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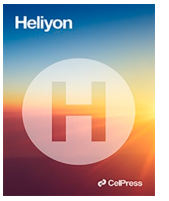

Research article

Interactions between genistein and Wnt pathway in colon adenocarcinoma and early embryos

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ABSTRACT

The Wnt signaling pathway is one of the most ancient and pivotal signaling cascades, governing diverse processes in development and cancer regulation. Within the realm of cancer treatment, genistein emerges as a promising candidate due to its multifaceted modulation of various signaling pathways, including the Wnt pathway. Despite promising preclinical studies, the precise mechanisms underlying genistein's therapeutic effects via Wnt modulation remain elusive. In this study, we unveil novel insights into the therapeutic mechanisms of genistein by elucidating its inhibitory effects on Wnt signaling through macropinocytosis. Additionally, we demonstrate its capability to curtail cell growth, proliferation, and lysosomal activity in the SW480 colon adenocarcinoma cell model. Furthermore, our investigation extends to the embryonic context, where genistein influences gene regulatory networks governed by endogenous Wnt pathways. Our findings shed light on the intricate interplay between genistein, Wnt signaling, membrane trafficking, and gene regulation, paving the way for further exploration of genistein's therapeutic potential in cancer treatment strategies.

1. Introduction

Genistein [5,7-dihyroxy-3-(-4-hydroxyphenyl)-4H-1-benzopyran-4-one], a bioflavonoid found in soy products [1], has sparked a growing interest in oncological research due to its potential ability to modulate crucial intracellular signaling pathways in cell proliferation and survival [2,3]. Among these pathways, genistein has been indicated to modulate Wnt signaling; and has emerged as a promising therapeutic target in cancer treatment, highlighting genistein's anti-proliferative and anti-metastatic effects in various cancer types [4]. The Wnt signaling pathway is known for its fundamental role in regulating cell growth, differentiation, and organ formation during embryonic development [5–8]. Wnt dysregulation contributes to the development and progression of various diseases [9]. Inappropriate activation of the Wnt pathway has been associated with uncontrolled cell proliferation, invasion, and metastasis in various cancer subtypes. Genistein has a demonstrable ability to interfere with the Wnt pathway through various

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mechanisms [10–13]. One key aspect is its ability to modulate the expression of essential components of the pathway, such as β-catenin. β-catenin is a central protein in the Wnt pathway that, when stabilized, translocates into the nucleus and activates the expression of genes involved in cell proliferation and survival.

Studies have shown that genistein can inhibit the accumulation of β-catenin in the nucleus by promoting its degradation [14]. Genistein also regulates the expression of other proteins and regulatory molecules in the Wnt pathway, such as GSK-3β (Glycogen Synthase Kinase 3 beta) and APC (Adenomatous Polyposis Coli) [15]. In addition to its direct influence on the stabilization of β-catenin, genistein interacts with other signaling pathways [16] that intersect with the Wnt pathway. For instance, genistein exhibits the ability to regulate the activity of the PI3K/Akt/mTOR pathway by interacting with the Wnt pathway to promote cell proliferation and cancer metastasis (16). The PI3K/Akt/mTOR pathway, a pivotal signaling cascade frequently dysregulated in cancer, plays a crucial role in multiple cancer hallmarks. It governs cell proliferation, survival, metabolism, and angiogenesis, all pivotal in cancer progression [17]. Dysregulated activation of this pathway, often stemming from genetic alterations in key components like PIK3CA, AKT, and PTEN, can fuel tumor growth and advancement [18]. Various inhibitors targeting different nodes of this pathway, including PI3K inhibitors, AKT inhibitors, and mTOR inhibitors, are currently under clinical evaluation across diverse cancer types. Targeting this pathway holds promise in managing pivotal cancer features, potentially enhancing treatment outcomes for cancer patients (19).

Furthermore, genistein plays a significant role in immune regulation, boasting anti-inflammatory properties capable of suppressing the production of proinflammatory cytokines like TNF-α, IL-1β, and IL-6. It exerts inhibitory effects on immune cell activation including dendritic cells, T cells, and NK cells, thereby modulating immune responses [20]. Additionally, genistein shows potential in enhancing certain aspects of immune function such as increasing antibody production and antioxidant capacity as well as the proliferation of B cells, T helper cells, and NK cells. Mechanistically, genistein's regulatory effects on immunity involve modulation of signaling pathways like the MAPK cascade and Toll-like receptor signaling, along with potential influences on immune-related gene expression [21].

Preclinical research has supported the role of genistein as a Wnt pathway modulator in colorectal, prostate, and breast cancer [22]. These studies have revealed that genistein can inhibit cell proliferation, induce apoptosis, and suppress metastasis by influencing the Wnt pathway and its interactions with other signaling pathways [22]. In the current study, we investigate the effects of genistein in the context of the emerging relationship between macropinocytosis and Wnt signaling [5,23–25]. We show that genistein blocks colon cancer proliferation by inhibiting macropinocytosis and Wnt signaling. In early embryonic development, genistein interferes with gene regulatory networks controlled by Wnt and inhibits head formation.

2. Results

2.1. Genistein inhibits Wnt signaling and cell proliferation

First, we sought to confirm that genistein affects the Wnt signaling pathway. We used LiCl because it inhibits GSK3β, triggers macropinocytosis, increases the number of lysosomes, and mimics Wnt signaling (Fig. 1A) [24].

