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The Effect of Fertilized Egg White on Growth, Migration, Differentiation and Genes Expression Profile in SW480 Colon Cancer Cell Line

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Research Article

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Abstract

Background: Embryonic microenvironments influence cancer stem cells properties, which leads to anti-cancer effects. Therefore, the current study investigates the effects of fertilized egg white, as an embryonic/fetal microenvironment, on survival, apoptosis, self-renewal characteristic, stemness properties, and migration capacity of SW480 colon cancer cells and 5-fluorouracil (5FU) resistant subgroup.

Methods: MTT and Flow cytometry was used to study the cell viability and cell cycle analysis. Clonogenic, spheroid formation, and wound healing assays were used to evaluate cancer cells' self-renewal, stemness properties, and migration capacity. RT-PCR was performed to analyze *NANOG*, *c-MYC*, *E-cadherin*, and *NDRG1* mRNA expression.

Results: The SW480 colon carcinoma cell line and SW480-5FU chemo-resistant subpopulation cells were subjected to Fertilized Egg White (FEW). FEW decreased cell viability and increased the percentage of the sub-G1 stage in both cell lines. In addition, colony and spheroid formations were decreased in both cells, and the FEW inhibited the migration. Expression of *NANOG* and *c-MYC* were reduced in both cells. *E-cadherin* and *NDRG1* expression increased in SW480 cells.

Conclusion: FEW decreased the SW480 colon cancer cell line and the SW480-5FU chemo-resistant subpopulation growth and migration. Also, by the changes observed in gene expression and spheroid formation, we suggest the possibility of decreased stemness properties and induction of differentiation following fertilized egg white treatment.

1 Introduction

Despite huge attempts to find cancer initiation and progression mechanisms, it remains the second leading cause of death globally [1]. Conventional cancer therapies, including chemo and radiotherapy, have adverse side effects and tumor cells resist them [2]. Evidence shows a compensatory mechanism that promotes proliferation in neighboring cells, enforced by apoptotic cells [3]. Even in successful therapies, remission may occur aggressively after an apparent cancer-free period [4, 5]. The adverse effects of traditional therapies persuaded scientists to search for new molecular targets and design new therapeutic approaches [4, 5].

According to the cancer stem cell paradigm, tumor cells arise from a subpopulation of tumor bulk, which drives tumorigenesis and progression [6]. Cells in this category share numerous characteristics with adult and embryonic human stem cells, specifically self-renewal, stemness, differentiation to other types of tumor cells, and resistance to conventional medications [6]. The Gene expression pattern of aggressive cancer stem cells shows similarities with adult and embryonic stem cells, particularly in stemness and differentiation-related genes expression [7]. Unlike stem cells, cancer cells are uncontrolled in expressing stemness and differentiation factors, such as c-Myc, NANOG, Sox 2, NDRG1, E-cadherin, and developing pathway including Notch, Hedgehog, and Wnt, resulting in the progression of cancer bulk [8, 9].

Despite similarities between embryos and cancer development, and between embryonic stem cells and cancer stem cells, cancer incidence in the embryo and fetus is sporadic [10, 11]. Some studies also have shown that embryonic niches could reverse the tumorigenic state of cancer cells [10, 11], and the malignancy of aggressive melanoma cells declines when transferred to chick embryos [12]. Also, the injection of cancer cells in the different fetal stages of mouse fetus impaired cancer cell growth [13].

Egg whites are well known for providing all the nutrients needed to form and grow normal embryos, including amino acids, lipids, vitamins, elements, and growth factors [14]. The protein within egg white migrates to the developing chick embryos, potentially establishing that eggs have a direct role in embryogenesis, in addition to nourishing and storing protein, and can create an embryonic niche that can be an anticancer [15, 16].

This study aimed to investigate the effect of fertilized egg white (FEW) as an *in vitro* embryonic/fetal microenvironment on human colorectal SW480 cell line growth, stemness and differentiation. Also, we looked at a subpopulation of SW480 that were made resistant to the chemotherapy agent 5-Fluorouracil (5-FU). We exposed cells to the FEW environments and studied the growth rate, cell cycle, colony, spheroid formations, migration, and expression of genes involved in stemness and differentiation.

