

## SHORT COMMUNICATION

### **Genistein does not inhibit TGF- $\beta$ 1-induced conversion of human dermal fibroblasts to myofibroblasts**

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**Short title:** Genistein and TGF- $\beta$ 1-induced myofibroblasts

## **Summary**

Transforming growth factor beta 1 (TGF- $\beta$ 1) is a pro-fibrotic cytokine with a key role in wound repair and regeneration, including induction of fibroblast-to-myofibroblast transition. Genistein is a naturally occurring selective estrogen receptor modulator with promising anti-fibrotic properties. In the present study we aimed to investigate whether genistein modulates TGF- $\beta$ 1 (canonical and non-canonical) signaling in normal dermal fibroblasts at the protein level (western blot and immunofluorescence). We demonstrated that TGF- $\beta$ 1 induces the myofibroblast-like phenotype in the studied fibroblast signaling via canonical (SMAD) and non-canonical (AKT, ERK1/2, ROCK) pathways. Genistein induced only ERK1/2 expression, whereas the combination of TGF- $\beta$ 1 and genistein attenuated the ERK1/2 and ROCK signaling. Of note, the other studied pathways remained almost unaffected. From this point of view, genistein does not impair conversion of normal fibroblasts to myofibroblast-like cells.

**Key words:** wound healing; selective estrogen receptor modulator; phytoestrogen

## Introduction

Ageing is associated with deterioration in physical condition (Hill *et al.* 2020) also related to estrogen deprivation during menopause. Estrogens have been shown to modulate a variety of biological processes (Herichova *et al.* 2019; Herichova *et al.* 2020) including wound healing (Ashcroft *et al.* 1997). In particular the age-related reduced rate of wound healing is associated with improved quality of scarring and reduced levels of transforming growth factor beta 1 (TGF- $\beta$ 1). TGF- $\beta$ 1 is a pro-fibrotic cytokine with a key role in wound repair and regeneration (Klass *et al.* 2009). TGF- $\beta$ 1 is transiently up-regulated in normal skin wounds, whereas its ability to induce fibroblast-to-myofibroblast conversion is crucial for the wound closure (Lichtman *et al.* 2016), even though myofibroblasts never represent the dominating cell population in contracting human wounds (Berry *et al.* 1998). Characteristically, these cells express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and secrete a variety of regulatory/signaling molecules, and they also actively participate in production and organization of the extracellular matrix (ECM) (Pakshir *et al.* 2020). TGF- $\beta$ 1 signaling occurs either via canonical (SMAD) or non-canonical (phosphoinositide-3-kinase (PI3K / AKT), mitogen activated protein kinases (MAPK / ERK1/2), Rho GTPase (ROCK) and p53) pathways (Shi *et al.* 2020) with the presence of further possible cross-talks (Derynck *et al.* 2003).

It has been shown that genistein (GEN), a naturally occurring isoflavone and selective estrogen receptor modulator (Diel *et al.* 2001), may act as an anti-fibrotic agent (Andugulapati *et al.* 2020; Ning *et al.* 2020) and effective skin wound healing modulator (Emmerson *et al.* 2010; Marini *et al.* 2010). Estrogen receptor (ER) signaling attenuates TGF- $\beta$ 1-induced activation of Sma and MAD-related protein 3 (SMAD3), whereas TGF- $\beta$ 1 signaling increases ER-mediated transcription activity (Matsuda *et al.* 2001). Since myofibroblasts persist in chronic inflammatory and fibroproliferative diseases, they contribute to the disease progress (Cannito *et al.* 2017). Thus, we were interested to answer the question whether genistein as a

potential anti-fibrotic molecule also modulates the TGF- $\beta$ 1-induced fibroblast-to-myofibroblast conversion in cells isolated from the normal healthy skin.

## **Methods**

### *Isolation of Human Dermal Fibroblasts (HDFs)*

HDFs were isolated (Dvorankova *et al.* 2019) from two healthy donors with the informed consent of the patient and Ethical Committee of the Third Faculty of Medicine, Charles University in Prague approval following the Helsinki declaration. Briefly, small pieces of residual skin specimens were enzymatically treated with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 30 min. Epidermis was peeled off and dermis specimens were cut into small pieces and seeded on cultivation dishes containing Dulbecco's medium (DMEM) with 10% fetal bovine serum (FBS) and antibiotics (all from Biochrom, Berlin, Germany) at 37°C and 5% CO<sub>2</sub>/95% air atmosphere. After 18/15 (donor 1/2) days, migrating cells were collected and further expanded by culturing. For the experiment passage 10 was used.

