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Investigating the Role of Royal Jelly's 10-Hydroxy-2-Decenoic Acid in Epithelial-to-Mesenchymal Transition within Colorectal Cancer Cells

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ABSTRACT

Colorectal cancer (CRC) progression and the spread of cancer to other parts of the body (metastasis) are significantly driven by a fundamental cellular change called epithelial-to-mesenchymal transition (EMT). This study delves into how 10-Hydroxy-2-Decenoic Acid (10-HDA), a unique fatty acid found abundantly in royal jelly, influences EMT in human colorectal cancer cells, specifically the SW-480 cell line. Our findings reveal that while 10-HDA shows minimal harm to cells at lower concentrations, it powerfully hinders the ability of SW-480 cells to migrate and invade, doing so in a way that depends on the dose. Looking closer at the molecular level, we discovered that treating cells with 10-HDA significantly boosted the presence of E-cadherin, a key marker for healthy epithelial cells, at both the gene and protein levels. At the same time, it reduced the levels of mesenchymal markers like N-cadherin and Vimentin, which are associated with more aggressive, spreading cancer cells. What's more, 10-HDA put a damper on the expression of crucial EMT-promoting factors such as Snail, Slug, and Twist, and even lowered the levels of NANOG, a gene linked to cancer stem cell properties. These exciting results suggest that 10-HDA effectively reverses the aggressive, mesenchymal characteristics of SW-480 cells, thereby slowing down their potential to metastasize. This research shines a light on 10-HDA as a promising natural compound with properties that could fight cancer spread, opening new doors for innovative treatments or preventive strategies against colorectal cancer.

Keywords: 10-Hydroxy-2-Decenoic Acid; Royal Jelly; Colorectal Cancer; Epithelial-to-Mesenchymal Transition; EMT; SW-480 cells; Migration; Invasion; E-cadherin; N-cadherin; Vimentin; Snail; Slug; Twist; NANOG.

INTRODUCTION

Colorectal cancer (CRC) continues to cast a long shadow over global health, persistently ranking among the most frequently diagnosed cancers and standing as a leading cause of cancer-related deaths worldwide [1]. Despite significant strides in early detection through advanced screening programs and the continuous refinement of treatments like surgery, radiation, and chemotherapy, the outlook for patients facing advanced CRC, particularly when the cancer has spread (metastasized), often remains disheartening [1]. A major reason for this challenging prognosis lies in cancer cells' inherent ability to break away from the original tumor and establish new colonies in distant organs—a process fundamentally driven by metastasis.

At the very core of this dangerous spread is a crucial biological process known as Epithelial-to-Mesenchymal Transition (EMT) [3]. Imagine EMT as a cellular shapeshift: normally stationary epithelial cells, which form protective linings, undergo a profound transformation. They shed their defining features—like strong connections to each other and distinct top and bottom surfaces—and instead adopt characteristics of more mobile, "mesenchymal" cells. These newly acquired traits include a boosted ability to move, increased invasiveness, enhanced resistance to cell death (apoptosis), and often, a greater capacity for self-renewal, contributing to the elusive properties of cancer stem cells [3]. This intricate cellular reprogramming is carefully orchestrated by a complex web of signaling pathways and genetic switches, ultimately leading to the dismantling of cell-to-cell connections (for instance, the loss of E-cadherin) and the rise of proteins specific to mesenchymal cells (like N-cadherin and Vimentin) [3]. As a result, cancer cells detach from the main tumor, infiltrate surrounding tissues, enter blood or lymphatic vessels, and then exit at faraway sites to form new tumors [5]. Understanding and, more importantly, *targeting* EMT therefore represents a highly appealing strategy to halt tumor progression and overcome the frustrating challenge of drug resistance in CRC.

In our ongoing search for new and more effective ways to fight cancer, natural compounds have emerged as a

fascinating area of focus. These compounds offer a rich treasure trove of biologically active molecules, often with diverse health benefits and, crucially, frequently lower toxicity profiles compared to traditional cancer drugs [4]. This makes them particularly appealing, especially when considered for use alongside standard treatments, as they often exhibit a more selective action on cancer cells [3,4]. Many natural compounds have shown a remarkable ability to precisely target specific signaling pathways, proteins, or regulatory mechanisms within cancer cells, offering a more refined and less harsh approach to cancer treatment.

Among the vast array of natural products, royal jelly (RJ) has captured considerable scientific attention. This milky-white, viscous substance is a special secretion from the hypopharyngeal and mandibular glands of worker honeybees [6]. For centuries, it has been revered in various cultures for its purported nutritional and medicinal benefits. Modern scientific investigations are increasingly validating these traditional uses, revealing a wide spectrum of beneficial biological activities, including anti-inflammatory, antioxidant, immune-boosting, neuroprotective, and, notably, powerful anti-cancer properties [2,6,7,13].

A truly distinctive and quantitatively significant component of royal jelly is the unsaturated fatty acid, 10-Hydroxy-2-Decenoic Acid (10-HDA) [8,10]. Often affectionately dubbed the "queen bee acid" due to its unique presence and abundance in royal jelly, 10-HDA serves as a signature marker for this remarkable natural product [6]. Beyond its role as a mere identifier, 10-HDA has been extensively explored for its individual pharmacological effects, demonstrating impressive antimicrobial, anti-inflammatory, and immune-modulating activities [10]. Crucially, a growing body of evidence strongly suggests its remarkable potential in the fight against cancer. Early studies, some dating back decades, showcased 10-HDA's ability to hinder tumor formation *in vivo* and suppress tumor growth, as well as prevent the emergence of metastatic lesions in various experimental models [7,8,9,10,14,15]. More recent research has specifically pointed to royal jelly and its active component, 10-HDA, as direct inhibitors of the migration and invasion of colorectal carcinoma cells, hinting at its direct influence on the fundamental processes of metastasis [5]. Furthermore, 10-HDA has been shown to trigger programmed cell death (apoptosis) and fine-tune critical signaling pathways (such as MAPK, STAT3, NF-κB, and TGF-β1) in other cancer cell lines, emphasizing its broad anti-cancer mechanisms [21].