2 Material And Methods

2.1 Preparation of Egg white (EW)

The fertilized 9-day-old chicken egg was purchased from the chicken incubation farm. Egg white was separated in a sterile condition. In all following experiments, EW was dissolved in the DMEM medium to reach a final concentration of 10% v/v concentration, similar to FBS concentration in media.

2.2 Cell culture

The SW480 human colorectal carcinoma line was obtained from the Cell Bank of Pasteur Institute's Tehran. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Life Technologies), cell culture medium containing 10% fetal bovine serum (FBS) (GIBCO, Life Technologies), 1% glutamine (Sigma), and 1% penicillin/streptomycin antibiotic (Sigma) at 37°C and 95% humidity in a 5% CO_2 humidified incubator. The cells were passaged after reaching appropriate confluency (80-90%), and media was changed every other day.

2.3 Establishment of 5FU resistant SW480 colon cancer cell line

According to our previous study, the MTT assay determined the IC50 value of 5-fluorouracil (5FU) [17]. In brief, SW480 cells were exposed to the increasing doses (0, 10, 50, 100, 150, 200, 300µM) of 5FU for 72h. MTT assay was performed, and the dose-dependent diagram was created by GraphPad prism. The IC50 dose for 5FU was calculated as 20 µM. After that, an almost fully confluent (90%) monolayer of SW480 cells was treated with 20 µM (the IC50 dose) of 5FU. Following 72 h incubation, the medium was

discarded, and the plates were cultured under the standard condition for two to three weeks. When cells reached their logarithmic phase, they were used for the experiments.

2.4 Flow cytometry cell analysis

To ascertain the effect of fertilized egg white on cell cycle and apoptosis, we used the Flow cytometry cell analysis. The cells were incubated overnight in a 12-well plate and then exposed to fertilized egg white for three days (72h). Then, cells were collected, rinsed with PBS two times, and fixed by 70% ethanol at -20˚C overnight. The following day, the cells were rinsed with PBS containing 0.5 µM/ml RNase (Sigma, St. Louis, MO, USA) for 30 min. Then, cells were stained with 50 µg/ml propidium iodide (Sigma, St. Louis, MO, USA) for 30 minutes in the dark. Finally, the FACScan flow cytometer (Becton Dickinson) analyzed the Cell cycle.

2.5 Colonospheres (spheroid) formation

10^4 cells per well were cultured in a 24-well plate covered with poly-HEMA to make it a low attachment. The media used was serum-free DMEM supplemented with 30 ng/mL epidermal growth factor (EGF) (Royan Institute, Iran), 30 ng/mL fibroblast growth factor (FGF) (Royan Institute, Iran), and pen/strep antibiotic 1% with and without FEW. In addition, EGF and FGF were added every other day for eight days. Finally, the produced colonospheres were counted under the microscope.

2.6 Clonogenic assay

100 cells/well were seeded in a 12-well plate in triplicate and incubated for 24h at 37°C. Cells were treated with 10% fertilized egg white. After two weeks, Acetic acid/methanol (1:7) was added to fix colonies, and they were stained using 0.05% crystal violet (Sigma Chemical Company, Louis, MO, USA) at 25°C for 2h. Plates were washed with water and kept at 25˚C to dry. Finally, Colonies that had greater than fifty cells were selected and counted under a microscope. The clonogenic capacity was determined based on the number of colonies formed in proportion to the number of seeded cells.

2.7 Scratch wound healing assay

The wound-healing assay was used to evaluate cell migration. The cells were cultured in a six-well plate for two days to reach 95% confluency, and then the plate was incubated overnight with no-FBS to inhibit proliferation. Gently and slowly, the monolayer was using a sterile tip. Cells were rinsed with PBS to eliminate debris, and the medium containing FEW was added. The images were captured at 0, 48 h, and 72 h following treatment and were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (10). Cell migration that was calculated as the percentage of gap closure:

$$\% \text{ of gap closure} = [(A_{t=0\,h} - A_{t=\Delta\,h}) / A_{t=0\,h}] \times 100\%$$

At =0h is the area of gap measured at time 0 after scratching, and At = Δh is the area of gap measured later [18].

