Laboratory Investigation (2008) 88, 1303–1315

Gene expression profiling of ABC transporters in dermal fibroblasts of pseudoxanthoma elasticum patients identifies new candidates involved in PXE pathogenesis

Doris Hendig[1], Thomas Langmann[2,3], Sarah Kocken[1], Ralf Zarbock[1], Christiane Szliska[4], Gerd Schmitz[2], Knut Kleesiek[1] and Christian Götting[1]

Mutations in the *ABCC6* gene, encoding the multidrug resistance-associated protein 6 (MRP6), cause pseudoxanthoma elasticum (PXE). This heritable disorder leads to pathological alterations in connective tissues. The implication of MRP6 deficiency in PXE is still unknown. Moreover, nothing is known about a possible compensatory expression of other ATP binding-cassette (ABC) transporter proteins in MRP6-deficient cells. We investigated the gene expression profile of 47 ABC transporters in human dermal fibroblasts of healthy controls ($n = 2$) and PXE patients ($n = 4$) by TaqMan low-density array. The analysis revealed the expression of 37 ABC transporter genes in dermal fibroblasts. *ABCC6* gene expression was not quantifiable in fibroblasts derived from PXE patients. Seven genes (*ABCA6*, *ABCA9*, *ABCA10*, *ABCB5*, *ABCC2*, *ABCC9* and *ABCD2*) were induced, whereas the gene expression of one gene (*ABCA3*) was decreased, comparing controls and PXE patients (with at least twofold changes). We reanalyzed the gene expression of selected ABC transporters in a larger set of dermal fibroblasts from controls and PXE patients ($n = 6$, each). Reanalysis showed high interindividual variability between samples, but confirmed the results obtained in the array analysis. The gene expression of ABC transporter genes, as well as lineage markers of PXE, was further examined after inhibition of *ABCC6* gene expression by using specific small-interfering RNA. These experiments corroborated the observed gene expression alterations, most notably in the ABCA subclass (up to fourfold, $P < 0.05$). We therefore conclude that MRP6-deficient dermal fibroblasts exhibit a distinct gene expression profile of ABCA transporters, potentially to compensate for MRP6 deficiency. Moreover, our results point to a function for *ABCC6*/MRP6 in sterol transport, as sterols are preferential regulators of ABCA transporter activity and expression. Further studies are now required to uncover the role of ABCA transporters in PXE.
Laboratory Investigation (2008) **88,** 1303–1315; doi:10.1038/labinvest.2008.96; published online 20 October 2008

KEYWORDS: ABCC6; dermal fibroblast; gene expression profiling; MRP6; pseudoxanthoma elasticum; PXE

Progressive calcification and fragmentation of elastic fibers are characteristic hallmarks of pseudoxanthoma elasticum (PXE), which is caused by mutations in the *ABCC6* gene encoding the multidrug resistance-associated protein 6 (MRP6,[1–6]). Recently, two different *abcc6* knockout mouse models were generated, that exhibited a PXE phenotype, strengthening *ABCC6*/MRP6 as the candidate gene for PXE.[7,8] MRP6 is a member of the large ATP-binding cassette (ABC) transporter superfamily. To date, 49 ABC transporter genes have been identified as belonging to this family of membrane transporter proteins performing various functions in human cells. The most prominent and well-characterized ABC transporter proteins are P-gp (*ABCB1*/MDR1) and *ABCC1*/MRP1, mediating multidrug resistance in cancer cells (for review see Haimeur *et al*[9] and Deeley *et al*[10] and http://nutrigene.4t.com/humanabc.htm). MRP6 belongs to subfamily C currently consisting of 13 members, of which 9 belong to the MRP family known to be organic anion transporters. Until now, nothing is known about the physiological function of MRP6 and its role in PXE manifestation. The protein is predominantly expressed in the liver and kidney, whereas very low expression has been observed in

[1]Institut für Laboratoriums—und Transfusionsmedizin, Herz—und Diabeteszentrum Nordrhein-Westfalen, Universitätsklinik der Ruhr-Universität Bochum, Bad Oeynhausen, Germany; [2]Institut für Klinische Chemie und Laboratoriumsmedizin, Universität Regensburg, Regensburg, Germany; [3]Institut für Humangenetik, Universität Regensburg, Regensburg, Germany and [4]Dermatologische Klinik, Krankenhaus Bethesda, Freudenberg, Germany
Correspondence: Dr C Götting, PhD, Institut für Laboratoriums—und Transfusionsmedizin, Herz—und Diabeteszentrum Nordrhein-Westfalen, Georgstraße 11, 32545 Bad Oeynhausen, Germany.
E-mail: cgoetting@hdz-nrw.de

Received 6 March 2008; revised 15 August 2008; accepted 18 August 2008

tissues primarily affected by PXE, for instance the skin.[2,11] It was assumed that MRP6 serves as an efflux pump on the basolateral surface of hepatocytes transporting as yet unidentified substrates from the intracellular milieu to the blood.[12,13] Indeed, several studies reported alterations in systemic blood components in PXE patients and mouse models due to MRP6 deficiency.[14–20] Accordingly, PXE was assumed to be primarily a metabolic disorder.[12,21–23] In contrast, results of several studies also point to a local role for *ABCC6*/MRP6 as expression of the protein was detected in dermal fibroblasts from healthy controls whereas it was found to be absent in fibroblasts from PXE patients.[24] Moreover, dermal fibroblasts expanded from PXE patients' biopsies exhibited pathological alterations compared to dermal fibroblasts from healthy controls.[25] These cells have been shown to synthesize abnormal proteoglycans and to exhibit a higher expression of elastin (ELN), which was further associated with pathological assembly of elastic fibers when cultured in the presence of PXE sera.[16,26] Quaglino *et al*[27] reported that PXE fibroblasts have a raised matrix metallopeptidase 2 mRNA and protein expression. In addition, a mild oxidative stress was observed in PXE fibroblasts with higher malondialdehyde levels, increased superoxide dismutase 2 and reduced catalase and glutathione peroxidase activities.[28] Therefore, it cannot be excluded that also low-level expression of *ABCC6*/MRP6 plays an important physiological role. MRPs are active in dermal fibroblasts as these cells exhibit a MRP efflux activity that can be blocked by specific inhibitors known to interfere with MRP function.[29–31] Furthermore, it was reported that PXE fibroblasts have a reduced MRP transporter activity compared to normal dermal fibroblasts.[31] Beside MRP6, other MRPs have also been reported to be associated with human diseases. Mutations in *ABCC2*/MRP2 lead to Dubin–Johnson syndrome, whereas mutations in *ABCC7*/CFTR are the cause for cystic fibrosis. A compensatory expression of *ABCC3*/MRP3 was previously observed in rats suffering from cholestasis due to MRP2 deficiency, as seen in patients with Dubin–Johnson syndrome and Eisai hyperbilirubinemic rats.[32,33] The question is whether a MRP6 deficiency has an effect on the expression of other ABC transporter proteins. A recent study by Li *et al*[34] reported no alterations in the gene expression of other ABC transporter subfamily C members in *abcc6* knockout mice. The aim of our present study was to investigate the expression profile of 47 ABC transporter genes, as well as the MRP transporter activity of dermal fibroblasts derived from skin biopsies of PXE patients and of healthy controls. Our results will provide new data implying a compensatory role for other ABC transporter proteins in PXE manifestation. Moreover, we show that the observed alterations in gene expression of other ABC transporters are associated with MRP6 deficiency due to inhibition of *ABCC6*/MRP6 synthesis. Finally, we will discuss a possible role for *ABCC6*/MRP6 in lipid metabolism.

MATERIALS AND METHODS

Skin Biopsies and Cell Culture Conditions

Dermal fibroblasts from seven PXE patients were expanded from skin biopsies. The diagnosis of PXE in all patients was consistent with the reported consensus criteria.[35,36] The status of the PXE patients was determined by the presence of dermal lesions and ocular findings. The dermal lesions were histologically confirmed by the observation of mineralized elastic fibers in biopsy samples following von Kossa staining. The study was approved by the institutional review board and all patients gave their informed consent. In detail: biopsy samples were digested with 0.5% pronase E (Sigma, Steinheim, Germany) for 30 min, and 1% collagenase/dispase (Roche, Penzberg, Germany) in Dulbecco's modified essential medium (DMEM) containing 10% fetal calf serum, 1% L-glutamine (200 mM) and 1% antibiotic/antimycotic solution (100 ×) at 37°C overnight. Afterward, the samples were washed twice with Dulbecco's phosphate-buffered saline (DPBS) and seeded in DMEM medium including all supplements. Media and supplements were obtained from PAA (Pasching, Austria) and Biowest (Nuaillé, France). Fibroblast cultures were grown out until they reached confluence and were then subcultured once a week with a ratio of 1:3. Dermal fibroblasts from six healthy controls (NHDF) were purchased from Promocell (Heidelberg, Germany), Genlantis (San Diego, USA) and Cambrex (Walkersville, USA). All cultures were checked for fibroblast-specific marker expression (Thy-1; CD90) by antibody staining following immunofluorescence, according to the manufacturer's protocol (Dianova, Hamburg, Germany). Main characteristics of PXE patients and healthy controls are summarized in Table 1. For all experiments subcultures of the 4th to 8th passage were used. Experiments were done on four different subcultures of each PXE patient and control subject to exclude variability from culture conditions. Measurements were done in duplicate or triplicate for each biological sample. HepG2 cells were maintained in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine (200 mM) and 1% antibiotic/antimycotic solution (100 ×), subcultured with a ratio of 1:3 once a week.

