

Original Paper

Microarray Analysis of Dupuytren's Disease Cells: The Profibrogenic Role of the TGF- β Inducible p38 MAPK Pathway

Ivana Ratkaj^a Maro Bujak^b Davor Jurišić^c Mirela Baus Lončar^b Krešo Bendelja^d
Krešimir Pavelić^a Sandra Kraljević Pavelić^a

^aUniversity of Rijeka, Department of Biotechnology, Rijeka; ^b"Rudjer Boskovic" Institute, Division of Molecular Medicine, Zagreb; ^cUniversity Hospital Center Rijeka, Clinic for Surgery, Department for Plastic and Reconstructive Surgery, Rijeka; ^dResearch & Development, Institute of Immunology, Zagreb

Key Words

Dupuytren's Disease • Myofibroblasts • Differentiation • p38, Akt • CD90

Abstract

Background: Dupuytren's disease (DD) is a nodular palmar fibromatosis that causes irreversible permanent contracture of fingers and results in the loss of hand function. Surgery still remains the only available solution for DD patients but cannot permanently cure the disease nor reduce high recurrence rates. With this rationale, we designed a study aimed at an improved understanding of the molecular mechanisms underlying DD. Our major focus was an analysis of the global gene expression profile and signalling pathways in DD cells with the aim of identifying novel biomarkers and/or therapeutic targets. **Methods:** Primary cells were cultured from surgically removed diseased and healthy tissue. Microarray expression analysis (HG-U133A array, Affymetrix) and qPCR was performed with total RNA isolated from primary DD cells. Mechanistic studies involving inhibition of p38 phosphorylation were performed on normal human fibroblasts' and primary DD cells' *in vitro* models. Expression of stem cell markers in primary fibroblasts/myofibroblasts was assessed as well. **Results:** We identified 3 p38MAPK signalling pathway regulatory genes, THBS1, GADD45 α and NUA1, all involved in cellular proliferation and production of the extracellular matrix proteins. Inhibition of the p38MAPK signalling pathway induced down-regulation of myofibroblast markers, α -smooth muscle actin and palladin. A stem-cell like subpopulation positive for CD90 marker was identified among primary DD cells. **Conclusion:** The study reveals involvement of the p38 MAPK pathway as a possible signalling cascade in the pathogenesis of Dupuytren's disease. Moreover, a particular stem cell-like CD90⁺ subpopulation was identified that might contribute to DD development.

Copyright © 2012 S. Karger AG, Basel

Introduction

Fibrotic changes can affect all tissues [1] leading to an impairment of organ function and structure caused by excessive deposition of extracellular matrix (ECM) produced by activated fibroblasts [2]. Dupuytren's disease (DD) is one of the most common fibroproliferative disorders, characterized as a nodular palmar fibromatosis that causes irreversible permanent contracture of fingers and results in the loss of hand function [3]. Similar to other fibrosis, the key molecular event that triggers the onset of disease is an aberrant proliferation of fibroblasts and their differentiation into α -smooth muscle actin (α -SMA) producing myofibroblasts [4]. Accumulation of myofibroblasts and their persistence after wound healing are believed to be the major inducers of structural deformations associated with fibrotic processes and fibroproliferative disorders in general [5-9]. Determining the cause or the underlying pathogenesis of DD would benefit the discovery of new treatment options. Indeed, surgical excisions are a gold treatment standard but are not a permanent solution, require a substantial patient effort during the post-operative period and end up with a high recurrence rate [10, 11].

A number of studies indicate that growth factors control the cellular growth of miofibroblasts. The role of several so called fibrogenic cytokines, i.e. FGF, IL-1, TGF- β and PDGF, has been proved in DD pathogenesis [12, 13]. Unlike several other fibrotic conditions, the origin of myofibroblasts in Dupuytren's disease has not been established yet. Recent hypotheses on the mechanism of fibrotic processes are specifically focused on the research of fibroblast origin that points to multipotent cell lineage. Precursors that express hematopoietic stem cell features with a fugacious expression of myeloid markers give rise under favourable conditions to various progenitor cells including mesenchymal cells known as fibrocytes [14]. Fibrocytes, as a circulating subpopulation of bone marrow derived cells, express the hematopoietic CD34 marker, have fibroblast-like properties [15] and are able to acquire a contractile phenotype and differentiate into myofibroblasts [16, 17]. They are known to drive the onset of various fibroses such as pulmonary, kidney and dermal fibrosis [18-22]. The complexity of fibroproliferative disorders like DD has been partly elucidated by a global approach using microarray analysis of gene expression of either primary DD cells or DD tissue samples [23-26]. These studies identified the expressional modulation of genes involved in cell proliferation/growth, apoptosis and lipid metabolism but failed to mechanistically validate the obtained results and propose novel biomarkers and/or therapeutic targets.

With still a great void in the insights of mechanistic hypotheses on disease progression, we focused on the global profiling of gene expression mechanisms in DD cells with the aim of identifying the key processes and signalling pathways underlying DD pathogenesis and the development of symptoms. The paper therefore presents the transcriptomic profiling results for primary DD cultures from affected vs. non-affected palmar fasciae. The results point to a role of specific signalling pathways driven by the p38 kinase in DD cells that might account for disease progression that was validated on a representative number of DD samples.

Materials and Methods

Primary cell cultures

Clinical specimens were collected at the Clinical Hospital "University Hospital Centre Rijeka" in Croatia in strict compliance with the clinic's chief pathologist and the ethics committee for research involving human subjects, with the informed consent of all patients and in compliance with EU laws. The cells were isolated according to established protocols [27, 28]. Primary DD cells were obtained from affected palmar fascia localized in the palmodigital and digital area (D). Patient matched control cell samples were cultured from macroscopically unaffected fascia that was immediately adjacent to the disease tissue (ND). Tissue samples

were collected from 45 patients (age range 54 to 75 years, males, including smokers and patients with diabetes as two common predisposing factors for DD) diagnosed with the last stage of DD. First and second passages were used for all experiments to assure uniformity and prevent loss of original cell phenotype.

Microarray data analysis and quantitative real-time PCR (qPCR)

Total RNA was isolated from primary D and ND cell samples by RNeasy spin columns (Qiagen) and processed for microarray analysis according to the protocol (Affymetrix, microarray HG-U133A). The results were uploaded in the Gene Expression Omnibus database, NCBI (reference number GSE31356) and analyzed by Affymetrix software Microarray Suite 5.0 (MAS5). Analysis of differentially expressed genes in D cells in comparison with ND cells was performed using GeneSpring GX9 software from Agilent (5 summarization algorithms, t-test paired, cut-off value of fold change >2, $p < 0.05$), Gene Ontology and GenMAPP (Gladstone Institutes, San Francisco, CA, USA).

Differentially expressed genes were monitored further by qPCR analyses of additional 16 patient samples. RNA (1.5 μg per sample) was reversely transcribed into cDNA by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) on GenAmp PCR System 2400 (Applied Biosystems). TaqMan Universal PCR Master Mix (Applied Biosystems) was used and fluorescence detection was performed on AB 7300 Real-Time PCR System (Applied Biosystems). Expression analysis of a panel of 11 fibrotic genes was done on a total RNA (1.5 μg per sample) isolated from ND cells treated with TGF- β 1 and TGF- β 1 together with p38 inhibitor (RNAeasy kit; Qiagen), by SYBR Green-based qPCR. TaqMan assays used are Hs 01046520_m1 for ABLIM1, Hs 00265081_s1 for ADRA2A, Hs 00214395_m1 for ASPN, Hs 00176121_m1 for BDKRB2, Hs 00189021_m1 for CALD1, Hs 00175188_m1 for CTSC, Hs 00355783_m1 for ELN, Hs 00197774_m1 for FBLN1, Hs 00169587_m1 for GADD45 α , Hs 00254699_m1 for GREM2, Hs 00220138_m1 for LXN, Hs 00921945_m1 for MBP, Hs 00185803_m1 for MFAP5, Hs 00981464_m1 for MYLK, Hs 00819630_m1 for NR2F2, Hs 00934234_m1 for NUA1, Hs 00153133_m1 for PTGS2, Hs 00170236_m1 for THBS1 and for the housekeeping genes Hs 99999905_m1 for GAPDH and Hs 99999901_s1 for s18. Primer pairs are presented in Table 1. All samples were done in triplicate and normalized with the most stable control gene. The results were processed with REST t-384-beta and REST 2008 V2.0.7 [29].

Treatment with TGF- β 1 and inhibitor of p38 phosphorylation

Normal skin fibroblasts were treated with TGF- β 1, prepared according to the manufacturer's recommendation (concentration: 3 ng/mL in serum-free medium) (R&D Systems), as a model for validation studies. The cells were deprived of serum for 24 hours prior to treatment that lasted from 2-96 hours. Gene expression was monitored by qPCR, and protein expression was analysed by Western blot and immunocytochemistry. In the same manner as with normal skin fibroblasts, we treated primary ND cell cultures with TGF- β 1 for 16 hours or co-incubated them with TGF- β 1 and an inhibitor of p38 phosphorylation. The inhibitor is a commercially available competitive inhibitor of ATP binding SB203580 (Calbiochem). Stock solutions of SB203580 were prepared in DMSO and diluted in a serum-free medium to a concentration of 10 μM . Total RNA was isolated from the treated ND cell samples to detect the expression of fibrotic genes, while protein lysates were used to ascertain the status of MK2 activation.

Western blot analysis

The cells were lysed in a buffer containing 50 mM Tris HCl (pH 8), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, a protease inhibitor cocktail (Roche) and halt phosphatase inhibitor cocktails (Thermo Scientific). A total of 35 μg of proteins were resolved on 9% SDS polyacrylamide gels using the Mini-protean cell (Bio-Rad), and analysis was performed by a previously established procedure [30]. The membranes were incubated with primary antibodies raised against palladin (1:700, antibody was a kind gift from Mikko Rönty, Department of Pathology, University of Helsinki, Finland), α -SMA (2 $\mu\text{g}/\text{mL}$, α -smooth muscle actin, Sigma), phosphorylated p38 (1:1000, phospho-p38 (Thr180/Tyr182) mouse mAb, Cell Signaling), phosphorylated Akt (1:800, phospho-Akt (Ser473) rabbit mAb, Cell Signaling), and phosphorylated MAPKAPK-2 (1:1000, phospho-MAPKAPK-2) at 4 $^{\circ}\text{C}$ overnight. Secondary antibodies linked to anti-mouse (Dako, 1:1000) or anti-rabbit (Dako, 1:1300) horseradish peroxidase were used. The signal was visualized by Western Lightening Chemiluminescence Reagent Plus Kit (Perkin Elmer, USA) on

Table 1. Detailed list of primer pairs used for the qPCR reaction. Housekeeping genes are marked with an asterisk (*).

Gene		Sequence 5'-3'	size	Conditions
Thrombospondin-1 (THBS-1)	For	CCC GTG GTC ATC TTG TTC TGT	133bp	59°C 3.5mM MgCl ₂
	Rev	TTT CTT GCA GGC TTT GGT CTCC		
Collagen 1A1 (COL1A1)	For	TGG CCC AGA AGA ACT GGT ACA TCA	87bp	60°C 3 mM MgCl ₂
	Rev	ACT GGA ATC CAT CGG TCA TGC TCT		
Rho GDP- dissociation inhibitor alpha (ARHGDI A)	For	CAG GAA AGG CGT CAA GAT TG	81bp	59°C 3 mM MgCl ₂
	Rev	GTC AGG AAC TCG TAC TCC TC		
Palladin (PALLD)	For	ATG GAT TGA GAC TGC ATG GTG GCA	182bp	60°C 3.5mM MgCl ₂
	Rev	GCA AAG TTT CAT GGC AGC AGG GAA		
Human Epidermal Growth Factor Receptor 2 (ERBB2)	For	GTG GCC ATC AAA GTG TTG AGG GAA	166bp	60°C 3.5 mM MgCl ₂
	Rev	AGC CAT AGG GCA TAA GCT GTG TCA		
Procollagen III A (PROCOLIIIA)	For	TGG CTA CTT CTC GCT CTG CTT	132bp	60°C 3.5mM MgCl ₂
	Rev	CGG ATC CTG AGT CAC AGA CAC A		
α -smooth muscle actin (α -SMA)	For	AAA CAG GAA TAC GAT GAA GCC GGG	160bp	60°C 3.5 mM MgCl ₂
	Rev	ACG AGT CAG AGC TTT GGC TAG GAA		
Fibronectin (FN)	For	GGA GAA TTC AAG TGT GAC CCT CA	73bp	60°C 3mM MgCl ₂
	Rev	TGC CAC TGT TCT CCT ACG TGG		
Fibronectin extra type III domain A (EDA)	For	AGG ACT GGC ATT CAC TGA TGT G	86bp	60°C 3m M MgCl ₂
	Rev	GTC ACC CTG TAC CTG GAA ACT TG		
Fibronectin extra type III domain B (EDB)	For	GGT GGA CCC CGC TAA ACT C	67bp	60°C 3.5 mM MgCl ₂
	Rev	ACC TTC TCC TGC CGC AAC TA		
Insulin-like growth factor type I receptor (IGFR1)	For	TGA AAG TGA CGT CCT GCA TTT C	75bp	60°C 3 mM MgCl ₂
	Rev	CGG TGC CAG GTT ATG ATG ATG		
18S rRNA*	For	GTA ACC CGT TGA ACC CCA TT	151bp	60°C 3.5mM MgCl ₂
	Rev	CCA TCC AAT CGG TAG TAG CG		

the VersaDoc Imaging System 4000 (Bio-Rad). α - tubulin was used as a loading control (1:1000, Sigma). The signal intensities of particular bands were normalized with the intensity of the loading control and compared in Quantity One software (Bio-Rad, USA). The values are expressed as the average \pm standard deviation. For statistical comparisons of the two groups, a paired, two-tailed Student's t-test was used. All of the relevant comparisons were considered to be significantly different at $p < 0.05$.

Table 2. Results of microarray gene expression analysis. List of 18 differentially expressed genes among primary cell cultures isolated from patient-matched diseased (D) and healthy tissue (ND) of DD patients obtained by microarray analysis (Affymetrix, HG-U133A). Gene expression for 10 out of 18 studied genes was additionally validated by qPCR (bold and marked with asterisk, $p=0.05$). (NSA – not significant). (statistically significant at $p<0.05$ (*)).

Gene symbol	Expression status in D	Fold change obtained
ARP1	UP	NS
ELN*	UP	7
GREM2*	UP	2.1
PTGS2	DOWN	NS
BDKRB2	DOWN	NS
GADD45 β *	UP	1.5
CALD1*	UP	1.5
FBLN*	DOWN	1.8
MFAP5	UP	NS
MYLK*	UP	1.8
THBS1*	UP	3.2
NUAK1*	UP	1.8
CTSC	UP	NS
ABLIM1*	DOWN	1.5
ADRA2A	DOWN	NS
MBP*	UP	2
LXN	DOWN	NS
ASPN	UP	NS

Immunocytochemistry

The cells were seeded (4×10^4 per well) in chamber slides (Lab-Tk II Chamber slide Nunc), washed with PBS, fixed and permeabilized with cold methanol for 8 minutes at -20°C . After fixation and incubation with 7% goat blocking serum (Dako), the cells were incubated with primary antibodies raised against palladin (1:100), α -SMA (5 $\mu\text{g}/\text{mL}$), phosphorylated p38 (1:100) and phosphorylated Akt (1:70) overnight at $+4^\circ\text{C}$. Incubation followed with biotinylated secondary antibody (biotinylated anti-mouse IgG and biotinylated anti-rabbit IgG, Dako, dilution 1:500) and streptavidin/FITC conjugated tertiary antibody (Streptavidin/FITC, Dako). For visualisation, the cells were incubated with propidium iodide for 5 min (50 ng/mL), and fixed with 10% solution of glycerol in PBS. Fluorescence was detected using an OLYMPUS DP50 fluorescent microscope (magnification 400X).

Flow cytometry

Flow cytometric analysis was performed using 300,000 viable cells derived from a total of 9 DD patients. The cells were washed with staining buffer (PBS with 0.1% NaN_3 , 1% FSC and 2mM EDTA), incubated with anti-human HESCA-1 primary antibody (Millipore) for 15 min at room temperature, washed again and incubated with goat anti-mouse IgG APC-conjugated secondary antibody (Invitrogen) for 15 min at room temperature. Alternatively, cells were incubated directly with CD34 PE-Cy7-conjugated antibody (BD Pharmingen), CD117 PE-Cy5-conjugated antibody (PD Pharmingen) and CD90 FITC-conjugated antibody (BD Pharmingen) simultaneously for 15 min at room temperature. For intracellular staining, cells were washed and incubated with fixation buffer (4% formaldehyde in PBS) for 30 min on ice, washed again with staining and permeabilization buffer (0.1% NaN_3 , 1% FSC, 0.1% saponine) and incubated with anti-human SMA PE-conjugated antibody (R&D Systems) for 30 min on ice. After incubation and washing, the cells were resuspended in staining buffer. Multiparametric analysis was performed by Becton Dickinson LSR II flow cytometer (BD Biosciences) acquiring 200,000 cells per sample. The data were analyzed with BD FACSDiva Software version 5.0.3 (BD Biosciences) and statistical differences were determined using a Student's two-tailed, paired t-test.

Fig. 1. Results of qPCR gene expression analyses. Microarray gene expression was additionally analysed on 16 primary patient-matched cell samples. Expression was confirmed for 10 presented genes (fold change = 2, $p < 0.05$).

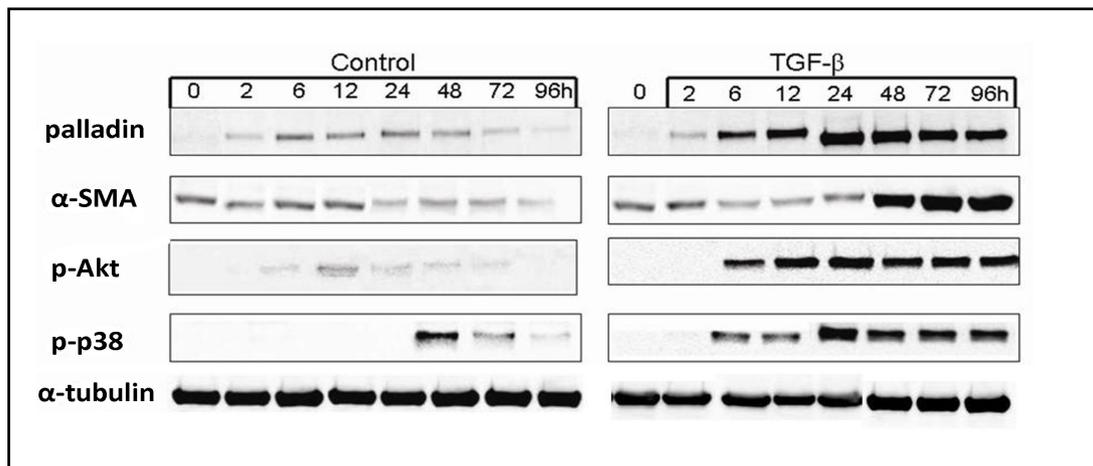
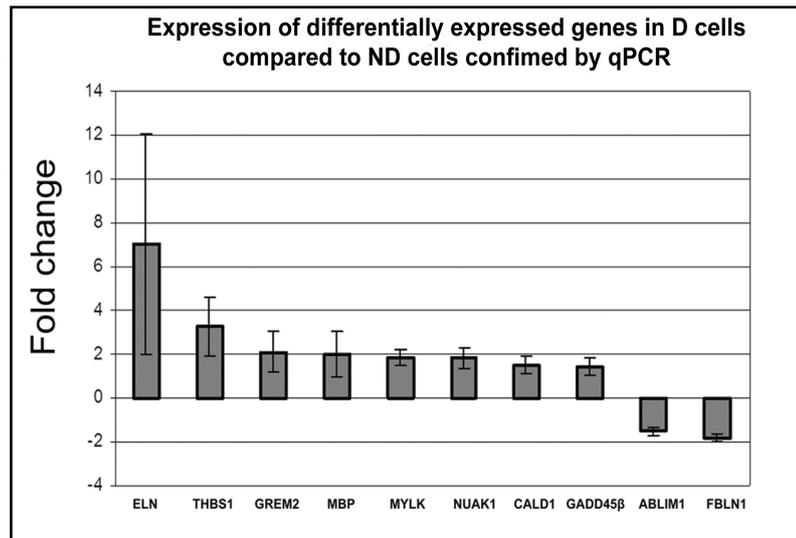


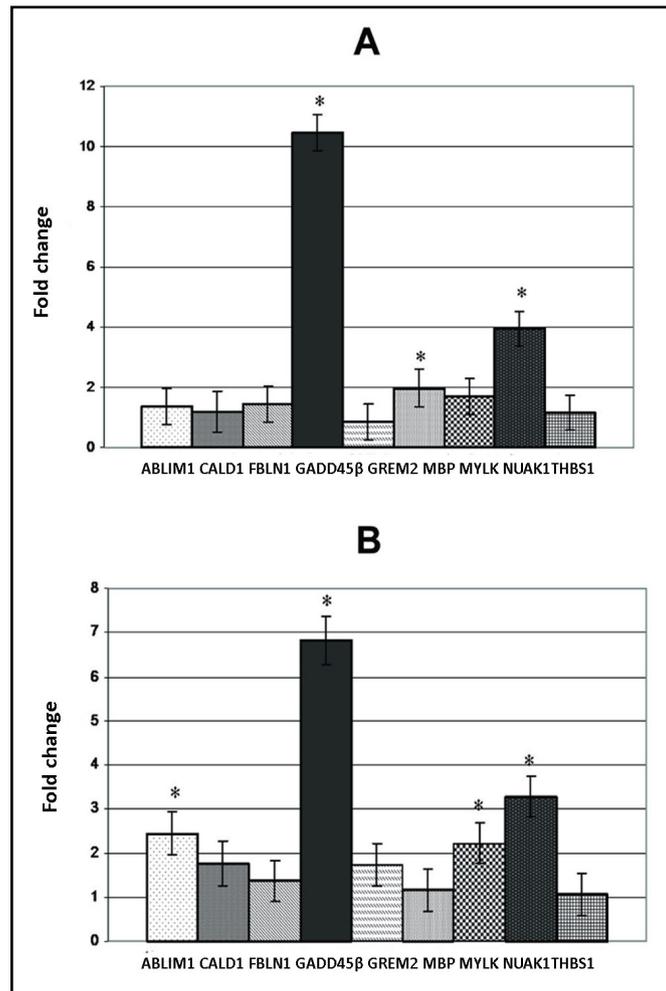
Fig. 2. Analysis of myofibroblasts markers (palladin and α -SMA) expression and activation of p38 and Akt kinases in normal skin fibroblasts upon 96 hours treatment with TGF- β 1. The expression of α -tubulin was used as loading control.

Results

Modulation of gene expression detects possible involvement of p38MAPK in profibrotic processes

Transcriptomic analysis of primary DD cells by Affymetrix Whole genome platform revealed 18 differentially expressed genes between the D cells obtained from affected fasciae and the ND cells derived from the patient-matched unaffected fasciae (Table 2). Part of the results related to cytoskeleton changes in the DD cells that are not discussed here are presented elsewhere [31]. The expression patterns of ten genes were further validated by qPCR. In particular, up-regulation of genes coding for elastin (ELN), caldesmon 1 (CALD1), gremlin 2 (GREM2), growth arrest and DNA-damage-inducible beta (GADD45 β), myelin basic protein (MBP), thrombospondin 1 (THBS1), myosin light chain kinase (MYLK) and NUAK family SNF1-like kinase 1 (NUAK1) was confirmed, with a concomitant decline in the expression level of genes coding for fibulin 1 (FBLN1) and actin binding LIM protein 1 (ABLIM1) (Table 2, Fig. 1). While over-expression of genes coding for proteins that have a dominant role in ECM regulation, like ELN, or that regulate actin dynamics, namely MYLK and

Fig. 3. Influence of TGF- β 1 treatment of normal skin fibroblasts on the expression of 10 microarray confirmed genes after 2 hours (A) and 6 hours (B). Genes GADD45 β and NUAK1 had the highest increase in expression after 2 and 6 hours of treatment. Statistically significant changes (fold change = 2, $p < 0.05$) are marked with an asterisk (*).

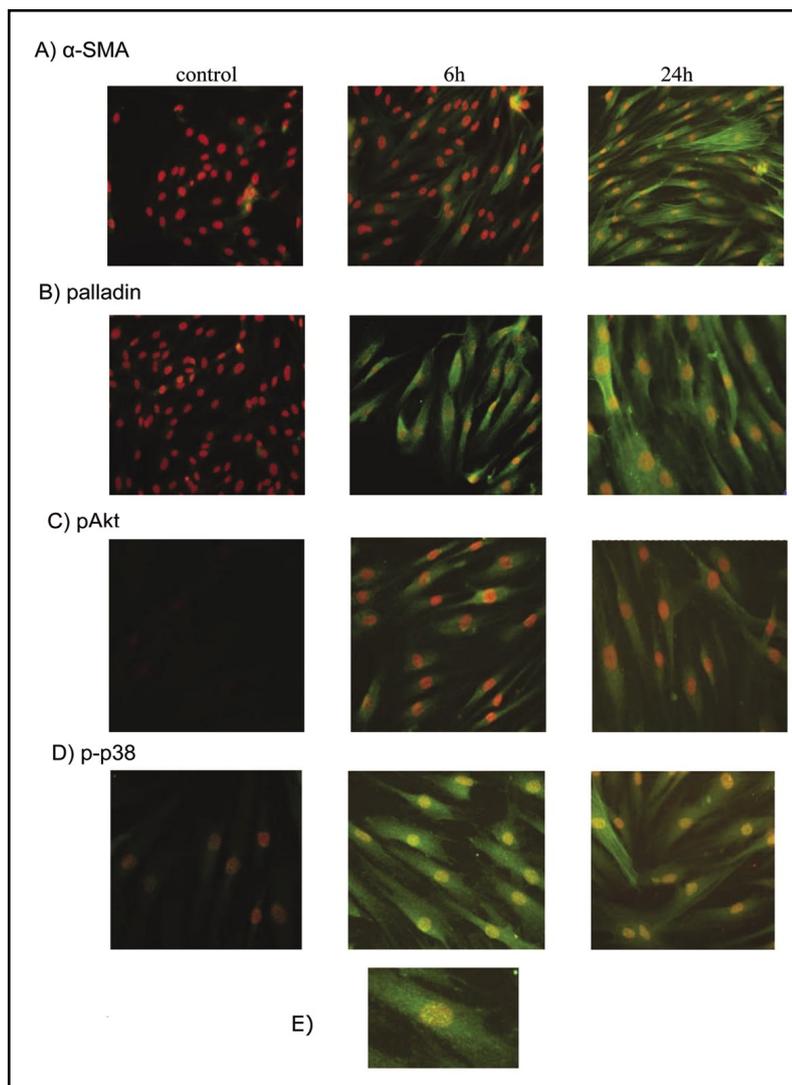


CALD1, is consistent with previous studies, over-expression of NUAK1, GADD45 β and THBS1 was of particular interest. THBS1 is an ECM protein [32] that can activate latent TGF- β [33], an already established important modulator of fibroblast differentiation [34-36] initiating cell signals that enable mitogen activated protein kinases such as p38 [37, 38]. Over-expression of THBS1 accompanied with increased expression of GADD45 β , a well-known regulator of p38 activity and consequently p38-regulated biology [39-41], led us to further investigate the p38 role in fibrogenesis on the *in vitro* models of normal skin fibroblasts treated with TGF- β 1 and primary DD cell cultures.

Activation status of p38 and Akt kinases during differentiation of normal skin fibroblasts treated with TGF- β 1

Based on microarray data that pointed to an important involvement of p38 MAP kinase pathway in the fibrogenic processes, we established an *in vitro* model of normal skin fibroblasts treated with TGF- β 1. This model allowed us to monitor the activation status of p38 during the differentiation process of fibroblasts to myofibroblasts. The differentiation status was determined through the expression of well-established myofibroblast markers, α -SMA and palladin. A marked increase in the palladin and α -SMA expression was detected 6 and 48 hours upon treatment with TGF- β 1, respectively (Fig. 2). Activation of p38 kinase was consistent with the expression dynamics of palladin. P38 (Fig. 2) kinase was phosphorylated after 6 hours of treatment, indicating that its activation occurs at the beginning of differentiation. The expression patterns of 10 confirmed genes during differentiation were also examined. Quantitative PCR analysis annotated that TGF- β treatment caused a 10 fold increase in the

Fig. 4. Immunocytochemistry analysis of normal skin fibroblasts treated with TGF- β 1. Normal skin fibroblasts were treated with TGF- β 1 for 6 and 24 hours. The green colour represents the expression of A) α -SMA, B) palladin, C) pAkt and D) p-p38 (nucleuses are coloured in red). Nuclear localization of p-p38 might be observed as yellow spots in nucleus, E) magnified picture indicating p-p38 nuclear localization (cells were photographed at 400x magnification).



expression of GADD45 β and a 4 fold increase in NUA1-1 after 2 hours (Fig. 3A), followed by a large increase after 6 hours of treatment as well (Fig. 3B). Since NUA1 codes for a kinase that is directly activated by Akt kinase [42, 43], we decided to examine the phosphorylation status of Akt during differentiation. The results reveal that Akt is phosphorylated after 6 hours of treatment (Fig. 2). The detected changes in myofibroblast markers expression and kinase activation were also confirmed by immunocytochemistry (Fig. 4), which additionally revealed that phosphorylated p38 had a nuclear localisation (Fig. 4E). The fibrotic *in vitro* model confirmed the importance of p38 and Akt kinases during the differentiation changes. Therefore, the next experimental step of our research was the examination of the identified fibrogenic profile in primary DD fibroblasts.

Phosphorylation status of p38 kinase in primary cell cultures and the influence of its inhibition on the activation status of Akt kinase and the expression of myofibroblast markers

We set out to determine if cells cultured from affected fascia (D) have an increased level of activated p38 and Akt kinases compared with cells cultured from healthy tissue samples (ND). We observed higher phosphorylation levels of p38 and Akt kinases in the D

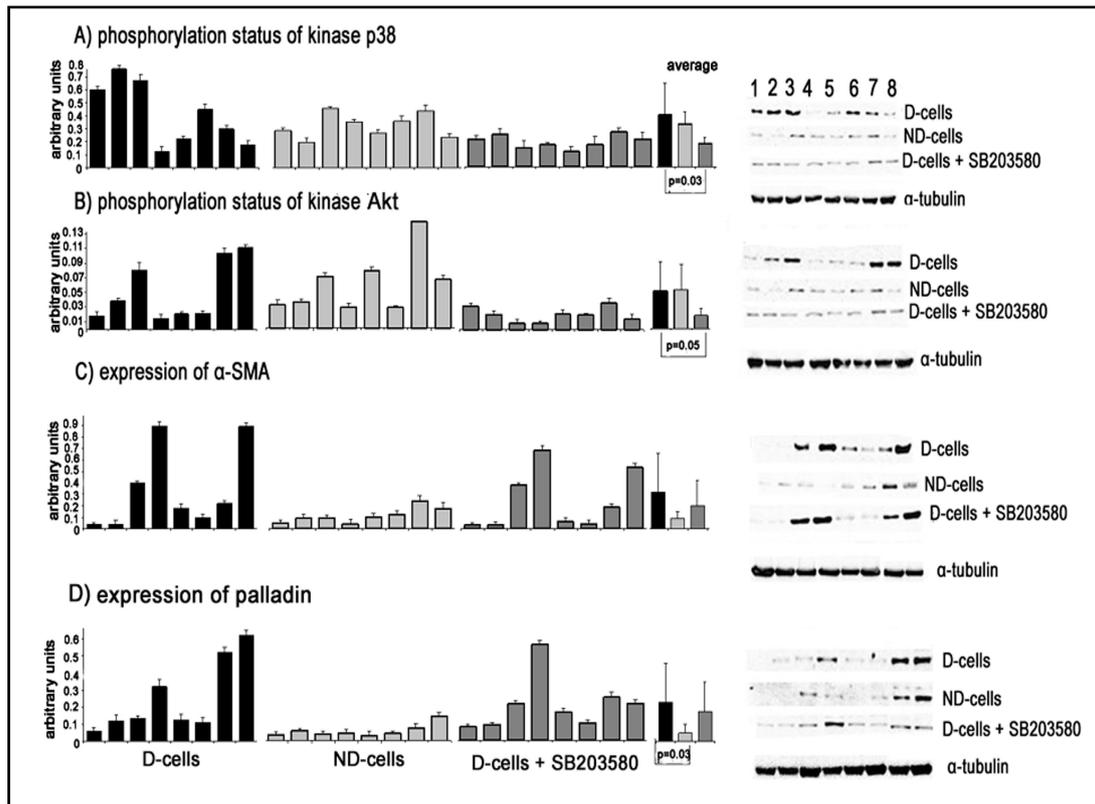
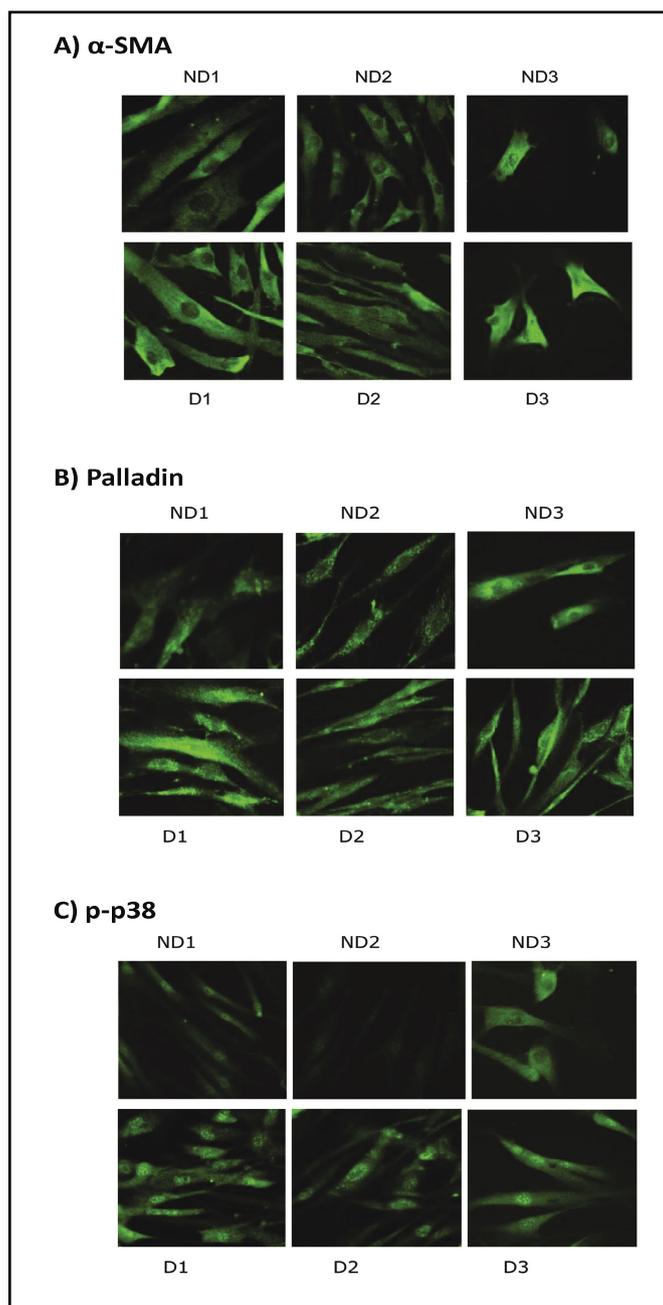


Fig. 5. Western blot analysis of primary DD cell cultures. Phosphorylation status of kinases A) p-p38 and B) pAkt as well as expression of myofibroblast markers C) α -SMA and D) palladin, in patient-matched primary cell cultures. D cells were additionally treated with an inhibitor of p38 phosphorylation, SB203580. Quantification and densitometric analyses of signals were normalized with the intensity of the loading control (α -tubulin). Protein expression level and activation status is shown for primary cell samples isolated from 8 diseased tissues (D) (black columns) and patient-matched healthy tissues (ND) (light gray), as well as D cells treated with SB203580 inhibitor (dark gray), represented by an average value as well. Results indicate that the p38 phosphorylation inhibitor SB203580 diminished levels of p-p38 in D cells, leading to a concomitant decrease in the expression of myofibroblasts markers and pAkt. Statistically significant changes are indicated with a p-value.

cells than in the control ND cells (Fig. 5). The myofibroblast markers, α -SMA and palladin, were over-expressed in the D cells as well (Fig. 5). Immunocytochemistry additionally proved these changes both in (D) and (ND) cells (Fig. 6). A prominent nuclear localisation of phosphorylated p38 was observed in the D cells. Taken together, these results speak in favour of the substantial role of the p38 kinase in pro-fibrogenic changes of DD cells. This result was mechanistically confirmed by use of a specific inhibitor of p38 kinase phosphorylation. The inhibitor SB20358 efficiently inhibited the activation of p38 kinase, which resulted in diminished activation of Akt kinase along with lowered expression of α -SMA and palladin (Fig. 5). Additionally, the SB203580 inhibitor decreased the expression of important fibrogenic genes, including THBS1, fibronectin (FN and FN-a) and collagen 1a (COL1A) (Fig. 7). The results, along with previously published data, prompted us to propose that a combination of cytokines intertwined with the activation of powerful p38 MAPK signalling cascades plays an important role in the onset of DD. Inhibition of p38 readily diminished the activation of Akt kinase, myofibroblasts markers, as well as down-regulates the expression of genes involved in fibroproliferative changes.

Fig. 6. Immunocytochemistry of primary DD cells isolated from diseased tissue (D) and patient-matched healthy tissues (ND). The expression of α -SMA (A), palladin (B) and p-p38 (C) is visible as a green colour. Among the studied DD cell samples, D cells had a higher expression of myofibroblasts markers (α -SMA, palladin) and p-p38 with prevailing nuclear localization. The cells were photographed at 400x magnification.



Specific subpopulation of CD90⁺ mesenchymal stem cells in the palmar region of DD patients might account for fibrosis

Microarray data revealed the over-expression of bone morphogenetic protein antagonist GREM2, known to inhibit differentiation and promote the self-renewal of stem cells in some stem-cell niches [44], and myelin basic protein (MBP), whose related transcripts are found in CD34⁺ bone marrow cells [45]. These results speak in favour of the stem cell like characteristics of DD myofibroblasts and prompted us to further analyses of stem cell markers.

Several studies showed that a substantial population of the tissue fibroblasts recruited to the site of injury are derived from the hematopoietic component of marrow [18, 46]. We therefore analysed the expression levels of typical mesenchymal stem cell markers of primary D and ND cells (Table 3). A lower percent of D cells (14%) than ND cells (23%) were positive for the CD34 marker. The opposite trend was noticed with the CD117 marker, where D cells expressed 2 percent more CD117 than ND cells. Surprisingly, about 1% of

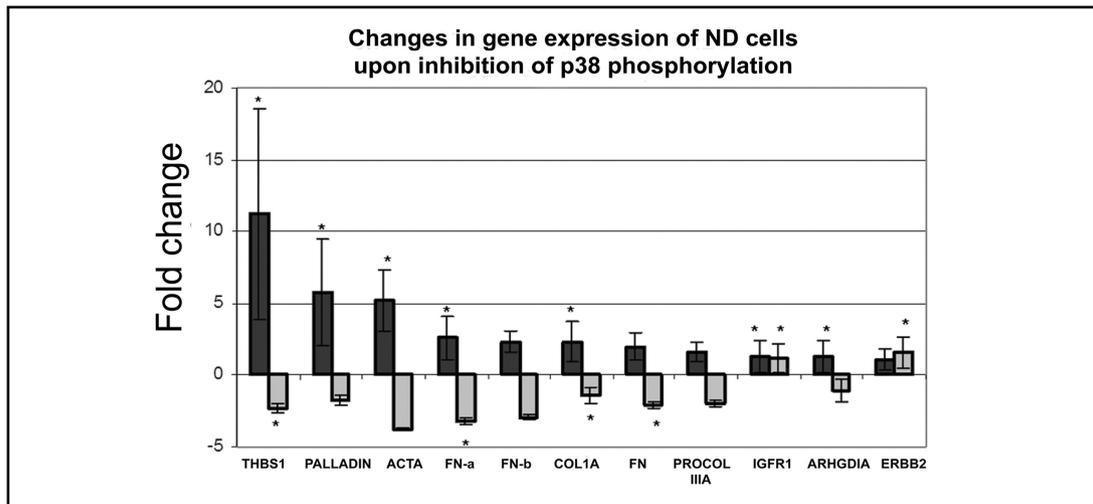


Fig. 7. Changes in gene expression of ND cells upon inhibition of p-p38 assessed by qPCR. The columns represent expression levels of cytoskeleton and ECM-regulating genes in ND cells treated with TGF-β1 (black columns) or TGF-β1 and SB203580 (grey columns) for 16 hours. Co-incubation with the inhibitor of p38 phosphorylation SB203580 caused down regulation of several studied genes. Statistically significant changes (fold change = 2, p=0.05) are marked with an asterisk (*).

Table 3. Expression of stem cell markers in primary DD cells. Cells grown from diseased (D) and healthy patient-matched tissues (ND) of DD were analyzed. Expression of CD34 was significantly lower in D cells than in ND cells. A subpopulation of CD90⁺ cells was identified that exerted a significantly higher expression of CD34, CD117 and HESCA-1 markers compared with the CD90⁻ cell population. (statistically significant at p=0.05 (*)).

Marker	Percentage of cells (% ± standard deviations)	
	D	ND
CD90	83.92±26.24	95.13±4.14
CD34	13.8±11.1*	23.21±19.02 *
CD117	4.29±5.08	2.43±2.01
HESCA-1	0.93±0.5	0.82±0.34
	CD90⁻ /CD90⁺	CD90⁻ /CD90⁺
CD34	1.04±1.6/12.8±12.05 *	0.58±0.96/22.8±18.85 *
CD117	0.48±0.79/2.1±1.2 *	0.18±0.22/2.6±3.06 *
HESCA-1	0.26±0.3/0.7±0.33 *	0.08±0.07/0.74±0.38 *

cell populations were positive for the highly specific stem cell marker HESCA-1. Among the tested cells, the specific stem cell-like CD90⁺ subpopulation contained a statistically higher percentage of CD34, CD117 and HESCA-1 markers compared with CD90⁻ cells. Altogether, the results revealed the presence of a specific subpopulation of cells derived from bone marrow in the affected palmar fascia of DD patients that might be important for fibrotic processes in general.

Discussion

Fibroproliferative diseases emerge from complex cellular events that lead to an accumulation of fibroblasts and an augmented deposition of the ECM proteins. A large

amount of research over the last thirty years [1, 47] indicate that fibrotic disorders often share common mechanisms and have a severely detrimental affect on human health. Although fibrotic tissue remodelling can be associated with neo-angiogenesis, which is of particular importance for cancer development [48], there is still no adequate treatment that directly targets the specific mechanisms of fibrotic processes.

DD is a common type of fibrosis affecting hand functions. Recent reports indicate that approximately 25 % of men over the age of 60 who are of North European origin are affected by this disease [49], which stresses the growing need to find a non-surgical solution that will stop the proliferating process that causes a high recurrence rate.

A number of studies have confirmed the activation of fibroblasts by TGF- β and other cytokines to be the major mechanism driving fibrotic processes in DD and other fibroses [35]. Transcriptomic studies of DD have reported on specific gene expression patterns that might be correlated with molecular events characteristic of DD [23-26] or even with other fibrotic disorders such as liver fibrosis [50]. Our transcriptomics results revealed an over-expression of genes that code for important signalling proteins associated with the activation of TGF- β , abundantly expressed in DD [12, 51], and the consequent ECM protein expression driven by p38 and Akt phosphorylation, namely THBS1, GADD45 β and NUA1 genes. Additionally, like previous studies, we found similar expression patterns of the ECM and myosin regulatory genes [25, 31]. Our previously published proteomics results proved the profibrogenic role of the phosphatidylinositol 3-kinase (PI3K)-Akt c-Jun N-terminal kinase signalling pathway in the onset of DD [30]. Direct interaction between Akt and NUA1 protein, coded by the gene that was over-expressed in primary D cells, may lead to impaired apoptosis as NUA1 inhibits caspase 8 and stimulates cell proliferation [42, 43, 52]. Finally, GADD45 β , a well-known regulator of the TGF- β -induced p38 activation [39, 40], is the primary TGF- β -responsive gene in normal human mammary epithelial cells that can mediate G₂/M progression and apoptosis [40, 41].

These three newly identified up-regulated genes, namely THBS1, GADD45 β and NUA1, induce activation of p38 and Akt kinases and have important roles during proliferation and differentiation of fibroblasts into myofibroblasts. Activated p38 and Akt kinases have numerous possible downstream targets driving the cell proliferation and the ECM deposition [53-56] and the importance of the p38 MAP kinase signalling pathway has been confirmed in other fibrotic diseases [57-62]. Similarly to our results, a recent study of molecular mechanisms of the DD pathogenesis has shown a connection between increased expression of TGF- β and the activation of another MAP kinase ERK 1/2, [63]. Moreover, we observed nuclear localization of p-p38, showing that activated p38 translocates to the nucleus of DD fibroblasts, which is in accordance with previous research [64, 65], where it can activate diverse targets in the cell, e.g. it may interact with transcriptional factors regulating the expression of α -SMA and ECM proteins [55]. Indeed, higher levels of phosphorylated p38 and Akt kinases along with typical myofibroblast markers were found in primary D cells than in ND cells, which is in agreement with our previously published data [27]. Moreover, we propose that the receptor tyrosine kinase, CD117, that we found to be over-expressed in D cells, may contribute to cell proliferation in DD as well. This receptor is linked with the activation of mitogen activated kinase and PI-3 kinase pathways resulting in proliferation and antiapoptotic signalling [66]. Abolishment of these processes driven by activation of p38 and Akt kinases was successfully obtained upon treatment of DD cells with the SB203580 inhibitor of p38 phosphorylation, which is in accordance with similar studies [67]. Additionally, co-treatment of ND cells with TGF- β and the SB203580 inhibitor induced pronounced down-regulation of several fibrotic genes (THBS1, fibronectin genes and COL1A1).

The microarray results showing over-expression of GREM2 and MBP genes prompted us to investigate stem cell features of DD cells and their possible origin. We examined the expression of hematopoietic stem cell marker CD34, CD117, CD90 and HESCA-1 in primary DD cell cultures. As expected, we detected a lower expression of CD34⁺ and CD90⁺ markers in D cells than in ND samples. Since CD34⁺ fibroblasts, present in many organs [68], are thought to represent uncommitted cells capable of multidirectional mesenchymal differentiation,

our results support the findings that D cells had already commenced the differentiation process [69] during which expression of CD34 is reduced. Thus it might be hypothesized that the loss of CD34 might be in correlation with an accentuated terminal differentiation of cells into myofibroblasts, which is typical of DD pathogenesis. Similarly, CD90 is a marker known to be involved in various biological processes, such as migration, the formation of actin stress bundles and the differentiation of cells into myofibroblasts. In particular, CD90-induced expression in a fibroblast population has been associated with wound healing and fibrosis. Its lower expression in D cells additionally supports the evidence of accentuated myofibroblast phenotype of D cells. Bayat and co-workers [70, 71] report similar results. They also detected CD34 and CD90 positive cell populations in cells isolated from the cord and nodule of DD patients. These reports differ from our results in that the total percentage of CD34 and CD90 positive cell populations was lower in affected DD tissues in their results than in our results. While we narrowed our analysis to cells isolated directly from the affected palmodigital fascia tissue of DD patients, Bayat and co-workers analysed heterogeneous histological layers and consequently diverse cell types. [72]. The identified stem cell markers point to a possible mesenchymal origin of cells involved in the DD pathogenesis. This assumption was further corroborated by the detection of HESCA-1 in 1% of analyzed cells, which is one of the markers for bone-marrow derived cells. It is therefore plausible to assume that the proliferation/differentiation potential of cells in DD patients relates to a specific subpopulation of CD90⁺ cells in palmar fascia expressing a panel of stem-cell related markers (CD34, CD117 and HESCA-1) at a higher rate than the CD90⁻ cells.

In conclusion, our study reveals a specific gene-expression pattern for cells isolated from affected DD fascia that is not found in cells isolated from patient-matched unaffected fascia and that account for a higher proliferation/differentiation potential of cells involved in the development of DD symptoms. The over-expression of fibrotic-related genes ELN, THBS1, GREM2, MBP, MYLK, NUA1, CALD1 and GADD45 β as well as the presence of particular membrane receptors in primary DD cells (CD90⁺, CD34, CD117 and HESCA-1) might be important molecular players in the onset of DD. In particular, mechanistic analyses of the results revealed the role of the p38 MAPK pathway in the phenotype changes underlying the differentiation of DD fibroblasts.

Disclosure/Conflict of Interest

The authors declare no competing interests or other interests that might be perceived to influence the results and discussion reported in this paper.

Acknowledgements

This work was supported by grants from the Croatian Ministry of Science, Education and Sports (grants no. 335-0982464-2393 and 335-0000000-3532) and the National Employment and Development Agency (grant no. 14V09809 "Development of a drug intended for treating the Dupuytren's disease patients").

References

- 1 Wynn TA: Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest* 2007;117:524-529.
- 2 Kalluri R, Zeisberg M: Fibroblasts in cancer. *Nat Rev Cancer* 2006;6:392-401.
- 3 Sedic M, Jurisic D, Stanec Z, Hock K, Pavelic K, Kraljevic Pavelic S: Functional genomics in identification of drug targets in Dupuytren's contracture. *Front Biosci* 2010;15:57-64.

- 4 Black EM, Blazar PE: Dupuytren's disease: an evolving understanding of an age-old disease. *J Am Acad Orthop Surg* 2011;19:746-757.
- 5 Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG: Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest* 2002;110:341-350.
- 6 Chilosi M, Poletti V, Zamo A, Lestani M, Montagna L, Piccoli P, Pedron S, Bertaso M, Scarpa A, Murer B, Cancellieri A, Maestro R, Semenzato G, Doglioni C: Aberrant Wnt/beta-catenin pathway activation in idiopathic pulmonary fibrosis. *Am J Pathol* 2003;162:1495-1502.
- 7 Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R: Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. *J Biol Chem* 2007;282:23337-23347.
- 8 Rygiel KA, Robertson H, Marshall HL, Pekalski M, Zhao L, Booth TA, Jones DE, Burt AD, Kirby JA: Epithelial-mesenchymal transition contributes to portal tract fibrogenesis during human chronic liver disease. *Lab Invest* 2008;88:112-123.
- 9 Humphreys BD, Lin SL, Kobayashi A, Hudson TE, Nowlin BT, Bonventre JV, Valerius MT, McMahon AP, Duffield JS: Fate tracing reveals the pericyte and not epithelial origin of myofibroblasts in kidney fibrosis. *Am J Pathol* 2010;176:85-97.
- 10 Shaw RB Jr, Chong AK, Zhang A, Hentz VR, Chang J: Dupuytren's disease: history, diagnosis, and treatment. *Plast Reconstr Surg* 2007;120:44e-54e.
- 11 Rayan GM: Dupuytren disease: Anatomy, pathology, presentation, and treatment. *J Bone Joint Surg Am* 2007;89:189-198.
- 12 Badalamente MA, Hurst LC: The biochemistry of Dupuytren's disease. *Hand Clin* 1999;15:35-42, v-vi.
- 13 Bayat A, Watson JS, Stanley JK, Alansari A, Shah M, Ferguson MW, Ollier WE: Genetic susceptibility in Dupuytren's disease. TGF-beta1 polymorphisms and Dupuytren's disease. *J Bone Joint Surg Br* 2002;84:211-215.
- 14 Mattoli S, Bellini A, Schmidt M: The role of a human hematopoietic mesenchymal progenitor in wound healing and fibrotic diseases and implications for therapy. *Curr Stem Cell Res Ther* 2009;4:266-280.
- 15 Roderfeld M, Rath T, Voswinckel R, Dierkes C, Dietrich H, Zahner D, Graf J, Roeb E: Bone marrow transplantation demonstrates medullar origin of CD34+ fibrocytes and ameliorates hepatic fibrosis in Abcb4-/- mice. *Hepatology* 2010;51:267-276.
- 16 Schmidt M, Sun G, Stacey MA, Mori L, Mattoli S: Identification of circulating fibrocytes as precursors of bronchial myofibroblasts in asthma. *J Immunol* 2003;171:380-389.
- 17 Aiba S, Tagami H: Inverse correlation between CD34 expression and proline-4-hydroxylase immunoreactivity on spindle cells noted in hypertrophic scars and keloids. *J Cutan Pathol* 1997;24:65-69.
- 18 Moore BB, Kolodsick JE, Thannickal VJ, Cooke K, Moore TA, Hogaboam C, Wilke CA, Toews GB: CCR2-mediated recruitment of fibrocytes to the alveolar space after fibrotic injury. *Am J Pathol* 2005;166:675-684.
- 19 Barth PJ, Westhoff CC: CD34+ fibrocytes: morphology, histogenesis and function. *Curr Stem Cell Res Ther* 2007;2:221-227.
- 20 Naik-Mathuria B, Pilling D, Crawford JR, Gay AN, Smith CW, Gomer RH, Olutoye OO: Serum amyloid P inhibits dermal wound healing. *Wound Repair Regen* 2008;16:266-273.
- 21 Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM: Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 2004;114:438-446.
- 22 Wada T, Sakai N, Matsushima K, Kaneko S: Fibrocytes: a new insight into kidney fibrosis. *Kidney Int* 2007;72:269-273.
- 23 Pan D, Watson HK, Swigart C, Thomson JG, Honig SC, Narayan D: Microarray gene analysis and expression profiles of Dupuytren's contracture. *Ann Plast Surg* 2003;50:618-622.
- 24 Qian A, Meals RA, Rajfer J, Gonzalez-Cadavid NF: Comparison of gene expression profiles between Peyronie's disease and Dupuytren's contracture. *Urology* 2004;64:399-404.
- 25 Satish L, LaFramboise WA, O'Gorman DB, Johnson S, Janto B, Gan BS, Baratz ME, Hu FZ, Post JC, Ehrlich GD, Kathyu S: Identification of differentially expressed genes in fibroblasts derived from patients with Dupuytren's Contracture. *BMC Med Genomics* 2008;1:10.
- 26 Shih B, Wijeratne D, Armstrong DJ, Lindau T, Day P, Bayat A: Identification of biomarkers in Dupuytren's disease by comparative analysis of fibroblasts versus tissue biopsies in disease-specific phenotypes. *J Hand Surg Am* 2009;34:124-136.

- 27 Kraljevic Pavelic S, Bratulic S, Hock K, Jurisic D, Hranjec M, Karminski-Zamola G, Zinic B, Bujak M, Pavelic K: Screening of potential prodrugs on cells derived from Dupuytren's disease patients. *Biomed Pharmacother* 2009;63:577-585.
- 28 Tse R, Howard J, Wu Y, Gan BS: Enhanced Dupuytren's disease fibroblast populated collagen lattice contraction is independent of endogenous active TGF-beta2. *BMC Musculoskelet Disord* 2004;5:41.
- 29 Pfaffl MW, Horgan GW, Dempfle L: Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res* 2002;30:e36.
- 30 Kraljevic Pavelic S, Sedic M, Hock K, Vucinic S, Jurisic D, Gehrig P, Scott M, Schlapbach R, Cacev T, Kapitanovic S, Pavelic K: An integrated proteomics approach for studying the molecular pathogenesis of Dupuytren's disease. *J Pathol* 2009;217:524-533.
- 31 Kraljević Pavelić S, Ratkaj I: Microarray expression analysis of primary Dupuytren's contracture cells; in Eaton C SM, Bayat A, Gabbiani G, Werker P, Wach W (eds): Dupuytren's disease and related hyperproliferative disorders. Berlin, Heidelberg : Springer Verlag, 2012, pp 109-113.
- 32 Bornstein P: Thrombospondins: structure and regulation of expression. *FASEB J* 1992;6:3290-3299.
- 33 Murphy-Ullrich JE, Poczatek M: Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 2000;11:59-69.
- 34 Vaughan MB, Howard EW, Tomasek JJ: Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast. *Exp Cell Res* 2000;257:180-189.
- 35 Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 2002;3:349-363.
- 36 Hinz B: Formation and function of the myofibroblast during tissue repair. *J Invest Dermatol* 2007;127:526-537.
- 37 Bornstein P: Thrombospondins as extracellular modulators of cell function. *J Clin Invest* 2001;107:929-934.
- 38 Donnini S, Morbidelli L, Tarabozetti G, Ziche M: ERK1-2 and p38 MAPK regulate MMP/TIMP balance and function in response to thrombospondin-1 fragments in the microvascular endothelium. *Life Sci* 2004;74:2975-2985.
- 39 Yoo J, Ghiassi M, Jirmanova L, Balliet AG, Hoffman B, Fornace AJ, Jr., Liebermann DA, Bottinger EP, Roberts AB: Transforming growth factor-beta-induced apoptosis is mediated by Smad-dependent expression of GADD45b through p38 activation. *J Biol Chem* 2003;278:43001-43007.
- 40 Takekawa M, Tatebayashi K, Itoh F, Adachi M, Imai K, Saito H: Smad-dependent GADD45beta expression mediates delayed activation of p38 MAP kinase by TGF-beta. *EMBO J* 2002;21:6473-6482.
- 41 Major MB, Jones DA: Identification of a gadd45beta 3' enhancer that mediates SMAD3- and SMAD4-dependent transcriptional induction by transforming growth factor beta. *J Biol Chem* 2004;279:5278-5287.
- 42 Suzuki A, Kusakai G, Kishimoto A, Lu J, Ogura T, Esumi H: ARK5 suppresses the cell death induced by nutrient starvation and death receptors via inhibition of caspase 8 activation, but not by chemotherapeutic agents or UV irradiation. *Oncogene* 2003;22:6177-6182.
- 43 Suzuki A, Lu J, Kusakai G, Kishimoto A, Ogura T, Esumi H: ARK5 is a tumor invasion-associated factor downstream of Akt signaling. *Mol Cell Biol* 2004;24:3526-3535.
- 44 Klapholz-Brown Z, Walmsley GG, Nusse YM, Nusse R, Brown PO: Transcriptional program induced by Wnt protein in human fibroblasts suggests mechanisms for cell cooperativity in defining tissue microenvironments. *PLoS One* 2007;2:e945.
- 45 Marty MC, Alliot F, Rutin J, Fritz R, Trisler D, Pessac B: The myelin basic protein gene is expressed in differentiated blood cell lineages and in hemopoietic progenitors. *Proc Natl Acad Sci USA* 2002;99:8856-8861.
- 46 Forbes SJ, Russo FP, Rey V, Burra P, Rugge M, Wright NA, Alison MR: A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. *Gastroenterology* 2004;126:955-963.
- 47 Wynn TA: Cellular and molecular mechanisms of fibrosis. *J Pathol* 2008;214:199-210.
- 48 Pinzani M: Welcome to fibrogenesis & tissue repair. *Fibrogenesis Tissue Repair* 2008;1:1.
- 49 Hindocha S, McGrouther DA, Bayat A: Epidemiological evaluation of Dupuytren's disease incidence and prevalence rates in relation to etiology. *Hand (NY)* 2009;4:256-269.
- 50 Rehman S, Salway F, Stanley JK, Ollier WE, Day P, Bayat A: Molecular phenotypic descriptors of Dupuytren's disease defined using informatics analysis of the transcriptome. *J Hand Surg Am* 2008;33:359-372.

- 51 Al-Qattan MM: Factors in the pathogenesis of Dupuytren's contracture. *J Hand Surg Am* 2006;31:1527-1534.
- 52 Suzuki A, Kusakai G, Kishimoto A, Lu J, Ogura T, Lavin MF, Esumi H: Identification of a novel protein kinase mediating Akt survival signaling to the ATM protein. *J Biol Chem* 2003;278:48-53.
- 53 Bujor AM, Pannu J, Bu S, Smith EA, Muise-Helmericks RC, Trojanowska M: Akt blockade downregulates collagen and upregulates MMP1 in human dermal fibroblasts. *J Invest Dermatol* 2008;128:1906-1914.
- 54 Hu Y, Peng J, Feng D, Chu L, Li X, Jin Z, Lin Z, Zeng Q: Role of extracellular signal-regulated kinase, p38 kinase, and activator protein-1 in transforming growth factor-beta1-induced alpha smooth muscle actin expression in human fetal lung fibroblasts in vitro. *Lung* 2006;184:33-42.
- 55 Meyer-Ter-Vehn T, Gebhardt S, Sebald W, Buttmann M, Grehn F, Schlunck G, Knaus P: p38 inhibitors prevent TGF-beta-induced myofibroblast transdifferentiation in human tenon fibroblasts. *Invest Ophthalmol Vis Sci* 2006;47:1500-1509.
- 56 Wang J, Fan J, Laschinger C, Arora PD, Kapus A, Seth A, McCulloch CA: Smooth muscle actin determines mechanical force-induced p38 activation. *J Biol Chem* 2005;280:7273-7284.
- 57 Kolosova I, Nethery D, Kern JA: Role of Smad2/3 and p38 MAP kinase in TGF-beta1-induced epithelial-mesenchymal transition of pulmonary epithelial cells. *J Cell Physiol* 2011;226:1248-1254.
- 58 Matsuoka H, Arai T, Mori M, Goya S, Kida H, Morishita H, Fujiwara H, Tachibana I, Osaki T, Hayashi S: A p38 MAPK inhibitor, FR-167653, ameliorates murine bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L103-112.
- 59 Moran N: p38 kinase inhibitor approved for idiopathic pulmonary fibrosis. *Nat Biotechnol* 2011;29:301.
- 60 Nishida M, Okumura Y, Sato H, Hamaoka K: Delayed inhibition of p38 mitogen-activated protein kinase ameliorates renal fibrosis in obstructive nephropathy. *Nephrol Dial Transplant* 2008;23:2520-2524.
- 61 Li J, Campanale NV, Liang RJ, Deane JA, Bertram JF, Ricardo SD: Inhibition of p38 mitogen-activated protein kinase and transforming growth factor-beta1/Smad signaling pathways modulates the development of fibrosis in adriamycin-induced nephropathy. *Am J Pathol* 2006;169:1527-1540.
- 62 Zhang S, Weinheimer C, Courtois M, Kovacs A, Zhang CE, Cheng AM, Wang Y, Muslin AJ: The role of the Grb2-p38 MAPK signaling pathway in cardiac hypertrophy and fibrosis. *J Clin Invest* 2003;111:833-841.
- 63 Krause C, Kloen P, Ten Dijke P: Elevated transforming growth factor beta and mitogen-activated protein kinase pathways mediate fibrotic traits of Dupuytren's disease fibroblasts. *Fibrogenesis Tissue Repair* 2011;4:14.
- 64 Ballif BA, Blenis J: Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ* 2001;12:397-408.
- 65 Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ: Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J Biol Chem* 1995;270:7420-7426.
- 66 Edling CE, Hallberg B: c-Kit--a hematopoietic cell essential receptor tyrosine kinase. *Int J Biochem Cell Biol* 2007;39:1995-1998.
- 67 Cabane C, Coldefy AS, Yeow K, Derijard B: The p38 pathway regulates Akt both at the protein and transcriptional activation levels during myogenesis. *Cell Signal* 2004;16:1405-1415.
- 68 Barth PJ, Schenck zu Schweinsberg T, Ramaswamy A, Moll R: CD34+ fibrocytes, alpha-smooth muscle antigen-positive myofibroblasts, and CD117 expression in the stroma of invasive squamous cell carcinomas of the oral cavity, pharynx, and larynx. *Virchows Arch* 2004;444:231-234.
- 69 Hagood JS, Prabhakaran P, Kumbala P, Salazar L, MacEwen MW, Barker TH, Ortiz LA, Schoeb T, Siegal GP, Alexander CB, Pardo A, Selman M: Loss of fibroblast Thy-1 expression correlates with lung fibrogenesis. *Am J Pathol* 2005;167:365-379.
- 70 Hindocha S, Iqbal SA, Farhatullah S, Paus R, Bayat A: Characterization of stem cells in Dupuytren's disease. *Br J Surg* 2011;98:308-315.
- 71 Iqbal SA, Manning C, Syed F, Kolluru V, Hayton M, Watson S, Bayat A: Identification of mesenchymal stem cells in perinodular fat and skin in Dupuytren's disease: a potential source of myofibroblasts with implications for pathogenesis and therapy. *Stem Cells Dev* 2012;21:609-622.
- 72 Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO: Diversity, topographic differentiation, and positional memory in human fibroblasts. *Proc Natl Acad Sci USA* 2002;99:12877-12882.