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Research article

Function and mechanism of exogenous AGR2 in colorectal cancer cells

Chao Zheng *, Yu Mao, Jianping Ye, Miaolong Zhang, Yongfeng Chen

Department of General Surgery, The People's Hospital of Yuhuan, Taizhou, 317600, Zhejiang, China

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ABSTRACT

Background: Anterior gradient 2 (AGR2) is highly enriched in several malignant tumors and can boost tumor metastasis. Whereas, AGR2 role in colorectal cancer (CRC) is not clear.
Methods: AGR2 expression in the GEPIA database was studied, and the results were confirmed by Western blot in CRC cell lines (SW480, SW620, and HT-29). The impact of AGR2 on the multiplication, migration, invasion and EMT of CRC cells were studied by CCK-8 assay, as well as clone formation, wound healing and transwell assays. The protein concent related to the AKT/β-catenin signaling pathway were accessed via Western blot.
Results: AGR2 concent in CRC tissues was notablely boosted versus normal colorectal tissues. Exogenous AGR2 boosted the multiplication of CRC cells. In addition, exogenous AGR2 induced EMT, which demonstrated that ZEB1, N-cadherin, Vimentin, Slug, Snail protein concent boosted and E-cadherin protein abated in CRC cells. In terms of mechanism, exogenous AGR2 upgulated *p*-AKT/AKT, *p*-GSK3β/GSK3β and β-catenin concent. Exogenous AGR2 combined with AKT agonist IGF- I can further enhance the multiplication, migration and invasion of CRC cells.
Conclusion: Exogenous AGR2 enhances the multiplication of CRC cells and induces EMT process, the mechanism of which is related to AKT/β-catenin signal pathway.

1. Introduction

Colorectal cancer (CRC) has high morbidity and mortality, as the third most common malignant tumor on a world-wide scale [1]. Regarded as the main therapy for CRC, surgical resection has a good containment effect. Whereas, there are limited therapy options for metastatic CRC sufferers, and chemotherapy is commonly used [2,3]. But chemotherapy has many side effects, including cytotoxicity, therapy resistance and pain in sufferers [4]. Hence, developing new molecular targets for CRC is pressing.

Anterior gradient 2 (AGR2) belongs to the protein disulfide isomerase family and participates in protein folding in endoplasmic reticulum [5]. The role of AGR2 in tumorigenesis, development, progression and therapy of drug resistance has aroused widespread interest in AGR2. AGR2 is highly expressive in different types of solid tumors [6]. AGR2 concent is boosted in lung adenocarcinoma cells, and the overexpression and hypomethylation of AGR2 enhance the multiplication, invasion and migration of lung adenocarcinoma cells, and boost the development of lung adenocarcinoma cells [7]. AGR2 is the most important prognostic biomarker in bone metastasis of breast cancer and conducts a vital part in the progression of breast cancer [8]. AGR2 is highly expressed in stomach, colon and duodenum [9]. Metformin dependent CRC cells increase sensitivity to chemotherapy through AGR2 silencing [10]. Whereas, AGR2 in CRC and its potential mechanism not clear.

* Corresponding author. No. 18, Changle Road, Yucheng Street, Yuhuan, Taizhou City, Zhejiang Province, China.
E-mail address: zhengchao1990@126.com (C. Zheng).

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Most colorectal tumors originate from the epithelium. In the process of tumorigenesis, intercellular contact is abated owing to the loss of intercellular connexins, such as E-cadherin and β-catenin. Malignant cells further acquire mesenchymal characteristics and boost invasion and movement in connective tissue. This cellular plasticity, known as Epithelial-mesenchymal transition (EMT), is a critical time for distant metastasis and spread, and is also one of the markers of tumor [11,12]. Targeting EMT-related molecular pathways is considered to be a new strategy for effective therapy of metastatic CRC [13].

Therefore, this research was devoted to determining the outcome of AGR2 on CRC. Exogenous AGR2 was supplemented to evaluate its impact on CRC cells. Furthermore, the mechanism of changes in related signal pathways caused by exogenous AGR2 addition was also discussed.