2.8 RNA extraction and Real-time PCR analysis

According to the manual, the total RNA of cells was isolated with RNX-plus Solution (Sinaclon, Tehran, Iran). Purification was evaluated by determining the 260/280 nm absorbance ratio at 260 nm and 260/280 nm. Then, 3 µg extracted RNA was used to generate cDNA by Frist Strand cDNA Synthesis Kit (2-step RT-PCR kit, Vivantis, USA). The real-time PCR was performed for *NANOG*, *c-MYC*, *E-cadherin*, *NDRG1*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (as the endogenous control) mRNA expression using SYBR Green (Bio-Rad). Table 1 lists the sequences of the primers used.

Table 1
Primer sequences

Gene	Primer sequence
NANOG	F:AACTCTCCAACATCCTGAACCTC R: CGTCACACCATTGCTATTCTTCG
c-MYC	F:GCATACATCCTGTCCGTCCAAG R:TTCCTTACGCACAAGAGTTCCG
E-cadherin	F:TGCTAATTCTGATTCTGCTGCTC F:TCAAGTCAAAGTCCTGGTCCTC
NDRG1	F: TTCCTGCTCCCTAACCTTTCG R: ATTCACTCTGACCAAACTTCCTATG
GAPDH	F:CATCAAGAAGGTGGTGAAGCAG R:GCGTCAAAGGTGGAGGAGTG

c-MYC, highly expressed in the colon cancer stem cells, causes greater tumor sphere-forming efficiency, apoptosis resistance, invasion, migration, and tumorigenesis in cancer cells [19]. *NANOG* induces stem-like phenotype in colon cancer cells, similar to *Nanog* in prostate cancer, *Oct4* in melanoma, and *Sox2* in breast cancer [20].

2.9 Statistical analysis

Results are presented as mean ± SD. Data were analyzed with a Student t-test, and $P<0.05$ indicates a statistically significant difference. Gap closure in wound healing assay was measured by ImageJ software. Figures were generated using GraphPad Prism version 4 software (GraphPad Software, Inc., La Jolla, CA, USA).

3 Results

3.1 Effect of fertilized egg white Treatment on Cell Viability

The colorimetric MTT assay was performed to assess the effect of FEW on SW480 and SW480-5FU cellular viability, using MTT assay after 72h treatment. As demonstrated in Figure 1, fertilized egg white significantly decreased SW480 cells (51%) and SW480-5FU cells (46%) viability compared to control.

3.2 Effect of fertilized egg white on Cell Cycle

To further investigate the inhibitory effect of fertilized egg white on SW480 and SW480-5FU, we used flowcytometry cell cycle analysis using propidium iodide (PI) (Sigma, USA) staining. The results demonstrated that treatment increased the percentage of the sub-G1 stage in both SW480 cell line (59 ± 26.87 vs. 17.5 ± 5.26) ($P<0.05$) and SW480-5FU (67 ± 0.0 vs. 6.0 ± 2.82) (mean ± SEM) (Figure 2).

3.3 Effect of fertilized egg white on colonosphere (spheroid) formation

To evaluate the presence and self-renewal of cancer stem cells, the effect of fertilized egg white on SW480 and SW480-5FU cells self-renewal was assessed by spheroid formation following 8 days incubation. The result showed that fertilized egg white decreased spheroid formation in both SW480 cells (% 35.96) and SW480-5FU cells (% 30.05) (Figure 3).

3.4 Effect of fertilized egg white on colony formation

The colony formation assay was used to semi-quantitatively study the effects of fertilized egg white on self-renewal and prolonged cell proliferation. SW480 and SW480-5FU cells were treated with FEW for two weeks, and colonies greater than fifty cells were counted. Fertilized egg white significantly reduced the colony formation after 13 days by 76% and % 80.5 in SW480 and SW480-5FU cells, respectively ($P<0.05$) (Figure 4).

3.5 Effect of Fertilized egg white on cell migration

We performed the scratch wound healing assay to evaluate the cell migration induced with FEW treatments. The percentage of wound area closure was calculated at 48 and 72h. FEW caused approximately 50% gap area after 72 h compared to control in both cells, inhibiting cell migration. The results are presented in Figure 5.