A stably transfected HEK293 BAR-luciferase/Renilla cell line [26] was used to measure β-catenin activity, which marks the

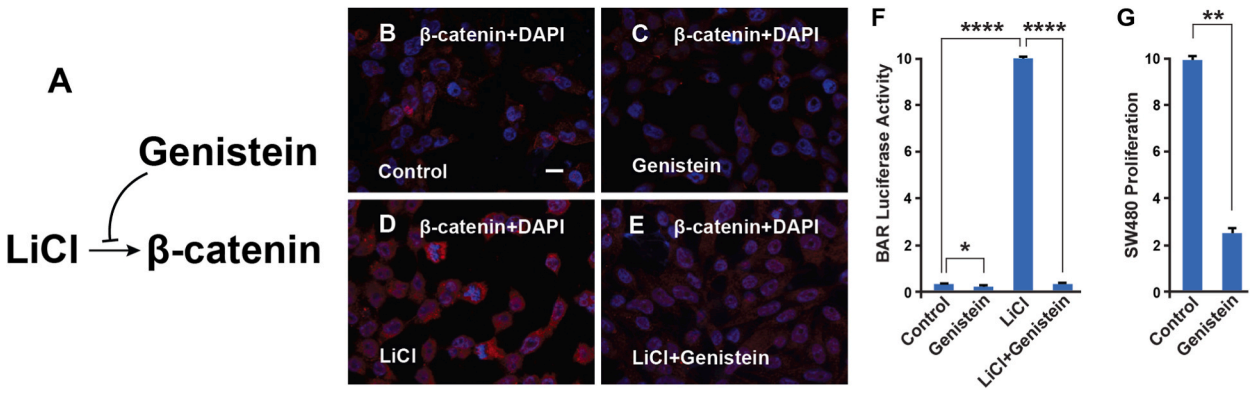

Fig. 1. Genistein Inhibits Activation of Wnt Pathway and Cell Proliferation
(A-F) Genistein blocks activation of the Wnt pathway by LiCl and inhibits β-catenin stabilization in HEK 293 cells
(B) Control (untreated cells).
(C) Genistein treatment (500 μM for 3 h).
(D) LiCl treatment (40 mM for 3 h) results in increased β-catenin levels.
(E) Genistein + LiCl treatment. No increase in β-catenin levels.
(F) BAR Luciferase assay of β-catenin activity levels for the same experiments as inB-E.
(G) Cell proliferation assay in SW480 cells was reduced after genistein treatment (250 μM).
All experiments with cultured cells were repeated in at least 3 biological replicates. Red: β-catenin; blue: nuclei. Scale bar, 10 μm. Error bars denote SEM ($n \geq 3$) (*$P < 0.05$,**$P < 0.01$, ****$P < 0.0001$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

activation of the Wnt pathway. Cells were incubated overnight with LiCl in the presence or absence of genistein. The LiCl treatment activates Wnt signaling at a 40 mM concentration [23–25]. This effect was completely abolished by adding genistein (Fig. 1B–F). Following the effect of genistein on externally induced Wnt signaling, its effect on endogenous Wnt signaling was investigated. The human colorectal adenocarcinoma SW480 cell line, which expresses a truncated version of APC and has constitutively active Wnt signaling, was used to test the effect of genistein on cell proliferation with the Vi-CELL XR Cell Viability Analyzer. After 24 h of incubation with the genistein inhibitor, a reduction in cell proliferation was observed with no effect on cell viability (Fig. 1G). SW620 cells, originating from a metastatic site of the same tumor as SW480, were also employed in the study to corroborate the effect of genistein in the Wnt pathway (Supp. Fig. 1).

