



Impact and significance of EGCG on Smad, ERK, and β -catenin pathways in transdifferentiation of renal tubular epithelial cells

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Genet. Mol. Res. 14 (1): 2551-2560 (2015)

Received March 6, 2014

Accepted July 2, 2014

Published March 30, 2015

DOI <http://dx.doi.org/10.4238/2015.March.30.14>

ABSTRACT. We investigated the impact and signal transduction mechanisms of epigallocatechin-3-gallate (EGCG) on transdifferentiation of renal tubular epithelial cells. Rat renal tubular epithelial cells (NRK-52E) were randomly divided into a normal control group, transforming growth factor (TGF)- β 1-induced group (10 ng/mL), and intervention groups with 200 μ g/L EGCG + 10 ng/mL TGF- β 1 and 400 μ g/L EGCG + 10 ng/mL TGF- β 1. Tested cells were collected after 48 h. Levels of α -smooth muscle actin (α -SMA) and cytokeratin-18 were detected using immunohistochemical methods. Western blotting was used to detect cytoplasmic Pi-extracellular receptor kinase 1/2 (ERK1/2), Pi-Smad3 protein, and nuclear β -catenin protein. mRNA expression of *ERK2*, *Smad3*, and *β -catenin* was measured by reverse transcription-polymerase chain reaction. After induction by TGF- β 1, cytokeratin-18 expression in the renal tubular epithelial cells decreased and α -SMA

expression appeared. mRNA expression of cytoplasmic *Pi-Smad3* and *Pi-ERK1/2*, *Smad3*, *ERK2*, and β -catenin protein expression increased, while *β -catenin* mRNA decreased. These changes were reduced after intervention by EGCG. EGCG may be helpful for maintaining the renal tubular epithelial cell phenotype and reducing the degree of TGF- β 1-induced cell transdifferentiation, which may be related to the signal transduction pathway of ERK, *Smad3*, and β -catenin.

Key words: α -Smooth muscle actin; Cytokeratin-18; Epithelial cells; Epigallocatechin-3-gallate; Transforming growth factor

INTRODUCTION

The epithelial mesenchymal transdifferentiation (EMT) of tubular epithelial cells (TECs) is an important component of renal interstitial fibrosis. EMT is a process during which fully differentiated epithelial cells undergo a phenotypic change to fibroblasts through diverse intracellular signaling pathways (Kim et al., 2013a). Approximately 36% of the renal tubule cells in renal interstitial fibroblasts are derived from local EMT when renal interstitial fibrosis occurs (Liu, 2010). Cytokeratin-18 (CK-18) is a marker for the occurrence of transdifferentiation of epithelial cells, while α -smooth muscle actin (α -SMA) is a marker for the appearance of mesenchymal cells. As a recognized pro-fibrotic factor, transforming growth factor- β 1 (TGF- β 1) can stimulate the transformation of mesenchymal cells to epithelial cells (Rastaldi, 2006; García-Sánchez et al., 2010; Zeisberg and Duffield, 2010). EMT in the kidneys is driven by regulation of the expression of fibrogenic cytokines such as TGF- β (Liu et al., 2012). The development of effective drugs for preventing and treating fibrosis is difficult and has attracted attention in recent studies.

Epigallocatechin-3-gallate (EGCG) was found to have a role in anti-hepatic fibrosis (Nakamuta et al., 2005; Yumei et al., 2006; Zhen et al., 2007) as well as in reducing oxidative stress and inflammatory factors, and these effects were associated with pathways such as TGF/Smad and PI3 K/Akt/FoxO1 (Xiao et al., 2013). Whether or not EGCG inhibits the transdifferentiation of renal tubular epithelial cells and the mechanism of this process have not been reported.

In this study, TGF- β 1 was used to induce the transdifferentiation of rat renal tubular epithelial cell (RTEC) line NRK cells. EGCG was used to intervene with the transdifferentiation process of NRK cells. The role of EGCG on TEC transdifferentiation and the signal transduction mechanisms were evaluated by detecting changes in the expression of CK-18, α -SMA and phosphorylated extracellular receptor kinase 1/2 (*Pi-ERK1/2*), phosphorylated *Smad3* (*Pi-Smad3*), and nuclear β -catenin in NRK cells.