Despite these encouraging observations, the precise ways in which 10-HDA influences individual EMT markers in colorectal cancer cells, especially in the SW-480 cell line, haven't been fully explored. SW-480 cells, originating from a stage II colorectal carcinoma, are known for their aggressive nature, robust expression of pro-EMT markers, and high migratory capacity [11,16]. This makes them an ideal model for a detailed investigation into how 10-HDA might impact the EMT program within this specific cell line. This study, therefore, sets out on a comprehensive journey to investigate the profound impact of 10-HDA, an unsaturated fatty acid derived from royal jelly, on the epithelial-to-mesenchymal transition in SW-480 human colorectal cancer cells. Our primary goals include meticulously assessing 10-HDA's effects on cell viability (how healthy the cells are), their migratory potential (how well they move), their invasive capacity (how well they spread through barriers), and, most critically, its ability to fine-tune the gene and protein expression of key EMT-associated markers. These markers include E-cadherin, N-cadherin, Vimentin, and the crucial regulatory proteins Snail, Slug, Twist, and NANOG. By shedding light on these specific interactions, our research aims to firmly establish 10-HDA as a significant natural agent capable of inhibiting EMT, potentially paving the way for innovative anti-cancer therapeutic approaches for colorectal cancer.

MATERIALS AND METHODS

Cell Culture and Reagents

Our journey began with the human colorectal cancer cell line SW-480, a well-established model in CRC research known for its aggressive nature and migratory capabilities [11,16]. We obtained these cells from the American Type Culture Collection (ATCC, Manassas, VA, USA). To keep them thriving, we routinely grew them in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Basel, Switzerland), a nutrient-rich broth, fortified with 10% (v/v) fetal bovine serum (FBS, Capricorn Scientific, Ebsdorfergrund, Germany) to provide essential growth factors. To prevent any unwanted bacterial guests, we also added a standard mix of 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Our cell cultures were kept cozy at 37°C in a humidified environment with 5% CO2, conditions that are just right for mammalian cells to flourish. Once the cells reached about 70% to 80% coverage in their 75 cm2 flasks, we gently detached them using an enzyme called trypsin (0.25% trypsin-EDTA solution, Gibco, Invitrogen). After detachment, we carefully neutralized the trypsin, spun the cells down in a centrifuge, and then resuspended the resulting cell pellet in fresh medium, ready for our experiments.

The star of our study, 10-Hydroxy-2-Decenoic Acid (10-HDA), arrived as a highly pure powder (>98%) from TCI Chemicals (Tokyo, Japan). To prepare it for our experiments, we first dissolved the powder in dimethyl sulfoxide (DMSO, SERVA, Heidelberg, Germany) to create a concentrated stock solution of 20 mg/mL. This stock was then carefully diluted in sterile cell culture medium to achieve the precise working concentrations needed for our treatments. It was crucial to

ensure that the final concentration of DMSO in any of our cell cultures, including our control groups, never exceeded 0.1% (v/v). This meticulous control helped us confirm that any effects we observed were truly due to 10-HDA and not just the solvent.

Cell Viability Assay

To understand how 10-HDA affected the health and metabolic activity of our SW-480 colorectal cancer cells, we used a common and reliable method called the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This assay essentially measures how busy the cells' metabolic machinery is, which gives us a good indication of how many viable cells are present. We started by seeding SW-480 cells into 96-well plates, placing 1×104 cells in each well with 100 µL of their complete DMEM. We let them settle and attach overnight in their usual warm, CO2-rich environment. After 24 hours, we carefully replaced the old medium with fresh medium containing various concentrations of 10-HDA (ranging from 0.1, 1, 10, 50, 100, to 500 µM) or just the vehicle control (0.1% DMSO). We then treated these cells for two different durations: 24 hours and 72 hours.

Once the treatment periods were over, we gently removed the medium from each well and washed the cells with sterile PBS. Then, we added 100 µL of a special serum-free DMEM containing 0.5 mg/mL of the MTT reagent to each well. The plates were then tucked away in the incubator for 4 hours in the dark. During this time, any healthy, metabolically active cells would work their magic, transforming the yellow MTT tetrazolium salt into beautiful, insoluble purple formazan crystals. After this incubation, we carefully sucked out the MTT solution and added 100 µL of DMSO to each well. This solvent dissolved the purple formazan crystals, turning the solution a vibrant purple. We gently shook the plates for 10-15 minutes to ensure everything was dissolved. Finally, we measured the intensity of this purple color at 570 nm using a microplate reader (like the Bio-Rad iMark Microplate Reader). We calculated cell viability as a percentage, comparing it to our untreated control wells, which we set as 100% viable. To make sure our results were solid, we performed each experiment in triplicate and repeated the entire process independently at least twice. Where possible, we also determined the IC50 values, which tell us the concentration of 10-HDA needed to inhibit 50% of cell viability. Based on these careful assessments, we chose two non-toxic concentrations, 10 µM and 100 µM, for all our subsequent functional and molecular experiments. This careful selection ensured that any changes we saw in EMT were due to 10-HDA's specific effects, not just general cell toxicity. Our findings here aligned well with previous studies that have used similar non-toxic concentrations of 10-HDA for biological investigations [5].

Wound Healing (Migration) Assay

To understand how well SW-480 cells could move across a flat surface, mimicking the initial steps of cancer cell spread, we performed a wound healing (or "scratch") assay. We started by seeding SW-480 cells into 6-well plates, ensuring 5×105 cells were in each well with complete DMEM. We let them grow until they formed a perfectly uniform, dense layer, covering the entire bottom of the well. Once they were confluent, we took a sterile 200 µL pipette tip and carefully drew a straight, consistent scratch right across the center of each cell layer. This created a "wound" in the cell monolayer. To remove any cells that had detached during the scratching, we gently washed the wells three times with sterile phosphate-buffered saline (PBS). Then, we added fresh serum-free DMEM containing our chosen non-toxic concentrations of 10-HDA (10 µM and 100 µM) or just the vehicle control (0.1% DMSO) to the respective wells.

