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# *Aesculus hippocastanum* L. extract differently modulates normal human dermal fibroblasts and cancer-associated fibroblasts from basal/squamous cell carcinoma

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Fibroblasts are actively involved in the formation of granulation tissue and/or tumor stroma. These cells possess the potential to differentiate into myofibroblasts acquiring a highly contractile phenotype characterized by the expression of α-smooth muscle actin (SMA). Considering TGF-β1 as the main inducer of myofibroblast differentiation and horse chestnut extract (HCE) as an effective modulator of the wound healing, we have new evidence to demonstrate canonical TGF-β1/SMAD and non-canonical/non-SMAD signaling in normal fibroblasts, isolated from healthy human skin (human dermal fibroblasts - HDFs), and their malignant counterparts (CAFs) isolated from basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) using western blot and immunofluorescence. Our study revealed that HCE stimulated the deposition of fibronectin by BCC fibroblasts (BCCFs), an effect not seen in other studied fibroblasts. Moreover, HCE in combination with TGF-β1 showed a synergic effect on the presence of polymerized SMA-stress fibers, particularly visible in CAFs. Interestingly, the TGF-β1 exposure led to activation of the canonical SMAD signaling in HDFs and BCCFs, whereas treatment of SCC fibroblasts (SCCFs) resulted in activation of the non-canonical AKT and/or ERK1/2 signaling. In conclusion, we observed specific differences in signaling between HDFs and CAFs that should be considered when developing new therapeutic approaches targeting wound/tumor microenvironments.

*Key words: wound healing, cancer growth, phytotherapy, microenvironment*

The process cascades of wound healing and tumor progression share common characteristics such as the appearance of phenotypically similar cell populations, including (adult/cancer) stem cells [1, 2], as well as immune/inflammatory cells (predominantly neutrophils and macrophages) [3–6] and activated (cancer-associated) fibroblasts [7–10]. Fibroblasts are the main cell population of connective tissue involved in the formation of granulation tissue and/or tumor stroma [7–9, 11] by producing structural macromolecules (fibronectin, collagen) and proteolytic enzymes (matrix metalloproteinases) responsible for the deposition and remodeling of the extracellular matrix (ECM) [7–9, 12, 13]. Moreover, fibroblasts can differentiate into myofibroblasts acquiring a highly contractile phenotype characterized by the expression of α-smooth muscle actin (SMA) [14] and are involved in the process of wound contraction [15]. In tumors, cancer-associated fibroblasts (CAFs) resemble the myofibroblast-like phenotype, which typically involves the expression of SMA and the deposition of fibronectin [16, 17]. It has been well shown that CAFs modulate the biological properties of tumors [8], for example, by secretion of growth factors [*e.g.*, HGF [18], FGF-2, VEGF, platelet-derived growth factor [19, 20]) and cytokines/chemokines (e.g., TGF-β, IL-6, IL-8, CXCL1) [13].

A renewed strategy directed towards using natural agents isolated from plants might represent a promising adjunctive therapy to conventional treatment, as they are able to exert pleiotropic effects by modulating the activity of several proteins and signaling pathways [21, 22]. In this context, we previously described [23] that *Aesculus hippocastanum*

L. water extract (horse chestnut extract-HCE), a member of the *Hippocastanaceae* family, is able to modulate ECM production by fibroblasts, leading to increased wound stiffness *in vivo*. Detailed phytochemical analysis revealed that the extract contains bioflavonoids, triterpenoid saponins, proanthocyanidin A2, and coumarins [24]. In addition, HCE exerts further biological effects, the most important being generation of contraction force in fibroblasts [25], but without induction of the fibroblast-to-myofibroblast switch, which is characterized by the presence of SMA fibers [23]. Besides that, attention has recently been directed towards β-escin, the major active component of HCE [24], because of its anticancer properties [26–29], such as the induction of cell cycle arrest or apoptosis in various cancer cells (*e.g.*, colon, hepatocellular, cholangiocarcinoma) [30–32]. Several studies suggest that its anti-proliferative activities are mainly mediated by inhibition of transcription factors JAK, STAT, NF-κB, and activator protein-1 [28, 33–35]. Moreover, it was further observed that β-escin treatment inhibited FGF-2-induced AKT activation in human umbilical vein endothelial cells, also indicating an anti-angiogenic effect of β-escin [36].