### *HDFs treatment*

HDFs were seeded on Petri dishes / cover slips at the density of 3,000 cells/cm<sup>2</sup> in standard cultivation medium. Next day, the medium was changed and cells were cultivated for nine days (medium was changed on day 3 and 7) in the presence (10, 100 and 1000 nM/mL) or absence (control) of genistein (Tocris Bioscience, Abingdon, UK). TGF- $\beta$ 1 (PeproTech, London, UK) at a final concentration of 30 ng/mL was used as positive control (Brenmoehl *et al.* 2009). To assess the genistein effect on TGF- $\beta$ 1-mediated myofibroblast differentiation, cells were treated with medium containing a combination of TGF- $\beta$ 1 and genistein.

### *Western blot (WB)*

Cells cultivated on Petri dishes were scratched and collected in Laemmli lysis buffer (0.1 M Tris/HCl (pH ~ 6.8), 20% glycerol, 10% SDS (sodium dodecyl sulfate)) containing protease and phosphatase inhibitors (Sigma-Aldrich). Afterwards, samples were sonicated (QSonica, Newtown, CT, USA, 40% amplitude, 15 s) to ensure complete cell lysis. Before loading into SDS-PAGE gel (10% Bis-Tris), samples were shortly boiled (95 °C, 5 min). Following separation, proteins were dry blotted to PVDF membrane using the iBlot 2 (Thermo Fisher Scientific) system and blocked in 5% NFDM/BSA (non-fat dry milk/bovine serum albumin) dissolved in TBS (tris-buffered saline) with 0.1% Tween at room temperature. After overnight incubation at 4 °C with primary antibody, membranes were incubated with appropriate HRP-conjugated secondary antibodies for 1 h at room temperature. Protein bands were detected as chemiluminescent signal from ECL (SuperSignal West Pico PLUS chemiluminescent Substrate, Thermo Fisher Scientific) acquired at MF-ChemiBis 2.0 (DNR Bio Imaging Systems, Israel).  $\beta$ -Actin was used to verify equal sample loading. The list of antibodies applied in western blotting is shown in Table 1.

### *Immunofluorescence staining (IF) of HDFs*

Cells cultivated on cover slips were shortly fixed (2% buffered paraformaldehyde, pH ~ 7.2, 5 min) and washed with phosphate-buffered saline (PBS). Afterwards, cell membranes were permeabilized by Triton X-100 (Sigma-Aldrich) and blocked by porcine serum albumin (DAKO, Glostrup, Denmark). Following 1h incubation at room temperature with primary antibody, samples were washed and incubated with appropriate secondary antibody. Cell nuclei were stained by 4', 6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich). All coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) and investigated by a fluorescence microscope (Eclipse Ni-E, Nikon, Tokyo, Japan) equipped with filter cubes

for fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), DAPI, digital camera C11440 ORCA-flash 4.0 (Hamamatsu, Hamamatsu City, Japan), and NIS-Elements software (Nikon). The list of antibodies used for the immunofluorescent analysis is shown in Table 1.

## **Results**

The capability of HDFs to differentiate into myofibroblasts following TGF- $\beta$ 1 exposure was affirmed by increased  $\alpha$ -SMA and fibronectin expressions (Fig 1A) and further confirmed by immunofluorescence (Fig 1B). In detail, clearly visible SMA-positive stress fibers were present in TGF- $\beta$ 1 treated groups. Moreover, TGF- $\beta$ 1 treatment induced deposition of a more prominent fibronectin-rich ECM scaffold. In contrast, WB showed that GEN affects neither  $\alpha$ -SMA nor fibronectin expressions; thus, it did not induce fibroblast-to-myofibroblast differentiation. Instead ECM deposition was rather inhibited by GEN. Interestingly, this effect persisted also in the presence of TGF- $\beta$ 1.

WB analysis (Fig. 1A) further revealed that TGF- $\beta$ 1 activated each studied signaling pathway to a different level. In particular, pSMAD and pERK1/2 were affected the most. On the other hand, GEN induced only pERK1/2 expression. Interestingly, the combination of GEN and TGF- $\beta$ 1 attenuated the TGF- $\beta$ 1-induced pERK1/2 and ROCK1 protein levels. Of note, GEN slightly decreased MLCK expression, but the effect was abolished in the presence of TGF- $\beta$ 1.