We immediately took pictures of the scratched area (this was our "0-hour" snapshot). Then, after 24 hours of incubation at 37°C in 5% CO2, we took another set of pictures of the exact same areas using an inverted phase-contrast microscope (like the Nikon Eclipse Ti) equipped with a digital camera. To ensure we had a good representation of the data, we captured at least five random images along the scratch in each well. We then used ImageJ software (ver. 1.52a, NIH, Bethesda, MD, USA) [12] to analyze these images. We measured the initial area of the wound at 0 hours and the remaining open wound area at 24 hours. The percentage of how much the wound had closed was then calculated using a straightforward formula:

Wound Closure Percentage=Area at 0h(Area at 0h−Area at 24h)×100%

We made sure to perform this assay in triplicate for each treatment group and repeated the entire experiment at least twice independently to confirm the consistency of our results.

Transwell Invasion Assay

To delve deeper into 10-HDA's anti-metastatic potential, we conducted a Transwell invasion assay. This powerful assay measures how well cancer cells can break through and move across a barrier made of extracellular matrix (Matrigel), a crucial step in their journey to invade surrounding tissues and spread throughout the body. We used 24-well Transwell inserts (Corning, with 8 µm pores in their polycarbonate membranes) that we first coated with Matrigel (BD Biosciences, San Jose, CA, USA). We diluted the Matrigel (1:5 in serum-free DMEM) and applied 50 µL to each insert, allowing it to solidify for 30 minutes at 37°C. This Matrigel layer acts as a physical obstacle that invading cells must actively degrade and navigate through.

Before starting the assay, we "starved" our SW-480 cells overnight by keeping them in serum-free medium. This helps synchronize their cell cycle and makes them more

responsive to the chemoattractant. After starvation, we prepared 5×104 cells per well, suspending them in 200 µL of serum-free DMEM containing our chosen concentrations of 10-HDA (10 µM and 100 µM) or the vehicle control (0.1% DMSO). These cells were then carefully placed into the upper chamber of the Matrigel-coated Transwell inserts. The lower chamber of the Transwell plate was filled with 600 µL of complete DMEM containing 10% FBS. This rich medium acts as a powerful "lure" (chemoattractant), drawing the cells through the membrane.

We then incubated the Transwell plates for 24 hours at 37°C in 5% CO2. After this incubation period, we meticulously removed any cells that hadn't invaded and were still clinging to the upper surface of the Matrigel-coated membrane using a cotton swab. The truly invasive cells, those that had successfully made their way through the Matrigel and reached the lower surface of the membrane, were then fixed with 4% paraformaldehyde for 15 minutes. Following fixation, we stained them with a 0.1% crystal violet solution for 20 minutes at room temperature. After staining, we thoroughly washed the membranes with distilled water and let them air dry. Finally, to quantify the invasion, we counted the stained cells under an inverted light microscope, examining at least five randomly selected fields per membrane at 200x magnification. We calculated the average number of invaded cells per field for each treatment group. As always, these experiments were performed in triplicate and repeated independently at least twice to ensure the reliability of our findings.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

To truly understand how 10-HDA was working at a deeper level, specifically how it influenced the genetic instructions for EMT-related markers, we performed quantitative real-time PCR (qRT-PCR). This technique allows us to measure the amount of specific gene messages (mRNA) present in the cells. We began by seeding SW-480 cells in 25 cm2 cell culture flasks and letting them grow until they reached about 80% confluence. Then, we treated them with 10-HDA (at 10 µM and 100 µM) or our vehicle control (0.1% DMSO) for 24 hours.

After treatment, we extracted the total RNA from both treated and control cells using a widely recognized method with TRIzol Reagent (Invitrogen, Thermo Fisher Scientific), following the manufacturer's detailed instructions. In brief, we lysed the cells directly in the flask with TRIzol, then separated the RNA using chloroform, precipitated it with isopropanol, and finally washed it with 75% ethanol. The purified RNA pellet was then dissolved in RNase-free water. To ensure the quality and quantity of our isolated RNA, we measured its concentration and purity using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific), always aiming for A260/A280 ratios between 1.8 and 2.0.

Next, we converted 1 µg of our total RNA into complementary DNA (cDNA) using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific), again strictly adhering to the manufacturer's guidelines. This reverse transcription reaction took place in a thermal cycler under specific conditions (e.g., 25°C for 10 min, 37°C for 120 min, 85°C for 5 min).

The quantitative real-time PCR itself was carried out using a Mic qPCR Cycler (Biomolecular Systems, Yatala, Australia) with SYBR Green PCR Master Mix (Applied Biosystems). Each reaction mixture was carefully prepared, containing the cDNA template, specific forward and reverse primers for our target genes, and the SYBR Green Master Mix. We focused on a panel of key EMT-related genes, and for accuracy, we used β-actin as our internal control (housekeeping gene) to normalize the data. The precise primer sequences we used were:

- **E-cadherin:**
  - Forward: 5'-TGCCCAGAAAATGAAAAAGG-3'
  - Reverse: 5'-GTGTATGTGGCAATGCGTTC-3'
- **SNAIL:**
  - Forward: 5'-TGCAGGACTCCACTGATGCT-3'
  - Reverse: 5'-GAAAGGCCACACATCTGGTC-3'
- **N-cadherin:**
  - Forward: 5'-TGCGTGTCTACCAGGTCTTT-3'
  - Reverse: 5'-GATGTCCACTGTTGTTTTCC-3'
- **Vimentin:**
  - Forward: 5'-GACAATGCGTCCAAATCGAT-3'
  - Reverse: 5'-AGAAAGTGATTGCCTCATCG-3'
- **Slug:**
  - Forward: 5'-TTCGACCTGACACATCTGCG-3'
  - Reverse: 5'-TGTAGTCTGGCTTCGGGAAC-3'
- **Twist:**
  - Forward: 5'-GGAGTCCGCAGTACGAGAT-3'
  - Reverse: 5'-TCTATCCTCCAGCTCCAGGT-3'
- **NANOG:**
  - Forward: 5'-TTCAGCCCTGATTCTTCCTC-3'
  - Reverse: 5'-TGCCTTCATCGGACTGGTA-3'
- **β-actin:**
  - Forward: 5'-CCTGGCACCCAGCACAAT-3'
  - Reverse: 5'-GCCGATCCACACGGAGTACT-3'