MATERIAL AND METHODS

Cell culture and grouping

Rat RTECs (NRK-52E) were purchased from the Type Culture Collection cell bank of the Sciences Committee of Chinese Academy, established by the American Type Culture Collection. L-EGCG was purchased from Sigma (St. Louis, MO, USA) and TGF- β 1 was purchased from Peprotech Company (Rocky Hill, NJ, USA). NRK-52E rat RTECs were routinely

cultured, synchronously treated with serum-free Dulbecco's modified Eagle's medium for 24 h, and then the cultured cells were randomly divided into 4 groups: the normal control group (taking the medium culture solution as a blank control), the TGF- β 1-induced group (10 ng/mL TGF- β 1 in the culture medium), the 200 μ g/L EGCG intervention group (10 ng/mL TGF- β 1 and 200 μ g/L EGCG in culture medium), and the 400 μ g/L EGCG intervention group (10 ng/mL TGF- β 1 and 400 μ g/L EGCG in the culture medium). Cells in each group were collected for testing after treatment for 48 h.

Immunohistochemical determination of cells climbing slices

Paraformaldehyde (4%) was purchased from Sigma. α -SMA and CK-18 rabbit anti-rat antibodies were purchased from Santa Cruz Biotechnology Company (Santa Cruz, CA, USA). The SP immunohistochemical-staining kit and DAB reagent were purchased from Beijing Zhongshan Jinqiao Biotechnology (Beijing, China). Cell climbing slices were washed with phosphate-buffered saline, and then fixed in 4% paraformaldehyde and stored at -20°C. An immunohistochemical method was used to detect α -SMA and CK-18. Eight fields of view of each set of pictures were selected. The average optical density values were measured using an Olympus camera system and the MetaMorph/BX41 image data analysis system (Olympus Corporation, Tokyo, Japan). A larger mean optical density value indicated higher target protein content in the representative samples.

Western blotting analysis of cells

Differential centrifugation was used to extract nuclear proteins and cytoplasmic proteins. Pi-ERK1/2, Pi-Smad3, and β -catenin-specific antibodies (primary antibodies) were purchased from Cell Signaling Technology Company (Danvers, MA, USA). Equipment such as the Centrifuge 5417R type low-temperature high-speed centrifuge (Eppendorf, Hamburg, Germany), electrophoresis tank (Miniprotein Bio-Rad, Hercules, CA, USA), and transfer film equipment (Trans-blot Cell; Bio-Rad) were used.

Cytoplasmic levels of Pi-ERK1/2, Pi-Smad3, and nuclear β -catenin were analyzed. Absorbance scanning was analyzed by Flour Chem V 2.0 using the gel image analysis software (ProteinSimple, Santa Clara, CA, USA). β -actin was included on the same film as an internal reference, and protein content was expressed based on the absorbance ratio of the target protein and the internal reference. A larger ratio indicated higher protein content.

Relative mRNA expression of *ERK1/2*, *Smad3*, and *β -catenin* determined by real-time polymerase chain reaction (PCR)

Trizol total RNA extraction solution was purchased from Shanghai Sangon Biological Engineering Co., Ltd. (Shanghai, China). PrimeScript RT Reagent Kit and the real-time quantitative PCR (SYBRPremix Ex Taq) kit were purchased from TaKaRa (Shiga, Japan). The ABI7500 system was used for quantitative PCR (Invitrogen, Foster City, CA, USA). Total cellular RNA was extracted and reverse transcribed using synthetic primers to cDNA using real-time quantitative PCR; primer sequences are shown in Table 1. The SYBR Green I fluorescent dye-embedded method was used to prepare the standard curve of the target gene and housekeeping gene [glyceraldehyde 3-phosphate dehydrogenase (GAPDH)], which was used

to quantify the target gene and housekeeping gene in the samples. The relative expression of the target gene in each group was determined by comparison with the housekeeping gene.

Table 1. Primer sequence.

ERK2-F	5'-CGTTCAGATGTCGGTGTC-3'
ERK2-R	5'-AAAGGAGTCAAGAGTGGG-3'
Smad3-F	5'-GATGCTTCGTGACATGGAACCTA-3'
Smad3-R	5'-TTCCACGTTTAAATGCTGCTG-3'
β -catenin-F	5'-CCCCAAGCCTTAGTAAACA-3'
β -catenin-R	5'-GACAGACAGCACCTTCAGC-3'
GAPDH-F	5'-GCACCGTCAAGGCTGAGAAC-3'
GAPDH-R	5'-ATGGTGGTGAAGACGCCAGT-3'

Statistical analysis

Data were analyzed using the SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA). Measurement data are reported as means \pm standard deviation. One-way analysis of variance was used for normally distributed data that showed homogeneity of variance. Comparisons between the 2 groups were performed using the *q*-test. $P < 0.05$ indicated statistical significance.