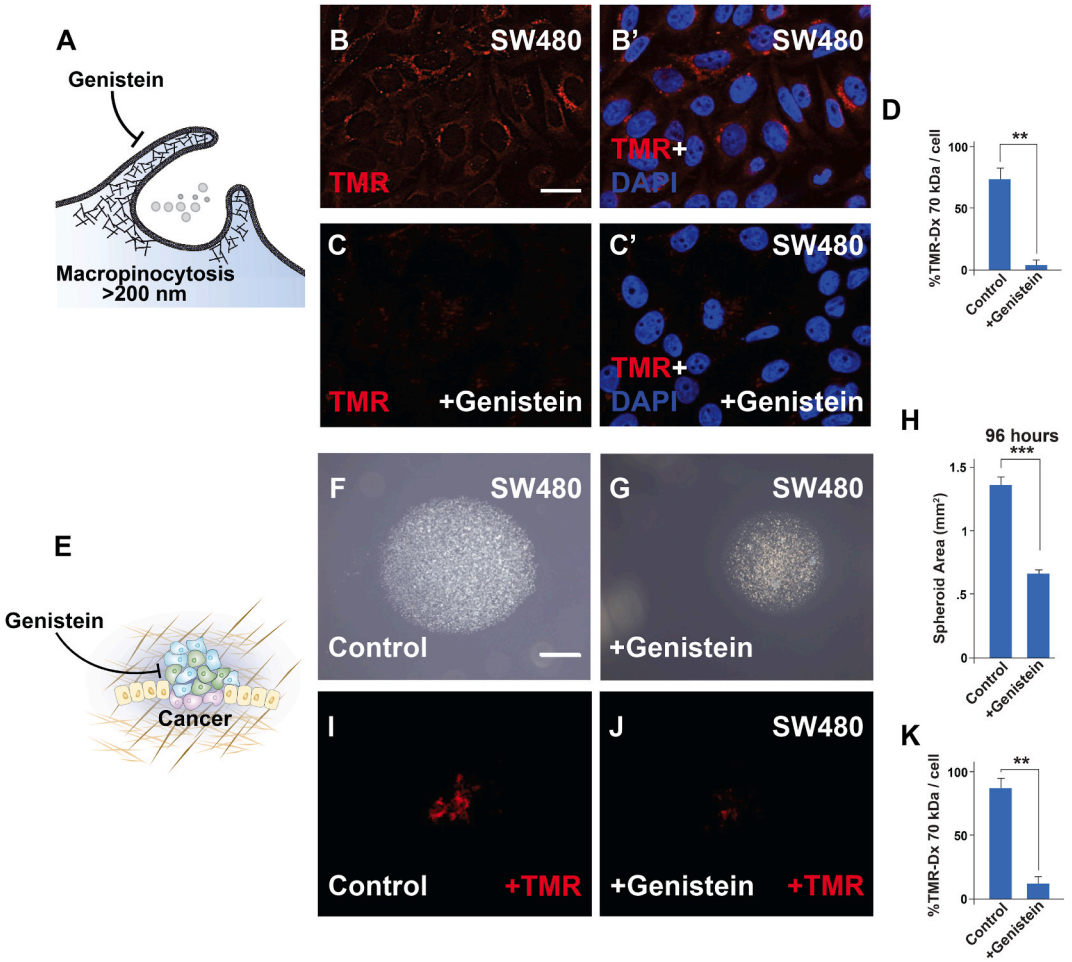

Fig. 2. Genistein Inhibits Macropinocytosis in Colon Cancer Cells
(A-D) Illustration of the cell membrane with genistein inhibiting macropinocytosis.
(B–B′) TMR-dx (red, 1 mg/mL) uptake in SW480 cells after 1 h of incubation (control)
(C–C′) Same as B–B′ after overnight genistein treatment (250μM). The TMR-dx uptake was reduced.
(D) TMR-dx uptake quantification for B–C'. Percentage of cells with detectable TMR-dx signal.
(E–K) Genistein reduces cell growth in SW480 spheroids
(F) SW480 spheroids at 96 h of inverted drop culture visualized in bright-field (see Methods).
(G) SW480 spheroids with the same conditions as F after the overnight genistein treatment (250 μM), the sizes of spheroids in 3D culture were reduced.
(H) Quantification of the spheroid area from F and G.
(I) TMR-dx (red, 1 mg/mL) uptake in spheroids.
(J) After the overnight genistein treatment (250 μM), the TMR-dx uptake was reduced in cells treated with the same conditions as I.
(K) TMR-dx uptake quantification for I and J.
All experiments with cultured cells were repeated in at least 3 biological replicates.Eight spheroids were plated per replicate. Scale bars, B–C′10 μm; F-J 500 μm. Error bars denote SEM ($n \geq 3$) (**$P < 0.01$, ***$P < 0.001$).). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.2. Genistein Inhibits Macropinocytosis in Colon Cancer Cells

A connection has emerged between Wnt signaling, lysosomal activity, and macropinocytosis in the Wnt pathway [5,23–26]. Macropinocytosis is an actin-driven pinocytosis of large endocytic vesicles with diameters greater than 200 nm (Fig. 2A) [27]. It has been demonstrated that SW480 cells are capable of sustaining macropinocytosis and Wnt signaling [23,24,26,28]. To investigate the effects of genistein on these cellular processes, we measured the uptake of the high-molecular-weight dextran TMR-dx (Tetramethylrodamine, 70 kDa dextran) [26]. The short 1-**h** incubation of SW480 cells with TMR-dx resulted in elevated uptake of this macropinocytosis marker (Fig. 2B-B′**).** The treatment with genistein completely abolished the TMR-dx uptake (Fig. 2C-C').

SW480 cells have a characteristic circular ruffle membrane formation at the leading edge that reflects active macropinocytosis. The addition of genistein rapidly reduces the formation of macropinosomes on the surface of SW480 cells (Supplemental Movie S1**).** Next, we tested the effects of genistein on spheroid formation in hanging drop cultures using SW480 cells with a malignant phenotype **(**Fig. 2E**).** Genistein treatment decreased the size of cell spheroids **(**Fig. 2F–H), uptake of TMR-dextran (Fig. 2I–K), and reduced cell proliferation.

2.3. Genistein Reduces Lysosome Formation and β-catenin Levels in Colon Cancer Cells

Lysosomes have been associated with oncogenic transformation in Wnt-driven cancers [29–32]. Changes in volume and subcellular localization point to lysosomes as a marker for early cancer detection and progression [18–22]. To investigate the effects of genistein on lysosome formation (Fig. 3A), we visualized MVBs/lysosomes in SW480 cells using marker genes (tetraspanin CD63 (Fig. 3), and LAMP1 (lysosomal associated membrane protein 1) (Supp. Fig. 2**)** [23]**).** After the overnight incubation with genistein, we observed reduced levels of both marker genes, which indicates inhibition of lysosome formation. In the same experiment, we also observed a reduction in nuclear β-catenin levels, confirming parallel dysregulation of Wnt signaling **(**Fig. 3B-**C**″).

