# Inhibition of Apoptosis in Dairy Cow Mammary Epithelial Cells by Factor Signaling Pathway: An In Vitro Study (Postprint)

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## Abstract

The present study was designed to investigate whether the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is involved in the regulation of umbilical cord mesenchymal stem cells (UC-MSCs) inhibiting apoptosis of bovine mammary epithelial cells (BMECs) via insulin-like growth factor-I (IGF-I). UC-MSCs and BMECs were co-cultured using a Transwell™ co-culture system, with BMECs cultured alone serving as the control. The insulin-like growth factor-I receptor (IGF-IR) inhibitor AG1024 was administered for intervention, and the signal blocker AG490 was used to treat the cells. After 24 h, real-time quantitative PCR was employed to determine the relative expression levels of B-cell lymphoma/leukemia-2 (Bcl-2), B-cell lymphoma/leukemia-associated x protein (Bax), and cysteine protease-3 (Caspase-3) genes in each group, while flow cytometry was used to assess cell apoptosis. The results demonstrated that the apoptosis rate of BMECs in the UC-MSCs and BMECs co-culture group was extremely significantly lower than that in the other groups ($P<0.01$). The relative expression level of the Bcl-2 gene in the UC-MSCs and BMECs co-culture group was extremely significantly upregulated compared with the BMECs group ($P<0.01$), whereas the relative expression levels of Caspase-3 and Bax genes were significantly or extremely significantly downregulated ($P<0.05$ or $P<0.01$). Treatment with AG1024 and AG490, either alone or in combination, increased the apoptosis rate of BMECs cultured alone and BMECs co-cultured with UC-MSCs, upregulated the relative expression levels of Bax and Caspase-3 genes, and downregulated the relative expression level of the Bcl-2 gene, with all differences being statistically significant ($P<0.05$ or $P<0.01$). It was concluded that UC-MSCs can regulate the expression of apoptosis-related genes in BMECs and reduce the apoptosis rate of BMECs through the IGF-I-mediated JAK/STAT signaling pathway.

## Full Text

# An in Vitro Study of Umbilical Cord Mesenchymal Stem Cells Inhibiting Bovine Mammary Epithelial Cell Apoptosis via Insulin-Like Growth Factor-I-Mediated Janus Kinase/Signal Transducer and Activator of Transcription Signaling Pathway

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**Abstract:** This study aimed to investigate whether the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway is involved in the regulation of umbilical cord mesenchymal stem cells (UC-MSCs) inhibiting bovine mammary epithelial cell (BMEC) apoptosis through insulin-like growth factor-I (IGF-I). UC-MSCs and BMECs were co-cultured using a TranswellTM double-chamber system, with BMECs cultured alone serving as the control. Cells were treated with the IGF-I receptor (IGF-IR) inhibitor AG1024 and the JAK inhibitor AG490. After 24 hours, real-time quantitative PCR (RT-qPCR) was used to detect the relative expression levels of B-cell lymphoma/leukemia-2 (Bcl-2), Bcl-associated x protein (Bax), and cysteine aspartic acid-specific protease (Caspase-3) genes, while flow cytometry was employed to assess apoptosis. The results demonstrated that the apoptosis rate of BMECs in the UC-MSC and BMEC co-culture group was significantly lower than in all other groups ($P<0.01$). The relative expression of the Bcl-2 gene in the co-culture group was significantly upregulated compared to the BMEC-only group ($P<0.01$), whereas the expression levels of Caspase-3 and Bax genes were significantly or extremely significantly downregulated ($P<0.05$ or $P<0.01$). Treatment with AG1024 and/or AG490 increased the apoptosis rate of both singly cultured BMECs and BMECs co-cultured with UC-MSCs, upregulated the expression of Bax and Caspase-3 genes, and downregulated Bcl-2 expression, with all differences being statistically significant ($P<0.05$ or $P<0.01$). These findings indicate that UC-MSCs can regulate the expression of apoptosis-related genes in BMECs and reduce their apoptosis rate through IGF-I-mediated JAK/STAT signaling pathway activation.