RESULTS

Protein expression changes of CK-18 and α -SMA in NRK cells

CK-18 in NRK cells was highly expressed in the control group, while α -SMA was not expressed. After induction for 48 h, the expression of cytosolic CK-18 protein in NRK cells decreased and the expression of α -SMA protein increased. After treating NRK cells with TGF- β 1 combined with EGCG for 48 h, CK-18 protein expression in the cytoplasm clearly decreased, and α -SMA protein expression was lower than that in the TGF- β 1-induced group (Table 2 and Figure 1).

Table 2. Expression changes of CK-18 and α -SMA after EGCG intervention (average integrated optical density value; N = 8, means \pm SD).

Group	CK-18	α -SMA
Control group	0.671 \pm 0.024	0
TGF- β 1-induced group	0.225 \pm 0.056*	0.857 \pm 0.045
EGCG intervention group (400 μ g/L)	0.531 \pm 0.078 [#]	0.342 \pm 0.028*

*Indicates that $P < 0.05$ compared with the control group, [#]indicates that $P < 0.05$ compared with the TGF- β 1 group.

Pi-Smad3 protein and *Smad3* mRNA expression in the cytoplasm of NRK cells

After NRK cells were induced by TGF- β 1 for 48 h, Pi-Smad3 protein and *Smad3* mRNA expression significantly increased. After treatment of NRK cells with TGF- β 1 and EGCG, these changes were reduced. The effect of alleviating the proteins and mRNA expression by EGCG with a concentration of 400 μ g/L was better than that observed when using a concentration of 200 μ g/L (Table 3 and Figure 2).

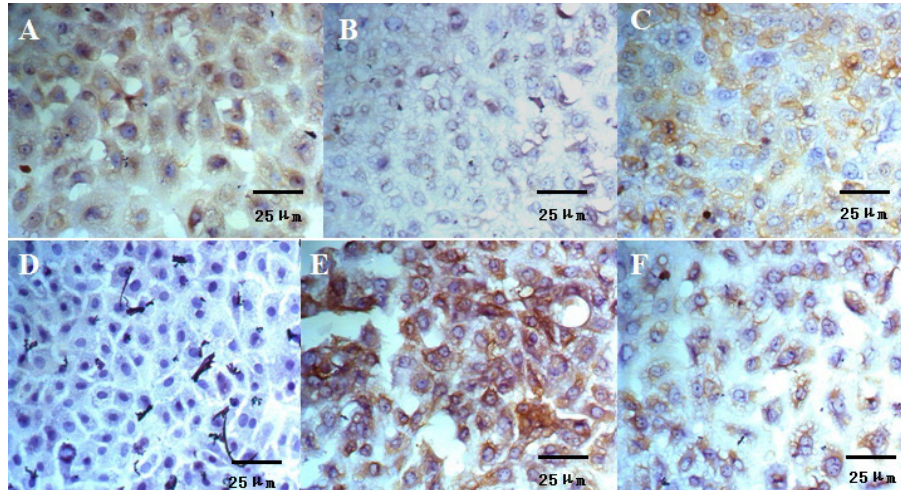


Figure 1. Immunohistochemical results of CK-18 and α -SMA protein in NRK cells. **A.** CK-18 in the control group; **B.** CK-18 in the TGF- β 1-induced group; **C.** CK-18 in the TGF- β 1+EGCG 400 μ g/L group; **D.** α -SMA in the control group, **E.** α -SMA in the TGF- β 1-induced group, **F.** α -SMA in the TGF- β 1+EGCG 400 μ g/L group.

Table 3. Expression changes of Pi-Smad3 and Smad3 mRNA in NRK cytoplasm before and after EGCG intervention (N = 8, means \pm SD).