2.4. Genistein Inhibits Head Formation in Early Embryos via Wnt signaling

The Xenopus embryo serves as a premier *in vivo* model system for studying developmental processes and diseases [24], providing insights into fundamental molecular mechanisms of Wnt signaling that operate universally across various tissues and animal systems. The Wnt pathway is one of the earliest developmental signals that coordinates the formation of the primary head-to-tail axes [33,34] in Xenopus embryos. RNA microinjection of canonical Wnt signaling activators such as dominant-negative GSK3 can induce secondary axis formation when injected ventrally [35]. Due to the significant impact of aberrant Wnt signaling in promoting tumor progression, targeting this pathway has become an appealing strategy in chemotherapy. The Xenopus axis duplication assay stands out as a highly effective method for screening potential inhibitor candidates. Understanding the implications of Wnt signaling modulation in cancer progression and developmental processes is pivotal to evaluating the therapeutic potential of genistein. To investigate how genistein treatment affects early development, we incubated *Xenopus* embryos with genistein at the 4-cell stage and cultured them until the tadpole stage. This treatment results in ventralized embryos without head structures (Fig. 4). The genistein treatment phenocopies the effects of inhibition of the Wnt pathway and reduces the expression domains of the anterior neural markers *sox2* and the *otx2* (Fig. 5). These results confirm that the Wnt pathway is the primary target of the genistein treatment in both normal and aberrant developmental processes.

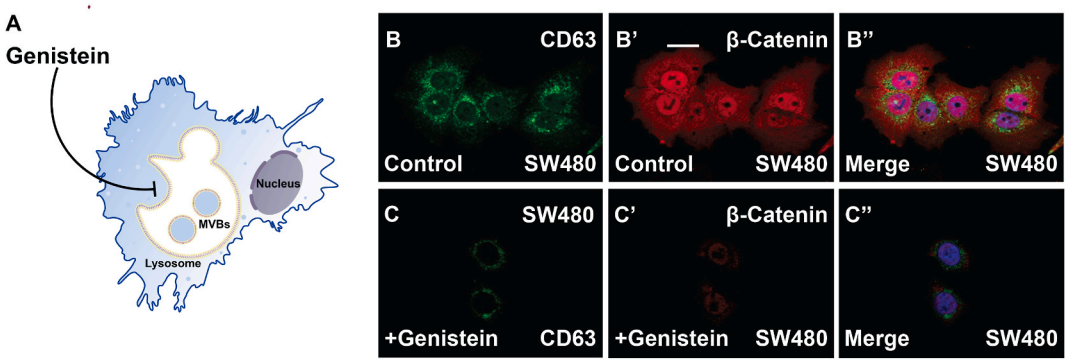

Fig. 3. Genistein Reduces Lysosome Formation and β-catenin Levels in Colon Cancer Cells
(A) Genistein reduces multivesicular body formation (MVB) in SW480 cells.
(B–B″) Untreated (control) SW480 cells have high levels of lysosomes visualized by MVB marker CD63 (green) and high levels of nuclear β-catenin (red).
(C–C″) Cells with identical treatment as (B)-(B″) after the overnight genistein treatment (250 μM). Genistein treatment reduces both CD63 and β-catenin and CD63 levels.
All experiments with cultured cells were repeated in at least 3 biological replicates. Blue: nuclei. Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

