoma cells exposed to egg-derived microenvironment modify their shape, adhesive pattern and migratory behaviour: a molecular and morphometric analysis. PLoS One 2013; 8: e76192.

7) Allegrucci C, Rushton MD, Dixon JE, Sottile V, Shah M, Kumari R, Watson S, Alberio R, Johnson AD Epigenetic reprogramming of breast cancer cells with oocyte extracts. Mol Cancer 2011; 10: 7.

8) Hansis C, Barreto G, Maltry N, Niehrs C. Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. Curr Biol 2004; 14: 1475-1480.

9) Tuynder M, Susini L, Prieur S, Besse S, Fiucci G, Amson R, Telerman A. Biological models, and genes of tumor reversion: cellular reprogramming through tpt1/TCTP and SIAH-1. Proc Natl Acad Sci U.S.A. 2002; 99: 14976-1481.

10) D'Anselmi F, Cucina A, Biava PM, Proietti S, Coluccia P, Frati L, Bizzarri M. Zebrafish stem cell differentiation stage factors suppress Bcl-xL release and enhance 5-Fu-mediated apoptosis in colon cancer cells. Curr Pharm Biotechnol 2011; 12: 261-267.

11) Livraghi T, Meloni F, Frosi A, Lazzaroni S, Bizzarri M, Frati L, Biava PM. Treatment with stem cell differentiation stage factors of intermediate-advanced hepatocellular carcinoma: an open randomized clinical trial. Oncol Res 2005; 15: 399-408.

12) Proietti S, Cucina A, Giuliani A, Verna R, Palombi E, Biava PM, Pensotti A. Fish protein extract enhances clinical response to salvage chemotherapy in colon cancer patients. Or J Biol Sci 2018; 2: 81-90.

13) Livraghi T, Ceriani R, Palmisano A, Pedicini V, Pich MG, Tommasini MA, Torzilli G. Complete response in 5 out of 38 patients with advanced hepatocellular carcinoma treated with stem cell differentiation stage factors: case reports from a single centre. Curr Pharm Biotechnol 2011; 12: 254-260.

14) Hao Y, Baker D, Ten Dijke P. TGF-β-Mediated Epithelial-Mesenchymal Transition and Cancer Metastasis. Int J Mol Sci 2019; 20: 2767.

15) Yilmaz M, Christofori G. EMT, the cytoskeleton, and cancer cell invasion. Cancer Metastasis Rev 2009; 28: 15-33.

16) Amson R, Karp JE, Telerman A. Lessons from tumor reversion for cancer treatment. Curr Opin Oncol 2013; 25: 59-65.

17) Amson R, Pece S, Lespagnol A, Vyas R, Mazzarol G, Tosoni D, Colaluca I, Viale G, Rodrigues-Ferreira S, Wynendaele J, Chaloin O, Hoebeke J, Marine JC, Di Fiore PP, Telerman A. Reciprocal repression between P53 and TCTP. Nat Med 2011; 18: 91-99.

18) Liu CY, Lin HH, Tang MJ, Wang YK. Vimentin contributes to epithelial-mesenchymal transition cancer cell mechanics by mediating cytoskeletal organization and focal adhesion maturation. Oncotarget 2015; 6: 15966-15983.

19) Scanlon CS, Van Tubergen EA, Inglehart RC, D'Silva NJ. Biomarkers of epithelial-mesenchymal transition in squamous cell carcinoma. J Dent Res 2013; 92: 114-121.

20) Karagiannis GS, Condeelis JS, Oktay MH. Chemotherapy-induced metastasis: mechanisms and translational opportunities. Clin Exp Metastasis 2018; 35: 269-284.

21) Rho SB, Lee JH, Park MS, Byun HJ, Kang S, Seo SS, Kim JY, Park SY. Anti-apoptotic protein TCTP controls the stability of the tumor suppressor p53. FEBS Lett 2011; 585: 29-35.

22) Skrypek N, Goossens S, De Smedt E, Vandamme N, Berx G. Epithelial-to-mesenchymal transition: epigenetic reprogramming driving cellular plasticity. Trends Genet 2017; 33: 943-959.

23) D'Alterio C, Scala S, Sozzi G, Roz L, Bertolini G. Paradoxical effects of chemotherapy on tumor relapse and metastasis promotion. Semin Cancer Biol 2020; 60: 351-361.

24) Xu W, Coll JL, Adamson ED. Rescue of the mutant phenotype by reexpression of full-length vinculin in null F9 cells; effects on cell locomotion by domain deleted vinculin. J Cell Sci 1998; 111: 1535-1544.

25) Singh A, Settleman J. EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer. Oncogene 2010; 29: 4741-4751.

26) Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat Rev Clin Oncol 2017; 14: 611-629.

27) D'Anselmi F, Dinicola S, Pasqualato A, Lisi E. Embryonic morphogenetic field induces phenotypic reversion in cancer cells. Review article. Curr Pharm Biotechnol 2011; 12: 243-253.

28) Proietti S, Cucina A, Pensotti A, Biava PM, Minini M, Monti N, Catizone A, Ricci G, Leonetti E, Harrath AH, Alwasel SH, Bizzarri M. Active Fraction from Embryo Fish Extracts Induces Reversion of the Malignant Invasive Phenotype in Breast Cancer through Down-regulation of TCTP and Modulation of E-cadherin/β-catenin Pathway. Int J Mol Sci 2019; 20:2151.

29) Rosel D, Fernandes M, Sanz-Moreno V, Brábek J. Migrastatics: Redirecting R&D in Solid Cancer Towards Metastasis? Trends Cancer 2019; 5:755-756.