Group	Pi-Smad3/actin	Smad3 mRNA/GAPDH
Control group	0.41 \pm 0.03	0.0031 \pm 0.0004
TGF- β 1-induced group	0.87 \pm 0.05*	0.0122 \pm 0.0008*
EGCG intervention group (200 μ g/L)	0.63 \pm 0.02 [#]	0.0072 \pm 0.0006 [#]
EGCG intervention group (400 μ g/L)	0.52 \pm 0.02 [#]	0.0052 \pm 0.0004 [#]

*Indicates that $P < 0.05$ compared with the control group, [#]indicates that $P < 0.05$ compared with the TGF- β 1 group.

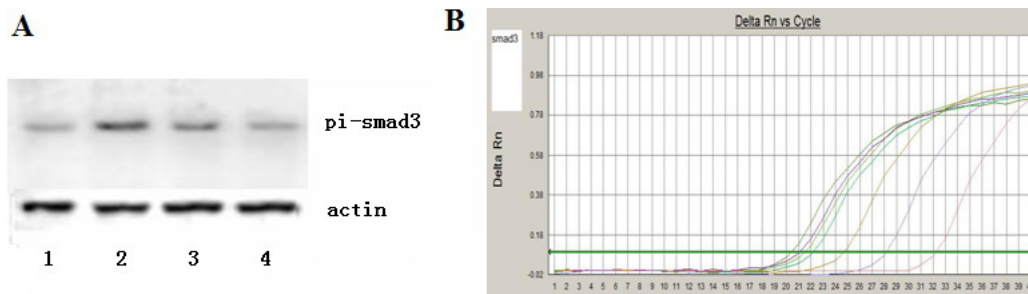


Figure 2. Western blot results of Pi-Smad3 (**A**) protein and RT-PCR results of Smad3 mRNA expression (**B**) in the NRK cytoplasm. *Lane 1* = the normal control group; *lane 2* = the TGF- β 1-induced group; *lane 3* = the TGF- β 1+EGCG 200 μ g/L group; *lane 4* = the TGF- β 1+EGCG 400 μ g/L group.

Pi-ERK1/2 protein and *ERK2* mRNA expression in the cytoplasm of NRK cells

After NRK cells were induced with TGF- β 1 for 48 h, Pi-ERK1/2 protein and *ERK2*

mRNA expression were significantly increased. After treatment of NRK cells with TGF- β 1 and EGCG, these changes were reduced. The effect of alleviating the proteins and mRNA expression by EGCG with concentration of 400 μ g/L was better than that observed when using a concentration of 200 μ g/L (Table 4 and Figure 3).

Table 4. Expression changes of Pi-ERK1/2 and ERK2 mRNA in NRK cytoplasm before and after EGCG intervention (N = 8, means \pm SD).

Group	Pi-ERK/actin	ERK mRNA/GAPDH
Control group	0.56 \pm 0.03	0.0042 \pm 0.0003
TGF- β 1-induced group	1.42 \pm 0.07*	0.0182 \pm 0.0007*
EGCG intervention group (200 μ g/L)	0.85 \pm 0.03 [#]	0.0071 \pm 0.0004 [#]
EGCG intervention group (400 μ g/L)	0.71 \pm 0.02 [#]	0.0052 \pm 0.0004 [#]

*Indicates that P < 0.05 compared with the control group, [#]indicates that P < 0.05 compared with the TGF- β 1 group.

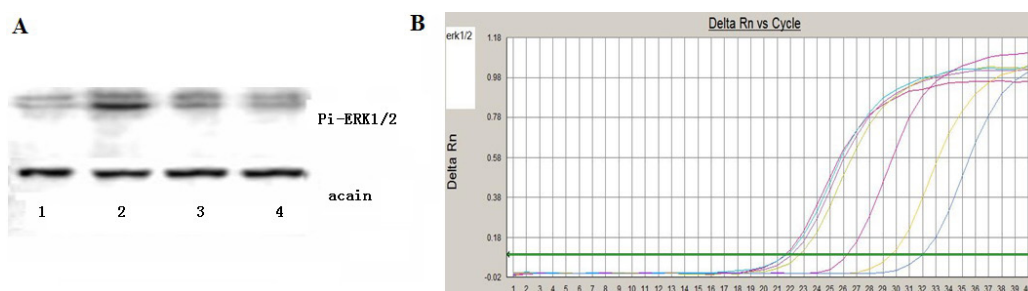


Figure 3. Western blot results of Pi-ERK1/2 protein (A) and RT-PCR results of ERK2 mRNA expression (B) in the NRK cytoplasm. Lane 1 = the normal control group; lane 2 = the TGF- β 1-induced group; lane 3 = the TGF- β 1+EGCG 200 μ g/L group; lane 4 = the TGF- β 1+EGCG 400 μ g/L group.