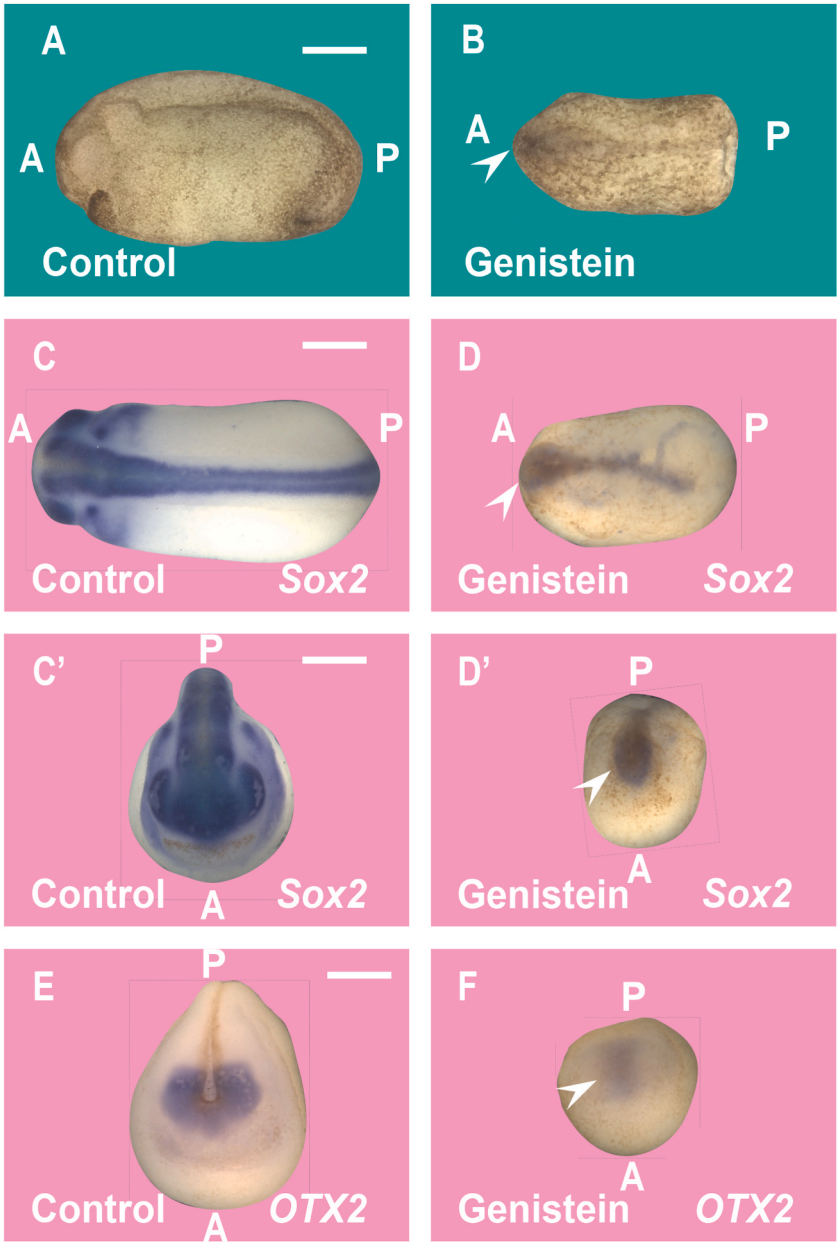

Fig. 4. Genistein Inhibits Head Formation in Early Embryos via Wnt signaling
(A) *Xenopus* embryos at tadpole stage. A, anterior; P, posterior.
(B) Same as A after genistein treatment (500 μM) from the 4-cell stage until the 9.5 stage (early gastrula). Genistein treatment results in ventralized embryos.
(C and D) Same conditions as A and B. A pan-neuronal marker *Sox2* is visualized by *in situ* hybridization.
(C′ and D′) Same as C and D. Frontal view showing the reduction in the head formation (white arrow).
(E–F) Same conditions as A and B. A forebrain/midbrain marker *Otx2* is visualized by *in situ* hybridization.
All experiments were repeated five times. Total numbers of embryos analyzed for each condition, and percentage of embryos with reported phenotypes: A = 130, 100 %; B = 138, 93 %; C = 50, 100 %; D = 62, 91 %; E = 60, 100 %; F = 65; 92. Scale bars, 500 μm.

2.5. Genistein Treatment Has Broad Effects on Gene Regulation in Early Embryos

Next, we investigated how genistein treatment affects gene regulatory networks during development. We treated embryos with genistein at the 4-cell stage and collected them at stage 9.5 (early gastrula) for RNA extraction and sequencing (RNA-Seq). In addition to control and genistein conditions, we used two established Wnt activators: BIO (6-bromoindirubin-3′-oxime) and Chiron (CHIR 99021). BIO and Chiron activate the Wnt pathway via inhibition of GSK3, resulting in dorsalized embryos [25]. All four conditions