## **Discussion**

In the present study, we demonstrated that TGF- $\beta$ 1 induces the myofibroblast-like phenotype in the studied primary culture of HDFs. It has been previously shown that GEN inhibits secretion of ECM proteins and expression of TGF- $\beta$ 1 at both protein and mRNA levels in

renal mesangial cells (Yuan *et al.* 2009) and keloid fibroblasts (Jurzak *et al.* 2014). However, the GEN ability to decrease TGF- $\beta$ 1-induced  $\alpha$ -SMA expression was not observed in the present study. In contrast to a previously shown inhibitory effect of GEN on MAPK signaling in keloid fibroblasts (Cao *et al.* 2008), we observed induction of the MAPK signaling pathway (phosphorylation of ERK1/2) in HDFs. Although we showed that TGF- $\beta$ 1 induced both canonical (SMAD) and non-canonical (MAPK, AKT, ROCK) signaling, a combination of GEN and TGF- $\beta$ 1 attenuated the ERK1/2 and ROCK signaling in HDFs, whereas the other studied pathways remained almost unaffected. From this point of view, GEN should not impair the wound contraction induced by TGF- $\beta$ 1 in normal/healthy subjects.

Even though the exact mechanism is still not fully understood, several cytokines have been identified (IL-6, IL-8, IL-10, and TGF- $\beta$ ) to play major regulatory roles in the pathophysiology of keloids and hypertrophic scar development (Berman *et al.* 2017). In particular, genistein at high concentration (100  $\mu$ M) arrested hypertrophic fibroblasts proliferation and decreased collagen deposition, whereas normal skin fibroblasts remained rather unaffected (Cao *et al.* 2009). Our IF analysis of normal HDFs revealed decreased fibronectin ECM deposition indicating certain level of GEN action specificity.

In conclusion, our results indicate that genistein does not impair conversion of normal dermal fibroblasts to myofibroblast-like cells. Accordingly, further research using an experimental animal as *in vivo* model is warranted by present set of *in vitro* data.

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### **Abbreviation**

ER - estrogen receptor; GEN, genistein; HDFs, human dermal fibroblasts; IL, interleukin; MAPK, mitogen activated protein kinases; MLCK, myosin light-chain kinase; ROCK, Rho GTPase;  $\alpha$ -SMA, alpha smooth muscle actin; TGF- $\beta$ 1, transforming growth factor beta 1



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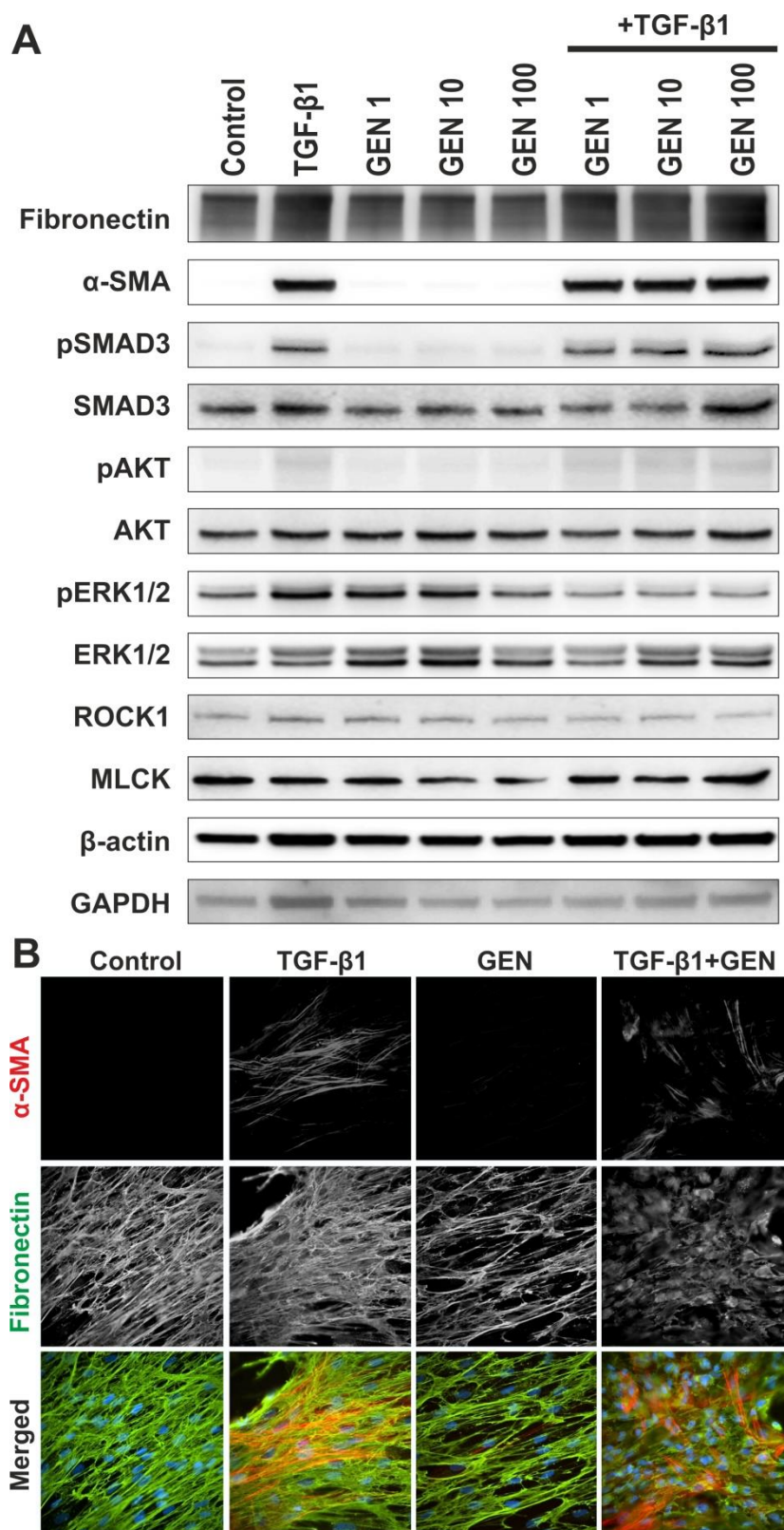
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**Fig. 1** A) Western blot analysis of human dermal fibroblasts exposed to genistein (GEN – 1, 10 and 100 nM) and/or TGF- $\beta$ 1 (30 ng/mL); A) Immunofluorescence analysis of human dermal fibroblasts (HDF) exposed to genistein (GEN – 100 nM) and/or TGF- $\beta$ 1 (30 ng/mL);  $\alpha$ -SMA - alpha-smooth muscle actin (red signal); Fibr - fibronectin (green signal); nuclei were labeled with DAPI (blue signal) (magnification 600x).

**Table 1.** Antibodies used for western blot and immunofluorescence.**Antibodies used for western blot**

<b>Primary Antibody</b>	<b>Abbreviation</b>	<b>Host</b>	<b>Isotype</b>	<b>Clonality</b>	<b>Produced by</b>
$\alpha$ -Smooth muscle actin	$\alpha$ -SMA	rabbit	IgG	monoclonal	CST, Danvers, MA, USA
Fibronectin	Fibr	rabbit	IgG	monoclonal	Abcam, Cambridge, UK
Phospho-ERK1/2	pERK1/2	rabbit		polyclonal	CST, Danvers, MA, USA
Phospho-AKT	pAKT	rabbit	IgG	monoclonal	CST, Danvers, MA, USA
Phospho-SMAD3	pSMAD3	rabbit	IgG	monoclonal	Abcam, Cambridge, UK
ERK1/2	ERK1/2	rabbit	IgG	monoclonal	CST, Danvers, MA, USA
SMAD3	SMAD3	rabbit	IgG	monoclonal	CST, Danvers, MA, USA
AKT	AKT	rabbit		polyclonal	CST, Danvers, MA, USA
$\beta$ -Actin	$\beta$ -actin	rabbit	IgG	monoclonal	CST, Danvers, MA, USA
D-Glyceraldehyde 3-phosphate	GAPDH	rabbit	IgG	monoclonal	CST, Danvers, MA, USA

<b>Secondary Antibody</b>	<b>Host</b>	<b>Isotype</b>	<b>Produced by</b>
Anti-rabbit, HRP-linked	goat	IgG	CST, Danvers, MA, USA

**Antibodies used for immunofluorescence**

<b>Primary Antibody</b>	<b>Abbreviation</b>	<b>Host</b>	<b>Produced by</b>	<b>Secondary Antibody</b>	<b>Produced by</b>	<b>Channel</b>
$\alpha$ -Smooth muscle actin	$\alpha$ -SMA	mouse monoclonal	DakoCytomation, Denmark	goat anti-mouse	Sigma-Aldrich, St. Louis, MO, USA	TRITC-red
Fibronectin	Fibr	rabbit polyclonal	DakoCytomation, Denmark	goat anti-rabbit	Sigma-Aldrich, St. Louis, MO, USA	FITC-green