3.6 Effect of fertilized egg white treatment on gene expression

Growth and migration inhibition of SW480-5FU cells persuaded us to check the stemness and differentiation genes expression induced by 72h exposure to FEW. *c-MYC*, highly expressed in the colon cancer stem cells, causes greater tumor sphere-forming efficiency, apoptosis resistance, invasion, migration, and tumorigenesis in cancer cells [19]. *NANOG* induces stem-like phenotype in colon cancer

cells, similar to *Nanog* in prostate cancer, *Oct4* in melanoma, and *Sox2* in breast cancer [20]. *E-cadherin* is one of class of type-1 transmembrane protein that originated from the epithelia and has an key role in the epithelial to mesenchymal transition in cancer progression [21].

As shown in Figure 6, our data demonstrated that FEW reduced *c-MYC* and *NANOG* expression in SW480 cells compared to the control cells. At the same time, the expression of *E-cadherin* and *NDRG1* (3.73 fold) increased. In the SW480-5FU cells, *NANOG* and *c-MYC* expression were also decreased following FEW treatment. However, *E-cadherin* and *NDRG1* expression did not change after treatment with FEW.

4 Discussion

The present study provides preliminary data showing that fertilized egg white, as an embryonic/maternal microenvironment, exerted an inhibitory effect on the growth of colorectal carcinoma cell line (SW480), induced apoptosis, and decreased self-renewal and stemness-related colony and spheroid formation, inhibited migration in both SW480 cells and SW480-5FU cells. In addition, the genes involved in the stemness and differentiation of cells were modulated.

The Stemness properties of cancer cells are an essential feature in the prolonged proliferation, invasion, and metastasis of cancer [22]. We demonstrated that FEW inhibited the proliferation of SW480 cells by ceasing cells at the G0/G1 cell cycle and decreasing the stemness-related sphere formation and self-renewal characteristics of both SW480 and SW480-5FU cells. The analysis of stemness genes of *c-MYC* and *NANOG* in colorectal cancer cells also allowed us to suggest the possibility of a decrease in stemness properties by FEW. Some evidence indicated that abnormal expression of stemness genes promotes tumor growth, increases chemo-resistance, and enhances tumor invasion [23]. Previous studies have reported that the unfertilized egg white did not significantly affect the survival rate of the breast cancer cells (MCF-7, MDA-MB-231) [24] and TCam-2 seminoma cells [25]. However, similar to the present study, *Klf4*, *c-MYC*, and *NANOG* expression was diminished following egg white treatment [24]. Furthermore, ovomucin and lysozyme, two components of egg white, have been shown to reduce proliferation, metastasis, and angiogenesis of cancer cells [26–30]. Ovotransferrin increased caspase 9, and 6 changed the morphology of cell and mitochondrial membranes resulting in inhibition of proliferation and induction of apoptosis in colon cancer cells (HCT-116), in contrast to breast cancer cells (MCF-7) [26]. Therefore, the reduction of proliferation, induction of cell cycle arrest, and reduction of stemness induced by FEW in the present study could be partially due to the components of egg white mentioned above.

Metastasis is a major cause of death from cancer. Metastatic primer cells belong to a subset of stem cells that migrate to other tissues by expressing epithelial to mesenchymal transition (EMT) markers and other superficial markers involved in the invasiveness and metastasis of cancer cells [31]. Embryonic microenvironments can inhibit cell invasion and metastasis by inducing differentiation and increasing the expression of differentiation markers [32]. In the present study, FEW decreased migration in both SW480 and SW480-5FU cells. *E-cadherin* and *NDRG1* levels were also significantly higher in SW480 cells,

indicating that differentiation was induced in the subpopulation of SW480 cells that escaped the apoptosis that was induced by FEW. The results from this study are similar to findings in earlier studies using unfertilized egg white in seminoma and breast cancer cells MCF-7 and MDA-MB-231[24, 25], which demonstrated that egg white microenvironment can inhibit EMT [24].

