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Chapter 1

Zuojin Pill, a Classical Traditional Chinese Medicine Formula, Inhibits the Tafs-Induced Phenotype Transition and Migration in Normal Colonic Epithelial Cells Through TGF-B/Smads Signaling

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Abstract

Application of Traditional Chinese Medicine in clinic can go back more than 2,000 years. Recently, Chinese Medicine is reported to regulate remolding of tumor microenvironment (TME). However, the definite mechanisms are still unclear. Increasing studies have indicated that tumor-associated fibroblasts (TAFs) are crucial player in the TME remolding through inducing transdifferentiation of stromal cells. In our study, conditioned medium (CM) from TAF-like CCD-18Co cells stimulated the epithelial-mesenchymal transition (EMT) changes and enhanced the migration in colon epithelial cell line HCoEpiC, and these changes were suppressed by LY364947, a TGFβ receptor kinase I (TβRI) inhibitor. Consistently, Zuojin Pill-containing serum (ZJPCS), a prescription of Traditional Chinese Medicine, also abolished the EMT and migration in the CCD-18Co CM-induced HCoEpiCs. Importantly, ZJPCS also de-

creased the expressions of p-Smad2, p-Smad3 and Smad4 or the ratios of p-Smad2/Smad2 and p-Smad3/Smad3. In conclusion, Our findings indicate that zuojin Pill can mediate the TAF-induced differentiation state of colon epithelial cells, and this process is likely associated with modulation of TGF-β/Smads pathway.

Key words: Traditional Chinese Medicine; Zuojin Pill; Tumor microenvironment; Epithelial-mesenchymal transition; Transforming growth factor-β; Colon

1. Introduction

Cumulative evidence suggests that cancer-associated fibroblasts (CAFs) or TAFs, main manufacturer of ECM and multiple soluble factors, are the dominant contributor to cancer [1-2]. Although CAFs/TAFs are often deemed as the interaction factor with tumor cells, these cells also communicate with other stromal cells including epithelial cells [3]. Current researches think that activation of stromal cells in colon tissues is the result of local microenvironment or niche changes such as chronic colitis or in response to soluble factors including transforming growth factor beta (TGF-β) [4]. When activated or induced, these stromal components including epithelial cells present myofibroblast-like phenotypes, resulting from over-expressed α-smooth muscle actin (α-SMA) and losses of epithelial markers including E-cadherin and cell polarity. In such a context, these epithelial cells changed in cell phenotypes, commonly named as epithelial-mesenchymal transition (EMT), are considered to be the important origination of TAF-like stromal cells [5-6]. Therefore, transdifferentiation occurs between stromal cells in microenvironment [7]. On the other hand, TAFs/CAFs secrete a great many active cytokines such as collagen and TGF-β1 [8]. The increased deposition of such molecules inevitably affects the states of differentiation in colon epithelium. Many studies have shown involvement of TGF-β signaling in the induction and maintenance of EMT [9]. Therefore, orchestration of the TGF-β pathway activated by TAFs is likely to be a potential strategy in the regulation of cellular differentiation.

Zuojin Pill (ZJP), a Traditional Chinese Medicine formula, consists of *Coptis chinensis Franch*. and *Evodia rutaecarpa (Juss.) Benth*. in a ratio of 6:1 (w/w) [10] . Originally, ZJP was reported to treat multiple gastrointestinal dysfunction in the 15th century [10]. Previous study had found that ZJP also repressed the expression of inflammatory mediators including iNOS, COX-2, IL-6, IL-1β, and TNF-α [11]. In the context of cancer, ZJP is demonstrated to have *in vitro* pro-apoptosis effects on human gastric cancer cell line SGC-7901 [12]. Hence, we hypothesize whether the ZJP has modulatory influence on the phenotypes of stromal cells. Interestingly, ZJP-containing serum inhibited the TAF-induced EMT in colon epithelial HCoEpiC cells, which is likely associated with the mediation of TGF-β/Smads signaling. These data maybe provide a novel view for understanding the regulation of Traditional Chinese Medicine in the transdifferentiation of stromal cells.