β -catenin protein and mRNA expression in the NRK cell nucleus

β -catenin protein expression in the TGF- β 1-induced group significantly increased compared with that in the normal group. Expression in the EGCG intervention group was lower than that in the TGF- β 1-induced group. The reduction in expression was lower in the 400 μ g/L EGCG intervention group compared to that of the 200 μ g/L EGCG intervention group. β -catenin mRNA expression in the TGF- β 1-induced group was decreased compared with that in the normal group. Expression in the EGCG intervention group was higher than that in the TGF- β 1-induced group, but still lower than that in the normal group (Table 5 and Figure 4).

Table 5. Expression changes of β -catenin mRNA in NRK cytoplasm before and after EGCG intervention (N = 8, means \pm SD).

Group	β -catenin/actin	β -catenin mRNA/GAPDH
Control group	0.27 \pm 0.03	0.071 \pm 0.002
TGF- β 1-induced group	0.60 \pm 0.04*	0.045 \pm 0.006*
EGCG intervention group (200 μ g/L)	0.46 \pm 0.02 [#]	0.058 \pm 0.004 [#]
EGCG intervention group (400 μ g/L)	0.37 \pm 0.02 [#]	0.060 \pm 0.003 [#]

*Indicates that P < 0.05 compared with the control group, [#]indicates that P < 0.05 compared with the TGF- β 1 group.

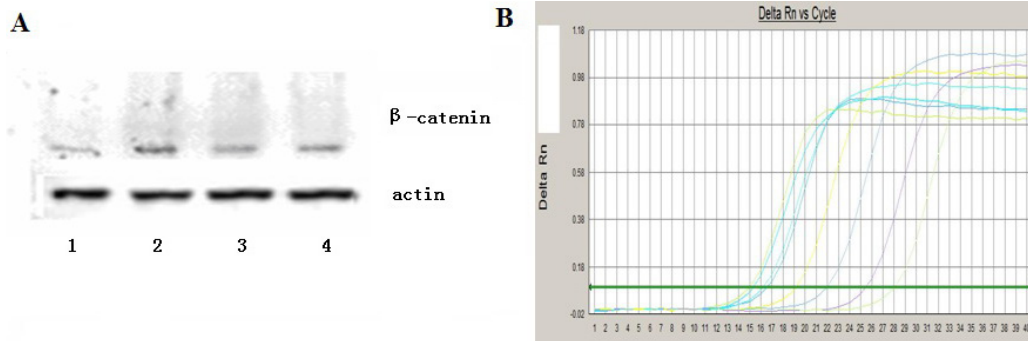


Figure 4. Western blot results of β -catenin protein (A) and RT-PCR results of the mRNA expression (B) in the NRK cytoplasm. Lane 1 = the normal control group; lane 2 = the TGF- β 1-induced group; lane 3 = the TGF- β 1+EGCG 200 μ g/L group; lane 4 = the TGF- β 1+EGCG 400 μ g/L group.

DISCUSSION

In this study, we found that expression of α -SMA occurred in NRK cells after induction with TGF- β 1, while the expression of CK-18 decreased, indicating that transdifferentiation had occurred in NRK cells. These changes were reduced after intervention with EGCG, suggesting that EGCG maintained the NRK phenotype and reduced epithelial cell transdifferentiation.

TGF- β 1 is a pro-fibrotic factor that can stimulate the transformation of mesenchymal cells to epithelial cells (Rastaldi, 2006; García-Sánchez et al., 2010; Zeisberg and Duffield, 2010). A key signaling molecule in the TGF- β 1 signaling pathway is the cytoplasmic protein Smad. Phosphorylation kinase activity in the internal segment of the TGF- β 1 receptor membrane can phosphorylate Smad2 and Smad3 in cells as well as enhance Smad3 binding to Smad4 (Shyu et al., 2010) and promote entry of phosphorylated Smad4 into the nucleus (Shi and Massague, 2003). This regulates the target gene activity with other transcription proteins such as lymphocytes/T cell factors to cause transdifferentiation. Previous studies showed that Smad molecules are involved in TGF- β 1-induced renal interstitial fibrosis (Poncelet et al., 1999; Poncelet and Schnaper, 2001; Li et al., 2002). Complete blockage of TGF- β 1/Smad3 signaling had a protective effect on unilateral ureteral obstruction-induced renal interstitial fibrosis (Sato et al., 2003). Phosphorylated Smad (Pi-Smad3) is the active form; in the rat model of renal interstitial fibrosis, an increase in Pi-Smad was accompanied by an increase in total Smad and its mRNA (Ning et al., 2013).