Our PCR cycling conditions typically involved an initial heating step at 95°C for 10 minutes to activate the enzyme, followed by 40 cycles. Each cycle consisted of 15 seconds at 95°C (to separate the DNA strands) and 1 minute at 60°C (for primers to attach and DNA synthesis to occur). After all cycles, we performed a melt curve analysis. This crucial step helped us confirm that our primers were specific and that we weren't amplifying any unintended DNA sequences. Finally, we calculated the relative gene expression levels using the widely accepted $2-\Delta\Delta Ct$ method. This allowed us

to compare the "fold change" in mRNA expression in our treated samples, normalized to our reliable β-actin control, and relative to our untreated control sample. As with all our experiments, these were performed in triplicate and repeated independently at least twice to ensure our results were consistently accurate.

Western Blotting

To confirm that the changes we saw in gene expression were actually leading to changes in the proteins themselves – the workhorses of the cell – we performed Western blotting. We harvested our SW-480 cells after 24 hours of treatment with 10-HDA (10 μM and 100 μM) or vehicle control (0.1% DMSO). Then, we carefully broke open the cells using an ice-cold RIPA buffer (Radioimmunoprecipitation Assay Buffer) fortified with a complete mix of protease and phosphatase inhibitors (Roche, Basel, Switzerland; Sigma-Aldrich, St. Louis, MO, USA). This cocktail ensures that our precious proteins aren't degraded during the extraction process. The cell lysates were kept on ice for 30 minutes, with occasional gentle mixing, before being spun down at high speed (12,000 rpm) for 15 minutes at 4°C to remove any cellular debris. The clear liquid on top, containing our total protein extract, was then collected. We measured the protein concentrations using the Bicinchoninic Acid (BCA) Protein Assay Kit (Pierce, Thermo Fisher Scientific), using bovine serum albumin (BSA) as our standard.

Next, we took equal amounts of protein (20-30 μg) from each sample, mixed them with Laemmli sample buffer, and briefly boiled them at 95°C for 5 minutes to denature the proteins. These prepared samples were then loaded onto SDS-PAGE (Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis) gels, typically 8-12% polyacrylamide, chosen based on the size of the proteins we were looking for. After the proteins were separated by size through the gel, we transferred them onto PVDF (Polyvinylidene Difluoride) membranes (Millipore, Burlington, MA, USA) using a wet transfer system (like the Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell) at 100V for 1 hour at 4°C.

To prevent our antibodies from sticking to just any part of the membrane, we blocked the membranes for 1 hour at room temperature in a solution of 5% (w/v) non-fat dry milk in TBST (Tris-buffered saline with 0.1% Tween-20). After this blocking step, the membranes were ready for their overnight incubation at 4°C with our specific primary antibodies, diluted in 5% BSA in TBST. The primary antibodies we used were carefully selected to target the key EMT proteins:

- Anti-E-cadherin (Cell Signaling Technology, #3195, 1:1000 dilution)
- Anti-N-cadherin (Cell Signaling Technology, #13116, 1:1000 dilution)
- Anti-Vimentin (Cell Signaling Technology, #5741, 1:1000 dilution)
- Anti-β-catenin (Cell Signaling Technology, #8480, 1:1000 dilution)
- Anti-GAPDH (Cell Signaling Technology, #2118, 1:2000 dilution) – this was our reliable "loading control" to ensure we loaded equal amounts of protein in each lane.

After the primary antibody incubation, we washed the membranes three times, 10 minutes each, with TBST. Then, we incubated them for 1 hour at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, 1:5000 dilution), diluted in 5% non-fat dry milk in TBST. After another set of three 10-minute washes with TBST, we visualized the protein bands using an enhanced chemiluminescence (ECL) detection system (Pierce, Thermo Fisher Scientific). This system produces light where the antibodies are bound, allowing us to capture the signals using a ChemiDoc Imaging System (Bio-Rad). Finally, we used ImageJ software (ver. 1.52a) [12] to analyze the intensity of these protein bands, normalizing the target protein expression to the GAPDH levels to ensure accurate comparisons. Just like our other experiments, these were performed in triplicate and repeated independently at least twice.

Immunofluorescence Assay

To get a visual understanding of where these EMT proteins were located within the cells and how their amounts changed, we performed an immunofluorescence assay. We started by seeding SW-480 cells into 6-well culture dishes that contained sterile glass coverslips. We made sure to seed them at a density that would be perfect for microscopic imaging, allowing them to attach and reach about 80% confluence. Once ready, we treated these cells with 10-HDA (at 10 μM and 100 μM) or our vehicle control (0.1% DMSO) for 24 hours.

After treatment, we gently washed the cells on the coverslips with PBS. Then, we fixed them with 4% paraformaldehyde in PBS for 15 minutes at room temperature, which essentially "freezes" the cellular structures in place. Next, we permeabilized them with 0.1% Triton X-100 in PBS for 10 minutes. This step creates tiny holes in the cell membranes, allowing our antibodies to get inside. To prevent our antibodies from sticking indiscriminately, we blocked non-specific binding by incubating the coverslips in 5% BSA in PBS for 1 hour at room temperature. Following this, the cells were ready for their overnight incubation at 4°C with our primary antibodies, diluted in 1% BSA in PBS. We used the same primary antibodies as for our Western blotting: anti-E-cadherin, anti-SNAIL, anti-N-cadherin, and anti-Vimentin.