2. *Zuojin* Pill (ZJP) inhibits the TAF-like CCD-18Co cell-induced EMT in colon epithelial cell HCoEpiCs

Tumor stroma is consist of a complex mixture of inflammatory cells, extracellular matrix protein, and tissue cells such as fibroblasts and endothelial cells. Normal fibroblasts work as a sentinel cell to maintain colon epithelium homeostasis and to prevent initiation of tumorigenesis. However, fibroblasts in the stroma, which are named as myofibroblasts or cancer-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs) [13], are involved in remodeling of microenvironment, leading to initiation and progress of tumor cells through ECM deposition. These TAFs/CAFs can regulate secretion of many paracrine factors [13-14], causing the modulation of transdifferentiation of tumor cells or other stroma cells [15].

Because CCD18-Co can also remodel the stromal microenvironment [16]. Considering the functions of TAFs in tumor microenvirnment remodeling [17], we selected a myofibroblast cell line CCD-18Co [18]. First, preparations of CCD18-Co-derived conditioned medium (CM) was established. A colon epithelial cell line HCoEpiC was then treated with the 25% CM (diluted with serum-free RPMI 1640) for 24 h. The results were shown in **Figure 1**. CCD-18Co CM stimulated the information of EMT-like changes in HCoEpiCs, comparing to the control. The HCoEpiCs presented a spindle-like cellular morphology, with prolonged cellular longitudinal axis. These changes were greatly repressed by LY364947, a TGF-β receptor kinase I inhibitor.

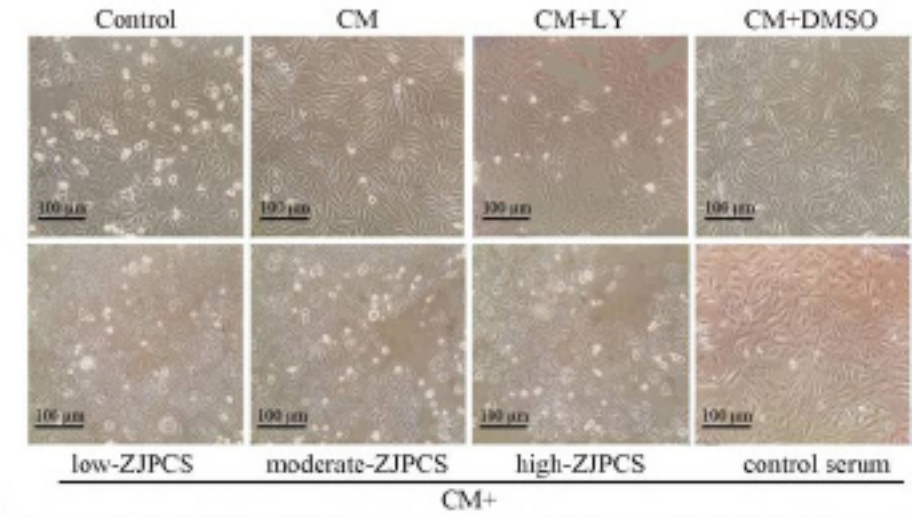

Figure 1: Morphological observation of HCoEpiC treated with different conditions for 24 h. The cells weretreated with 25% CM or 18 nMol of LY364947 (LY) or zuojin Pill-containing serum (ZJPCS) for 24 h. ZJPCS with different doses is treated by gavage of Zuojin Pill with different concentration according to 0.96, 1.91 and 3.82 g-1•kg-1•d, respectively (the same below). All morphological observations were acquired using a phase contrast microscope (Olympus). Bars = 100 μm for × 200 magnifications.