ABCC6 Genotyping

Genotyping of the c.3421C>T. mutation and mutational analysis of *ABCC6* was performed as previously described.[6]

Analysis of ABC Transporter Transcript Levels by TaqMan Low-Density Array

Transcript levels for 47 human ABC transporters and the reference gene 18S rRNA were analyzed by a well-established real-time PCR-based TaqMan low-density array (TLDA), as described.[37] Total RNA was extracted from 1×10^6 cells using the RNeasy Midi Kit (Qiagen, Hilden, Germany). Genes that were regulated more than twofold (≥ 2.0 and ≤ 0.5)

Table 1 Main characteristics of dermal fibroblasts derived from PXE patients and healthy controls used in the present study

Sample ID	Gender	Age[a]	Biopsy source	*ABCC6* genotype[b]			Status[c]	Age at disease onset[a]	Number of involved organs
PXE patients									
P60F	Female	58	Axilla	c.37-1G>A (SSM)	c.37-1G>A (SSM)		hm	56	3
P229F	Female	50	NA	c.1171A>G (p.R391G)	c.1208C>A (p.A413N)	c.2252T>A (p.M751K)	cht	NA	NA
P265F	Female	62	Cervix	c.1132C>T (p.Q378X)	c.3421C>T (p.R1141X)		cht	16	3
P3M	Male	57	Cervix	c.3421C>T (p.R1141X)	c.3883-6G>A (SSM)		cht	46	5
P128M	Male	51	Cervix	c.3769_3770insC (p.L1259fsX1277)	c.3769_3770insC (p.L1259fsX1277)		hm	48	3
P308M	Male	42	NA	c.3421C>T (p.R1141X)	c.-90ins14		(c)ht	NA	NA
P341M	Male	41	NA	c.1552C>T (p.R518X)	ND		ht	NA	NA
Healthy controls									
F37A	Female	37	Abdomen	—	—	—	wt	—	—
F42A	Female	42	Abdomen	—	—	—	wt	—	—
F52C	Female	52	Cheek	—	—	—	wt	—	—
M2FS	Male	2	Foreskin	—	—	—	wt	—	—
M45D	Male	45	Face	—	—	—	wt	—	—
M56D	Male	56	Face	—	—	—	wt	—	—

hm, homozygote; cht, compound heterozygote; ht, heterozygote; wt, wild type; SSM, splice site mutation; NA, not applicable; ND, nondetected.

[a]Age in years.

[b]Nucleotide numbering refers to the cDNA sequence with the A of the ATG translation initiation start site as nucleotide +1 (GenBank accession number NM_001171.2).

[c]Genotype status.

were considered significantly regulated.[37] NHDF served as calibrators to which gene expression values of the PXE fibroblasts were compared.

Validation of ABC Transporter Transcript Levels and PXE Lineage Marker Gene Expression by Quantitative Real-Time PCR

Total RNA was extracted from 1×10^6 cells using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions and then stored at -80°C. The integrity of the RNA samples was checked by assessing ribosomal RNA bands on a denaturing agarose gel. Quantity and quality of the RNA samples were measured spectrophotometrically. Total RNA (1 μg) was transcribed to cDNA using oligo (dT) primers and Superscript II Reverse Transcriptase according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). cDNA (equivalent to 12.5 or 25 ng of total RNA) served as a template for measurement of mRNA levels in quantitative real-time PCR (qPCR). Exon-intron-boundary-spanning PCR primers covering the major transcript forms were designed according to the published mRNA sequences (Table 2). QPCRs were performed using the Platinum Sybr-Green PCR Supermix UDG kit from Invitrogen on Eppendorf Mastercycler Realplex^2 System (Hamburg, Germany). PCR conditions were: 120 s incubation at 55°C, 120 s incubation at 95°C, followed by 45 cycles of degradation at 95°C for 10 s, primer-specific annealing for 15 s (Table 2), elongation and detection of the amplicon at 72°C for 20 s. Finally, a melting curve analysis of the amplicon was performed. Specificity of the amplicon was verified by standard methods. Data were analyzed using the method of Vandesompele *et al* to minimize interindividual sample variability.[38] Initially, we defined the number of housekeeping genes necessary for accurate normalization by analyzing different housekeeping genes using Genorm software.[38] Then, a normalization factor was determine by calculating a geometric mean of the expression values from three housekeeping genes (β-2-microglobulin = β-2m, glyceraldehyde-3-phosphate dehydrogenase = GAPDH, hypoxanthine phosphor-ribosyltransferase 1 = HPRT1). Relative expression values were calculated by considering PCR efficiency and dividing by the normalization factor.

Table 2 Primer annotation and sequence, GenBank accession number, annealing temperature, amplicon size and melting temperature used in quantitative real-time PCR

Primer annotation[a]		Sequence 5′-3′	Reference sequence[b]	Product size[c]	Annealing (T_M)[d]	Melting (T_M)[d]
hABCC1	E12/1760U18	CTGGCATTCAAGGACA	NM_004996	168	58.0	88
	E13/1910L18	CAGGATGTTGTTCTCGTC				
hABCC2	E23/3206U18	CACAAGCAACTGCTGAAC	NM_000392	226	56.5	85
	E24/3431L18	TGCCAAGAGGAATGACGA				
hABCC3	E8/993U18	GCTCCAGCTTCCTCATCA	NM_003786	159	60.5	86
	E9/1151L18	TGGAGCACAGGAACATCA				
hABCC4	E21/2684U18	GCTGTGGCCGTGATTCCT	NM_005845	185	59.5	83
	E22/2851L17	TTGTATGCCCGGATGGTC				
hABCC5	E18/2732U17	CCTTGGCATTCCTGGTT	NM_005688	171	58.0	85
	E19/2913L18	ATGGAGAGGGCGTAGATG				
hABCC6	E23/3218U18	CCTGCTGATGTACGCCTT	NM_001171	267	59.5	89
	E24/3484L18	ACGCGAGCATTGTTCTGA				
hABCC9	E12/1677U18	GTATGCCAGTGGAAACAA	NM_005691	181	59.5	83
	E13/1841L17	CCAACTGTCGTCACCAA				
hABCA3	E27/4972U18	CGGGAAGACCACGACTTT	NM_001089	110	57.0	85
	E27+28/5067L15	GCTGCCGCACCTTTC				
hABCA6	E22/3168U18	AGTCAAGCCCATTTCCTC	NM_080284	188	59.6	80
	E24/3339L17	CCCACACCAGTAAGCAG				
hABCA9	E17/2300U18	AAGGTGTGATCCAGAGAG	NM_080283	308	58.0	79
	E19/2607L18	GCTCAACAAGGCTTCCTA				
hABCA10	E27/4127U18	TCACTTTGCTGGGGTATG	NM_080282	245	55.0	78
	E30/4354L18	CGGATTGGGATGAGTTTC				
hABCD2	E8/2390U18	AGGGGAAAAGCAAAGAAT	NM_005164	217	55.0	81
	E10/2591L16	CCAACCTCCTTCACCA				
hB2M	E1/84U22	TGTGCTCGCGCTACTCTCTCTT	NM_004048	137	61.0	80
	E2/200L21	CGGATGGATGAAACCCAGACA				
hGAPDH	E2/116U18	AGGTCGGAGTCAACGGAT	NM_002046	223	59.5	83
	E4/338L18	TCCTGGAAGATGGTGATG				
hHPRT1	E3/311U18	GCTGACCTGCTGGATTAC	NM_000194	258	59.5	79
	E6/568L18	TGCGACCTTGACCATCTT				
hELN[e]	E17+18/929U23	CCGCTAAGGCAGCCAAGTATGGA	NM_000501	275	57.0	89
	E20/1180L24	AGCTCCAACCCCGTAAGTAGGAAT				
hSOD2	E3/471U19	GCCCTAACGGTGGTGGAGA	NM_000636	135	61.0	84
	E4/587L19	CAAGCCAACCCCAACCTGA				

[a]Genes were annotated to the nomenclature used in GenBank. Target exons and sequence positions are presented, whereas numbering refers to the cDNA sequence with the A of the ATG translation initiation start site as nucleotide +1.