5 Conclusion

As the embryonic/maternal microenvironment, fertilized egg white might perform a fundamental role in decreasing growth, migration, self-renewal and stemness, downregulation of stemness genes, and upregulation of differentiation genes in SW480 colon cancer cells. However, additional experiments are required to elucidate the exact mechanism of growth and metastatic inhibitory effect of FEW microenvironments. It also needs to be investigated which components of FEW mediated these stemness mitigation and differentiating induction by fertilized egg white.

Declarations

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Conflict of Interest: No potential conflict of interest was reported by the authors.

Ethical Review: This study does not involve any human or animal testing.

Authorship: conception and design of the study: Hamid Zand, Katayoun Pourvali, acquisition of data: Hadi Monji. Analysis and interpretation of data: Hadi Monji, Katayoun Pourvali, Arman Ghorbani. Drafting the article: Hadi Monji, revising: Katayoun Pourvali.

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Figures

Figure 1

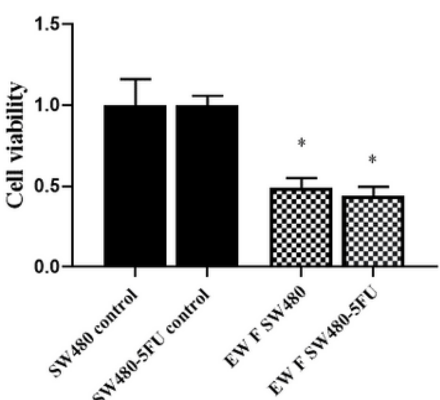

Figure 1

Effect of fertilized egg white on cell viability in the SW480 and SW480-5FU cells determined by MTT assay after 72h treatment. The values are presented as mean±SD and normalized to control. *p<0.05 is considered significant.