In the present study, considering the important role of fibroblasts in tumors and wounds, we further extended the previously published evidence [23] on canonical TGF-β1/SMAD and non-canonical/non-SMAD (Rho, MAPK, PI3K) signaling in normal fibroblasts, isolated from healthy human skin (human dermal fibroblasts-HDFs) and their malignant counterparts (CAFs) isolated from basal cell carcinoma (BCCFs) and squamous cell carcinoma (SCCFs) through a series of *in vitro* experiments.

## Patients and methods

**Plant material.** Horse chestnut (*Aesculus hippocastanum* L.) water extract (HCE) was a gift from Calendula a.s. (Nová Ľubovňa, Slovak Republic) and was provided in the form of a dry powder. Detailed analysis of the tested extract was shown in our previous report [23].

**Primary cultures of human dermal fibroblasts (HDFs).** HDFs were isolated from two healthy donors after reduction mammaplasty in the Department of Plastic Surgery, Third Faculty of Medicine and Kralovske Vinohrady University Hospital in Prague and cultured as previously described [37] with the informed consent of the patient and approval of the Ethics Committee of the Third Faculty of Medicine according to the rules of the Declaration of Helsinki. In order to separate the epidermis from the dermis, small pieces of split-thickness skin grafts were treated enzymatically with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 15 minutes. Small pieces of dermis were seeded on culture dishes and covered with DMEM, 10% FBS, and antibiotics (all from Biochrom, Berlin, Germany) at 37 °C and 5% $CO_2$/95% air atmosphere. A few days later, the migrating cells were collected and expanded by further cultivation.

**Primary cultures of cancer-associated fibroblasts.** Primary cultures of basal cell carcinoma fibroblasts (BCCFs) and squamous cell carcinoma fibroblasts (SCCFs) were obtained from basal cell carcinoma covering the skin of the upper limb and squamous cell carcinoma located in the root of the tongue, as previously mentioned [37], with the informed consent of the patient (in full compliance with the Helsinki Declaration after approval by the local ethics committee) in the Department of Dermatology and Venerology and the Department of Stomatology (both Charles University, First Faculty of Medicine and General University Hospital in Prague), respectively. Briefly, small pieces of tumor biopsies were collected and expanded by culturing in DMEM with 10% FBS and antibiotics (all from Biochrom, Berlin, Germany) at 37 °C and 5% $CO_2$/95% air atmosphere. Vimentin expressing cells that were negative for CD45 (leukocyte marker), keratins (epithelial marker), and CD31 (endothelial marker) were considered as fibroblasts.

**MTS assay.** MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell viability assay was performed according to a standard protocol with minimal modifications [38]. Briefly, cells were seeded (10,000 cells/cm$^2$/well) in 96-well-plates in DMEM containing 10% FBS and ATB. Twenty-four hours after seeding the cells, the medium was replaced with medium without HCE (untreated control), medium containing 30 ng/ml of TGF-β1, and medium containing different concentrations (0.1, 1, and 10 μg/ml) of HCE in the presence or absence of 30 ng/ml of TGF-β1. After 48 hours of incubation, the MTS was added to each well and cells were incubated for another 2 hours. Then, cell proliferation was evaluated by measuring the absorbance at 495 nm wavelength using an automated Cytation™ 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT, USA). Three independent experiments were performed for each assay. Cell proliferation was calculated as the percentage of viable cells in each well compared to the untreated control well.