[b]Reference sequence taken from GenBank. Accession numbers are presented (September 2007).

[c]Annealing temperature in °C.

[d]Melting temperature of the amplicon in °C.

[e]Primer sequences were taken from RT Primer database (http://medgen.ugent.be/rtprimerdb/).

Measurement of MRP Efflux Activity by Flow Cytometry

Functional activity of MRPs was measured by quantifying calcein efflux as recently described.[31] The principle of the assay in brief: cells were loaded with 0.1 μM of the non-fluorescent membrane-permeable calcein-acetoxymethyl (caAM) ester for 30 min at 37°C. The AM ester is cleaved by

Table 3 Sequences of used *ABCC6*-specific siRNA-oligo and targeting sites

siRNA oligonucleotide	siRNA ID[a]	Target site[b]	Sense[c]	Antisense[c]
1	118143	Exon 23	CCCAUUGGUCACCUGCUAAtt	UUAGCAGGUGACCAAUGGGtg
2	106395	Exon 21	GGAAAGGACAGCAUCCAAUtt	AUUGGAUGCUGUCCUUUCCtg
3	106396	Exon 21	GGACAGCAUCCAAUACGGCtt	GCCGUAUUGGAUGCUGUCCtt
Silencer negative control	#1 siRNA	NA	NA	NA

NA, not applicable.

[a]According to Ambion Inc. (Cambridgeshire, UK).

[b]Reference sequence: GenBank accession number NM_001171.

[c]Sequence 5′-3′.

intracellular esterases to release the fluorochrom calcein (ca). Calcein is a specific substrate for MRPs, thus allowing MRP efflux activity to be determined. The cells were washed twice with DPBS afterward and then incubated at 37°C in DMEM depleted of FCS. Calcein efflux was quantified after 0, 60, 120, 240 and 360 min using Epics XL flow cytometer (Beckman Coulter, Krefeld, Germany). Data are presented as remaining intracellular calcein (cellular fluorescence) at time point 360 min (% intracellular $ca = ((ca)^{0\,min} - (ca)^{360\,min})/(ca)^{0\,min} \times 100$).

Silencing of *ABCC6*/MRP6 Expression Using *ABCC6*-Specific Small-Interfering RNA

ABCC6-specific small-interfering RNA (siRNA) and FAM-labeled control siRNA oligonucleotides were purchased from Ambion (Cambridgeshire, UK; Table 3). NHDFs and PXE fibroblasts were reverse transfected using 4 μl/ml Lipofectamine 2000 (Invitrogen). Medium was replaced 12 h past transfection after washing cells twice with DPBS.

Data and Statistical Analysis

All values are given as mean ± s.e.m. Normality testing for Gaussian distribution of values was performed using Kolmogorov–Smirnov test. Statistical analysis was performed using Student's *t*-test and Mann–Whitney *U*-test where appropriate. *P*-values of less than 0.05 were considered significant. All tests were executed with GraphPad Prism 4.0 (GraphPad Prism Software, San Diego, CA, USA).

RESULTS

ABCC6 Genotyping

ABCC6 genotypes for the seven PXE patients investigated in the present study are shown in Table 1. Two patients were found to carry one *ABCC6* mutation in homozygous state (P60F and P128M). Three patients were identified as being carriers of two different *ABCC6* nonsense or splice-site mutations and one patient (P229F) was identified as carrying two missense mutations revealing a compound heterozygous status. Patient P308M revealed a novel 14-bp insertion in the promoter region in addition to a nonsense *ABCC6* mutation. We failed to detect a second *ABCC6* mutation for patient P341M.

Gene Expression Profile of ABC Transporter Genes in NHDFs

Characterization of the ABC transporter gene expression profile revealed 37 genes to be expressed in dermal fibroblasts from healthy controls ($n = 2$) and PXE patients ($n = 4$, Table 4). NHDFs revealed high expression levels (ΔC_t 12–16) for *ABC-A1*, *A2*, *A5*, *A6*, *B7*, *B8*, *B9*, *B10*, *C1*, *C4*, *C9*, *D1*, *D3*, *D4*, *E1*, *F1*, *F2*, *F3* and *TAP1* and *2*. Medium expression values (ΔC_t 16.5–20) were observed for *ABC- A3*, *A4*, *A7*, *A8*, *A9*, *A10*, *B4*, *B6*, *C3*, *C5*, *C10*, *D2* and *G2*. Low expression (ΔC_t 20.5–25) was noticed for *ABC- B5*, *C2*, *C6* and *G4*. TLDA analysis detected no gene expression for *ABC- A12*, *A13*, *B1*, *B11*, *C7*, *C8*, *C11*, *G1*, *G5* and *G8* in NHDFs.

NHDF-Specific Gene Expression Profile of ABC Transporters Compared to HDFs of PXE Patients

No *ABCC6* transcripts were quantifiable in fibroblast samples from PXE patients by TLDA analysis. Seven genes were found to be expressed more than twofold higher in PXE patients' fibroblasts (Table 4, depicted in dark gray). These include *ABC- A6*, *A9*, *A10*, *B5*, *C2*, *C9* and *D2*. The relative mRNA expression of *ABCA3* was found to be twofold reduced ($\leq$0.5-fold) in the PXE patient's samples.

Validation of Altered ABC Transporter Transcript Levels Due to *ABCC6*/MRP6 Deficiency by Quantitative Real-time PCR

To verify the observed mRNA expression differences, we developed real-time PCR assays for selected members of the ABCC and ABCA subclasses, as well as for *ABCD2*, and re-analyzed gene expression in a larger group of PXE patients and healthy controls (each $n = 6$). Samples of patient P60F were exchanged with samples of patient P308M in this analysis, due to a lack of material.

We developed a real-time PCR assay that permits relative quantification of *ABCC6* mRNA expression. Our assay detects very low levels of ABCC6 mRNA without amplification

Table 4 Gene expression profile of ABC transporters in dermal fibroblasts of healthy controls and PXE patients[a]