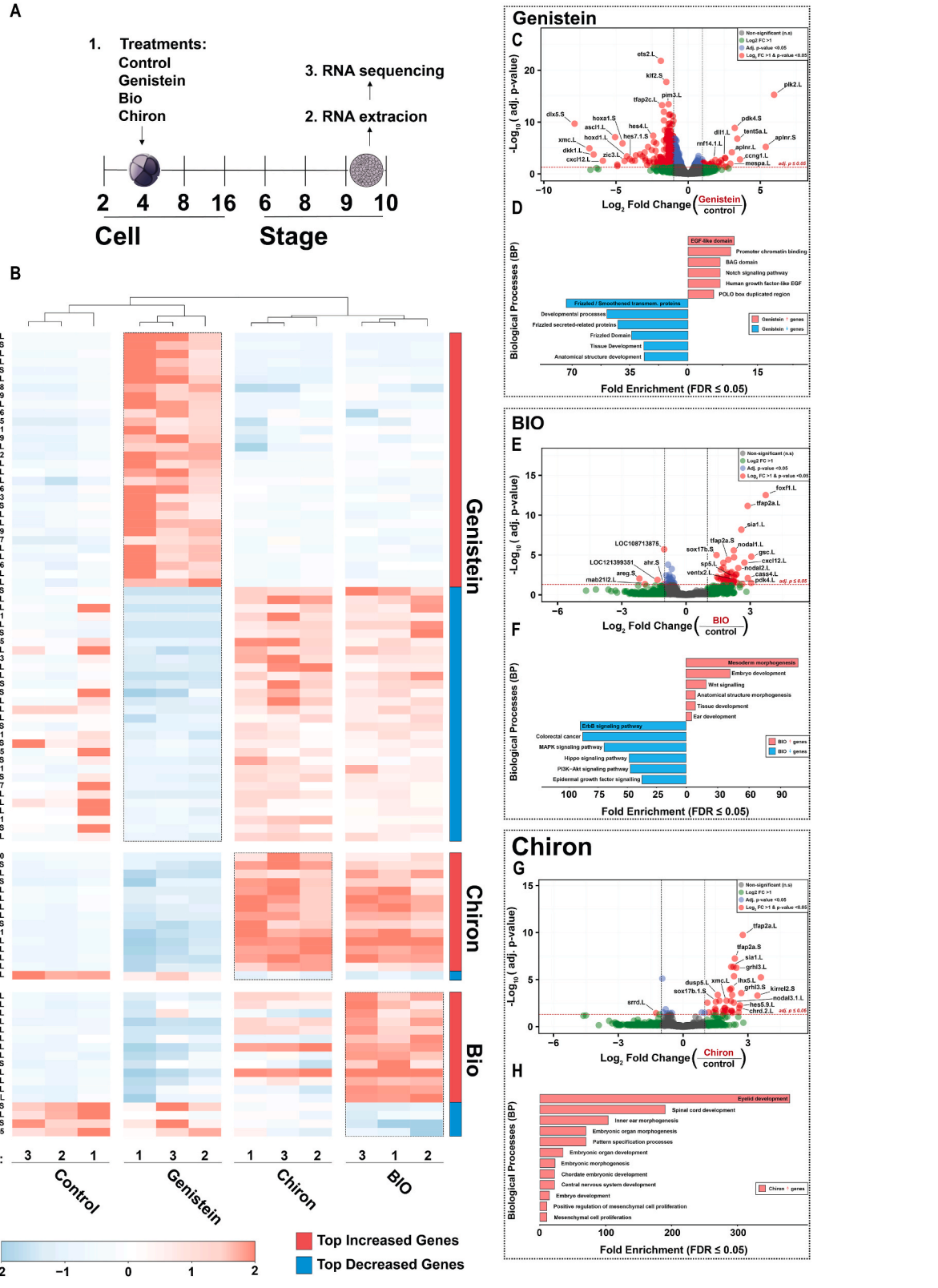

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Fig. 5. Genistein Treatment Has Broad Effects on Gene Regulation in Early Embryos
(A) Experimental design. *Xenopus* embryos were treated with different drugs at the 4-cell stage. Total RNA was extracted at the 9.5 stage and used for RNA sequencing. Three replicates with three embryos per replicate were used for each condition.
(B) A heatmap of expression patterns of top DEGs in each condition. For genistein, we show the top 30 upregulated and downregulated DEGs. For BIO and Chiron, we show the top 15 upregulated DEGs (few genes were downregulated; see D and F). The pink and blue bars represent up-regulated and down-regulated genes, respectively.
(C) Volcano plot of the differential expression analysis between control and genistein treatments. Log_2-fold-change values (x-axis) and adjusted *P*-values (y-axis) are shown for each gene. Differentially expressed genes (DEGs, adjusted $P < 0.05$ and log_2-fold-change >1) are in red. Gene names are shown for top DEGs.
(D) Gene Ontology enrichment analysis of DEGs from panel B. The x-axis summarizes the fold enrichments of DEGs for the biological process on the y-axis. Significance levels were computed to adjusted for multiple hypotheses testing via the $-log_{10}$ of the false-discovery rate (FDR ≤ 0.05).
(E and F) Same as B and C for the comparison of control and BIO (30 mM, a specific inhibitor of GSK-3)
(G and H) Same as B and C for comparing control and Chiron (CHIR 99021, 60 μM). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

were analyzed in triplicates. The analysis of RNA-Seq data revealed significant effects of genistein on transcriptomes. 36 genes were upregulated, and 247 genes were downregulated compared to controls (adjusted p-value <0.05 and minimum 2-fold-change, Fig. 5C). The downregulated genes were enriched with proteins associated with Wnt and Hedgehog pathways, while the upregulated genes were enriched with proteins associated with EGF and Notch pathways (Fig. 5D). Fewer genes were differentially regulated in response to both the BIO and Chiron treatments. As expected, many of the differentially expressed genes were shared between both conditions and included canonical Wnt targets such as Siamosis and Chordin (Fig. 5E–H) [36]. The observed broad effects of genistein on embryonic transcriptomes suggest that it may interfere not only with Wnt signaling but also with other cell signaling pathways. The top differential genes identified in our analysis provide targets for future studies of possible mechanisms underlying the therapeutic effects of genistein (Fig. 5B).

3. Discussion

Soy foods have been reported to be among the healthiest for human consumption due to their potent anticancer effect [37]. Genistein, a component of soy, was first isolated from the plant Genista tinctoria in 1899 and chemically synthesized in the 1920s. It is an isoflavone and phytoestrogen found predominantly in soy products that has been studied for its potential role in cancer prevention and treatment [38]. Genistein (4′,5,7-trihydroxyisoflayine) is one of the most common natural isoflavones [39,40] containing a 3-phenylchromen-4-one skeleton without hydroxyl group substitution on position 2. Its concentration in soy foods is between 1.9 and 229 mg/g, and can also be found in chickpeas and other soy-based foodstuff [41].

Genistein has been demonstrated to reduce several types of cancers, such as breast [42], prostate [43], gastric [44], and colon cancers [45] by modulation of a variety of different cell signaling cascades. It has been shown that genistein can suppress cell growth in colon cancer by decreasing the activity of the PI3K/Akt pathway [46,47]. Genistein has also been implicated in epigenetic regulation of Wnt pathway genes [48] and reduced colonic tumor growth [48], as well as inactivating Wnt-signaling by targeting GSK3-β [49].

Recently, we have demonstrated that mechanisms of Wnt activity extend beyond the regulation of gene expression to central metabolic pathways, such as acquiring nutrients through actin-driven endocytic mechanisms [5,24]. The connection between Wnt signaling and macropinocytosis opens new opportunities and targets for cancer treatment [42–46]. Here, we show how genistein interacts with the Wnt pathway in a multi-faceted fashion, from regulation of macropinocytosis and lysosomal activity in cancer models to modulation of gene expression in normal development. Furthermore, our transcriptomic analysis revealed other potential cell signaling pathways that can be modulated by genistein, providing insights into other possible mechanisms underlying its therapeutic effects.

4. Concluding remarks

Our study presents compelling evidence for the multifaceted effects of genistein on Wnt signaling, macropinocytosis, and gene regulation in cancer cells and embryonic development. However, our findings also highlight several avenues for further research and exploration. Mechanistic elucidation is imperative to fully comprehend the underlying molecular mechanisms by which genistein modulates Wnt signaling and macropinocytosis. The employment of advanced molecular biology techniques, such as proteomics and transcriptomics, holds promise in unraveling the intricacies of associated signaling pathways and identifying key molecular targets of genistein. Furthermore, the clinical validation of our preclinical findings is paramount to evaluate the feasibility and efficacy of genistein-based therapies in cancer patients. Conducting well-designed clinical trials will be essential in translating our preclinical observations into clinically relevant applications, ultimately advancing the field of cancer therapeutics.