**Western blot (WB) of HDFs, BCCFs, and SCCFs.** HDFs, BCCFs, and SCCFs (passages 9–11) were seeded on Petri dishes at the density of 3,000 cells/cm$^2$. Cells were cultured for ten days in the presence (0.1, 1, and 10 μg/ml) or absence (control) of the tested HCE extract. TGF-β1 (PeproTech, London, UK) at a final concentration of 30 ng/ml was used as a positive control to induce the expression of SMA and fibronectin [39, 40]. In addition, cells were stimulated with a medium containing a combination of TGF-β1 (30 ng/ml) and HCE at different concentrations (0.1, 1, and 10 μg/ml). The primary and secondary antibodies used in the analysis are listed in Table 1. Protein lysates were prepared according to the previously described method [23] using Laemmli lysis buffer [0.1M Tris/HCl (pH 6.8), 20% glycerol, 10% SDS (sodium dodecyl sulfate)] containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA) and a sonication procedure (QSonica, 40% amplitude, 15 s). Protein concentration was

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

**Antibodies used for western blot**

| Primary antibody | Abbreviation | Host | Isotype | Clonality | Produced by |
|---|---|---|---|---|---|
| Fibronectin | Fibr | rabbit | IgG | monoclonal | Abcam, Cambridge, UK |
| α-smooth muscle actin | SMA | rabbit | IgG | monoclonal | CST, Danvers, MA, USA |
| phospho-SMAD3 | pSMAD3 | rabbit | IgG | monoclonal | Abcam, Cambridge, UK |
| SMAD3 | SMAD3 | rabbit | IgG | monoclonal | CST, Danvers, MA, USA |
| phospho-AKT | pAKT | rabbit | IgG | monoclonal | CST, Danvers, MA, USA |
| AKT | AKT | rabbit | | polyclonal | CST, Danvers, MA, USA |
| phospho-ERK1/2 | pERK | rabbit | | polyclonal | CST, Danvers, MA, USA |
| ERK1/2 | ERK | rabbit | IgG | monoclonal | CST, Danvers, MA, USA |
| ROCK1 | ROCK1 | rabbit | IgG | monoclonal | Thermo Fisher Scientific, Waltham, MA, USA |
| MLCK | MLCK | rabbit | IgG | polyclonal | Thermo Fisher Scientific, Waltham, MA, USA |
| β-actin | β-actin | rabbit | IgG | monoclonal | CST, Danvers, MA, USA |

| Secondary antibody | Host | Isotype | Produced by |
|---|---|---|---|
| Anti-rabbit, HRP-linked | goat | IgG | CST, Danvers, MA, USA |

**Antibodies Used for Immunofluorescence**

| Primary antibody | Abbreviation | Host | Produced by | Secondary antibody | Produced by | Channel |
|---|---|---|---|---|---|---|
| α-smooth muscle actin | SMA | mouse monoclonal | DakoCytomation, Glostrup, Denmark | Goat anti-mouse | Sigma-Aldrich, St. Louis, MO, USA | FITC-green |
| Fibronectin | Fibr | rabbit polyclonal | DakoCytomation, Glostrup, Denmark | Goat anti-rabbit | Sigma-Aldrich, St. Louis, MO, USA | TRITC-red |

determined using a Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and measured using an automated Cytation™ 3 Cell Imaging Multi-Mode Reader (Biotek, Winooski, VT, USA) at a wavelength of 570 nm. After boiling (95 °C, 5 min), samples were separated onto SDS-PAGE gel (10% Bis-Tris) and transferred to the PVDF membrane using the iBlot 2 (Thermo Fisher Scientific, Waltham, MA, USA) dry blot system. The membranes were then blocked in 5% NFDM/BSA (non-fat dry milk/bovine serum albumin) dissolved in TBS (tris-buffered saline) with 0.1% Tween for 1 hour at room temperature and incubated overnight with primary antibody at 4 °C. The next day, membranes were incubated with appropriate HRP-conjugated secondary antibodies. Following 1 h of incubation at room temperature, protein bands were detected using ECL (SuperSignal West Pico PLUS Chemiluminescent Substrate, Thermo Fisher Scientific, Waltham, MA, USA) and a signal was recorded using MF-ChemiBis 2.0 (DNR Bio Imaging Systems, Israel). β-actin was used as a sample loading control.

**Immunofluorescence (IF) analysis of HDFs, BCCFs, and SCCFs.** HDFs, BCCFs, and SCCFs (passages 9–11) were seeded at a density of 3,000 cells/cm² on coverslips and cultured for ten days in the presence (0.1, 1, and 10 μg/ml) or absence (control) of the tested HCE. A medium containing 30 ng/ml of TGF-β1 was used as a positive control of the myofibroblast differentiation ability. Additionally, a medium containing a combination of TGF-β1 (30 ng/ml) and HCE extract (0.1, 1, and 10 μg/ml) was used to determine the effect of HCE on TGF-β1-induced fibroblast-to-myofibroblast differentiation. Briefly, cells were fixed with 2% buffered paraformaldehyde (pH 7.2) for 5 min and washed with PBS (phosphate-buffered saline). Cell membranes were permeabilized with Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and sites for antigen-independent binding of antibodies were blocked with porcine serum albumin (DAKO, Glostrup, Denmark). Commercial antibodies were diluted according to the manufacturer's instructions. Antibodies used for immunofluorescence are listed in Table 1. The specificity of immunocytochemical staining was controlled by replacing the first-step antibody with an irrelevant antibody and testing positive control samples. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO, USA). All samples were embedded in Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined with an Eclipse 90i fluorescence microscope (Nikon, Tokyo, Japan) equipped with filter cubes for FITC, TRITC, and DAPI and C11440 ORCA-flash 4.0 digital camera (Hamamatsu, Hamamatsu City, Japan) NIS-elements computer-assisted image analysis software (Nikon, Tokyo, Japan).

**Statistical analysis.** Data from the viability assay (MTS assay) were expressed as the mean ± standard deviation (SD) of three independent experiments (each performed in technical triplicates) and compared using one-way analysis of variance followed by Dunnett's post-hoc test. Significance was accepted at a p-value less than 0.05.

## Results

**MTS assay of HDFs, BCCFs, and SCCFs.** The results of the cell viability assay are shown in Figure 1 and clearly demonstrate that none of the tested concentrations exhibited a significant level of toxicity. Specifically, treatment with TGF-β1 (positive control) had rather no effect on the proliferation of the studied cells whereas the stimulatory effect of HCE was mostly apparent in SCCFs. Although the intermediate tested concentration of HCE and TGF-β1 tended to attenuated cell proliferation, the other two tested concentrations showed no effect.

**Western blot analysis of HDFs, BCCFs, and SCCFs.** The results of the WB analysis are shown in Figure 2 and summarized in Table 2. TGF-β1 (30 ng/ml) was used as a positive control for fibroblast-to-myofibroblast transition.

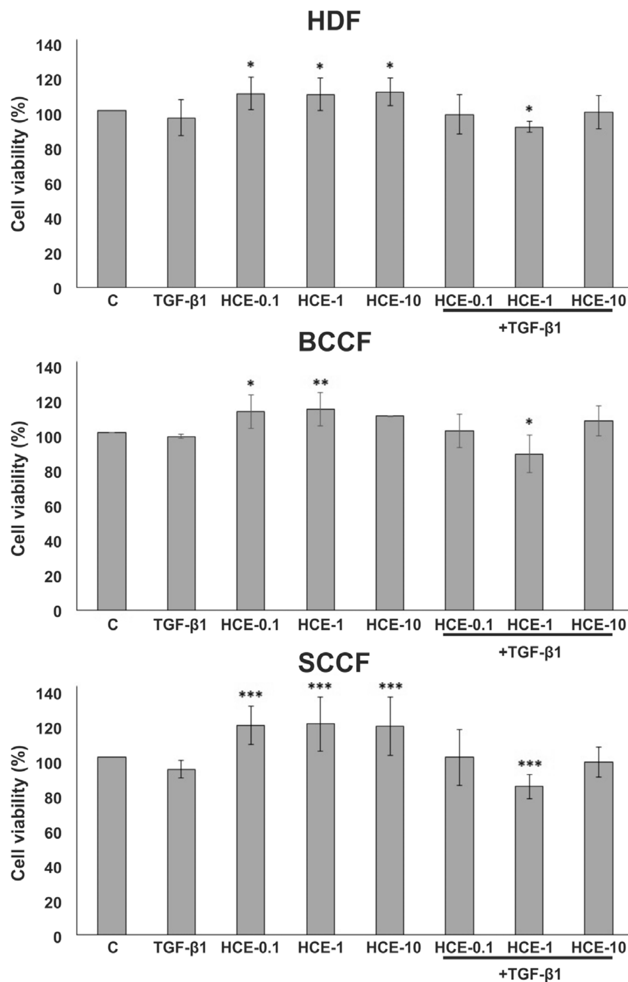


**Figure 1. MTS metabolic assay of human dermal fibroblasts (HDF), basal cell carcinoma fibroblasts (BCCF), and squamous cell carcinoma fibroblasts (SCCF). Statistical comparison (one-way ANOVA followed by Dunnett's post-hoc test) revealed significant differences between the treatment conditions (*p<0.05).**

As expected, TGF-β1 stimulated the production of SMA in HDFs and both studied CAFs. Untreated BCCFs expressed SMA and this phenotype was even promoted in the presence of TGF-β1. Treatment with HCE had no effect on the production of SMA in HDFs and SCCFs. Interestingly, the expression of SMA BCCFs slightly decreased following HCE treatment in a concentration-dependent manner.

The regulation of HDFs and SCCFs revealed a common pattern. In detail, TGF-β1 stimulated fibronectin expression whereas HCE treatment slightly decreased fibronectin levels in HDFs and SCCFs. Moreover, HCE extract was able to slightly attenuate the production of fibronectin in both HDFs and SCCFs stimulated by TGF-β1 (not observed by IF). Intriguingly, an inverse signaling effect was observed in BCCFs as TGF-β1 decreased and HCE increased fibronectin expression. Of note, the effect of TGF-β1 prevailed after co-treatment.

TGF-β1 treatment induced canonical (SMAD3) signaling in HDFs and BCCFs. HCE was able to deteriorate observed signaling only in BCCFs. In contrast, TGF-β1 rather induced the non-canonical signaling (AKT/ERK1/2) in SCCFs, an effect that was not modulated by HCE.

Even though HCE slightly induced the expression of ROCK1 in the studied fibroblasts, the down-regulatory effect of TGF-β1 prevailed when co-treatment was administered. Of note, TGF-β1 slightly decreased the expression of MLCK in HDFs, but increased its expression in CAFs, without any remarkable modulatory effect of the HCE co-treatment being observed.

**Immunofluorescence of HDFs, BCCFs, and SCCFs.** Immunofluorescent staining of fibronectin showed that all studied fibroblasts formed ECM, which in the case of BCCFs and SCCFs had a higher fibronectin density (Figure 3). TGF-β1 treatment resulted in an over-production of fibronectin in HDFs and SCCFs, whereas ECM synthesis was rather reduced in the culture of BCCFs. In contrast, HCE did not remarkably alter fibronectin production in HDFs and SCCFs but in BCCFs, the treatment resulted in a large increase in matrix deposition. In all studied cells the effect of TGF-β1 prevailed over HCE.

**Table 2. Overview of TGF-β1/HCE/HCE+TGF-β1 effects on normal (HDFs) and cancer associated fibroblasts isolated from BCC (BCCFs) and SCC (SCCFs).**

| Expression (Fibr/SMA) | HDFs | BCCFs | SCCFs |
|---|---|---|---|
| TGF-β1 | ↑ / ↑ | ↓ / ↑ | ↑ / ↑ |
| HCE | ↘ / – | ↑ / ↘ | ↘ / – |
| HCE+TGF-β1 | ↑ / ↑ | ↓ / ↑ | ↑ / ↑ |
| **Signaling (C/N)** | **HDFs** | **BCCFs** | **SCCFs** |
| TGF-β1 | ↑C / ↓N | ↑C / ↓N | ↓C / ↑N |
| HCE | –C / –N | –C / –N | –C / –N |
| HCE+TGF-β1 | ↑C / ↓N | ↓C / ↓N | ↓C / ↑N |

Notes: - – no effect; ↑ – up-regulation; ↓ – down-regulation; ↘ – mild down-regulation. Abbreviations: C-canonical (SMAD); N-non-canonical (non-SMAD); Fibr-fibronectin; SMA-α-smooth muscle actin

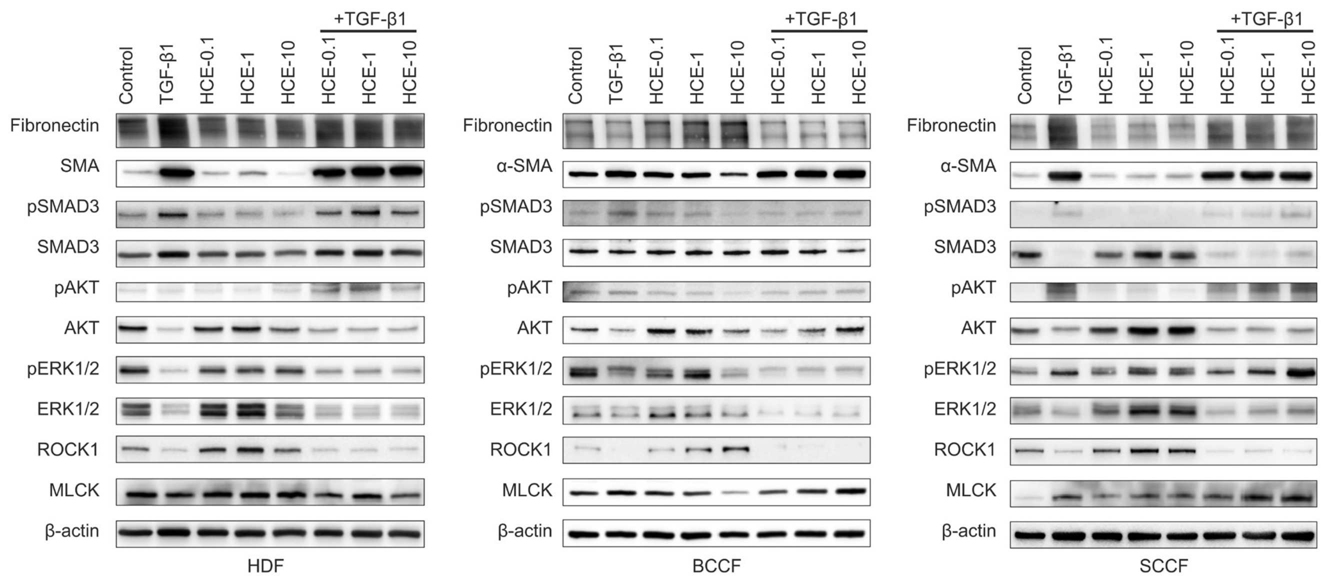


**Figure 2. Western blot analysis of human dermal fibroblasts (HDF), basal cell carcinoma fibroblasts (BCCF), and squamous cell carcinoma fibroblasts (SCCF) following treatment with TGF-β1 (30 ng/ml), HCE, and combination of TGF-β1 and HCE; SMA (α-smooth muscle actin); TGF-β1-transforming growth factor β-1; HCE-horse chestnut extract.**

**Figure 3. Immunofluorescence analysis of human dermal fibroblasts (HDF), basal cell carcinoma fibroblasts (BCCF), and squamous cell carcinoma fibroblasts (SCCF). SMA (α-smooth muscle actin; green signal), fibronectin (red signal), cell nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI; blue signal) (scale bars 100 μm).**

HDFs and SCCFs were SMA-negative whereas BCCFs expressed high levels of polymerized SMA indicating a myofibroblast-like phenotype. Accordingly, TGF-β1 promoted fibroblast-to-myofibroblast transition only in HDFs and SCCFs. On the other hand, HCE treatment only affected BCCFs, where a concentration-dependent decrease in SMA polymerization was observed. Of note, the expression of polymerized SMA in HDFs and SCCFs remained relatively unaffected. The combination of TGF-β1 and HCE treatments revealed a synergistic effect on myofibroblast-like cell formation that was particularly evident in BCCFs and SCCFs. Intriguingly, the highest tested concentration of HCE resulted in SMA inhibition in HDFs and SCCFs but not in BCCFs.