ID	Gene[b]	Healthy Controls ΔC_t (s.e.m.)[c]	PXE patients ΔC_t (s.e.m.)[c]	RQ Difference (range)[d]	
				Healthy controls	PXE patients
#	18S rRNA	14.28 (0.04)	14.54 (0.04)	—	—
1	ABCA1	14.06 (0.09)	13.82 (0.16)	1.01 (0.76–1.20)	1.40 (0.44–4.24)
2	ABCA2	14.53 (0.17)	14.06 (0.08)	1.05 (0.63–1.59)	1.44 (0.81–2.84)
3	**ABCA3**	**18.62 (0.29)**	**20.66 (0.24)**	**1.15 (0.52–1.96)**	**0.33 (0.01–1.34)**
4	ABCA4	20.62 (0.32)	21.47 (0.18)	1.16 (0.34–1.87)	0.63 (0.18–1.25)
5	ABCA5	13.99 (0.29)	14.11 (0.09)	1.15 (0.53–2.05)	0.98 (0.45–2.15)
6	**ABCA6**	**15.01 (0.63)**	**11.92 (0.15)**	**1.63 (0.15–3.22)**	**9.69 (2.14–20.13)**
7	ABCA7	20.38 (0.26)	20.25 (0.18)	1.12 (0.44–2.48)	1.31 (0.34–3.13)
8	ABCA8	17.90 (1.33)	15.68 (0.18)	5.78 (0.09–12.65)	7.79 (1.73–19.41)
9	**ABCA9**	**16.88 (0.91)**	**13.35 (0.23)**	**2.42 (0.07–5.27)**	**15.14 (1.35–40.66)**
10	**ABCA10**	**20.58 (0.47)**	**18.71 (0.15)**	**1.33 (0.25–2.34)**	**4.21 (1.18–9.20)**
11	ABCA12	NE	NE	—	—
12	ABCA13	NE	NE	—	—
13	ABCB1	NE	NE	—	—
14	ABCB4	16.86 (0.13)	16.46 (0.14)	1.03 (0.76–1.49)	1.52 (0.52–3.20)
15	**ABCB5**	**25.19 (2.1)**	**16.70 (0.44)**	**0.57 (0.0–1.60)**	**14.49 (0.19–63.53)**
16	ABCB6	17.02 (0.11)	16.42 (0.10)	1.02 (0.75–1.28)	1.61 (0.54–3.01)
17	ABCB7	14.89 (0.05)	14.78 (0.08)	1.00 (0.90–1.14)	1.12 (0.70–2.35)
18	ABCB8	16.34 (0.14)	15.89 (0.07)	1.03 (0.60–1.36)	1.32 (0.68–1.80)
19	ABCB9	18.25 (0.17)	18.94 (0.12)	1.05 (0.64–1.74)	0.68 (0.23–1.42)
20	ABCB10	14.7 (0.07)	14.41 (0.07)	1.01 (0.83–1.22)	1.26 (0.83–1.98)
21	ABCB11	NE	NE	—	—
22	ABCC1	12.93 (0.10)	12.52 (0.15)	1.02 (0.71–1.26)	1.51 (0.39–3.42)
23	**ABCC2**	**20.77 (0.31)**	**20.05 (0.17)**	**1.05 (0.69–1.45)**	**2.25 (0.35–4.66)**
24	ABCC3	16.75 (0.15)	17.84 (0.45)	1.04 (0.64–1.39)	1.40 (0.04–5.9)
25	ABCC4	14.21 (0.14)	14.35 (0.13)	1.03 (0.64–1.47)	1.00 (0.32–2.00)
26	ABCC5	18.47 (0.21)	17.84 (0.11)	1.08 (0.58–1.80)	1.69 (0.73–4.20)
27	**ABCC6**	**21.37 (0.25)**	**NE**	**1.13 (0.57–2.76)**	**0.002 (0.002–0.004)**
28	ABCC7	NE	NE	—	—
29	ABCC8	NE	NE	—	—
30	**ABCC9**	**16.05 (0.43)**	**12.75 (0.24)**	**1.29 (0.23–2.70)**	**14.00 (2.62–42.13)**
31	ABCC10	18.37 (0.16)	17.94 (0.07)	1.04 (0.56–1.54)	1.38 (0.73–2.10)
32	ABCC11	NE	NE	—	—
33	ABCD1	15.82 (0.08)	15.63 (0.08)	1.01 (0.84–1.33)	1.19 (0.57–1.72)
34	**ABCD2**	**20.39 (0.60)**	**18.96 (0.23)**	**1.40 (0.11–3.20)**	**3.44 (0.60–10.80)**
35	ABCD3	13.27 (0.05)	13.11 (0.06)	1.01 (0.85–1.15)	1.15 (0.71–1.99)
36	ABCD4	15.65 (0.16)	15.21 (0.06)	1.04 (0.69–1.60)	1.40 (1.02–2.23)
37	ABCE1	13.17 (0.05)	13.19 (0.07)	1.00 (0.92–1.16)	1.01 (0.61–1.74)
38	ABCF1	15.06 (0.10)	15.05 (0.08)	1.02 (0.75–1.37)	1.05 (0.49–1.97)
39	ABCF2	13.44 (0.06)	13.23 (0.05)	1.01 (0.78–1.18)	1.17 (0.88–1.61)
40	ABCF3	14.44 (0.05)	14.45 (0.06)	1.00 (0.86–1.15)	1.01 (0.66–1.75)
41	ABCG1	NE	NE	—	—

Table 4 Continued

ID	Gene[b]	Healthy Controls ΔC_t (s.e.m.)[c]	PXE patients ΔC_t (s.e.m.)[c]	RQ Difference (range)[d]	
				Healthy controls	PXE patients
42	ABCG2	18.91 (0.24)	19.55 (0.21)	1.10 (0.52–2.04)	0.87 (0.22–3.11)
43	ABCG4	20.96 (0.29)	21.11 (0.28)	1.16 (0.64–2.09)	1.25 (0.12–4.02)
44	ABCG5	NE	NE	—	—
45	ABCG8	NE	NE	—	—
46	TAP1 (ABCB2)	16.22 (0.10)	15.67 (0.06)	1.02 (0.72–1.29)	1.50 (0.97–2.42)
47	TAP2 (ABCB3)	15.62 (0.09)	15.50 (0.08)	1.01 (0.82–1.26)	1.14 (0.59–1.91)

NE, not expressed; PXE, pseudoxanthoma elasticum.

[a]Genes that are regulated more than twofold (≥ 2.0 or ≤ 0.5) are considered as significantly regulated. Induced genes are depicted in dark gray, downregulated genes in light gray.

[b]Gene annotation according to the Human ATP-binding Cassette Transporter TaqMan Low-Density Array.[37]

[c]ΔC_t values values were calculated by normalization to the expression value of 18S rRNA.

[d]Gene expression values were normalized to the expression value of 18S rRNA and calculated based on the comparative cycle threshold (C_t) method ($2\text{-}\Delta\Delta C_t$,[37,54]). NHDF served as calibrators to which gene expression values of the PXE fibroblasts were compared.

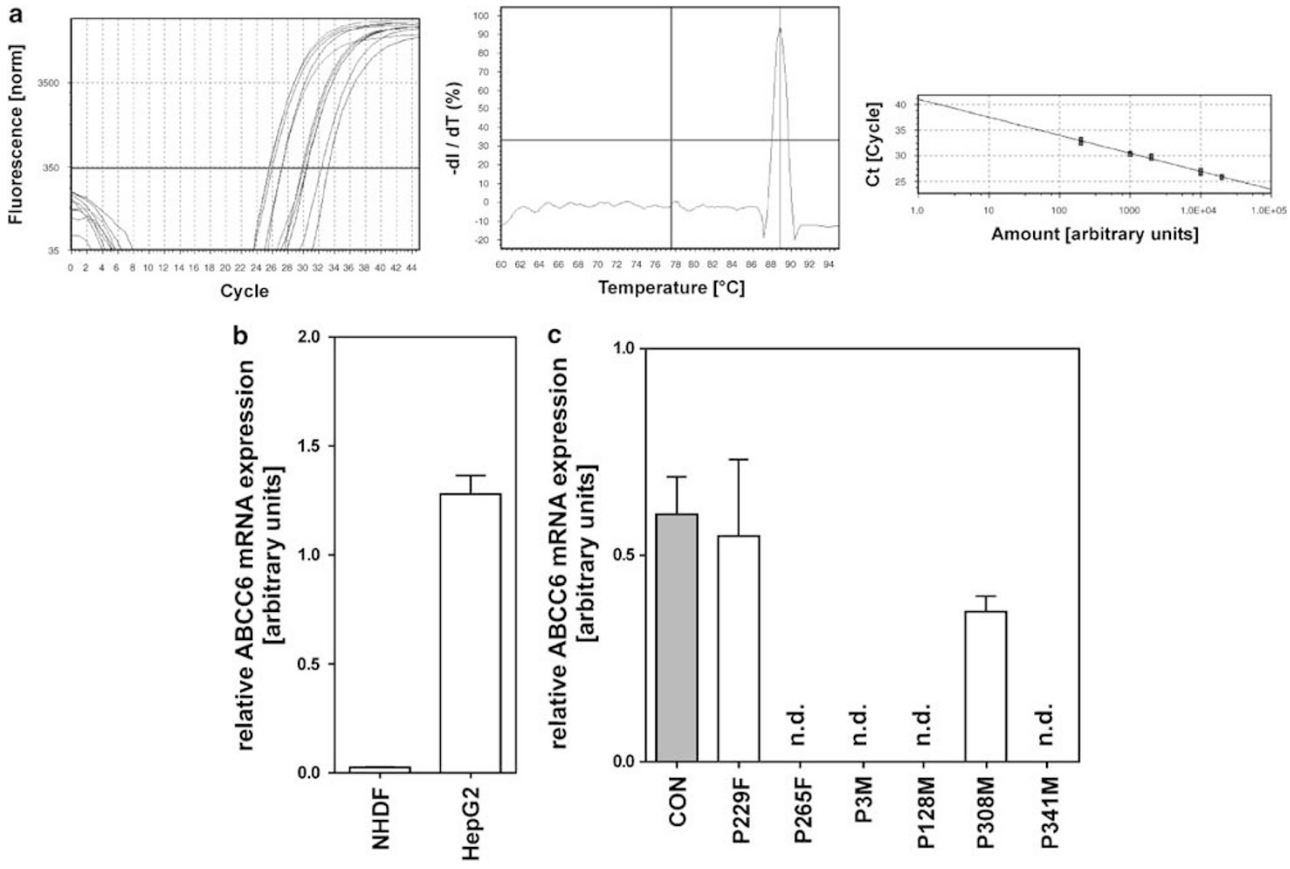

Figure 1 Quantification of ABCC6 mRNA expression. (**a**) Quantitative real-time PCR assay for detection of human *ABCC6* transcripts. The figure shows the amplification plot, a corresponding melting curve of the amplicon and a standard curve generated with diluted NHDF cDNA (Slope −3.51; PCR efficiency 1.93). (**b**) Validation of the assay with cDNA synthesized from RNA samples of NHDF and HepG2 cells. (**c**) Quantification of *ABCC6* mRNA expression in HDFs from healthy controls (CON, gray column; $n = 6$) and PXE patients (white columns). Data are presented in arbitrary units as means with corresponding standard error; n.d.: not detectable.