2. Materials and methods

2.1. Cell culture

The Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) were regarded as cell lines source, which included normal colonic epithelial cell line (CCD-841 CoN) and CRC cell lines (SW480, SW620 and HT-29). DMEM high glucose medium (11960044) containing 10% FBS (16140071) was used for culturing CCD-841 CoN cells. SW480 and SW620 cells were cultured in Leibovitz's L-15 medium (11415064) with 10% FBS. HT-29 cells were cultured in McCoy's 5A medium (16600082) with 10% FBS. All the cells were cultured in a 5% CO_2 incubator at 37 °C.

Every 2–3 days, the liquid was changed, and for follow-up experiments, the cells in logarithmic growth phase were chosen. All reagents were obtained from Wuhan Punosai Life Technology (Hubei, China).

2.2. Bioinformatics analysis

Gene Expression Profiling Interactive Analysis Gene Expression Profiling Interactive Analysis (GEPIA, http://gepia.cancer-pku.cn/index.html) database was applied for analyzing AGR2 content in normal colorectal tissues and CRC tissues.

2.3. Cell viability assay

With the number of 5×10^3 cells per well, the cells were inoculated into 96-well plates. The culture medium was discarded after 24 h culture. The cells were divided into three subgroups and cultured in the cell culture medium comprising 0,80,160 μg/mL AGR2 for 12,24,48,72,96 h. After the intervention, the culture medium was abandoned and 10 μL CCK-8 solution (Solarbio,CA1210,Beijing, China) was supplemented to each well and cultured at 37 °C for 4 h. Microplate reader (Multiskan FC, Shanghai, Beijing, China) was applied for measuring absorbance.

2.4. Clone formation assay

Leibovitz's L-15 medium comprising 10% FBS was supplemented with logarithmic growth phase cells to prepare suspension. The cells in each subgroup were inoculated into the dish comprising 10 mL 37 °C preheated culture medium at the gradient density of 50,100,200 cells per plate, respectively, and rotated gently to make the cells disperse evenly, cultured in 37 °C 5% CO_2 and saturated humidity cell incubator for 2 weeks. When a clone visible to the naked eye appeared in the Petri dish, the culture was terminated. The supernatant was discarded and washed twice with phosphate buffer (PBS, Solarbio, P1020, Beijing, China). The cells were fixed with 5 mL of 4% tissue cell fixation solution (Solarbio, P1110, Beijing, China) for 15 min. Then the fixed solution was removed, and an appropriate amount of crystal violet staining solution (Solarbio, G1062, Beijing, China) was supplemented to dye for 15 min. The staining solution was slowly washed away with running water in the dry air, and the cloned cells were counted and photographed.

2.5. Wound healing assay

Before the experiment, the marker pen was applied for marking the back of the 6-hole plate, and 1×10^6 cells were inoculated in each hole. After the cells were covered with the bottom of the plate, The 200 μ L pipette tip was used for straight scratches behind the vertical orifice plate. The cut cells were washed off by PBS and cultured in serum-free medium for 48 h, and the wound space was observed.

2.6. Transwell assay

For cell migration assay, cell suspension was adjusted to 1×10–5 cells/mL, 0.5 mL cell suspension was inoculated into transwell chamber. 0.75 mL medium comprising 10% FBS was supplemented to the lower 24-well plate and cultured in a 37 °C, 5% CO_2 incubator for 48 h. The culture plate was removed, 4% formaldehyde solution (Macklin, F111934, Shanghai, China) was supplemented, and it was fixed for 20 min. The fixed solution was discarded, crystal violet solution (Leagene Biotechnology, DA0061, Beijing, China) was supplemented and stained for 30 min. After staining, it was observed and counted under the microscope.

For cell invasion assay, before inoculation, 80 μ L Matrigel glue (Shanghai Fusheng Industrial Co., Ltd., FS-79064, shanghai, China) was spread in the chamber and placed in the incubator at 37 °C for 30 min. The rest were the same as migration assay.