In addition, we prepared ZJP-containing serum (ZJPCS). The preparation of ZJPCS is described below. Male Sprague-Dawley (SD) rats (weighing 220-250 g) were purchased from Guangzhou Jennio Biotech Co., Ltd (Guangzhou, China). Components of ZJP, *Coptis chinensis Franch*. and *Evodia rutaecarpa (Juss.) Benth*., were obtained form Affiliated Bao'an Hospital of Traditional Chinese Medicine of Shenzhen (Shenzhen, China), and the weight was

12 g and 2 g, respectively. These rats were treated with gavage of ZJP for 1 week, according to 0.96, 1.91 and 3.82 $g^{-1} \cdot kg^{-1} \cdot d$. The control was treated with normal saline (NS). These rats were killed with anesthesia by intraperitoneal injection of chloral hydrate. Then the serum was respectively obtained, centrifugalized at 5000 rpm, and inactivated at 56 ℃. These serums were named as low-ZJPCS, moderate-ZJPCS, high-ZJPCS, and control serum, respectively. Finally, all the concentration of ZJPCS in the HCoEpiCs medium added with CM was 10 %. We observed that the effect of LY364947 (18 nMol) was similar to that of ZJPCS. We found that ZJPCS with different concentration significantly suppressed the CCD-18Co-triggered EMT changes in HCoEpiCs, comparing with the control serum. These data suggest that ZJPCS can inhibit the *in vitro* dedifferentiation of colon epithelial cells induced by TAFs.

3. ZJP Represses the Migration of Hcoepics Promoted By CCD-18Co Cells

Although migration is involved in embryonic development [19], promotion of invasion and metastasis of cancer is highly associated with EMT [20]. Several studies have found that EMT is responsible for enhanced cell motility [21]. As our findings shown above, CCD-18Co induced the EMT-like transformation in HCoEpiCs, suggesting probable promotion of migration. With the help of wound-healing test (Detecting points were 0 h, 6 h, 12 h, 18 h, 24 h, respectively), we found that, after treatment 12 h, CCD-18Co-derived CM obviously promoted the migration of HCoEpiCs, which was largely repressed by LY364947 (**Figure 2**).

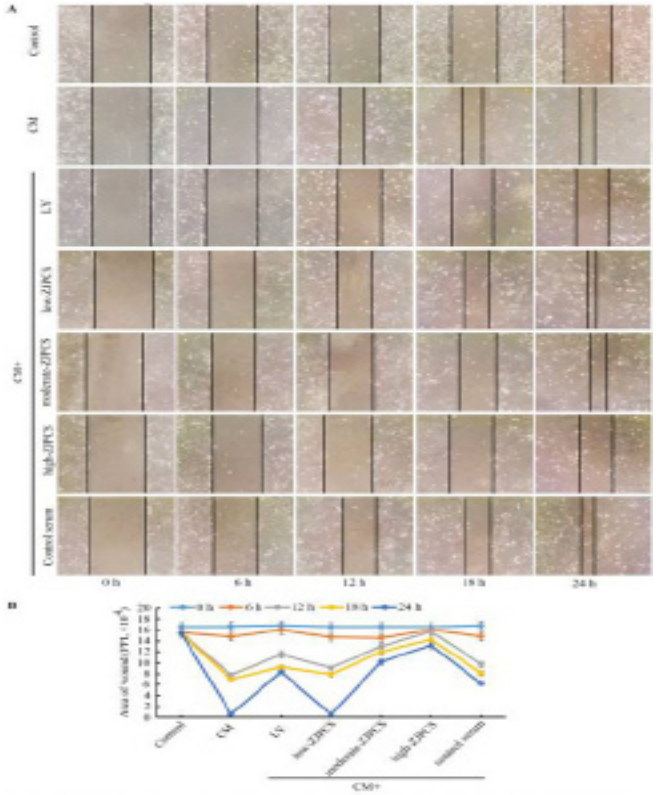

Figure 2: Detection of HCoEpiCs motility using wound-healing assay. **A** Cultured HCoEpiC were analysed by phase contrast microscopy (10×10). **B** Analysis of the wound area by Image J, with the area of the wound presented as pixels per inch (PPI).

Consistently, ZJPCS also inhibited the migration, comparing to the control serum, and this effect was dose-dependent. These results imply that both of ZJPCS and TGF-β receptor inhibitor can inhibit the CCD-18Co-stimulated migration, and the effect of ZJPCS is concentration-dependent.