## Discussion

Our study represents the first evidence that HCE modulates the expression of fibronectin/SMA even in CAFs of different origins [41]. This effect was seen both in the absence and presence of TGF-β1. Notably, fibronectin expression was remarkably increased in BCCFs following HCE treatment, an effect that was not observed in HDFs and SCCFs. Since high fibronectin levels are associated with increased adhesion, migration, and invasiveness of BCC lineages [42], we can speculate that HCE-induced fibronectin overproduction (which was not seen in the presence of TGF-β1) by BCCFs may contribute to an unfavorable infiltrative phenotype of the tumor. Interestingly, HCE resulted in decreased polymerization of SMA in myofibroblast-like BCCFs, but the combination of HCE and TGF-β1 (present in BCC) resulted in remarkably increased signal for SMA-positive stress fibers. Here, we observed a cell-type specific effect of HCE and TGF-β1 co-treatment on the presence of polymerized SMA-fibers, particularly visible in CAFs. The observed heterogeneity, which has also been observed previously [43, 44], suggests the influence of fibroblast origin/niche on the biological response to potential treatment and thus represents an important parameter for potential personalized tailor-made manipulation of the microenvironment.

TGF-β1 exposure led to activation of the SMAD3 pathway (canonical signaling) in HDFs and BCCFs. Although TGF-β1 treatment slightly increased pSMAD3 in SCCFs, total SMAD3 expression was decreased. In contrast, the increase in phospho-ERK1/2 and phospho-AKT indicated prevailed activation of non-canonical (non-SMAD) signaling in SCCFs [45]. These non-canonical pathways include various parts of MAPK (ERK1/2), Rho-like GTPase, and PI3K/AKT signalings [46]. Considering the negative/positive cross-talk between canonical and non-canonical TGF-β1 signaling described previously [47], we may hypothesize that (phospho/total) SMAD3 activation in BCCFs and HDFs may be a residuum of exposure to aggressive tumor microenvironment [48] perhaps associated with their myofibroblast-like phenotype [49] on the one hand and wound healing-like transient activation of normal cells [50, 51] on the other hand. Therefore, further research is needed to elucidate how normal fibroblasts differ from their cancer-associated counterparts, also taking into account their origin.

Consistent with previously reported data in normal fibroblasts [52], HCE induced the expression of ROCK1 in HDFs and SCCFs. It has been shown that ROCK (1 and 2) induction mediates the beneficial effect of HCE on wound healing [52], as it regulates calcium ion intake resulting in activation of myosin light-chain kinase (MLCK), which is responsible for cell contraction. Additionally, ROCK (1 and 2) inhibits the MLCK counterpart MYLP, resulting in further MLCK activation [53]. In the present study, we also observed different MLCK-related responses to HCE/TGF-β1 exposure between HDFs and CAFs. TGF-β1 in HDFs decreased MLCK expression, an effect that was enhanced when combined treatment with HCE was administered. In contrast, treatment with HCE and TGF-β1 in SCCFs acted rather in a synergic manner amplifying the MLCK levels. This observation further emphasizes the importance of fibroblast heterogeneity. In particular, the response (canonical vs. non-canonical) of cells to TGF-β1 revealed that SCCFs differ from normal HDFs and BCCFs, probably due to a more aggressive tumor phenotype that is prone to escape and form metastases [54].

In conclusion, we showed that HCE water extract differently modulated cell signaling in normal and cancer-associated fibroblasts. In detail, HCE did not stimulate the expression of SMA in HDFs but slightly increased TGF-β1-induced expression of SMA. However, HCE in combination with TGF-β1 showed a synergistic effect on the formation of SMA fibers in CAFs, which was particularly evident in BCCFs. Non-canonical signaling following TGF-β1 treatment prevailed in SCCFs (activation of non-canonical pERK1/2 and pAKT pathways), whereas canonical/SMAD signaling was rather activated in HDFs and BCCFs. In summary, we observed specific differences between the studied fibroblasts that should be considered in the development of novel therapeutic approaches targeting the wound/tumor microenvironment.

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