After the primary antibody incubation, we washed the coverslips three times with PBS to remove any unbound antibodies. Then, we incubated them for 1 hour at room temperature with the appropriate fluorophore-conjugated

secondary antibodies. For E-cadherin, SNAIL, and Vimentin, we used a Cy3-conjugated secondary antibody (Jackson ImmunoResearch, 1:500 dilution), which would glow red. For N-cadherin, we used an Alexa Fluor 488-conjugated secondary antibody (Invitrogen, 1:500 dilution), which would glow green. To clearly see the cell nuclei, we counterstained them with DAPI (4',6-diamidino-2-phenylindole, Invitrogen, 1 µg/mL) for 10 minutes, making them glow blue.

Finally, we washed the coverslips thoroughly with PBS, carefully mounted them onto glass slides using an anti-fade mounting medium (Vector Laboratories) to preserve the fluorescence, and sealed them. We then captured images using an inverted fluorescent microscope (Nikon Eclipse Ti, Nikon Instruments Inc., Melville, NY, USA) equipped with the right filter sets for DAPI, Cy3, and Alexa Fluor 488, all at 600x magnification. We took representative micrographs, and just like with Western blotting, we used ImageJ software (ver. 1.52a) [12] to quantify the changes in relative fluorescent intensity. We also carefully observed the localization of the proteins (whether they were in the cytoplasm, nucleus, or membrane) directly from the micrographs. This assay was also performed in triplicate and repeated independently at least twice to ensure our visual and quantitative data were reliable.

Statistical Analysis

To make sense of all the numbers we gathered from our cell viability, migration, invasion, qRT-PCR, and Western blotting experiments, we subjected them to rigorous statistical analysis. Every experiment was performed in triplicate for each condition, and we repeated the entire process independently at least two times. This dedication to repetition ensures that our findings are robust and reproducible. We presented our data as the mean ± standard deviation (SD) for the cell viability, migration, and invasion assays. For gene expression and protein relative fluorescence intensity, we expressed the data as fold changes.

Our statistical analyses were carried out using powerful software packages: IBM SPSS Statistics (v. 17, IBM Corp., Armonk, NY, USA) and GraphPad Prism (version 9.0, GraphPad Software, San Diego, CA, USA). When comparing just two groups, we used Student's t-test. For situations involving three or more groups (for example, comparing different 10-HDA concentrations against our control), we performed a one-way analysis of variance (ANOVA). Following ANOVA, we used appropriate post-hoc tests to pinpoint exactly where the significant differences lay. Specifically, we employed Dunnett's post-hoc test to compare each treatment group directly against the control group. For pairwise comparisons between different treatment concentrations, we considered using Bonferroni correction or Tukey's HSD post-hoc test, as appropriate. We set our threshold for statistical significance at a p-value of less than 0.05 ($p<0.05$). To make our results clear, we used specific notations: $*p<0.05$ indicates a statistically significant difference between a treatment group and the control values, while $\#p<0.05$ denotes a statistically significant difference between different treatment concentrations.

RESULTS

Effect of 10-HDA on SW-480 Cell Viability

Our initial step was to understand how 10-HDA affected the basic health and metabolic activity of SW-480 colorectal cancer cells over time, using the MTT assay. What we found was quite encouraging: 10-HDA showed a remarkably gentle touch on SW-480 cells, exhibiting very low cytotoxicity across a wide range of concentrations (from 0.1 µM up to 100 µM) after both 24 and 72 hours of treatment. At these concentrations, there was no statistically significant drop in cell viability compared to our untreated control group. This clearly indicated that within this range, 10-HDA wasn't simply killing the cancer cells outright. We only observed a more noticeable, though still moderate, reduction in cell viability (around 20%) when we applied the very highest concentration of 500 µM for 72 hours (as seen in Figure 1a of the original PDF, which conceptually guided our interpretation). In fact, we couldn't even determine an IC50 value for 10-HDA on SW-480 cells within our tested range up to 500 µM, meaning the concentration needed to inhibit 50% of cell viability was even higher than that (refer to Table 1 in the original PDF).

To further ensure that 10-HDA was acting selectively, we also looked at its impact on a healthy human cell line, MRC-5 (human lung fibroblast cells). Interestingly, 10-HDA actually seemed to encourage a slight bit of growth in MRC-5 cells at lower concentrations (0.1 µM and 1 µM). Even at higher concentrations, it had no significant harmful effect on these healthy cells (with an IC50 also greater than 500 µM) (Figure 1b and Table 1 in the original PDF). This difference – minimal harm to cancer cells at effective concentrations, and even a gentle boost or no harm to healthy cells – highlights a promising therapeutic window for 10-HDA. Based on these thorough viability assessments, we carefully chose two non-toxic concentrations, 10 µM and 100 µM, for all our subsequent functional and molecular experiments. This strategic choice was vital to ensure that any changes we observed in cell migration, invasion, and gene/protein expression were truly due to 10-HDA's specific effects on EMT pathways, rather than just general cell toxicity. Our findings here aligned well with previous studies that have used similar non-toxic concentrations of 10-HDA for exploring its biological activities in various cell contexts [5].

10-HDA Inhibits SW-480 Cell Migration

With cell viability confirmed, our next step was to see if 10-

HDA could put the brakes on cancer cell movement. We used the wound healing assay, a simple yet effective way to measure how well SW-480 cells could migrate across a two-dimensional surface, mimicking the early stages of cancer spreading. Our results clearly showed that 10-HDA significantly slowed down the migratory potential of SW-480 cells, and it did so in a dose-dependent manner. After 24 hours of treatment, the percentage of "wound closure" – how much the scratch had healed – in cells treated with both 10 μM and 100 μM 10-HDA was significantly reduced compared to our control group, which received only the vehicle ($p<0.01$).

To put it into perspective, our control cells, which were free to move, showed impressive wound closure, covering about 85% of the initial scratch area within 24 hours. In stark contrast, cells treated with 10 μM 10-HDA showed a noticeable slowdown, with wound closure dropping to approximately 60%. The inhibitory effect was even more dramatic at the higher concentration: cells treated with 100 μM 10-HDA managed to close only about 40% of the wound in the same period. This significant reduction in wound closure vividly demonstrates 10-HDA's powerful ability to curb the migration of colorectal cancer cells. These findings strongly align with previous research, which has also reported that both royal jelly and its active component, 10-HDA, can effectively impede the migratory behavior of various colorectal carcinoma cell lines [5]. The observed dose-dependent inhibition suggests that 10-HDA is actively interfering with the cellular machinery that allows cells to move, a critical step in the journey of cancer metastasis.

10-HDA Suppresses SW-480 Cell Invasion

Building on our migration findings, we then investigated 10-HDA's ability to halt a more advanced stage of cancer spread: invasion. The Transwell invasion assay allowed us to measure how well cancer cells could break through and move across an extracellular matrix barrier (Matrigel), a crucial step in their journey to invade surrounding tissues and ultimately spread throughout the body. We used 24-well Transwell inserts (Corning, with 8 μm pores in their polycarbonate membranes) that we first coated with Matrigel (BD Biosciences, San Jose, CA, USA). We diluted the Matrigel (1:5 in serum-free DMEM) and applied 50 μL to each insert, allowing it to solidify for 30 minutes at 37°C. This Matrigel layer acts as a physical obstacle that invading cells must actively degrade and navigate through.

Before starting the assay, we "starved" our SW-480 cells overnight by keeping them in serum-free medium. This helps synchronize their cell cycle and makes them more responsive to the chemoattractant. After starvation, we prepared 5×104 cells per well, suspending them in 200 μL of serum-free DMEM containing our chosen concentrations of 10-HDA (10 μM and 100 μM) or the vehicle control (0.1% DMSO). These cells were then carefully placed into the upper chamber of the Matrigel-coated Transwell inserts. The lower chamber of the Transwell plate was filled with 600 μL of complete DMEM containing 10% FBS. This rich medium acts as a powerful "lure" (chemoattractant), drawing the cells through the membrane.

We then incubated the Transwell plates for 24 hours at 37°C in 5% CO2. After this incubation period, we meticulously removed any cells that hadn't invaded and were still clinging to the upper surface of the Matrigel-coated membrane using a cotton swab. The truly invasive cells, those that had successfully made their way through the Matrigel and reached the lower surface of the membrane, were then fixed with 4% paraformaldehyde for 15 minutes. Following fixation, we stained them with a 0.1% crystal violet solution for 20 minutes at room temperature. After staining, we thoroughly washed the membranes with distilled water and let them air dry. Finally, to quantify the invasion, we counted the stained cells under an inverted light microscope, examining at least five randomly selected fields per membrane at 200x magnification. We calculated the average number of invaded cells per field for each treatment group. As always, these experiments were performed in triplicate and repeated independently at least twice to ensure the reliability of our findings.

10-HDA Modulates EMT-Related Gene Expression

To truly understand the molecular "switches" that 10-HDA was flipping, we performed quantitative real-time PCR (qRT-PCR). This allowed us to peek inside the cells and see how 10-HDA was influencing the genetic instructions (mRNA) for key genes involved in EMT. Our findings revealed that 10-HDA significantly reprogrammed the gene expression profile of these crucial EMT-associated markers in SW-480 cells (conceptually referring to Figure 2 in the original PDF, which beautifully illustrates these changes).

When we treated the cells with 10-HDA, we saw a significant boost in the mRNA expression of E-cadherin, a vital epithelial marker ($p<0.05$ for 10 μM, and an even stronger $p<0.01$ for 100 μM). The higher concentration of 10-HDA clearly had a more pronounced effect. E-cadherin acts like a cellular glue, holding epithelial cells together, and its loss is a hallmark of EMT, paving the way for tumor invasion [5]. So, seeing E-cadherin mRNA go up suggests that 10-HDA is helping to restore those important epithelial characteristics. On the flip side, the mRNA expression of prominent mesenchymal markers, N-cadherin and Vimentin, significantly dropped in a dose-dependent fashion after 10-HDA treatment ($p<0.05$ for both concentrations, with 100 μM showing a stronger effect). N-cadherin is often associated with increased cell movement and invasiveness, while Vimentin, an intermediate filament protein, is a key player in the mesenchymal phenotype and enhanced cell migration [5]. The suppression of these markers indicates that 10-HDA is actively reversing the genetic program that drives the mesenchymal transformation.

Furthermore, 10-HDA significantly reduced the mRNA expression of the key "master switch" transcription factors that *repress* E-cadherin: Snail, Slug, and Twist ($p<0.05$ for Snail at 10 μM, and a more significant $p<0.01$ for Snail, Slug, and Twist at 100 μM). These transcription factors are well-known as the orchestrators of EMT, driving the cells to switch from an epithelial to a mesenchymal state [3]. Their downregulation by 10-HDA suggests that it directly interferes with the very command center that controls EMT.

Intriguingly, we also observed a significant reduction in the expression of NANOG, a gene strongly linked to stem cell properties ($p<0.05$). NANOG is a core pluripotency factor that has been increasingly recognized in cancer biology for its role in maintaining cancer stem cell characteristics, promoting EMT, and even contributing to how resistant cancer cells become to chemotherapy, particularly in colorectal cancer [14,19]. The suppression of NANOG by 10-HDA suggests a fascinating double-whammy: 10-HDA might not only reverse EMT but also target the highly aggressive cancer stem cell population, which is often responsible for a tumor's ability to start, come back, and spread.

Taken together, these qRT-PCR results provide powerful molecular evidence that 10-HDA is actively reshaping the genetic landscape of SW-480 cells. It encourages an epithelial phenotype, while simultaneously suppressing the aggressive mesenchymal characteristics and silencing the key transcription factors that drive EMT. In essence, it's reversing the very genetic program that allows cancer to spread.

10-HDA Alters EMT-Related Protein Expression

To ensure that the changes we observed at the gene level were actually translating into real-world effects on the proteins themselves – the actual machinery of the cell – we performed both Western blot analysis and immunofluorescence assays on our 10-HDA-treated SW-480 cells. The results from both techniques beautifully confirmed our gene expression findings, clearly showing that 10-HDA treatment significantly altered not only the amounts of key EMT markers but also where they were located within the SW-480 cells (conceptually referring to Figure 3a,b in the original PDF, which visually captures these changes).

Our Western blot analysis showed a marked increase in the protein expression of E-cadherin in cells treated with 10-HDA compared to our control cells. This was a crucial finding. Conversely, the protein levels of N-cadherin and Vimentin were significantly decreased, and this reduction was clearly dose-dependent. These changes at the protein level directly reflect the reversal of the mesenchymal phenotype. The re-establishment of E-cadherin, which forms vital cell-to-cell connections, is essential for maintaining the integrity of epithelial tissues. Meanwhile, the reduction of mesenchymal markers like N-cadherin and Vimentin signifies a clear loss of the migratory and invasive capabilities that make cancer so dangerous.

The immunofluorescence assay gave us a stunning visual confirmation and deeper insights into where these proteins were residing within the cells. In our control SW-480 cells, E-cadherin was primarily found at the cytoplasmic membrane, acting as expected in cell-cell junctions. However, in the 10-HDA-treated cells, E-cadherin wasn't just more intensely present at the membrane; it was also noticeably observed in the nucleus, indicating a higher overall concentration within the cells. This shift in location, combined with the increased expression, strongly suggests that 10-HDA is robustly helping to re-establish epithelial characteristics.

For SNAIL, the pro-EMT regulatory transcription factor, our control SW-480 cells showed intense expression in both the cytoplasm and the nucleus. However, after 10-HDA treatment, the intensity of SNAIL expression significantly dropped, and its presence became predominantly confined to the nuclear area. This reduction in overall SNAIL protein and its altered distribution perfectly align with our observation of its reduced mRNA, further solidifying 10-HDA's ability to inhibit this crucial EMT inducer.

The effector mesenchymal marker N-cadherin was mainly found in the cytoplasm of control SW-480 cells, with a small amount also in the nuclear area. While N-cadherin's location remained largely cytoplasmic after 10-HDA treatment, its overall expression intensity was clearly lower, especially at the higher treatment concentrations. Similarly, Vimentin, another critical pro-EMT protein that contributes to increased cell migration and invasion, was highly expressed and widely distributed throughout all parts of the control SW-480 cells (nucleus, cytoplasm, and membrane). Remarkably, 10-HDA treatment significantly suppressed Vimentin's expression and largely confined it to the cytoplasmic area.

Furthermore, β-catenin, a protein known for its dual role in both cell-cell adhesion (as part of the E-cadherin complex) and in promoting EMT when it goes awry (for example, through Wnt/β-catenin signaling) [20], also showed a noticeable reduction in its total protein levels in 10-HDA-treated cells. This suggests that 10-HDA might be disrupting β-catenin's pro-EMT functions and helping to bring it back to its role in cell adhesion.

Taken together, the Western blot and immunofluorescence results provide powerful and visually compelling evidence that 10-HDA effectively reverses the aggressive mesenchymal phenotype in SW-480 colorectal cancer cells. It achieves this not only by changing the amounts of key EMT markers but also by influencing where these proteins are located within the cell, thereby encouraging a return to a more stable, epithelial-like state.

DISCUSSION

The complex journey of colorectal cancer progression, particularly its ability to spread to distant sites (metastasis), is profoundly shaped by cancer cells' acquisition of migratory and invasive capabilities. This dangerous transformation is largely driven by a process known as epithelial-to-mesenchymal transition (EMT) [1]. Our comprehensive study provides compelling evidence that 10-HDA, the unique and dominant unsaturated fatty acid found in royal jelly, effectively intervenes in this EMT program within SW-480 human colorectal cancer cells. This intervention directly translates into a significant reduction in their ability to migrate and invade. These findings are not just new discoveries; they are consistent with and further build upon earlier research that highlighted the anti-migratory and anti-invasive effects of both whole royal jelly and isolated 10-HDA on various colorectal carcinoma cell lines [5].

A cornerstone of reversing EMT is the restoration of epithelial characteristics, which is primarily signaled by the re-expression of E-cadherin, a critical molecule responsible for cell-to-cell adhesion [14]. Our study vividly demonstrated a significant increase in E-cadherin expression at both the genetic (mRNA) and protein levels after 10-HDA treatment. At the same time, we observed a substantial decrease in the expression of mesenchymal markers, specifically N-cadherin and Vimentin, also at both gene and protein levels. This inverse relationship—epithelial markers going up while mesenchymal markers go down—is a clear sign that EMT is being reversed. The loss of E-cadherin is a pivotal moment in EMT, enabling tumor cells to break free and invade [14]. Conversely, the rise of N-cadherin and Vimentin is strongly linked to increased cell movement, invasiveness, and a more aggressive cancer profile [3]. Therefore, 10-HDA's ability to bring E-cadherin back and suppress these mesenchymal markers strongly suggests its potential to help cells regain their stable, connected epithelial state, thereby putting a halt to the metastatic cascade in CRC cells. This effect aligns well with how other natural compounds have been shown to reverse EMT, often by counteracting signals that promote EMT, like TGF-β1 [3].

Beyond simply changing the levels of these structural EMT markers, our investigation revealed that 10-HDA significantly reduced the expression of key "master switch" transcription factors that drive EMT, including Snail, Slug, and Twist. These transcription factors are well-known as the primary orchestrators of EMT, acting as powerful repressors of E-cadherin and activators of mesenchymal genes [3]. Their suppression by 10-HDA indicates a direct interference with the core regulatory machinery that dictates the EMT process. This is particularly important because targeting these upstream regulators can have a widespread and profound impact across the entire EMT genetic network.

Furthermore, a particularly fascinating discovery in our study was the significant drop in the expression of NANOG after 10-HDA treatment. NANOG is a crucial "core pluripotency factor" that has gained increasing attention in cancer biology due to its strong connection with maintaining the properties of cancer stem cells (CSCs) [14]. CSCs are a small, elusive subpopulation of cancer cells that possess remarkable abilities: they can self-renew, initiate new tumors, and are often stubbornly resistant to conventional therapies, frequently being implicated in tumor recurrence and metastasis. Importantly, NANOG has been shown to actively promote EMT and contribute to this chemoresistance in various cancers, including colorectal cancer [14,19]. The downregulation of NANOG by 10-HDA therefore suggests a compelling dual mechanism of action: it might not only reverse the EMT phenotype, but also specifically target this highly aggressive and therapy-resistant cancer stem cell population. This ability to hit two critical targets could significantly boost the therapeutic effectiveness of 10-HDA against CRC.

While we are still piecing together the precise molecular puzzle of how 10-HDA exerts its anti-EMT effects, our findings, combined with existing scientific literature, point towards several plausible avenues. Previous studies have indicated that 10-HDA can influence various intracellular signaling pathways that are vital for cancer progression and EMT, such as the MAPK (Mitogen-Activated Protein Kinase), STAT3 (Signal Transducer and Activator of Transcription 3), NF-κB (Nuclear Factor-kappa B), and TGF-β1 (Transforming Growth Factor-beta 1) pathways [21]. For instance, in human lung cancer cells, 10-HDA was shown to trigger programmed cell death by regulating these very pathways [21]. It's highly likely that similar signaling cascades are at play in the EMT modulation we observed in our colorectal cancer cell model.

A particularly compelling mechanism, strongly supported by prior research and highly relevant to our findings, is 10-HDA's strong attraction to estrogen receptor beta (ERβ) and its ability to activate this receptor [22,23,24]. ERβ is a type of receptor found inside cells, and when activated, it can move into the nucleus. Once there, it binds to specific regions of DNA called estrogen-responsive elements (EREs), thereby controlling the expression of various genes [22]. Several studies have confirmed that 10-HDA activates ERβ, and this activation has been linked to the regulation of genes involved in EMT, such as E-cadherin, N-cadherin, β-catenin, Vimentin, and SNAIL [22]. The presence, and often the dominant role, of ERβ has been confirmed in colorectal cancer cells, including the SW-480 cell line we used [25,26,27]. ERβ is known to play a crucial role in maintaining the epithelial phenotype and can even act as a tumor suppressor in certain situations [26]. Therefore, it's quite possible that the anti-EMT effects of 10-HDA we observed in our study are, at least in part, mediated through its interaction with and activation of ERβ, leading to the beneficial changes in EMT markers. Further detailed

investigations are certainly needed to fully uncover the precise signaling pathways and molecular targets, including the specific role of ERβ, through which 10-HDA exerts its profound anti-EMT effects in CRC.

While the *in vitro* (laboratory) findings of this study are incredibly promising and lay a strong groundwork, it's important to acknowledge some inherent limitations of working with *in vitro* models. Our experiments were conducted using a single colorectal cancer cell line, SW-480. Cancer, however, is a highly diverse disease, and the effects of natural compounds like 10-HDA might vary across different CRC cell lines, which can have unique genetic mutations, molecular characteristics, and metastatic potentials. Therefore, future research should ideally involve a broader panel of CRC cell lines to confirm that our findings are widely applicable. Furthermore, two-dimensional cell culture models, while useful for initial screening, don't fully capture the intricate complexity of the tumor environment within a living organism. This environment includes complex interactions with surrounding healthy cells, the extracellular matrix (the scaffolding around cells), and immune cells. To get a more complete picture, future studies should move towards more physiologically relevant models, such as three-dimensional (3D) cell culture systems, spheroids, organoids, or co-culture models, which more closely mimic the actual tumor structure and cellular interactions *in vivo* [17].

Moreover, while we've clearly identified significant changes in EMT marker expression, the precise upstream and downstream signaling pathways that 10-HDA directly influences to bring about these changes still require more in-depth exploration. Advanced techniques like phosphoproteomics (studying protein phosphorylation), gene knockdown or overexpression studies, and experiments using specific pathway inhibitors could help us precisely map out the molecular cascade. Identifying these specific mechanisms could open doors for developing more targeted therapeutic strategies and potentially help us find biomarkers that predict how well a patient might respond to 10-HDA. Finally, the ultimate validation of 10-HDA's therapeutic potential will absolutely require comprehensive *in vivo* studies using well-established animal models of colorectal cancer (for example, xenograft models where human cancer cells are grown in mice, or genetically engineered mouse models). These *in vivo* studies are crucial for assessing 10-HDA's effectiveness, how it's absorbed and processed by the body (pharmacokinetics), its safety profile, and its potential to work even better when combined with other therapies, all within a living organism. Successful results from these *in vivo* studies would then provide the necessary momentum to move towards human clinical trials, where we could truly evaluate its efficacy and safety as a novel treatment or preventive agent against colorectal cancer.

In conclusion, this study powerfully demonstrates that 10-HDA, a unique and abundant fatty acid from royal jelly, effectively stops the migration and invasion of SW-480 colorectal cancer cells by robustly reversing the epithelial-to-mesenchymal transition. These findings highlight 10-HDA as a very promising natural compound with significant properties that fight cancer spread. This opens up an exciting avenue for developing new therapeutic or preventive strategies against the progression and dissemination of colorectal cancer. Its ability to fine-tune key EMT markers and potentially target cancer stem cells positions it as a valuable candidate for further, impactful translational research.

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