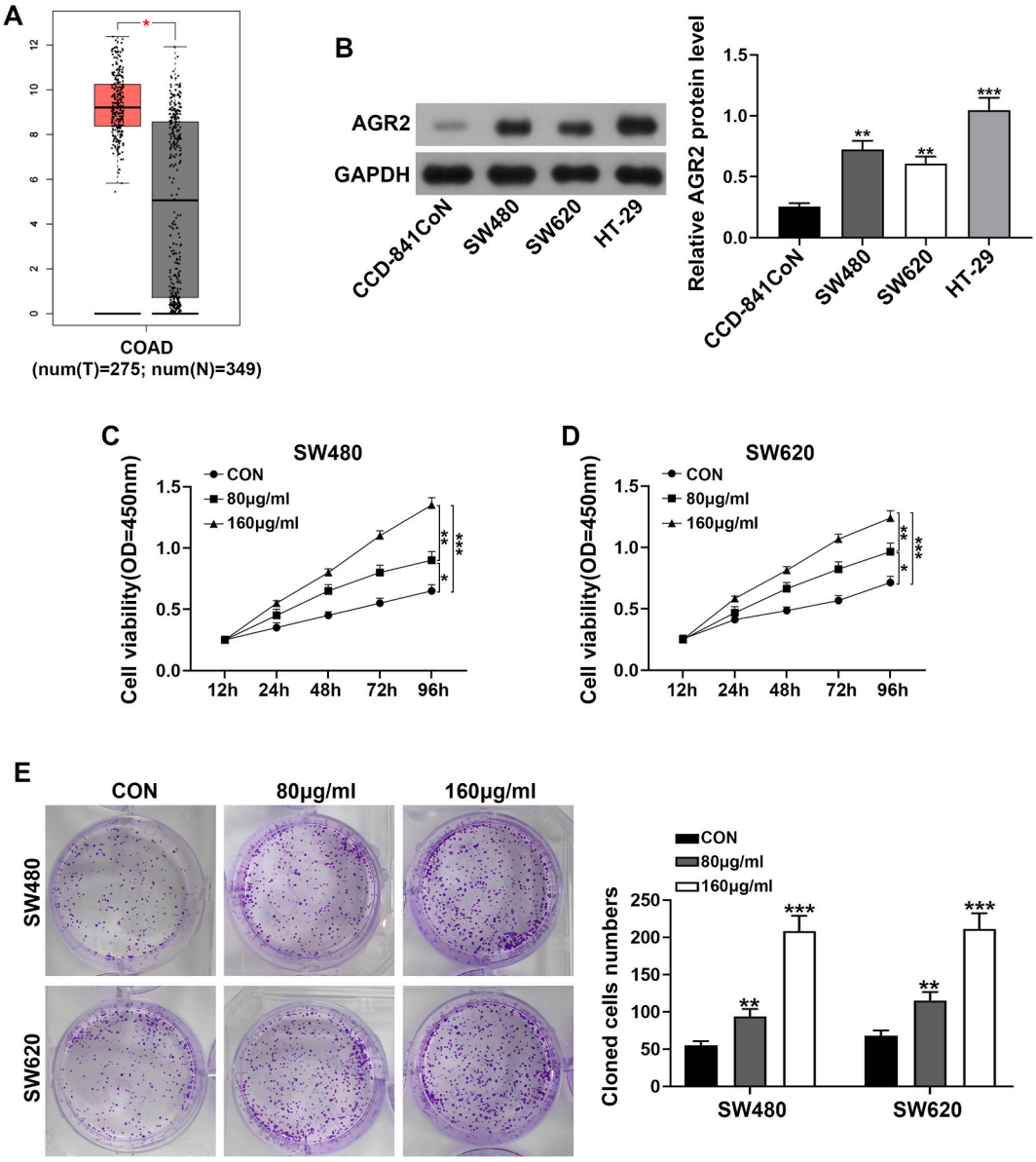

Fig. 1. Exogenous AGR2 concent was up-regulated in CRC cells (A) The up-regulated concent of AGR2 was appraised in colorectal cancer by GEPIA database. (B) western blot was aoolied to assess AGR2 concent in CCD-841CoN,SW480,SW620 and HT-29. (C,D) CCK-8 assay was used to evaluate the activity of SW480,SW620 cells. (E) Cell viability was evaluated by clone formation assay. **P < 0.01,***P < 0.001, versus CON subgroup.