Although the mechanisms of anti-migration are not reported, some studies have demonstrated that ZJP had the regulatory effect on several inflammatory cytokines in gastrointestinal tract by mediating the NF-KB signaling pathway [22]. In a study about endothelial progenitor cell, mechanism of TNF-α induced migration was found to be associated with up-regulation of CADM1 promoted by NF-kB [23]. In this scenario, we consider that regulation of signaling pathways such as NF-kB or cytokines may be one of the important mechanisms of ZJP against migration in TAF-induced HCoEpiCs.

4. TGF-B/Smads Signaling May be Involved in the CCD-18Co CM-Triggered EMT in Hcoepics and the Suppression Of ZJP

As a secretory pleiotropic factor, transforming growth factor β (TGF-β) play crucial functions in embryogenesis and adult tissue homeostasis [24]. Evidence has presented that TGF-β can orchestrate cell proliferation, differentiation and migration. During the signal transduction, type I and type II receptors (TβRI and TβRII) come into being a heteromeric complex, leading to activation of Smads family. As key signal transducers, Smads complexes including phosphorylated Smad2/3 (p-Smad2/3) and Smad4 are translocated into nucleus to induce multiple transcription of genes [24-25]. A good many studies have revealed that TGF-β signaling takes part in activation and maintenance of EMT [26-27]. For example, silence of Smad4 by miR-146a resulted in the attenuation the EMT in hepatocytes [26]. This suggests that TGF-β/Smads signaling is an important promoter for EMT.

We used western blotting to detect the Smads expressions. As demonstrated in Figure **3**, CCD-18Co significantly promoted the expressions of Smad2/3 and Smad4, as well as p-Smad2 and p-Smad3. Treatment of LY364947 obviously repressed their levels. In addition, CCD-18Co CM also stimulated the ratio of p-Smad2/Smad2 or p-Smad3/Smad3. These findings reveal high-activity of TGF-β/Smads signaling during the CCD-18Co-induced EMT. Although CCD-18Co may secrete multiple active molecules participating in the process of EMT, such as epidermal growth factor, TGF-β, hepatic growth factor and fibroblast growth factor [28], TGF-β signaling as a key mediator plays crucial functions owing to the contribution of 18 nMol LY364947. A new study indicated that α-SMA-positive CAFs produced TGF-β, which was in relation to dedifferentiation of cells, mediating the EMT in biliary tract cancer cells [29]. A previous study revealed that CM of CAFs promoted the expression of N-cadherin and vimentin, and down-regulated E-cadherin expression, with enhanced invasion and metastasis in endometrial cancer cells [28]. In fact, multiple studies have demonstrated that TGF-β treatment

induced the formation of mesenchymal phenotype in epithelial or tumoral cells [28, 30-32]. For example, TGF-β1 significantly up-regulated the p-Smad3 level during the induction of EMT in renal tubular epithelial cells (HK-2) [30]. Repressing TGF-β/Smad signaling by Ginsenoside Rb2 can abolish the EMT of colorectal cancer [33]. Consequently, TGF-β/Smad signaling may be a potential target to prevent the occurrence of EMT.