**Keywords:** umbilical cord mesenchymal stem cells; mammary epithelial cells; co-culture; IGF-I; JAK/STAT; apoptosis

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Umbilical cord mesenchymal stem cells (UC-MSCs) are important members of the mesenchymal stem cell (MSC) family that not only maintain the multipotent differentiation capacity of MSCs but also possess robust autocrine and paracrine functions. UC-MSCs secrete various cytokines including vascular endothelial growth factor (VEGF), insulin-like growth factor-I (IGF-I), and epidermal growth factor (EGF) [1], among which IGF-I plays a crucial role in regulating mammary gland development and lactation function, serving a key function in preventing mammary epithelial cell apoptosis [2]. Currently, the recognized anti-apoptotic effects of IGF-I are primarily mediated through the phosphatidylinositol-3-kinase (PI3K) [3] and mitogen-activated protein kinase (MAPK) signaling pathways [4], with their mechanisms well established. Previous studies have demonstrated that in vitro-cultured bovine mammary epithelial cells (BMECs), the PI3K regulatory subunit can induce the transcription factor STAT to regulate apoptosis [5]. Furthermore, the IGF-I receptor (IGF-IR) can activate multiple intracellular subunits including PI3K, MAPK, and Janus kinase (JAK) [6], triggering cascade signal transduction reactions that inhibit apoptosis through relevant signaling pathways. All three signaling pathways are essential for cell survival, and when any one is blocked, IGF-I cannot counteract apoptosis induced by various factors. Consequently, as research continues to explore the regulatory pathways of BMEC apoptosis, the JAK/STAT signaling pathway has emerged as a major focus in mammary gland research in recent years, playing important roles in regulating mammary tissue proliferation, metabolism, apoptosis, and lactation [7]. However, reports on IGF-I-mediated JAK/STAT signaling pathways in BMEC anti-apoptotic processes are lacking. Our previous work successfully established an optimal serum-free co-culture system for UC-MSCs and BMECs and found that co-culture attenuated apoptosis in the system [8], though the specific mechanism remained unclear. Therefore, this study simulated endogenous cytokine regulation of cell apoptosis in vivo by co-culturing UC-MSCs and BMECs using a TranswellTM chamber. Employing IGF-IR inhibitors and JAK/STAT signaling blockers, we investigated whether JAK/STAT participates in the regulatory effects of UC-MSCs on BMEC apoptosis and related gene expression through IGF-I, thereby revealing the impact of UC-MSC co-culture on BMEC apoptosis and its potential mechanisms.

## Materials and Methods

### Experimental Design and Grouping

Based on our previous study [8], we established a bilayer co-culture system using TranswellTM chambers (0.4 μm pore size) under optimal conditions for UC-MSC and BMEC co-culture. BMECs were seeded in the upper chamber at 1$×10^{5}$ cells/mL (1 mL per well), while UC-MSCs were seeded in the lower chamber at 1$×10^{5}$ cells/mL (2 mL per well), with BMECs cultured alone serving as the control. After 48 hours, cells were treated with the IGF-IR inhibitor AG1024 (10 μmol/L) and/or the JAK inhibitor AG490 (50 μmol/L) and incubated at 37°C with 5% $CO_2$ in serum-free medium (SFM) for 24 hours. The supernatant was then collected and cells were harvested by trypsinization and stored at -20°C for further analysis. The experiment comprised eight groups: (1) BMECs (BMECs cultured alone), (2) BMECs/UC-MSCs (co-cultured BMECs and UC-MSCs), (3) BMECs+AG1024 (AG1024-treated singly cultured BMECs), (4) BMECs/UC-MSCs+AG1024 (AG1024-treated BMECs co-cultured with UC-MSCs), (5) BMECs+AG490 (AG490-treated singly cultured BMECs), (6) BMECs/UC-MSCs+AG490 (AG490-treated BMECs co-cultured with UC-MSCs), (7) BMECs+AG490+AG1024 (BMECs treated with both AG1024 and AG490), and (8) BMECs/UC-MSCs+AG490+AG1024 (BMECs co-cultured with UC-MSCs and treated with both inhibitors). Each group was established in triplicate.

### Cell Sources

UC-MSCs were Holstein cow UC-MSCs previously isolated, cultured, and identified in our laboratory [8]. BMECs were purchased from Guangzhou Jennio Biotech Co., Ltd.

### Instruments and Reagents

The following instruments and reagents were used: inverted microscope (Motic-AE31), $CO_2$ incubator (HF151UV), TranswellTM chambers (Corning), AG1024 (Alexis), AG490 (Sigma), real-time quantitative PCR (RT-qPCR) kit (Shanghai Nuolun Biomedical Technology Co., Ltd.), Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Shanghai Bogu Biological Technology Co., Ltd.), and flow cytometer (Becton Dickinson Facscalibur).

### Measurements and Methods

Apoptosis was detected in each group using a flow cytometer with an Annexin V-FITC/PI detection kit. The relative expression levels of apoptosis-related genes including cysteine aspartic acid-specific protease (Caspase-3), B-cell lymphoma/leukemia-2 (Bcl-2), and Bcl-associated x protein (Bax) were measured by RT-qPCR, with $\beta$-actin serving as the internal reference gene. Gene sequences were obtained from GenBank, and primers were designed using

Primer 5.0 software and synthesized by Shanghai Bogu Biological Technology Co., Ltd. (see ). Apoptosis detection was performed using the Annexin V-FITC/PI double-staining method according to the manufacturer' s instructions, with flow cytometry used for quantification. The apoptosis rate was calculated as: Apoptosis rate (%) = [(apoptotic cells + secondary necrotic cells)/total cell count] × 100.

### Statistical Analysis

Data were analyzed using SPSS 18.0 software and expressed as mean ± standard deviation (mean ± SD). Differences were considered statistically significant at $P<0.05$.

## Results

### Effects of AG490 on Apoptosis Rate Under AG1024 Intervention

Representative apoptosis scatter plots for each group are shown in [Figure 1: see original paper], where the x-axis represents Annexin V-FITC and the y-axis represents PI. The Q1 quadrant contains mechanically damaged cells, Q2 represents late apoptotic or secondary necrotic cells, Q3 represents normal viable cells, and Q4 represents early apoptotic cells. As illustrated in [Figure 2: see original paper], the apoptosis rate of BMECs in the BMECs/UC-MSCs co-culture group was significantly lower than in all other groups ($P<0.01$). AG1024 treatment significantly increased the apoptosis rate of both singly cultured BMECs and BMECs co-cultured with UC-MSCs ($P<0.01$). AG490 treatment significantly elevated the apoptosis rate of singly cultured BMECs ($P<0.05$) and extremely significantly increased the apoptosis rate of BMECs co-cultured with UC-MSCs ($P<0.01$). Combined treatment with AG490 and AG1024 extremely significantly increased the apoptosis rate of both singly cultured BMECs and BMECs co-cultured with UC-MSCs ($P<0.01$).

### Effects of AG490 on Apoptosis-Related Gene Expression Under AG1024 Intervention

RT-qPCR results are presented in . The BMECs/UC-MSCs co-culture group exhibited significantly or extremely significantly lower relative expression levels of Caspase-3 and Bax genes compared to other groups ($P<0.05$ or $P<0.01$), while Bcl-2 expression was extremely significantly higher ($P<0.01$). Treatment with AG1024 or AG490 significantly or extremely significantly upregulated Caspase-3 and Bax expression in both singly cultured BMECs and BMECs co-cultured with UC-MSCs ($P<0.05$). Combined treatment with AG1024 and AG490 extremely significantly upregulated Caspase-3 and Bax expression in both culture conditions ($P<0.01$). Except for a slight but non-significant downregulation of Bax in AG1024-treated singly cultured BMECs ($P>0.05$), AG1024 treatment of co-cultured BMECs or AG490 treatment of both singly cultured and co-cultured BMECs significantly or extremely significantly downregulated Bcl-2 expression

($P<0.05$ or $P<0.01$). Combined AG1024 and AG490 treatment also significantly or extremely significantly downregulated Bcl-2 expression in both singly cultured and co-cultured BMECs ($P<0.05$ or $P<0.01$).

## Discussion

### IGF-I Involvement in UC-MSC-Mediated Inhibition of BMEC Apoptosis

Apoptosis is a genetically controlled form of programmed cell death. The Bcl-2 family members Bcl-2 and Bax represent the most important functionally antagonistic pair of regulatory genes known in apoptosis [9], while Caspase-3 serves as the primary executioner of apoptosis [10]. Our results demonstrated that compared to singly cultured BMECs, co-culture with UC-MSCs significantly reduced apoptosis rate and downregulated the expression of pro-apoptotic genes Caspase-3 and Bax while upregulating the anti-apoptotic gene Bcl-2. These findings indicate that UC-MSC co-culture can markedly suppress pro-apoptotic gene expression and enhance anti-apoptotic gene expression in BMECs, thereby inhibiting apoptosis, which aligns with the apoptosis rate data shown in [Figure 2: see original paper]. Our previous work confirmed that under serum-free culture conditions, UC-MSC and BMEC co-culture significantly increased IGF-I and IGF-IR levels, with IGF-I primarily localized in UC-MSCs. Since IGF-I must bind to IGF-IR to activate downstream signaling pathways and exert its biological functions [8], we investigated this mechanism further. Following 24-hour AG1024 treatment, BMECs showed significantly upregulated Caspase-3 and Bax expression, downregulated Bcl-2 expression, and a markedly increased apoptosis rate. However, co-culture with UC-MSCs reversed these effects, reducing pro-apoptotic gene expression and apoptosis rate. Numerous studies have confirmed that UC-MSCs can secrete various growth-promoting factors including IGF-I [11-12]. Research has established the critical role of IGF-I in regulating BMEC apoptosis [13-14], with reports indicating that IGF-I slows BMEC apoptosis during the declining phase of lactation in mice [15]. Gao et al. [16] also demonstrated that IGF-I significantly inhibits BMEC apoptosis. Our findings are consistent with these previous studies, with the distinction that our experiments employed serum-free medium to exclude exogenous factor interference, while the TranswellTM system accurately reflected the influence of UC-MSCs on BMECs. These results strongly suggest that UC-MSCs inhibit BMEC apoptosis through IGF-I secretion.

Wareski et al. [17] found that apoptosis-related factors including the apoptosis promoter Bax, apoptosis inhibitor Bcl-2, and apoptosis executor Caspase-3 are expressed throughout the lactation period, with upregulated Bax and Caspase-3 expression accompanying increased BMEC death and higher numbers of apoptotic cells during the dry period. Therefore, UC-MSCs can influence BMECs in the upper chamber through secreted IGF-I, which exerts endogenous protective mechanisms by downregulating pro-apoptotic genes Bax and Caspase-3 while upregulating the anti-apoptotic factor Bcl-2. This reduces damage from pro-

apoptotic factors and, in conjunction with anti-apoptotic factors, maintains or promotes BMEC growth, ultimately inhibiting apoptosis. Since BMECs cannot continuously secrete IGF-I and other growth factors themselves, in vitro co-culture of UC-MSCs with BMECs can substitute for exogenous IGF-I addition to suppress BMEC apoptosis, providing a novel approach for extending BMEC growth and proliferation-differentiation processes.

### Potential Pathways of UC-MSC-Mediated BMEC Apoptosis Inhibition via IGF-I

The initiation and progression of apoptosis is controlled by signal transduction. Apoptosis-inducing factors primarily enter cells through receptor-mediated mechanisms, transmit signals to central regulatory stages, activate corresponding apoptosis-related factors, and ultimately alter cell structure to cause death [18]. What, then, is the specific molecular mechanism by which IGF-I exerts its anti-apoptotic effects on BMECs? Previous reports have shown that IGF-I can activate PI3K/AKT and MAPK signaling pathways to promote proliferation and anti-apoptotic effects in various cell types [19], while the JAK/STAT signaling pathway participates in cell proliferation, differentiation, functional activity, and apoptosis, representing an important cytokine signal transduction pathway [20]. However, whether endogenous IGF-I secreted by UC-MSCs can activate the JAK/STAT pathway to exert anti-apoptotic effects on BMECs has not been reported. Therefore, this study built upon our previous work to explore this question further.

Our results showed that AG490 treatment significantly increased the apoptosis rate of singly cultured BMECs and extremely significantly elevated the apoptosis rate of BMECs co-cultured with UC-MSCs. As a JAK kinase inhibitor, AG490 effectively blocks JAK2 and JAK3 activation, thereby inhibiting JAK/STAT signal transduction [21]. This indicates that the JAK/STAT signaling pathway participates in BMEC apoptosis regulation in our experimental system. However, when BMECs were co-cultured with UC-MSCs in the presence of AG490, the apoptosis rate was significantly reduced, suggesting that UC-MSCs can effectively reactivate the blocked JAK/STAT pathway and decrease BMEC apoptosis. As shown in [Figure 2: see original paper], combined treatment with AG490 and AG1024 extremely significantly increased the apoptosis rate compared to untreated BMECs, and both individual treatments also significantly elevated apoptosis rates. Co-culture with UC-MSCs further increased apoptosis rates, indicating that inhibited IGF-I disrupts JAK/STAT signaling and promotes BMEC apoptosis, revealing that IGF-I mediates JAK/STAT pathway involvement in BMEC apoptosis regulation. The results in align with the apoptosis data: when IGF-I and JAK/STAT were blocked, Caspase-3 and Bax expression was upregulated while Bcl-2 expression was downregulated, decreasing the Bcl-2/Bax ratio and inducing BMEC apoptosis. Studies have shown that IGF-I deficiency triggers BMEC apoptosis and that STAT3 phosphorylation accompanies mammary gland involution [22]. During mammary gland

regression, Bax and Caspase-3 expression increases [23]. Xiong et al. [24] demonstrated that AG490 blocks the JAK/STAT3 pathway and downregulates Bcl-2 expression. Other reports indicate that leptin and prolactin may regulate mammary gland development by activating the JAK/STAT5 pathway [25-26], and blocking JAK/STAT5 induces BMEC apoptosis and impairs lactation function [27]. These findings suggest that UC-MSCs may secrete IGF-I that binds to its receptor IGF-IR, recruiting and phosphorylating JAK, which subsequently phosphorylates and activates STAT to trigger the JAK/STAT pathway. This activation upregulates anti-apoptotic genes and downregulates pro-apoptotic genes, thereby inhibiting BMEC apoptosis. However, whether IGF-I acts in concert with other hormones or growth factors or involves other pathways and synergistic effects remains unclear.

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*Note: Figure translations are in progress. See original paper for figures.*