Figure 2

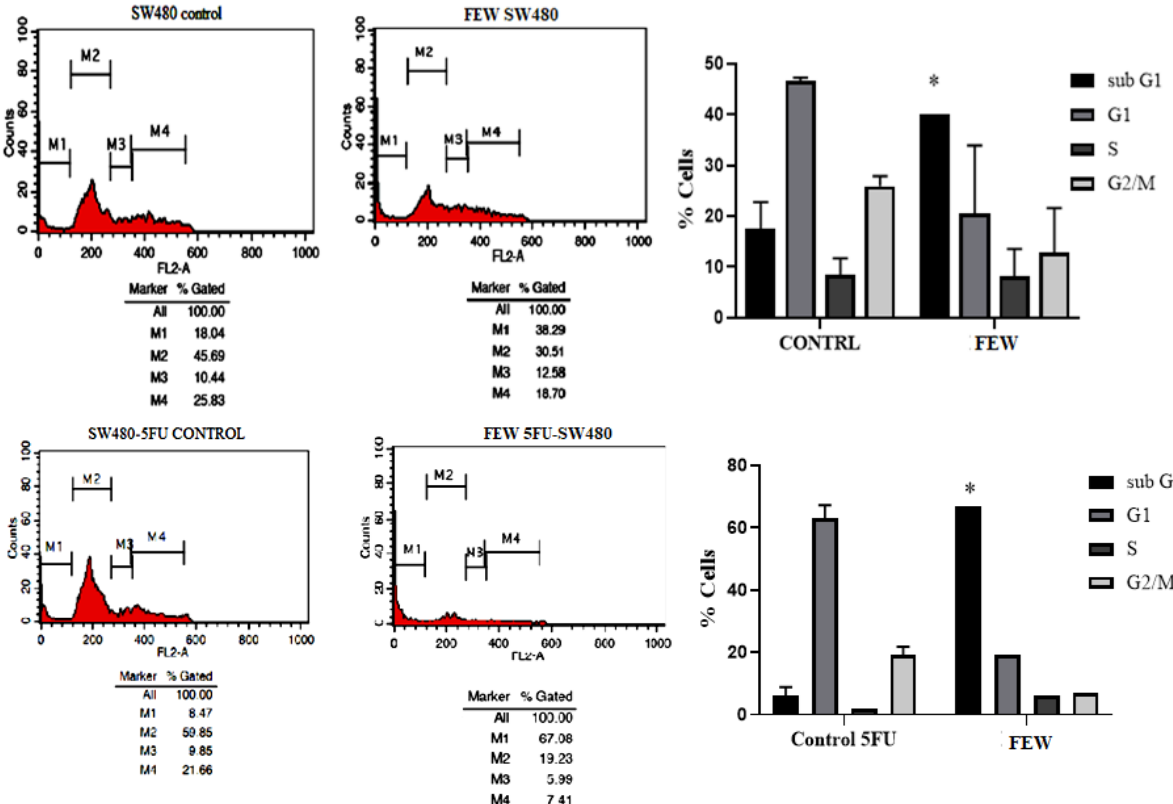

Figure 2

Effect of fertilized egg white on cell cycle in SW480 cells determined by flow cytometry assay after 72h treatment. The values are presented as Means of two experiments ± SD. M1, apoptosis; M2, G1; M3, S; M4, G2/M. *p<0.05 compared to control by student t-test.

Figure 3

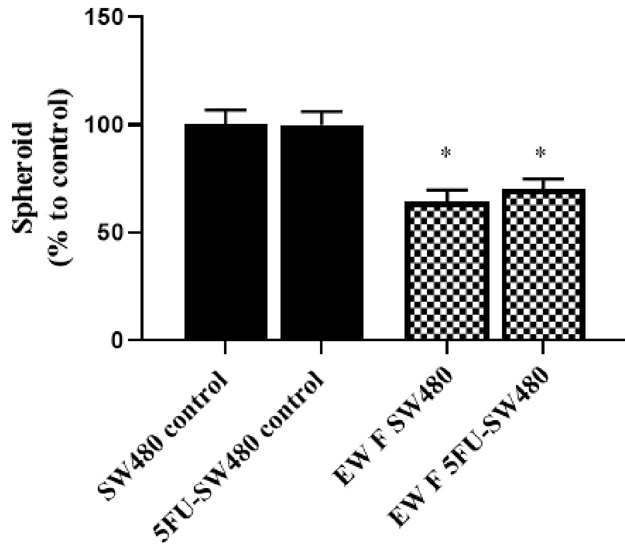

Figure 3

Effect of fertilized egg white on spheroid formation in SW480 cells and SW480-5FU cell. SW480 and SW480-5FU were treated with fertilized egg white in polyhema-coated-plates for 8-day. The normalized data shown are represented as mean ± SD of three separated tests. *p<0.05 compare with control by student t-test.

Figure 4

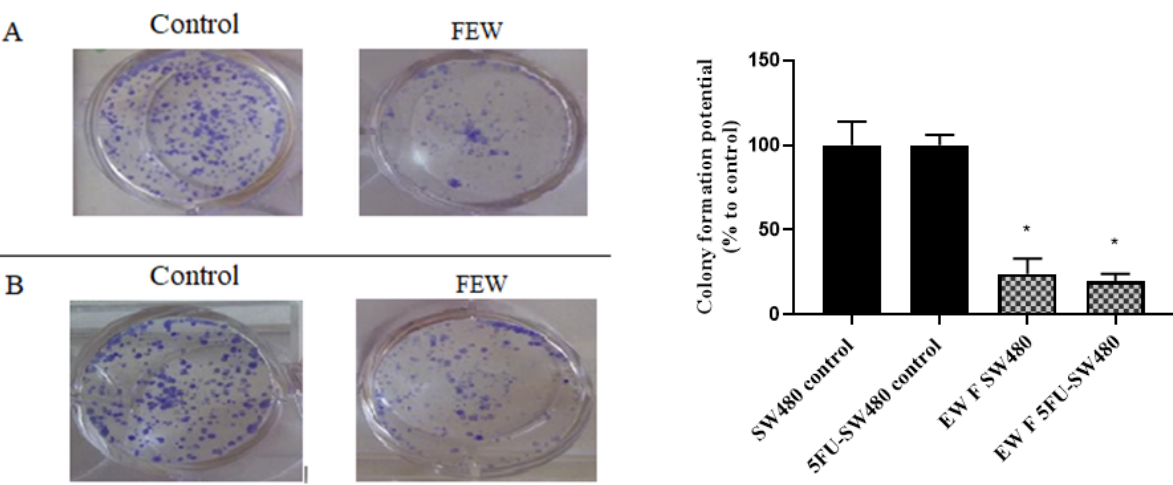

Figure 4

Effect of fertilized egg white on colony formation in SW480 and SW480-5FU cells. Cells were treated with fertilized egg white for two weeks and colonies greater than 50 cells counted. Normalized values are represented as mean ±SD of three separated test. *p<0.05 compared to control by student t-test.