After treating NRK cells with TGF- β 1 alone for 48 h, Pi-Smad3 levels significantly increased. After intervention with 200 μ g/L EGCG, Pi-Smad3 decreased, and the decrease was more significant with increasing doses of EGCG up to 400 μ g/L. This indicates that EGCG can reduce the expression of TGF- β 1-induced Pi-Smad3 in a dose-dependent manner. mRNA changes can indirectly reflect changes in the corresponding total protein. Our PCR results showed that EGCG inhibited the transcription of Smad3 mRNA.

ERK is a serine/threonine protein kinase that belongs to the mitogen-activated protein kinase (MAPK) family. Pi-ERK expression was found to be elevated in a variety of pulmonary fibrosis and liver fibrosis models, indicating that the ERK pathway is involved in the occurrence of fibrotic lesions to some extent (Robledo et al., 2000; Fubini and Hubbard, 2003; Kim et al., 2007; Cheng et al., 2013). TGF- β 1-induced fibrosis regulation was achieved by

acetylation of Smad3 (Kim et al., 2013b). *In vitro* experiments have demonstrated that ERK is involved in extracellular matrix formation induced by TGF- β 1 (Mucsi et al., 1996; Ramirez et al., 2006; Hu et al., 2006). The TGF- β 1-activated kinase, which activates the MAPK pathway, links the TGF- β 1 signaling pathway with the MAPK cascade pathway. TGF- β 1-activated kinase can be activated by exogenous TGF- β 1. Important substrates of activated ERK include transcription factors such as c-myc, nuclear factor- κ B, and signal transducers and activators of transcription, among others, ultimately leading to changes in the expression of fibrotic and transdifferentiated effector proteins such as type I collagen and α -SMA.

In this study, we found that EGCG downregulated the expression of TGF- β 1-induced Pi-ERK in NRK cells in a dose-dependent manner, in agreement with previous reports demonstrating that EGCG can activate the MAPK pathway (Gao et al., 2013). Additionally, PCR results showed that EGCG also could downregulate mRNA levels of ERK in a dose-dependent manner, indicating that EGCG could reduce the expression of total ERK at the transcription level, thus coordinating inhibition of EGCG on the ERK pathway.

β -catenin is a multifunctional protein, and β -catenin can bind to E-cadherin to form E-cad/ β -cat complexes, thereby maintaining the polarity of normal epithelial cells and adhesion between cells (Drees et al., 2005). Free β -catenin entered into the nucleus when E-cadherin decreased to bind to transcription factor lymphocytes/T cell factor family members as signaling molecules involved in cell proliferation and migration (Gottardi and Gumbiner, 2004). We found that EGCG reduced the expression of TGF- β 1-induced β -catenin in the nucleus in a dose-dependent manner. The PCR results showed that this effect was not caused by a reduction in β -catenin transcription; in fact, the transcription level in the EGCG intervention group was higher than that in the TGF- β 1 group, indicating that EGCG can increase expression of β -catenin to maintain the epithelial phenotype and inhibit nuclear translocation of β -catenin and its signaling.

The mechanism of EGCG on Pi-ERK, Pi-Smad3, β -catenin, and their mRNA is unknown, but may occur through 2 mechanisms: 1) EGCG may affect redox signal transduction. Reactive oxygen species spread signals by modifying the mercapto group or the heme iron in the active sites of target molecules that are modified by a redox reaction (Burdon, 1995; Iuchi and Weiner, 1996; Rhee, 1999; Barrett et al., 1999). Experimental results showed that the generation of reactive oxygen species was closely related to the process of ERK phosphorylation (Katiyar et al., 2001; Zhang et al., 2006). 2) EGCG affected intracellular signal transduction by trapping effects for some of the growth factor ligands (Weber et al., 2004), and indirectly influenced the ERK and Smad pathways through crosstalk among a variety of intracellular signal pathways. However, the exact mechanism requires further experimental validation.

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