2.7. Western blot

Radioimmunoprecipitation assay lysis buffer (Solarbio, R0030, Beijing, China) was used to extract total protein, Bicinchoninic acid kit (Solarbio, PC0020, Beijing, China) was used for protein quantification. The extracted 20 μ g protein was isolated and transferred to polyvinylidene fluoride membranes (Millipore, IPVH00010, MA, USA). The membranes were sealed in 5% skim milk powder (Solarbio, D8340, Beijing, China) overnight at 4 °C. Supplementary antibodies including GAPDH (5174), AGR2 (13062), ZEB1 (70512), E-cadherin (3195), N-cadherin (13116), Vimentin (5741), Slug (9585), Snail (3879), *p*-AKT (4060), AKT (4685), *p*-GSK3 β (9322), GSK3 β (5676), β-catenin (9582) were added. The primary antibodies were incubated for 12 h, then the goat anti-rabbit IgG second antibody (Solarbio, K1034G-AF594, Beijing, China) was incubated for 1 h. GAPDH was used as endogenous control, and all the primary antibodies were sourced from CST (Massachusetts, USA).

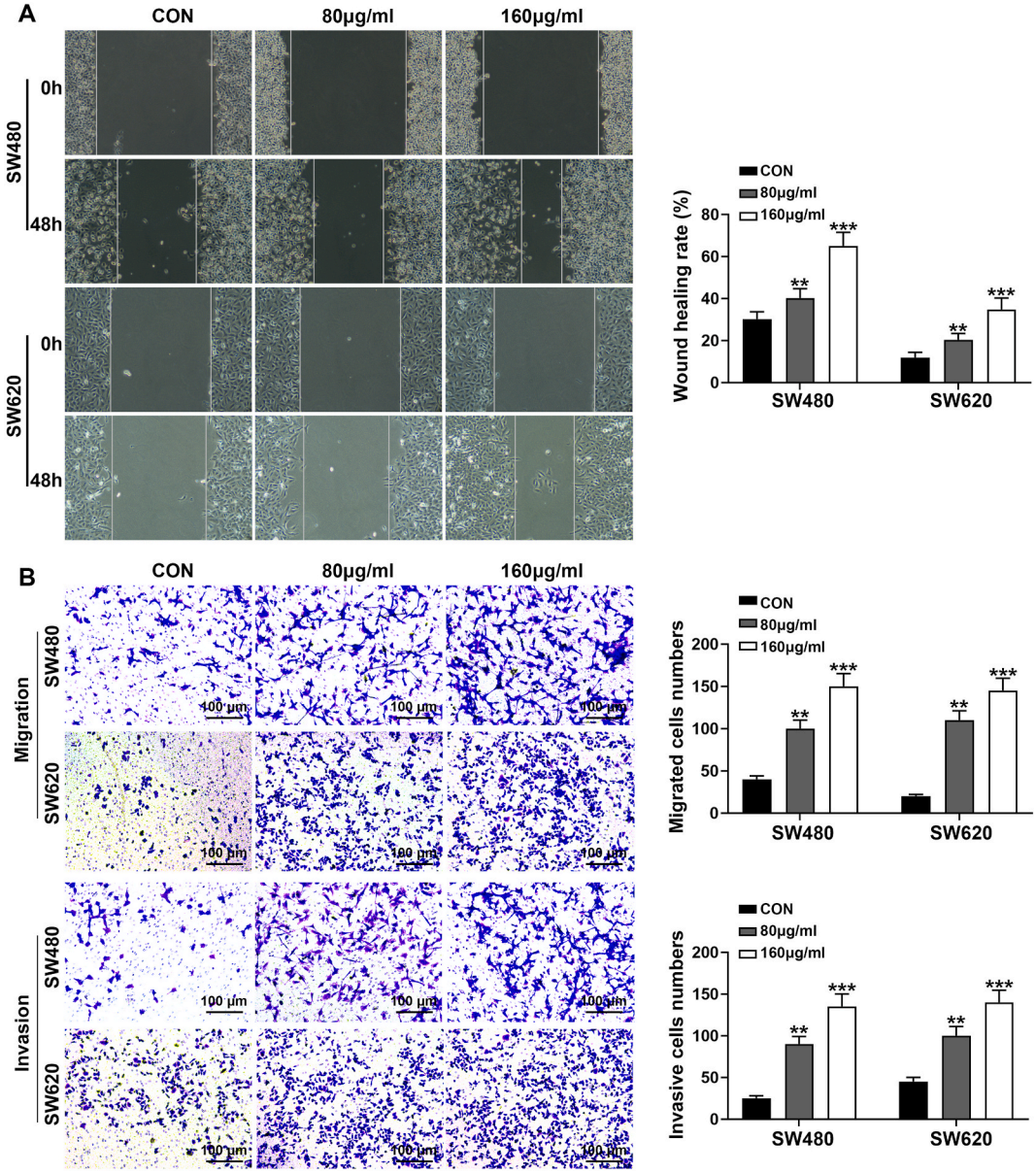

Fig. 2. Exogenous AGR2 boosted migration and invasion of CRC cells (A) scratch healing assay was used to evaluate cell migration. (B) Transwell assay was used to evaluate cell migration and invasion. **P < 0.01,***P < 0.001, versus CON subgroup.

2.8. Statistical analysis

Statistical analysis was conducted via SPSS 22.0 software (IBM, Armonk, NY, USA). The expression of data was analyzed using the mean and standard deviation (mean ± SD). Difference between subgroups were compared by one-way ANOVA. The significant difference was considered if the P-value was less than 0.05 (P < 0.05).

3. Results

3.1. The exogenous AGR2 concent was boosted in CRC cells

Bioinformatics analysis confirmed that the AGR2 concent in CRC tissues was boosted versus normal colorectal tissues (Fig. 1A). The results at cellular concent also confirmed that AGR2 concent in SW480, SW620, HT-29 cells were notablely boosted versus that in normal colonic epithelial cells (CCD-841 CoN) (Fig. 1B). Exogenous AGR2 could increase the viability of SW480 and SW620 cells and

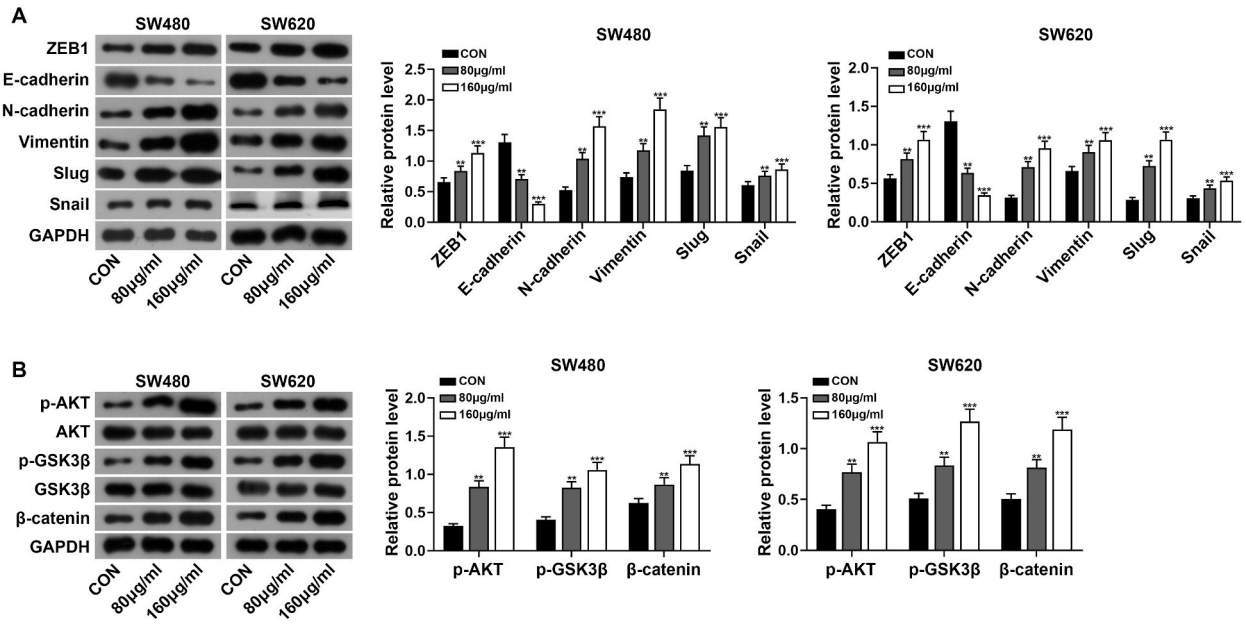

Fig. 3. Exogenous AGR2 boosted EMT in CRC cells (A) Western blot was applied to assess ZEB1,E-cadherin, N-cadherin,Vimentin, Slug and Snail concent. (B) western blot was applied to assess *p*-AKT, AKT, *p*-GSK3β, GSK3β and β-catenin concent. **P < 0.01,***P < 0.001, versus CON subgroup.

boost cell colony formation versus the control subgroup (Fig. 1C–E). It is suggested that exogenous AGR2 can increase the multiplication ability of CRC cells.

3.2. Exogenous AGR2 boosted migration and invasion of CRC cells

As confirmed, SW480 and SW620 cells migration rates in exogenous AGR2 subgroup were notablely boosted versus those in control subgroup (Fig. 2A). Migration and invasion of SW480 and SW620 cells in exogenous AGR2 subgroup was notablely boosted versus that in control subgroup (Fig. 2B).

3.3. Exogenous AGR2 boosted EMT in CRC cells

EMT plays a vital part in tumorigenesis and metastasis, CRC migration and invasion [14]. Exogenous AGR2 outcome on EMT-related proteins concent in CRC cells was appraised. ZEB1, N-cadherin, Vimentin, Slug, Snail protein concent in SW480 and SW620 cells was notablely boosted and E-cadherin protein concent was notablely abated in exogenous AGR2 subgroup versus the control subgroup (Fig. 3A). Then the possible mechanism of exogenous AGR2 was appraised. GSK3 β/GSK3 β and β-catenin concent in CRC cells of exogenous AGR2 subgroup was notablely boosted versus the control subgroup (Fig. 3B).

3.4. Exogenous AGR2 regulated CRC cell activity through AKT/β-catenin

In order to further confirm the possible mechanism of exogenous AGR2 on CRC cells, SW620 cells was treated with AKT agonist IGF- I (100 ng/ml). The protein concent of *p*-AKT/AKT, *p*-GSK3β/GSK3β and β-catenin in SW620 cells of exogenous AGR2 subgroup was notablely increased versus the control subgroup. Additionally, the protein concent of these factors in SW620 cells of exogenous AGR2+IGF-I subgroup were further boosted versus exogenous AGR2 subgroup (Fig. 4A). Exogenous AGR2 boosted cell colony formation versus the control subgroup, and the combination of exogenous AGR2 and IGF- I further boosted cell colony formation versus exogenous AGR2 subgroup (Fig. 4B). Exogenous AGR2 boosted cell migration and invasion versus the control subgroup. The combined therapy of exogenous AGR2 and IGF- I promoted cell migration and invasion versus exogenous AGR2 subgroup (Fig. 4C and D).

4. Discussion

In this study, AGR2 concent in CRC was boosted by GEPIA database analysis. At the cellular concent, AGR2 protein concent in CRC cell lines (SW480, SW620 and HT-29) was boosted versus that in normal colonic epithelial cells (CCD-841 CoN). This is also consistent with previous research results [15,16]. Then, the outcome of exogenous AGR2 on the multiplication of SW480 and SW620 cells was examined. Exogenous AGR2 increased the viability of SW480 and SW620 cells and boosted cell colony formation. This shows that exogenous AGR2 can increase the multiplication ability of CRC cells.

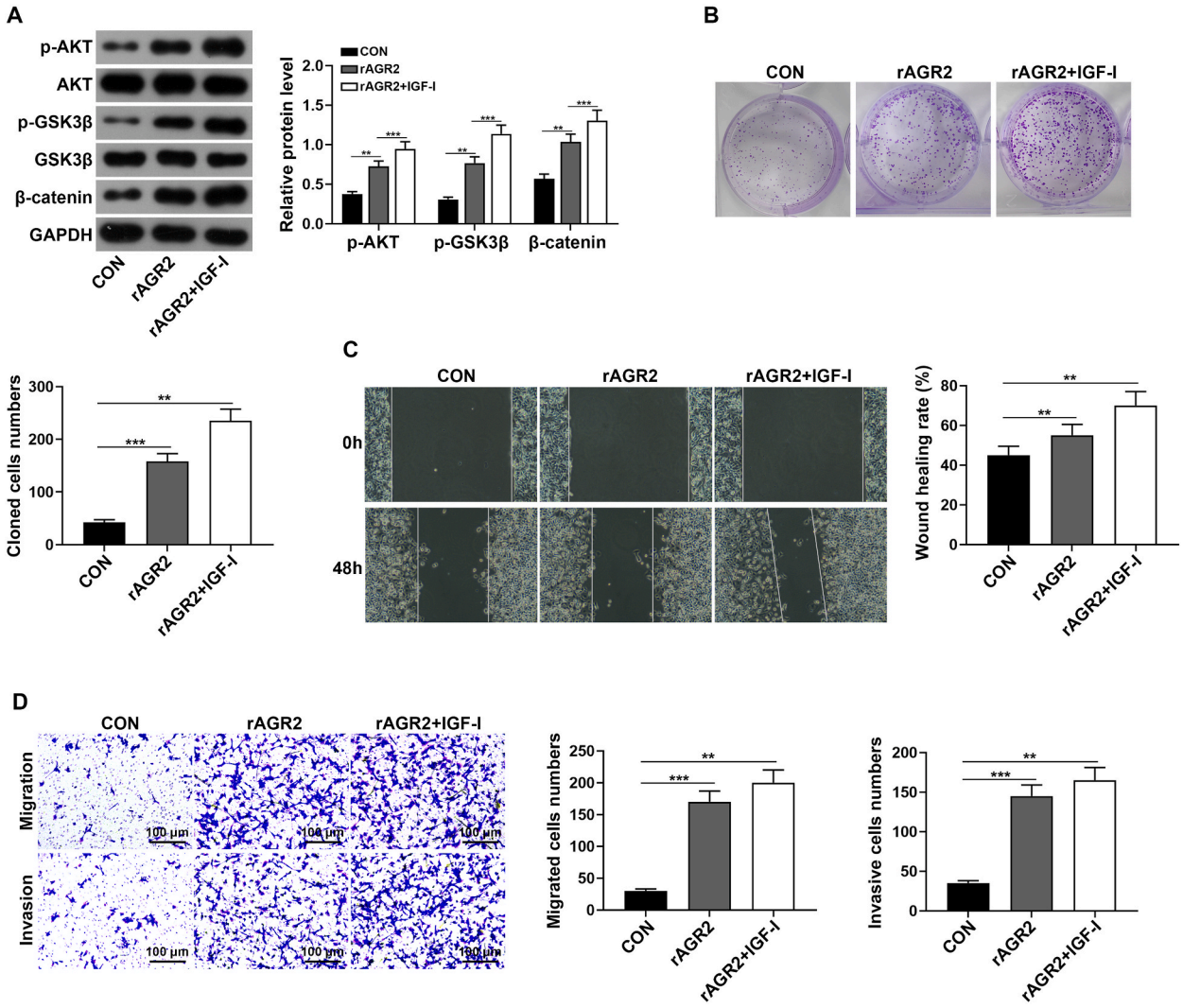

Fig. 4. Exogenous AGR2 regulated CRC cell activity through Akt/β-catenin (A) Western blot was applied to assess *p*-AKT, AKT, *p*-GSK3β, GSK3β and β-catenin concent. (B) The cell viability was evaluated by colony formation assay. (C) Scratch healing assay was used to evaluate cell migration. (D) Transwell assay was used to evaluate cell migration and invasion. **P < 0.01,***P < 0.001.

Metastasis is the main cause of cancer-related death. EMT conducts an essential part in the progress and metastasis of CRC [17]. EMT is an important process in which cells lose their epithelial characteristics and acquire mesenchymal characteristics, thus enhancing the fluidity and invasiveness of cancer cells and the characteristics of cancer stem cell-like cells, activating anti-apoptotic pathways, thus giving cancer cells metastases [18]. In this study, exogenous AGR2 boosted CRC cells migration and invasion ability. ZEB1 is an important transcription factor in the transformation of epithelial cells into mesenchymal cells and regulating cell differentiation and transformation [19]. E-cadherin is an epithelial marker. E-cadherin/β-catenin complex has a vital effect on the integrity and adhesion of epithelial cells [20]. In the process of EMT, zinc finger E-box-binding protein 1 (ZEB1) is a key regulatory transcription factor, which promotes tumor invasion and metastasis by inducing EMT in a variety of tumors. N-cadherin, Vimentin, Slug and Snail are mesenchymal markers, which are upregulated during EMT [21,22]. In this study, exogenous AGR2 increased the expression of ZEB1, N-cadherin, Vimentin, Slug and Snail protein and abated concent of E-cadherin in SW480 and SW620 cells. This indicates that exogenous AGR2 can induce EMT process.

AKT is a serine/threonine kinase that can be phosphorylated and activated. Activated AKT regulates cell proliferation, differentiation and apoptosis through multiple downstream targets. β-catenin is a multifunctional protein that plays a vital part in maintaining normal physiological function [23]. AKT is considered to be a carcinogenic gene and an upstream regulator of β-catenin. The nuclear transport and activation of β-catenin can be stimulated directly by phosphorylation of β-catenin or by phosphorylation and inactivation of GSK3 β [24]. AKT/β-catenin signal pathway participates in CRC Metastasis [25,26]. Therefore, this study was to investigate the outcome of exogenous AGR2 on AKT/β-catenin signal pathway related proteins concent in CRC cells. Exogenous AGR2 boosted GSK3 β/GSK3 β, β-catenin protein content in CRC cells. Furthermore, by using AKT agonist IGF- I to treat CRC cells, the results demonstrated

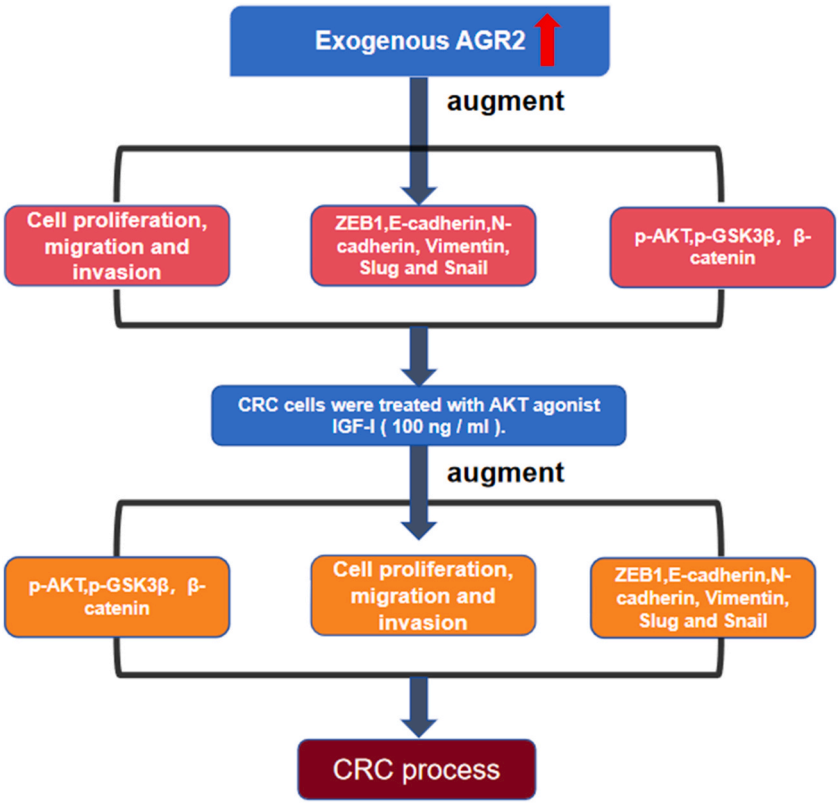

Fig. 5. A drawing showing the AGR2 work.

that IGF- I could further enhance the outcomes of exogenous AGR2 on the multiplication of CRC cells. However, there are some deficiencies in this study. Firstly, the role and mechanism of exogenous AGR2 in CRC need to be further confirmed in vivo models. Secondly, in addition to proliferation and metastasis, other cell phenotypes of exogenous AGR2 in CRC cells need to be evaluated. Besides, the mechanism by which exogenous AGR2 regulates ERK/MAPK signaling pathway to regulate a series of downstream effector molecules remains to be further confirmed in CRC.

5. Conclusion

Exogenous AGR2 enhances the ability of multiplication of CRC cells and induces EMT process, and its mechanism is related to AKT/β-catenin signal pathway (Fig. 5).

Ethic approval and consent to participate

Not applicable. This research adopted cell culture method, which are listed in the text.

CRediT authorship contribution statement

Chao Zheng: Writing – original draft, Methodology, Conceptualization. **Yu Mao:** Formal analysis, Data curation. **Jianping Ye:** Formal analysis, Data curation. **Miaolong Zhang:** Writing – review & editing, Supervision. **Yongfeng Chen:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e28175.

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