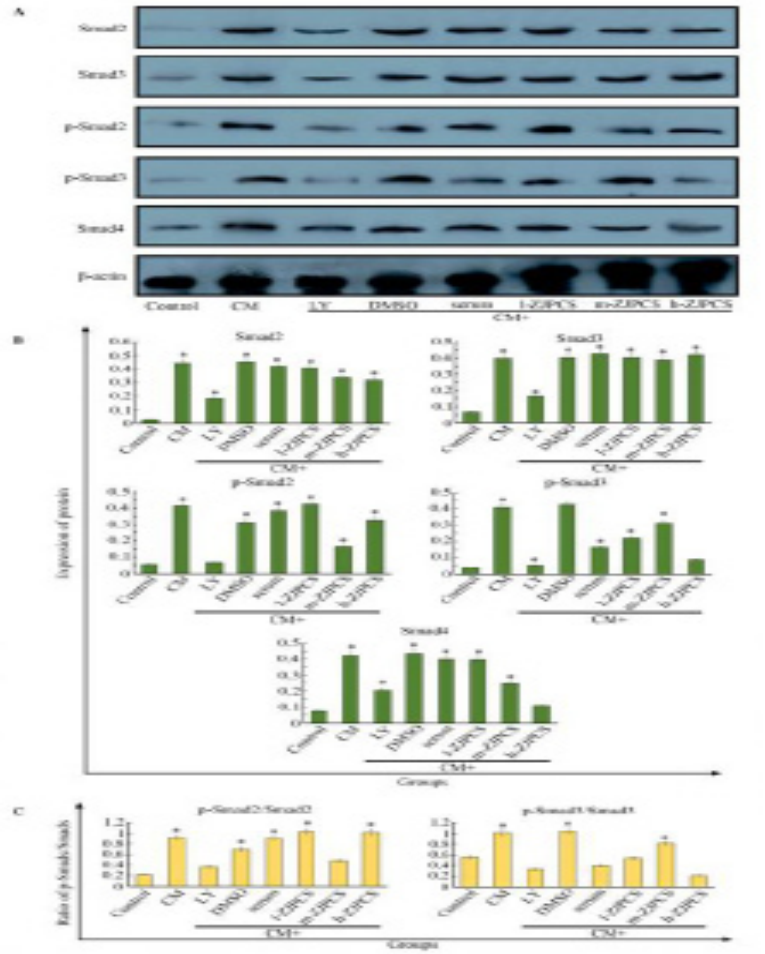

Figure 3: Detection of expression of Smads in TGF-β signaling by western blotting. A Protein bands of western blot analysis in the cells treated by different conditions. B Analysis of gray value for the protein bands. **C** The ratios of p-Smad2/Smad2 and p-Smad3/Smad3. l-ZJPCS: low-ZJPCS; m-ZJPCS: moderate-ZJPCS; h-ZJPCS: high-ZJPCS. The error bar represents the SD (n = 3). **P* <0.05 versus Control.

Zuojin Pill (ZJP), officially listed in the Chinese Pharmacopoeia as a prescription, has attracted more attention from the 15th century to the present. Because ZJP can effectively treat multiple conditions including gastric ulcer, pyloric obstruction and other disorders [34]. In addition to the modulation of inflammatory cytokines and NF-KB pathway mentioned above, ZJP was found for the first time that it also regulates the TGF-β/Smad signaling. As shown in our study, although ZJPCS did not effect the expression of Smad2/3, it obviously decreased the expression of p-Smad2/3 and the ratio of p-Smad2/Smad2 or p-Smad3/Smad3. However, this effect was dose-independent. We think that the dose-independent effect is likely to be associated with the instability or absorptivity of ZJP in serum. Alternatively, the targets of ZJPCS or the effect on the expression of Smads are different in different concentration. On the other hand, we observed that ZJPCS inhibited the expression of Smad4 in a concentration-dependent manner. This suggests that ZJPCS exerts the anti-EMT and anti-migration effects

through modulation of Smad4 expression, because Smad4 is required for EMT [35].

5. Prospects and Conclusions

So far, increasingly evidence has revealed that Chinese Medicine has greatly regulatory effect on biological behaviour of tumor cells through orchestrating various signaling pathways, including proliferation, cell death, differentiation, metastasis and angiogenesis [36]. Interestingly, some Chinese Medicines are reported to have the effect of mediation of tumor microenvironment. ZJP, one of the Chinese Medicine formulas, possesses multiple pharmacologic effects. Thus, researches about its mechanisms have recently became hot fields. Combining with previous study, we have found that ZJP can also regulate the transdifferentiation of stromal cells induced by TAFs, and these effects may be the results of modulation of TGF-β/Smads signaling. Our study suggests that ZJP can modulate the incongruous microenvironment around tumor cells.

6. Acknowledgments

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