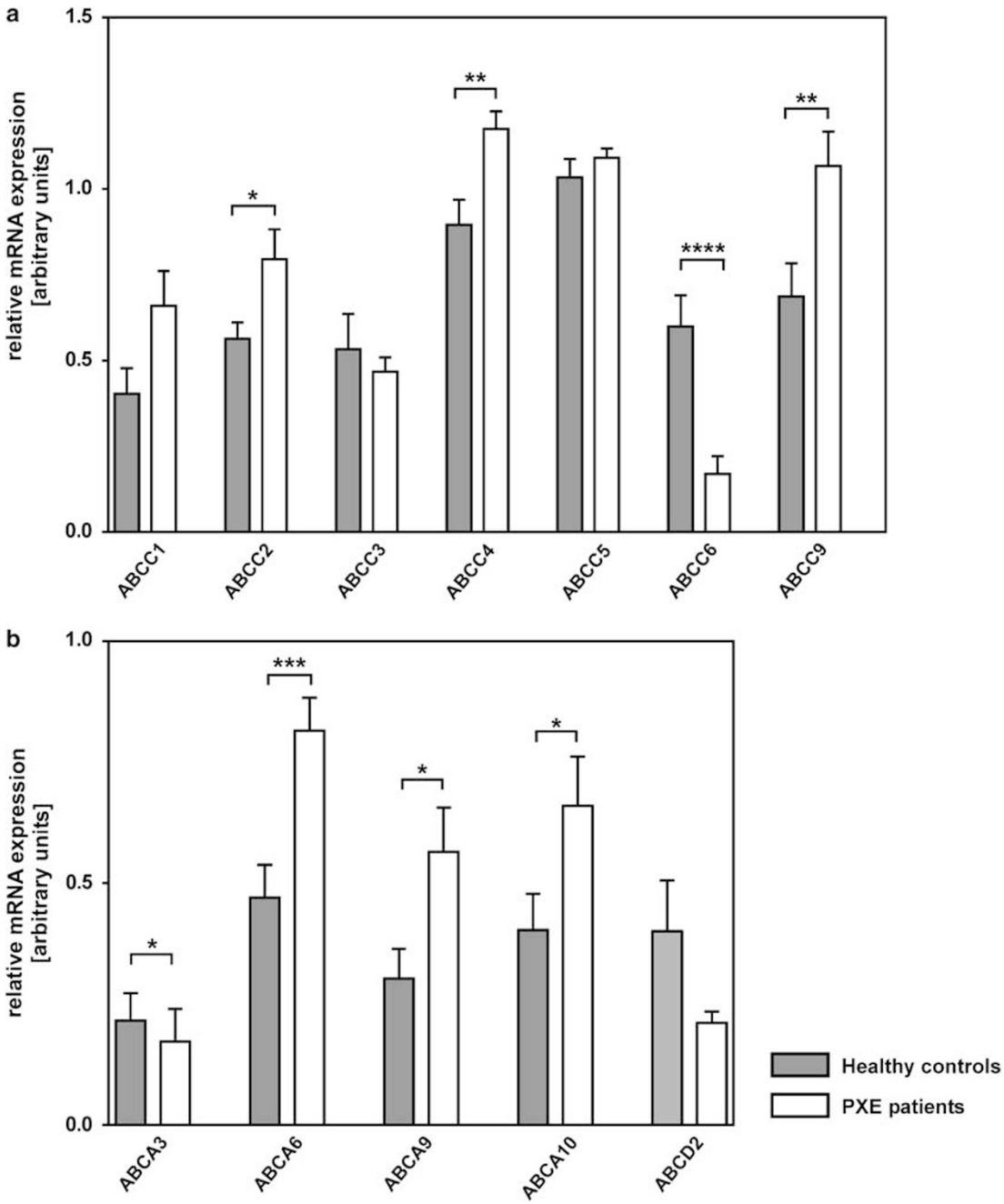

Figure 2 Validation of ABC transporter transcript levels by quantitative real-time PCR. (**a**) Validation of *ABCC* subclass members mRNA expression by quantitative real-time PCRs in dermal fibroblasts derived from skin biopsies from PXE patients (white columns) and healthy controls (gray bars; $n = 6$, each). (**b**) Validation of *ABCA* subclass members and *ABCD2* mRNA expression by quantitative real-time PCRs in dermal fibroblasts derived from skin biopsies from PXE patients and healthy controls ($n = 6$, each). Data are presented in arbitrary units as means with corresponding standard error. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

of competing primer dimers (Figure 1a). To verify our assay, we also analyzed *ABCC6* mRNA expression in HepG2 cells, a hepatocarcinoma cell line. *ABCC6* mRNA expression levels were expressed 50-fold higher in HepG2 cells compared to NHDFs (Figure 1b). We found significantly lower *ABCC6* mRNA expression in PXE patients compared to healthy controls (0.60 ± 0.09 and 0.17 ± 0.05, respectively, $P < 0.0001$; Figure 2a). No *ABCC6* transcripts were quantifiable in patients P265F, P3M, P128M and P341M (Figure 1c). Very low but clearly detectable *ABCC6* mRNA levels were measured in PXE patients P229F and P308M.

The mRNA expression of the selected other ABC transporter genes were highly variable between the analyzed individuals but reflect the findings of the TLDA analysis. We found moderately but significantly higher *ABCC2*, *ABCC4* and *ABCC9* mRNA expression in PXE patients (Figure 2a).

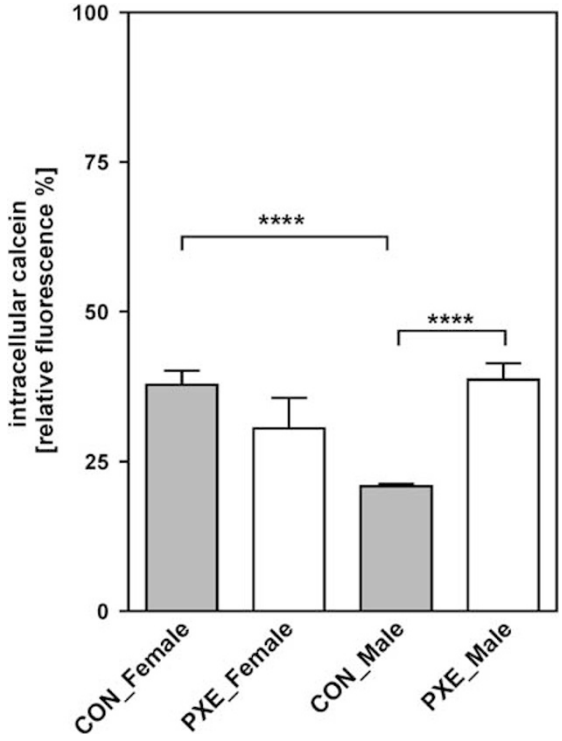

Figure 3 Analysis of MRP efflux activity of HDFs from healthy controls (gray bars) and PXE patients (white bars). Male HDFs show higher efflux rates than female HDFs. Male PXE patients exhibited a higher accumulation of calcein compared to male HDFs. Data are presented as remaining intracellular calcein (fluorescence) in % from default values at time point 0 min. CON = healthy controls. $^{****}P<0.0001$.

Significantly higher gene expression of *ABCA6*, *ABCA9* and *ABCA10* was detected in the patient's samples as already observed in the TLDA analysis (Figure 2b). *ABCA3* transcript levels were moderately but significantly reduced in PXE patients ($P<0.05$). Analysis of *ABCD2* mRNA expression revealed no significant difference comparing control and PXE fibroblasts.

Analysis of MRP Efflux Activity of Control and PXE Patient HDFs

Analysis of the MRP efflux activity revealed interesting results. We observed that dermal fibroblasts from male subjects exhibit significantly higher efflux rates. MRP efflux activities of dermal fibroblasts from male PXE patients were determined to be significantly reduced compared to male NHDFs ($P<0.0001$, Figure 3). In contrast, the dermal fibroblasts of female PXE patients showed no differences compared to female NHDFs (Figure 3).