Figure 5

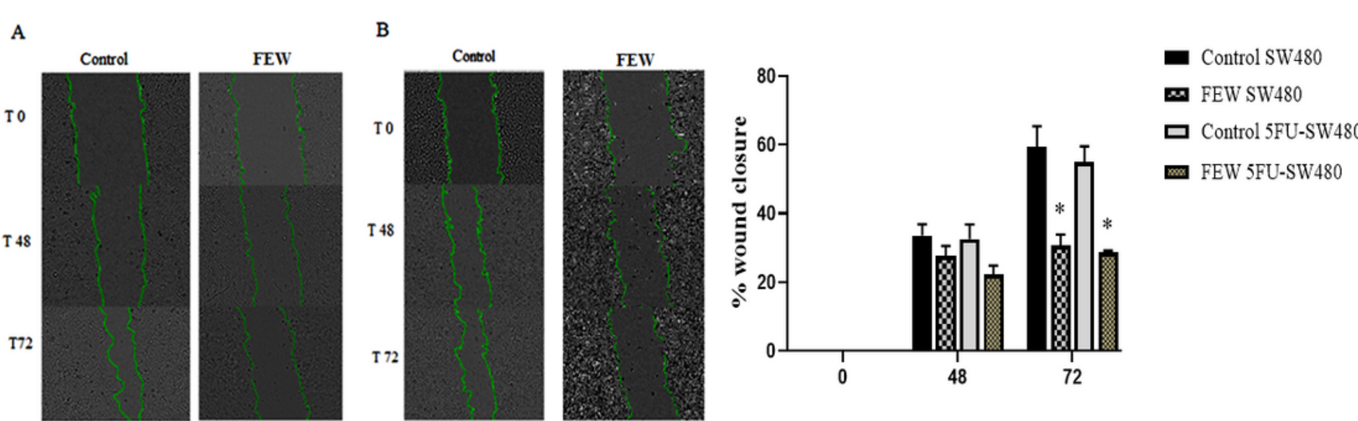

Figure 5

Effect fertilized egg white treatment on the migration of (A) SW480 cells, and (B) SW480-5FU cells was evaluated by scratch wound healing assay after 72h. Micrographs were captured at 0, 48 and 72h and gap closures were measured by ImageJ software. The results shown are represented as mean ± SD of three separated tests. *p<0.05 compared to control by student t-test.

Figure 6

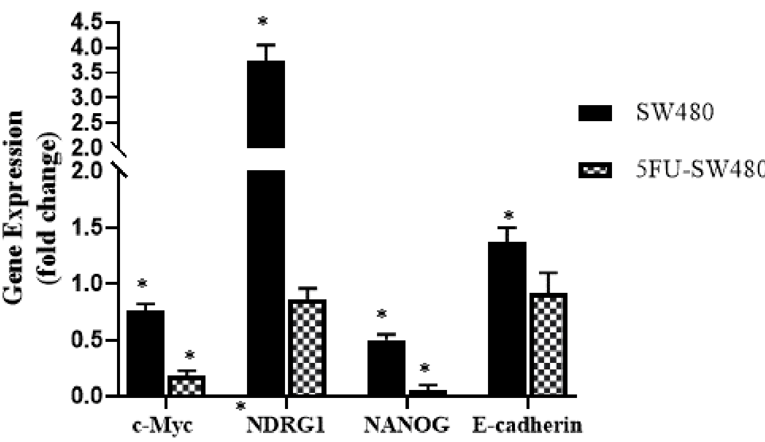

Figure 6

Effect of treatment with fertilized egg white after 72 h on relative gene expression of c-MYC, NDRG1, NANOG, E-cadherin in SW480 cells and SW480-5FU cells. Normalized gene expression relative to GAPDH (housekeeping gene) is presented as fold change (2-(ddCT)).