STAR Methods

KEY RESOURCES TABLE.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD63	Abcam	Cat# ab59479 RRID:AB_ 940915
β-catenin	Thermo Fisher Scientific	Cat# 71–2700; RRID:AB_ 2533982
GAPDH	EMD Millipore	Cat# AB2302; RRID:AB_10615768
Lamp1	Abcam	Cat# ab24170 RRID: AB_775978
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594) preadsorbed	Abcam	Cat# ab150084; RRID:AB_ 2734147
Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) preadsorbed	Abcam	Cat# ab150117; RRID:AB_ 2734147
Chemicals, peptides, and recombinant proteins		
Fibronectin	ThermoFisher	Cat#33016015
Dextran Tetramethylrhodamine (TMR-Dx) 70,000	ThermoFisher	Cat# D1818
5-(N-Ethyl-N-isopropyl) amiloride (EIPA)	Sigma	Cat# A3085
Lithium chloride (LiCl)	Sigma	Cat#L4408
10-cm dish	ThermoFisher	Cat#174903
Genistein	Tocris	Cat#1110
Glass-bottom chamber (#0)	Cell E&G	Cat#GBD00003-200
Circular coverslips	ibidi	Cat#10815
12-well dish	ThermoFisher	Cat#150628
DMEM	ThermoFisher	Cat#11965092
L-15	ThermoFisher	Cat#11415064
Glutamine	ThermoFisher	Cat#25030081
Fetal Bovine Serum (FBS)	ThermoFisher	Cat#16000044
Bovine Serum Albumin (BSA)	ThermoFisher	Cat#9048468
Pen-Strep antibiotics	ThermoFisher	Cat#15140122
Triton X-100	ThermoFisher	Cat#HFH10
Paraformaldehyde	Sigma	Cat#P6148
Fibronectin	Sigma	Cat# F4759
PBS	Gibco	Cat#10-010-023
PBS	Fisher Scientific	Cat#BP3994
Lipofectamine 3000	ThermoFisher	Cat#L3000001
Fluoroshield Mounting Medium with DAPI	Abcam	Cat# ab104139
RNAeasy Plus kit	QIAGEN	Cat#74034
Critical commercial assays		
Dual-Luciferase Reporter Assay System	Promega	Cat#E1500
Experimental models: Cell lines		
SW480	ATCC	RRID:CVCL_0546
Deposited data		
NCBI GEO		*in progress*
Experimental models: Organisms/strains		
Xenopus laevis	Xenopus I	
Recombinant DNA		
β-catenin Activated Reporter (BAR)	Addgene	RRID:Addgene_12456
Renilla reporter	Addgene	RRID:Addgene_62186
Software and algorithms		
ImageJ	NIH	http://imagej.nih.gov/ij/
Axiovision 4.8	Zeiss	http://Zeiss.com
Zen 2.3 imaging software	Zeiss	http://Zeiss.com
R	R Core Team	https://cran.r-project.org
Other		
Axio Observer Z1 Inverted Microscope with Apotome	Zeiss	N/A

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Nydia Tejeda Munoz.

Materials availability

This study did not generate new reagents. All materials used in this manuscript are publicly available and listed in the Key Resource Table.

Data and code availability

The RNA-Seq reads and processed data were deposited to NCBI GEO: GSE268980. The analysis of imaging and RNA-Seq data was performed using published tools and algorithms.

Experimental model and subject details

Tissue culture and transfection

HEK293T cells stably expressing BAR and Renilla reporters were cultured in DMEM (Dulbecco's Modified Eagle Medium CAT:11965092, Gibco) medium, supplemented with 10 % fetal bovine serum (FBS), 1 % glutamine, and penicillin/streptomycin. The human malignant SW480 cells [50] and SW620 cells [51] were cultured in DMEM/F12, supplemented with 5 % FBS, 1 % glutamine, and penicillin/streptomycin. The cells were seeded at a cell density of 20–30 %, and experiments were performed when cells reached between 70 and 80 % confluency. For transfection, lipofectamine 3000 was used following the instructions from the provider. Cells were seeded in a 12-well plate or chamber and transfected with the corresponding plasmid. Cells were cultured for 6–8 h in a medium containing 2 % FBS instead of 10 % before the treatments. All cells were cultured at 37 °C in 5 % CO_2/95 % humidity.

Genistein incubation and in situ hybridization in Xenopus embryo

The UCLA Animal Research Committee approved all animal experiments. *Xenopus laevis* embryos were fertilized *in vitro* using excised testis and staged as described [25,52]. *Xenopus* embryos at the 4-cell stage were incubated with genistein (10 μM in 20 % L-15 culture medium) and cultured overnight until the early tailbud tadpole stage. *In situ*, hybridizations were performed as described at http://www.hhmi.ucla.edu/derobertis using *sox2*, and *otx2* probes.

Antibodies and reagents

Total β-catenin antibody (1:100) was purchased from Thermo Fisher Scientific. CD63 and Lamp1 antibodies were obtained from Abcam (1:100; ab59479, ab24170). Secondary antibodies for immunostaining for cells (ab150084, ab150117) (1:300) were obtained from Abcam. Genistein (1110) was purchased from Tocris. TMR-dextran 70 kDa was obtained from ThermoFisher (D1818).

Method details

Immunostainings and Western blot

Immunofluorescence analyses were performed as described previously [26]. In short, HEK293 and SW480 cells were plated on glass coverslips and transferred to 2 % FBS 6–12 h before overnight treatments. Coverslips were acid-washed and treated with Fibronectin (10 μg/mL for 30 min at 37°C, Sigma F4759) to facilitate cell spreading and adhesion. Cells were fixed with 4 % para-formaldehyde (Sigma #P6148) for 15 min, permeabilized with 0.2 % Triton X-100 in phosphate-buffered saline (PBS; Gibco) for 10 min, and blocked with 5 % BSA in PBS for 1 h. Primary antibodies were added overnight at 4 °C. Cells were washed three times with PBS, and secondary antibodies were applied for 1 h at room temperature. After three additional washes with PBS, the coverslips were mounted with Fluoroshield Mounting Medium with DAPI (ab104139). Immunofluorescence was analyzed and photographed using a Zeiss Imager Z.1 microscope with Apotome.

Luciferase assay

HEK293T cells stably expressing BAR and Renilla reporters were incubated with LiCl (40 mM) or Wnt3a (100 ng/mL) with or without genistein (500 μM) for 5–6 h, and Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions, using the Glomax Luminometer (Promega). Luciferase values of each sample were normalized for Renilla activity.

3D spheroid cell culture and western blotting assay

SW480 cells were cultured in a Petri dish using DMEM: F-12 medium with 5%FBS. The top cover was removed from 60 mm tissue culture dishes, and 3 mL of PBS was placed in the bottom of the dish to act as a hydration chamber. Cells were counted, and 500 cells were seeded as 25 μL drops deposited onto the Petri dish cover and immediately inverted over the humid chamber. 6 drops per condition were plated, keeping enough distance between each other. The inverted drop cultures were incubated at 37 °C in 5 % CO_2/95 % humidity. The drops were monitored daily; after 4 days, aggregates had been formed, and genistein treatment was added to the spheroids. After 4 days, pictures for each condition were taken. Next, the spheroids were incubated with TMR-dx 70 kDa (1 mg/mL) for 1 h, and each spheroid was photographed with an Axio Zoom.V16 Stereo Zoom Zeiss microscope with apotome function. For Western blots, cells were lysed with RIPA buffer containing 0.1 % Nonidet P-40, 20 mMTris/HCl pH 7.5, 10 % glycerol. Additionally, protease inhibitors (Roche, 04693132001) and phosphatase inhibitors (Calbiochem, 524629) were included in the lysis buffer.

Time-lapse imaging

For live-cell analyses (as seen in Methods video S1), plate the cells on fibronectin in a glass-bottom chamber (#0 cover glass, Cell E&G: GBD00003-200) for 12–18 h. Images were collected in a green fluorescence filter, acquired every 30 s for 20 min, and acquired using the Zeiss Observer.Z.1 inverted microscope with Apotome.2. For more details, see Ref. [53].

RNA sequencing (RNA-seq)

RNA was isolated with the RNAeasy Plus kit (QIAGEN) from 3 *Xenopus* embryos of each condition. Strand-specific and barcode-indexed RNA-seq libraries were generated from 60 ng total RNA each after poly-A enrichment using the Kapa mRNA-seq Hyper kit (Kapa Biosystems, Cape Town, South Africa) following the manufacturer's instructions. The fragment size distribution of the libraries was verified via micro-capillary gel electrophoresis on a Bioanalyzer 2100 (Agilent, Santa Clara, CA). The libraries were quantified by fluorometry on a Qubit fluorometer (LifeTechnologies, Carlsbad, CA) and pooled in equimolar ratios. The pool was quantified by qPCR with a Kapa Library Quant kit (Kapa Biosystems) and sequenced on a partial lane of an Aviti (Element Bio, San Diego, CA) with paired-end 150 bp reads.

RNA-seq analysis

The reference genome and gene annotations of *Xenopus laevis* were downloaded from Xenbase (xenbase.org, v10.1). RNA-Seq reads were aligned to the reference genome (v10.1, xenbase.org) using STAR (2.5.2b, [49]). The total number of uniquely mapped reads was 13–19 million reads per sample (56 % of sequenced reads). Gene counts were normalized to CPM (counts-per-million) using edgeR [50]. Lowly expressed genes were filtered out; 18479 genes with CPM >1 in at least 3 samples were kept for downstream analysis.

The differential expression analysis was performed using edgeR/LRT [50]. Differentially expressed genes were detected at an FDR-adjusted p-value of 0.05 and a minimum fold-change value of 2. The results of differential expression analysis were visualized using volcano plots using the EnhancedVolcano package [51]. Expression patterns of the top upregulated and downregulated genes were scaled to Z-scores and visualized as a heatmap (maximum 30 genes for Genistein, and maximum 15 genes for BIO and Chiron).

Gene ontology (GO) analysis of differentially expressed genes and visualization of the results of this analysis was performed using ShinyGO [52]. We show the top 6 enriched GO terms (domain: biological processes) separately for upregulated and downregulated genes in each treatment (except for Chiron; only 1 gene was downregulated in this condition).

Quantification and statistical analysis

Quantification of imaging data is shown as means and standard errors of means (SEM). Statistical data analysis was performed using the student t-test; a *P*-value of $<0.01^{**}$ was considered statistically significant for differences between means. Fluorescence was quantified in control versus treated cells using ImageJ software analyses with $n > 30$ cells per condition. Fluorescence intensity was normalized in images compared in each condition, and results from three or more independent experiments were presented as the mean $\pm$ SEM.

CRediT authorship contribution statement

Yagmur Azbazdar: Methodology, Investigation, Formal analysis. **Eric A. Sosa:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Julia Monka:** Methodology. **Yerbol Z. Kurmangaliyev:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Nydia Tejeda-Muñoz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32243.

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