Silencing of *ABCC6*/MRP6 Expression by *ABCC6*-Specific siRNA

We further examined the influence of MRP6 deficiency through usage of *ABCC6*-specific siRNA. HDFs from four different donors were transfected with *ABCC6*-specific siRNA and nonsense siRNA oligonucleotides. Three different *ABCC6*-specific siRNA oligonucleotides were tested in preceding experiments (Table 3). *ABCC6*-specific siRNA oligonucleotides 1 and 2 yielded a knockdown of *ABCC6* mRNA expression of more than 90% (data not shown). *ABCC6* mRNA expression was significantly downregulated with 40 nM of *ABCC6*-specific siRNA oligonucleotide 1 for 48, 96 and 168 h (Figure 4). Preliminary results demonstrated a reduced MRP efflux activity 168 and 240 h after transfection, although the reduction was only negligible (12% in the female and ~5% in the male dermal fibroblast cultures compared to the scramble siRNA-transfected control culture, data not shown). Analysis of lineage markers of PXE reflected a PXE phenotype: *ELN* gene expression was significantly increased by up to 12-fold after 168 h incubation time in all four control fibroblast cultures (Figure 5a). Moreover, transcript levels of manganese superoxide dismutase (*SOD2*) were increased up to threefold (Figure 5b). Next, we analyzed the effect of the *ABCC6*-specific knockdown on the gene expression of other homologues ABCC transporter genes. We observed a slight effect of *ABCC6*-specific siRNA on *ABCC1* and *ABCC4* at early time points, returning to normal expression values after 168 h (data not shown). *ABCC3* transcript levels remained unaffected throughout the experiment. Moreover, moderately increased transcript levels up to 1.5-fold were detected for *ABCC5* at all time points analyzed (Figure 6b), whereas a significantly reduced gene expression was observed for *ABCC2* and *ABCC9* 48 and 96 h after transfection (Figure 6a and b). We found a significantly elevated gene expression of *ABCA3*, *ABCA9* and *ABCA10* (Figure 7). Gene expression of *ABCA6* was significantly reduced at early time points, returning to normal expression values after 168 h (data not shown). We performed the same experiments with *ABCC6*/MRP6-deficient cells from PXE patients P265F and P128M (Table 1). This experiments yielded interesting results: we observed similar significantly elevated *ELN* and *SOD2* expression as already seen in the *ABCC6*/MRP6-deficient control fibroblasts (Figure 5). We also found elevated gene expression levels for *ABCA3* and *ABCA10*, whereas transcript levels for *ABCA9* were decreased in the silenced PXE fibroblasts at 48 and 96 h, with a moderate increase at time point 168 h (Figure 7). Moderately increased transcript levels up to 1.5-fold were detected for *ABCC5* at all time points analyzed (Figure 6b). Futhermore, a significantly reduced gene expression was observed for *ABCC2* and *ABCC9* 48 and 96 h after transfection (Figure 6a and c).

DISCUSSION

MRP6 deficiency is the cause for PXE manifestation due to mutations in the *ABCC6* gene. To date, little is known about the implication of *ABCC6*/MRP6 in PXE pathogenesis. *ABCC6*/MRP6 is one of 49 more or less well-characterized ABC transporter genes. In the present study we investigated whether MRP6 deficiency leads to an altered gene expression profile of other ABC transporter genes. Several studies

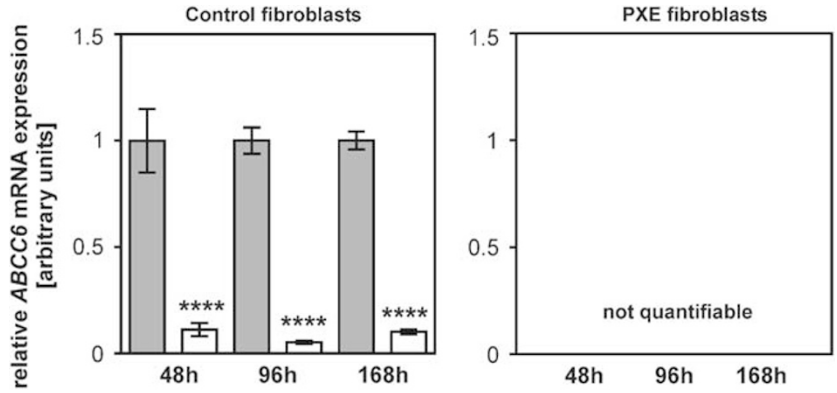

Figure 4 Effect of siRNA-mediated knockdown on *ABCC6* gene expression. Gray bars: cell cultures transfected with a scramble siRNA-negative control; white bars: *ABCC6*-specific siRNA-treated HDFs. Data are presented in arbitrary units as means with corresponding standard error at time points 48, 96 and 168 h. Experiments were performed with HDFs from healthy controls ($n=4$) and PXE fibroblasts ($n=2$). $^{****}P<0.0001$.

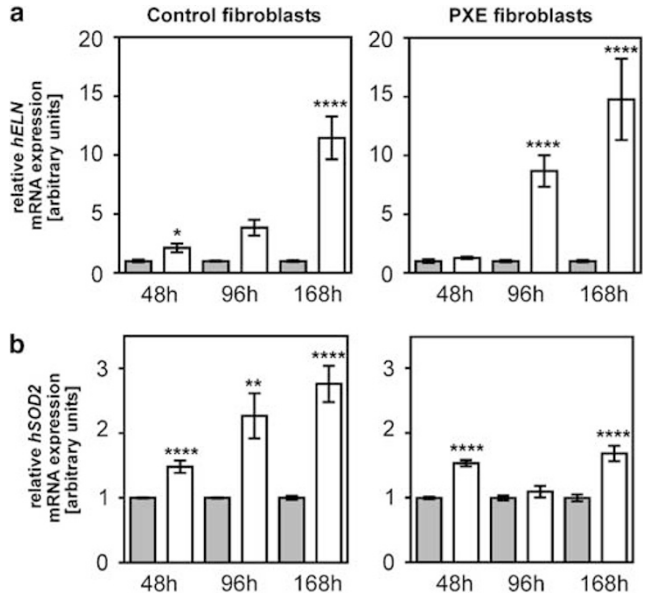

Figure 5 Effect of siRNA-mediated knockdown of *ABCC6* gene expression on PXE lineage markers. (**a**) Relative *ELN* mRNA expression. (**b**) Relative *SOD2* mRNA expression. Gray bars: cell cultures transfected with a scramble siRNA-negative control; white bars: *ABCC6*-specific siRNA-treated HDFs. Data are presented in arbitrary units as means with corresponding standard error at time points 48, 96 and 168 h. Experiments were performed with HDFs from healthy controls ($n=4$) and PXE fibroblasts ($n=2$). $^{*}P<0.05$; $^{**}P<0.01$; $^{****}P<0.0001$.

reported increased expression of related ABCC/MRP proteins as a mechanism to compensate a loss of function.[32,33]

We detected altered gene expression of ABCA transporters and the homologous ABCC subfamily members 2, 4 and 9 in dermal fibroblasts derived from PXE patient's skin biopsies. The observed alterations in gene expression and MRP efflux activity reveal high interindividual variability due to different *ABCC6* genotypes or genetic background. This may contribute to the great clinical variability observed in PXE patients. It is of important interest that *ABCC6*/MRP6-deficiency has a remarkable effect on the gene expression of *ABCA* subclass members. To date, *ABCA1* is the best characterized member of this subclass. It is mainly involved in HDL biosynthesis and cholesterol efflux.[39,40] Polymorphisms in the ABCA1 were associated with cardiovascular disease and low plasma HDL levels.[41–43] Wang *et al* previously reported that plasma HDL concentrations varied in a PXE patient and carriers of the *ABCC6*/MRP6 polymorphisms p.R1268Q.[44] Furthermore, *abcc6* knockout mice developed a 25% reduction in plasma HDL cholesterol.[8] *ABCA3* is a regulator of lamellar body metabolism and was reported to form lipid containing vesicles in human embryonic kidney cells.[45] Little is known about the physiological function of the ABCA6-like transporters including *ABCA6*, *ABCA9*, *ABCA10*. The members of this subclass were reported to be regulated by cholesterol inversely to *ABCA1* and therefore to act in opposed pathways.[42] Gene expression of *ABCA1* was previously shown to be positively and negatively regulated by products of the mevalonate pathway in different cell lines, as well in dermal fibroblasts.[46] This pathway supplies polyisoprenoid-conjugates as percursors to further synthesize dolichol, menaquinone (vitamin K_n), tocopherol, ubichinone and finally cholesterol.[47] It was previously hypothesized that vitamin K-conjugates might be possible *ABCC6* substrates.[48]

We further observed enhanced *ABCC2* gene expression in PXE patients samples; in female NHDFs and, most notably, in HDFs from female PXE patients (data not shown). These results are consistent with a previous study reporting gender differences in *abcc2*/mrp2 mRNA and protein expression in a mouse model for chronic kidney disease.[49] *Abcc2*/mrp2, *abcc3*/mrp3 and *abcc4*/mrp4 expression in mice were also reported to be induced in the context of oxidative stress.[50] Mild chronic oxidative stress might be involved in PXE manifestation.[28] *ABCC4*/MRP4 and *ABCC5*/MRP5 were demonstrated to transport cyclic nucleotides and nucleotide analogs, even if controversial results were obtained regarding the strength of substrate affinity.[51] Moreover, both transporters were thought to serve as overflow pumps under

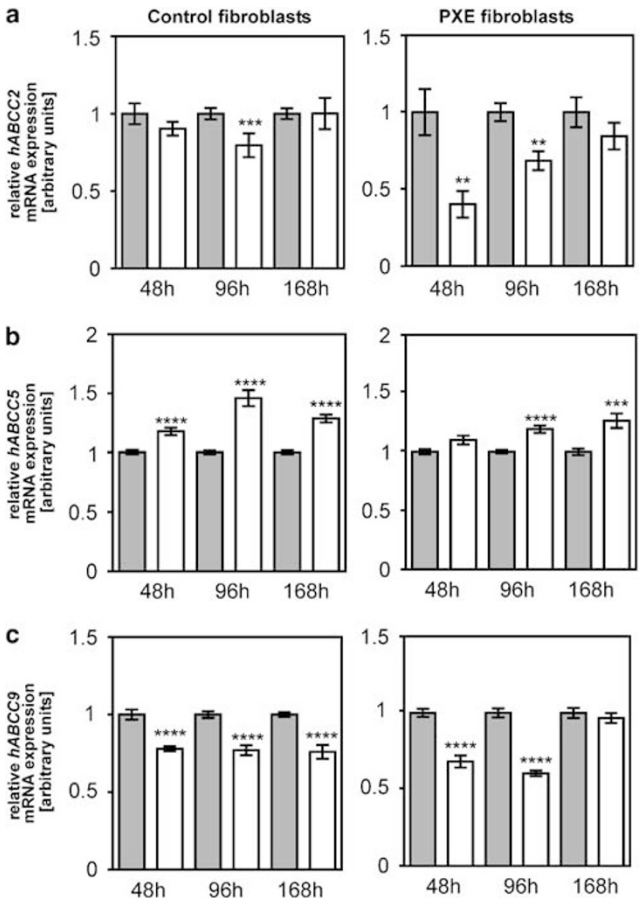

Figure 6 Effect of siRNA-mediated knockdown of *ABCC6* gene expression on gene expression of ABCC transporter proteins. (**a**) *ABCC2*, (**b**) *ABCC5* and (**c**) *ABCC9*. Experiments were performed with HDFs from healthy controls ($n=4$) and PXE fibroblasts ($n=2$). Gray bars: cell cultures transfected with a scramble siRNA-negative control; white bars: *ABCC6*-specific siRNA-transfected HDFs. Data are presented in arbitrary units as means with corresponding standard error at time points 48, 96 and 168 h. $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

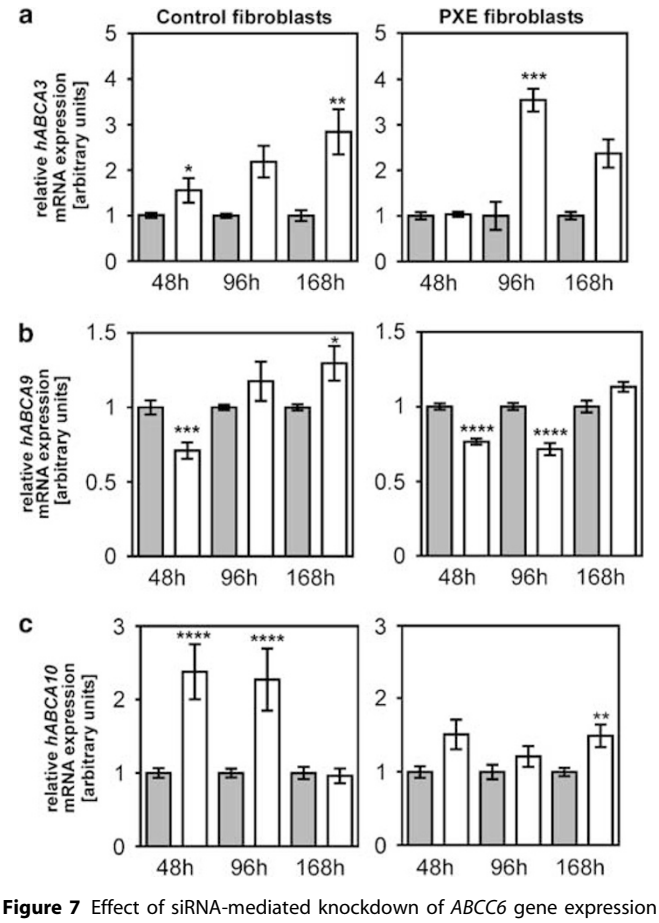

Figure 7 Effect of siRNA-mediated knockdown of *ABCC6* gene expression on gene expression of ABCA transporter proteins. (**a**) *ABCA3*, (**b**) *ABCA9* and (**c**) *ABCA10*. Experiments were performed with HDFs from healthy controls ($n=4$) and PXE fibroblasts ($n=2$). Gray bars: cell cultures transfected with a scramble siRNA-negative control; white bars: *ABCC6*-specific siRNA-transfected HDFs. Data are presented in arbitrary units as means with corresponding standard error at time points 48, 96 and 168 h. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

conditions where cGMP synthesis was impaired.[52] Induction of *ABCC4* and *ABCC5* gene expression suggests an increase in cAMP and cGMP accumulation/efflux due to MRP6 deficiency in PXE. In contrast to the other members of subclass C, *ABCC9*/sulfonylurea receptor (SUR) 2 has no identified transport function. SURs are ATP-sensitive potassium ion channels which form dimers with other postassium-channels of the K_{IR}-family. SURs are typical ABC transporter proteins due to their topology, and various compounds were demonstrated to modulate their activity as phosphoinositides and long-chain acyl coenzyme A derived from fatty acids.[53] The fact that most of the genes with altered expression due to *ABCC6*/MRP6 deficiency belong to the ABCA subclass points to a role of *ABCC6*/MRP6 in lipid metabolism. This assumption is underlined by the identification of *ABCC9*/SUR2 as further candidate in PXE pathogenesis since *ABCC9* is also affected by intermediates of lipid biosynthesis.

Our study design has several limitations due to the small sample size used and our major focus on investigating transcript levels. It is noteworthy that protein expression was not investigated due to study limitations, these may differ from mRNA expression. So, our new findings have to be confirmed on the level of protein expression which was behind the scope of our study. In order to investigate whether the impaired gene expression profile is really caused by MRP6 deficiency, and not by the high interindividual variability, we impaired *ABCC6*/MRP6 activity *in vitro*. Therefore, we knocked down *ABCC6*/MRP6 mRNA and protein expression by using target-specific siRNA to further analyze the influence of MRP deficiency on gene expression of other ABC transporter proteins and lineage markers of PXE manifestation. Indeed, specific downregulation of *ABCC6* gene expression led to increased gene expression of the PXE marker genes *ELN* and *SOD2*. We further observed increased gene

expression of *ABCA3*, *ABCA9* and *ABCA10* which corroborated the result from former experiments. In contrast, gene expression of *ABCC2*, *ABCC9* and *ABCA6* was controversially reduced. This needs to be further clarified and might be just due to off-target effects of siRNA silencing on highly homologous ABC transporter genes.

To our surprise, we observed similar siRNA-mediated effects on the gene expression profile in *ABCC6*/MRP6-deficient cells. To the best of our knowledge, siRNA silencing of target genes that cause genetic disorders has never been performed in cells, whose target expression is already disturbed. Therefore, it is quite difficult to evaluate the obtained results. The observed gene expression changes might be just due to off-target effects often mediated by siRNA silencing, particularly, in case of the PXE lineage markers *ELN* and *SOD2* that are affected by several conditions, including stress. Besides we could detect small amounts of *ABCC6* amplicons by performing standard PCRs pointing to a basal or residual *ABCC6* gene expression in PXE fibroblasts (data not shown). Hence, another possibility is that also residual target gene expression might be affected by siRNA silencing leading to an intensification of an already existing phenotype. These concerns have to be clarified for instance by performing complementation studies and using further *ABCC6*-specific siRNA oligonucleotides targeting other sequences of the *ABCC6* mRNA.

Our results show that the use of siRNA to downregulate *ABCC6*/MRP6 expression might be helpful for investigating PXE pathogenesis in cell culture models but that this strategy also reveals several limitations. We found no effect on the MRP efflux activity analyzing silenced control fibroblasts. The *ABCC6*/MRP6 knockdown mediated by siRNA might be accompanied by residual MRP6 protein. Moreover, we cannot exclude that *ABCC6*-specific siRNA has important off-target effects on other genes, particularly on highly homologues genes thus explaining the controversial results, especially obtained when analyzing samples from early time points after silencing. However, the usage of *ABCC6*-specific siRNA to knockdown *ABCC6*/MRP6 gene and protein expression is a valuable tool to determine what happens within the cell and to investigate the molecular mechanisms leading to PXE manifestation. Using different cell donors takes into account varying genetic backgrounds, which is important to detect common pathomechanisms induced by *ABCC6*/MRP6 deficiency. Therefore, our experimental design might be helpful in explaining the great clinical variability in PXE manifestation.

The observations of our study further support a role for *ABCC6*/MRP6 in transporting metabolites of sterol biosynthesis, as these function as important regulators of ABCA transporters. Many characteristic pathobiochemical hallmarks seen in PXE may arise from pathological changes in lipid metabolism, for instance, calcification, oxidative stress and enhanced matrix protein synthesis. It will be of great interest to uncover a possible role for *ABCC6*/MRP6 in this important metabolic pathway as this would be a major step toward disclosing the possible physiological substrate of *ABCC6*/MRP6.

ACKNOWLEDGEMENTS

We thank Manfred Haas, Veronika Schulz, Alexandra Adam and Christoph Lichtenberg for their excellent technical assistance and Sarah L Kirkby for her linguistic advice. We are very grateful to all the PXE patients, whose cooperation made this study possible. Furthermore, we thank Peter Hof, chairman of the Selbsthilfegruppe für PXE-Erkrankte Deutschlands 1999 e.V., and the members of the clinical outpatients department for PXE at the Bethesda hospital in Freudenberg, Germany. This work was supported by a grant from the 'Stiftung für Pathobiochemie und Molekulare Diagnostik' of the Deutsche Vereinte Gesellschaft für Klinische Chemie und Laboratoriumsmedizin.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

1. Kool M, van der Linden M, de Haas M, *et al*. Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. Cancer Res 1999;59:175–182.
2. Bergen AA, Plomp AS, Schuurman EJ, *et al*. Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet 2000;25:228–231.
3. Le Saux O, Urban Z, Tschuch C, *et al*. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet 2000;25:223–227.
4. Ringpfeil F, Lebwohl MG, Christiano AM, *et al*. Pseudoxanthoma elasticum: mutations in the MRP6 gene encoding a transmembrane ATP-binding cassette (ABC) transporter. Proc Natl Acad Sci USA 2000;97:6001–6006.
5. Neldner K, Struk B. Pseudoxanthoma elasticum. In: Royce PM, Steinmann B (eds). Connective Tissue and its Heritable Disorders. Wiley-Liss Inc.: New York, 2002, pp 561–583.
6. Schulz V, Hendig D, Henjakovic M, *et al*. Mutational analysis of the ABCC6 gene and the proximal ABCC6 gene promoter in German patients with pseudoxanthoma elasticum (PXE). Hum Mutat 2006;27:831.
7. Klement JF, Matsuzaki Y, Jiang QJ, *et al*. Targeted ablation of the abcc6 gene results in ectopic mineralization of connective tissues. Mol Cell Biol 2005;25:8299–8310.
8. Gorgels TG, Hu X, Scheffer GL, *et al*. Disruption of Abcc6 in the mouse: novel insight in the pathogenesis of pseudoxanthoma elasticum. Hum Mol Genet 2005;14:1763–1773.
9. Haimeur A, Conseil G, Deeley RG, *et al*. The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Metab 2004;5:21–53.
10. Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006;86:849–899.
11. Scheffer GL, Hu X, Pijnenborg AC, *et al*. MRP6 (ABCC6) detection in normal human tissues and tumors. Lab Invest 2002;82:515–518.
12. Jiang Q, Uitto J. Pseudoxanthoma elasticum: a metabolic disease? J Invest Dermatol 2006;126:1440–1441.
13. Madon J, Hagenbuch B, Landmann L, *et al*. Transport function and hepatocellular localization of mrp6 in rat liver. Mol Pharmacol 2000;57:634–641.
14. Götting C, Hendig D, Adam A, *et al*. Elevated xylosyltransferase I activities in pseudoxanthoma elasticum (PXE) patients as a marker of stimulated proteoglycan biosynthesis. J Mol Med 2005;83:984–992.
15. Hendig D, Schulz V, Arndt M, *et al*. Role of serum fetuin-A, a major inhibitor of systemic calcification, in pseudoxanthoma elasticum. Clin Chem 2006;52:227–234.
16. Le Saux O, Bunda S, VanWart CM, *et al*. Serum factors from pseudoxanthoma elasticum patients alter elastic fiber formation *in vitro*. J Invest Dermatol 2006;126:1497–1505.
17. Gheduzzi D, Boraldi F, Annovi G, *et al*. Matrix Gla protein is involved in elastic fiber calcification in the dermis of pseudoxanthoma elasticum patients. Lab Invest 2007;87:998–1008.

18. Jiang Q, Li Q, Uitto J. Aberrant mineralization of connective tissues in a mouse model of pseudoxanthoma elasticum: systemic and local regulatory factors. J Invest Dermatol 2007;127:1392–1402.
19. Li Q, Jiang Q, Schurgers LJ, *et al*. Pseudoxanthoma elasticum: reduced gamma-glutamyl carboxylation of matrix gla protein in a mouse model (Abcc6−/−). Biochem Biophys Res Commun 2007;364:208–213.
20. Meng H, Vera I, Che N, *et al*. Identification of Abcc6 as the major causal gene for dystrophic cardiac calcification in mice through integrative genomics. Proc Natl Acad Sci USA 2007;104:4530–4535.
21. Jiang Q, Dibra F, Wang K, *et al*. Cell-based therapy for Pseudoxanthoma elasticum: reduction of connective tissue mineralization in a mouse model ($Abcc6^{-/-}$) by transplantation of wild-type hepatocytes. J Invest Dermatol 2008, (Abstract).
22. Jiang Q, Endo M, Dibra F, *et al*. Pseudoxanthoma elasticum is a metabolic disease. J Invest Dermatol 2008.
23. Uitto J, Pulkkinen L, Ringpfeil F. Molecular genetics of pseudoxanthoma elasticum: a metabolic disorder at the environment-genome interface? Trends Mol Med 2001;7:13–17.
24. Hu X, Peek R, Plomp A, *et al*. Analysis of the frequent R1141X mutation in the ABCC6 gene in pseudoxanthoma elasticum. Invest Ophthalmol Vis Sci 2003;44:1824–1829.
25. Quaglino D, Boraldi F, Barbieri D, *et al*. Abnormal phenotype of *in vitro* dermal fibroblasts from patients with Pseudoxanthoma elasticum (PXE). Biochim Biophys Acta 2000;1501:51–62.
26. Passi A, Albertini R, Baccarani Contri M, *et al*. Proteoglycan alterations in skin fibroblast cultures from patients affected with pseudoxanthoma elasticum. Cell Biochem Funct 1996;14:111–120.
27. Quaglino D, Sartor L, Garbisa S, *et al*. Dermal fibroblasts from pseudoxanthoma elasticum patients have raised MMP-2 degradative potential. Biochim Biophys Acta 2005;1741:42–47.
28. Pasquali-Ronchetti I, Garcia-Fernandez MI, Boraldi F, *et al*. Oxidative stress in fibroblasts from patients with pseudoxanthoma elasticum: possible role in the pathogenesis of clinical manifestations. J Pathol 2006;208:54–61.
29. Belinsky MG, Chen ZS, Shchaveleva I, *et al*. Characterization of the drug resistance and transport properties of multidrug resistance protein 6 (MRP6, ABCC6). Cancer Res 2002;62:6172–6177.
30. Ilias A, Urban Z, Seidl TL, *et al*. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). J Biol Chem 2002;277:16860–16867.
31. Boraldi F, Quaglino D, Croce MA, *et al*. Multidrug resistance protein-6 (MRP6) in human dermal fibroblasts. Comparison between cells from normal subjects and from pseudoxanthoma elasticum patients. Matrix Biol 2003;22:491–500.
32. Hirohashi T, Suzuki H, Ito K, *et al*. Hepatic expression of multidrug resistance-associated protein-like proteins maintained in eisai hyperbilirubinemic rats. Mol Pharmacol 1998;53:1068–1075.
33. Kuroda M, Kobayashi Y, Tanaka Y, *et al*. Increased hepatic and renal expressions of multidrug resistance-associated protein 3 in Eisai hyperbilirubinuria rats. J Gastroenterol Hepatol 2004;19:146–153.
34. Li Q, Jiang Q, Larusso J, *et al*. Targeted ablation of Abcc1 or Abcc3 in Abcc6(−/−) mice does not modify the ectopic mineralization process. Exp Dermatol 2007;16:853–859.
35. Christiano AM, Lebwohl MG, Boyd CD, *et al*. