# Expression of protein kinase C isoforms in cultured human Tenon's capsule fibroblast cells

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**Abstract.** Members of the protein kinase C (PKC) family are involved in physiological and pathophysiological processes, and exert an important role in signal transduction. The aim of the present study was to determine which of the 12 protein kinase C (PKC) isoforms ($PKC_{\alpha}$, $PKC_{\beta I}$, $PKC_{\beta II}$, $PKC_{\gamma}$, $PKC_{\delta}$, $PKC_{\varepsilon}$, $PKC_{\eta}$, $PKC_{\theta}$, $PKC_{\mu}$, $PKC_{\zeta}$, $PKC_{\lambda}$ and $PKC_{\iota}$) were expressed *in vitro* in cultured human Tenon's capsule fibroblasts (HTFs). HTFs from cell passages three to five were investigated for the presence of the 12 PKC isoforms at the cellular, mRNA and protein levels using laser scanning confocal microscopy (LSCM), reverse transcription-polymerase chain reaction (RT-PCR) and western blot analysis, respectively. These analyses yielded similar results for several of the PKC isoforms ($PKC_{\alpha}$, $PKC_{\delta}$, $PKC_{\varepsilon}$, $PKC_{\eta}$, $PKC_{\mu}$, $PKC_{\zeta}$, $PKC_{\lambda}$ and $PKC_{\iota}$); however, $PKC_{\beta I}$, $PKC_{\beta II}$, $PKC_{\gamma}$ and $PKC_{\theta}$ were not expressed in the cultured HTFs. LSCM revealed that eight PKC isoforms, $PKC_{\alpha}$, $PKC_{\delta}$, $PKC_{\varepsilon}$, $PKC_{\eta}$, $PKC_{\mu}$, $PKC_{\zeta}$, $PKC_{\lambda}$ and $PKC_{\iota}$, appeared almost exclusively in the cytoplasm of the cells. Notably, $PKC_{\delta}$ was expressed particularly well in the cytoskeleton. The present study revealed that all 12 PKC isoforms were expressed and that eight of the isoforms were present in cultured HTFs. These results will be of value when determining specific roles for the PKC isoforms in HTF proliferation, which may provide a novel therapeutic target for bleb scarring in glaucoma filtering surgery.

## Introduction

Glaucoma, characterized by progressive optic neuropathy, is the second most commonly occurring disease causing blindness worldwide. Glaucoma filtration surgery (GFS) provides the gold standard for the management of intraocular pressure (IOP), after medication and laser surgery have failed to control IOP adequately. However, scar tissue, which can form under the conjunctiva, obstructs aqueous flow and causes the filter to fail. Previous studies have demonstrated that human Tenon's capsule fibroblasts (HTFs) located in the incision area exert a major role in scar formation by promoting the proliferation, migration and synthesis of the extracellular matrix (1). Several antimetabolites, including mitomycin C and 5-fluorouracil, have been used to prevent post-operative ocular scar tissue formation. However, a disadvantage is that these antimetabolites are associated with marked side-effects, including hypotony, endophthalmitis, bleb leakage and loss of vision (2,3). Several alternative methods have been demonstrated to reduce scar formation through the inhibition of the proliferation of the HTFs (4,5).

Protein kinase C (PKC) comprises a family of protein isoforms, which occupy a central role in cellular processes, including proliferation, differentiation, mitosis and inflammation. To date, at least 12 isoforms of PKC have been cloned, and these are divided into three major groups: Classical PKCs ($PKC_{\alpha}$, $PKC_{\beta I}$, $PKC_{\beta II}$ and $PKC_{\gamma}$), novel PKCs ($PKC_{\delta}$, $PKC_{\varepsilon}$, $PKC_{\eta}$, $PKC_{\theta}$ and $PKC_{\mu}$) and atypical PKCs ($PKC_{\zeta}$, $PKC_{\lambda}$ and $PKC_{\iota}$) (6). The differences in functionality among the specific PKC isoforms are predominantly due to their subcellular localization, activation or inhibition by different stimuli, and transcriptional regulation. It was reported that tranilast inhibits the proliferation and migration of HTFs *in vitro*, at least in part, by downregulating the expression of PKC (7,8). Alkylphosphocholines were identified as effective inhibitors of HTF proliferation and migration, and cell-mediated contraction of collagen gels at non-toxic concentrations. A previous study indicated that the mechanism of action appeared to involve the inhibition of the PKC pathway (9). It was therefore feasible that several of the PKC isoforms may exert a role in HTF proliferation, and the present study was focused on analyzing the expression of the 12 PKC isoforms in cultured HTFs with a view towards elucidating their role(s) in HTF proliferation.

## Materials and methods

*Culture of HTFs*. Fresh human Tenon's capsule tissues (48 h post-mortem) from donors, were obtained from the Eye Bank

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of Zhongshan Ophthalmic Center (Guangzhou, China). The HTFs were cultured in 6-well plates (BD Biosciences, Lincoln Park, NJ, USA) with explants in Dulbecco's modified Eagle's medium/F12 nutrient mixture (Invitrogen Life Technologies, Grand Island, NY, USA), containing 5% FBS (Hyclone, Logan, UT, USA), 100 U/ml penicillin G (Gibco Life Technologies, Carlsbad, CA, USA), 100 mg/ml streptomycin sulfate (Gibco Life Technologies) and L-glutamate (Invitrogen Life Technologies), as previously described (10). HTFs from cell passage 3 to 5 were subsequently used in the experiments. The cells were lysed for total RNA extraction to assess the mRNA expression levels, or to extract proteins and assess the protein expression levels.

*Immunofluorescence analysis*. HTFs ($1x10^6$/liter) grown on coverslips were fixed in 4% paraformaldehyde for 15 min at room temperature, prior to rinsing three times in phosphate buffered saline (PBS). The cell cultures were permeabilized with 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS at room temperature for 10 min. Indirect immunostaining was performed, as described previously (11). The primary antibodies, rabbit anti-PKCα polyclonal antibody (1:200; cat. no. sc-208), rabbit anti-PKCγ polyclonal antibody (1:100; cat. no. sc-211) and mouse anti-PKCη monoclonal antibody (1:100; cat. no. sc-136036) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); mouse anti-PKCδ monoclonal antibody (1:100; cat. no. 610397), mouse anti-PKCε monoclonal antibody (1:200; cat. no. 610086), mouse anti-PKCθ monoclonal antibody (1:50; cat. no. 612734), mouse anti-PKCι monoclonal antibody (1:50; cat. no. 610175) and mouse anti-PKCλ monoclonal antibody (1:50; cat. no. 610208) were purchased from BD Biosciences; rabbit anti-PKCβI polyclonal antibody (1:200; cat. no. p3078), rabbit anti-PKCβII polyclonal antibody (1:100; cat. no. p8371), rabbit anti-PKCζ polyclonal antibody (1:30; cat. no. SAB2104776) and rabbit anti-PKCμ polyclonal antibody (1:50; cat. no. SAB1306354) were from Sigma-Aldrich. The cells were incubated with the antibodies overnight in a solution of PBS at a temperature of 4˚C. Following washing with PBS, Alexa Fluor 488-conjugated secondary antibodies [1:200 (cat. nos. A28175 and A27034); Invitrogen Life Technologies] were applied for 1 h and 1 μg/ml Hoechst 33342 (Sigma-Aldrich) was used for nuclear counterstaining. Either secondary antibody alone, without primary antibodies, or isoform immunoglobulin G (BD Biosciences) served as the negative control. The staining was imaged using a laser-scanning confocal microscope (LSCM510META; Zeiss, Thornwood, NY, USA). Each antibody was used in a minimum of three separate experiments.

*Total RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)*. The total RNA was isolated from the cells using an RNeasy Mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The RNA concentration was quantified spectrophotometry, using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) prior to storage at -80˚C. The RNA (5 μg) was reverse-transcribed using the SuperScript™ first-strand synthesis system, according to the manufacturer's instructions (Invitrogen Life Technologies). cDNAs (2 μg) encoding the PKC isoform genes were amplified by PCR as follows: Denaturation for 30 sec, followed by annealing (56˚C for $PKC_{\alpha}$, $PKC_{\beta II}$, $PKC_{\gamma}$, $PKC_{\varepsilon}$, $PKC_{\eta}$, $PKC_{\theta}$, $PKC_{\iota}$, $PKC_{\zeta}$ and $PKC_{\mu}$; 60˚C for $PKC_{\delta}$; 52˚C for $PKC_{\beta I}$) for 30 sec, and elongation at 72˚C for 60 sec, for 30 cycles. The resulting PCR products were analyzed by 2% gel electrophoresis. Primer sequences for 11 PKC isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were published previously (12), and are listed in Table 1 (PKCλ cannot be detected due to the absence of its human PKC cDNA in GeneBank; http://www.ncbi.nlm.nih.gov/genbank/). Each PCR experiment was performed a minimum of three times with each set of primers.

*Western blot analysis*. The culture medium was removed and washed twice with ice-cold PBS. The HTFs were lysed using sample buffer [60 mM Tris/HCl (pH 6.8), 2% (w/v) sodium dodecyl sulfate (SDS), 100 mM 2-mercaptoethanol and 0.01% (w/v) Bromophenol Blue] (13). The lysates were incubated on ice for 30 min and the extracts were harvested using a cell scraper, and boiled for 5 min prior to storage at -20˚C.

Subsequently, western blotting was performed, as previously described (14). Briefly, 40 μg protein per well, measured using a bicinchoninic acid protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA), was loaded onto a 12% SDS-polyacrylamide gel for SDS-polyacrylamide gel electrophoresis. The proteins were separated and electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA) for 1 h at 350 mA. The membranes were blocked with 5% non-fat milk disolved in TTBS buffer, containing, 50 mM Tris/HCl (pH 7.5), 0.9% NaCl and 0.1% Tween-20, for 1 h at room temperature and incubated with primary antibodies overnight at 4˚C, prior to subsequent incubation with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The signals were detected using an enhanced chemiluminescence kit (GE Healthcare, Inc., Piscataway, NJ, USA), according to the manufacturer's instructions. Each PKC isoform was detected in a minimum of three independent experiments. Rat brain lysate (BD Biosciences) served as a positive control, as recommended by the manufacturer's instructions for the primary antibody.

## Results

*Immunofluorescence analysis of the PKC isoforms in HTFs*. Using laser scanning confocal microscopy (LSCM), eight PKC isoforms ($PKC_{\alpha}$, $PKC_{\delta}$, $PKC_{\varepsilon}$, $PKC_{\zeta}$, $PKC_{\eta}$, $PKC_{\iota}$, $PKC_{\lambda}$ and $PKC_{\mu}$) were identified in the cultured HTFs, predominantly localized in the cytoplasm of the cells, as revealed by immunofluorescence staining. In particular, $PKC_{\delta}$ was expressed in the cytoskeleton. However, no staining was identified for the $PKC_{\beta I}$, $PKC_{\beta II}$, $PKC_{\gamma}$ or $PKC_{\theta}$ isoforms in the HTFs (Fig. 1). Note that the primary antibody replaced with β-actin, and the primary antibody replaced with IgG isotype (positive and negative controls, respectively), are not shown in Fig. 1.

*mRNA expression levels of the PKC isoforms in HTFs*. The results from the 2% agarose gel electrophoresis experiment revealed the presence of mRNAs coding for seven of the

Table I. Primers and reverse transcription-polymerase chain reaction conditions.

| Primer | Sequence (5'→3') | Amplicon (bp) |
|---|---|---|
| PKCα | Forward: ATCCGCAGTGGAATGAGTCCTTTACAT<br>Reverse: TTGGAAGGTTGTTTCCTGTCTTCAGAG | 327 |
| PKCβI | Forward: CTGTGGAACTGACTCCCACTG<br>Reverse: ATACTGAAGCATTTTGGTATC | 404 |
| PKCβII | Forward: GACCGTTTTTCACCCGCCA<br>Reverse: CCATCTCATAGAGATGCTCC | 309 |
| PKCγ | Forward: CACGAAGTCAAGAGCCACAA<br>Reverse: TAGCTATGCAGGCGGAACTT | 233 |
| PKCδ | Forward: CAACTACATGAGCCCCACCT<br>Reverse: GAGGCTCTCTGGGTGACTTG | 189 |
| PKCε | Forward: GATGCAGAAGGTCACTGCAA<br>Reverse: GTCGTCATGGAGGATGGACT | 249 |
| PKCζ | Forward: GTTATCGATGGGATGGATGG<br>Reverse: GCACCAGCTCTTTCTTCACC | 166 |
| PKCη | Forward: GAACAGAGGTTCGGGATCAA<br>Reverse: ATATTTCCGGGTTGGAGACC | 239 |
| PKCθ | Forward: ACAAACAGGGCTACCAGTGC<br>Reverse: ATGCCACATGCATCACACTT | 250 |
| PKCι | Forward: TACGGCCAGGAGATACAACC<br>Reverse: TCGGAGCTCCCAACAATATC | 169 |
| PKC*μ* | Forward: ACGGCACTATTGGAGATTGG<br>Reverse: TGACCACATTTTCTCCCACA | 206 |
| GAPDH | Forward: ACCCAGAAGACTGTGGATGG<br>Reverse: TGCTGTAGCCAAATTCGTTG | 415 |

PKC, protein kinase C.

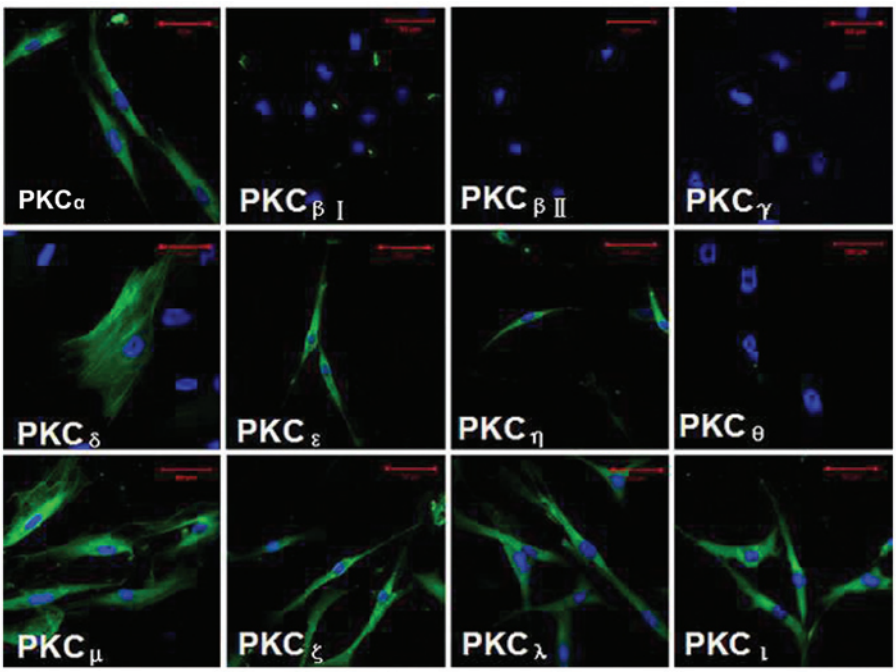


Figure 1. Immunofluorescence staining of the 12 PKC isoforms in HTFs using light-scanning confocal microscopy (magnification, x100). The nuclei of the HTFs were labeled with Hoechst 33342 and appear blue. A green signal indicates the positive staining of the PKC isoforms. PKC, protein kinase C; HTF, human Tenon's capsule fibroblasts. Scale bar, 50 *μ*m.

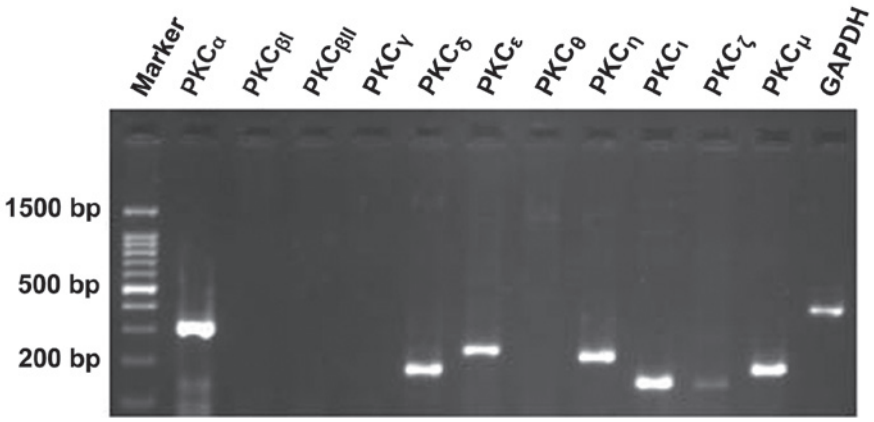


Figure 2. Reverse transcription-polymerase chain reaction analysis of 11 protein kinase C (PKC) isoforms in human Tenon's fibroblasts. GAPDH (415 bp) was used as a control. The molecular size markers are indicated on the left. GADPH, glyceraldehyde-3-phosphate dehydrogenase.

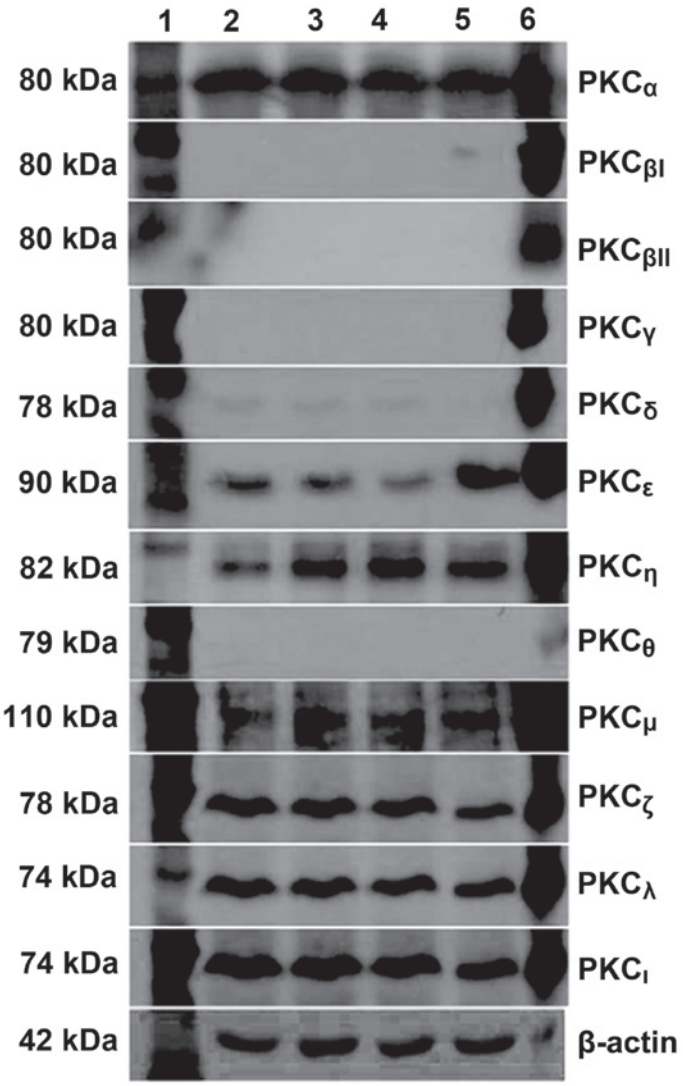


Figure 3. Western blot analysis of the 12 PKC isoforms in HTFs. Each gel was loaded with 40 $\mu$g protein. Lanes 2-5 were HTF protein samples derived from independent cell lines. Lane 6 was a positive control. The markers are indicated on the left. β-actin (42 kDa) was used for protein normalization. PKC, protein kinase C; HTF, human Tenon's capsule fibroblast.

PKC isoforms. $PKC_{\alpha}$ (327 bp), $PKC_{\delta}$ (189 bp), $PKC_{\varepsilon}$ (249 bp), $PKC_{\eta}$ (239 bp), $PKC_{\iota}$ (169 bp) and $PKC_{\mu}$(206 bp) were present at a higher level compared with $PKC_{\zeta}$ (166 bp), which only produced a weak signal. $PKC_{\beta I}$, $PKC_{\beta II}$, $PKC_{\gamma}$ and $PKC_{\theta}$ were not detected at the mRNA level (Fig. 2). GAPDH was used as a positive control.

*Protein expression levels of the PKC isoforms in HTFs*. An analysis of the protein expression levels of the PKC isoforms in HTFs was also performed. Using western blotting, seven PKC isoforms [$PKC_{\alpha}$ (80 kDa), $PKC_{\delta}$ (78 kDa), $PKC_{\varepsilon}$ (90 kDa), $PKC_{\zeta}$ (78 kDa), $PKC_{\eta}$ (82 kDa), PKCι (74 kDa) and $PKC_{\mu}$ (110 kDa)] were observed to be expressed in the HTFs, corroborating the results of the mRNA expression level analysis. In addition, $PKC_{\lambda}$ (74 kDa) was also expressed in the HTFs. However, the other four isoforms, $PKC_{\beta I}$, $PKC_{\beta II}$, $PKC_{\gamma}$ and $PKC_{\eta}$, were not detected (Fig. 3). Lanes 2-5 feature HTF protein lysates derived from independent cell lines. Rat brain lysate was used as a positive control (lane 6) and β-actin (42 kDa) was used for protein normalization.

## Discussion

Scarring is the predominant reason for the failure of GFS. Successful filtration surgery depends directly on an individual's wound-healing response. HTFs are crucially important in this process. Previous studies have revealed that subconjunctival scarring of the filtering bleb site is predominantly mediated by HTF proliferation, migration and contraction (15,16). In order to assess the role of PKCs in the biological function of HTFs, the specific expression of the 12 PKC isoforms were characterized. The present study assessed for the first time, to the best of our knowledge, the expression levels of the 12 PKC isoforms in HTFs at the cellular, mRNA and protein level, using LSCM, RT-PCR and western blotting, respectively.

A similar expression pattern of the PKC isoforms was identified by each of the three methods. Eight of the PKC isoforms, $PKC_{\alpha}$, $PKC_{\delta}$, $PKC_{\varepsilon}$, $PKC_{\zeta}$, $PKC_{\eta}$, $PKC_{\iota}$, $PKC_{\lambda}$ and $PKC_{\mu}$, were expressed in cultured HTFs; however, no expression was observed for the four other isoforms, $PKC_{\beta I}$, $PKC_{\beta II}$, $PKC_{\gamma}$ and $PKC_{\theta}$. With the exception of $PKC_{\lambda}$, the protein expression levels of the other 11 isoforms were consistent with their gene expression levels. A previous report revealed that 8 of 10 of the PKC isoforms were expressed in rat subconjunctival fibroblasts, as determined using western blot analysis, including $PKC_{\alpha}$, $PKC_{\beta}$, $PKC_{\gamma}$, $PKC_{\delta}$, $PKC_{\varepsilon}$, $PKC_{\eta}$, $PKC_{\iota}$ and $PKC_{\lambda}$. $PKC_{\zeta}$ was not examined (17). The differences identified between the two studies are likely to be species-specific.

It has been suggested that isoform-specific functions may be conferred by the subcellular localization of the PKCs. Using immunofluorescence staining, the present study revealed that the subcellular localization of the PKC isoforms occurs predominantly in the cytoplasm of the cells. In addition, $PKC_{\delta}$ was localized specifically to the cytoskeleton, which may be associated with its function in HTFs.

Although the PKC isoforms exhibit very few differences in terms of their structures, substrate preferences, expression and localization, individual PKC isoforms still appear to be tissue- and cell-specific. Epidermal growth factor activates the $PKC_{\alpha}$ phosphorylation pathway to stimulate goblet cell proliferation (18). Hepatocyte growth factor induces the migration of retinal pigment epithelial cells by enhancing the activation of $PKC_{\delta}$ and the phosphorylation of ERK (19). However, the majority of studies to date have focused on the activation of PKC in general (17). Therefore it is necessary to elucidate which PKC isoforms exert specific roles in HTFs proliferation.

In conclusion, the present study demonstrated that 8 of the 12 PKC isoforms were expressed in HTFs at both the protein and the mRNA level. This represents an important step in understanding their precise physiological role, and how they are regulated during the process of proliferation. To elucidate whether one or several of the isoforms are involved in HTF proliferation, due to their role in cellular signal transduction of scarring post-GFS, remains to be elucidated by further experiments.

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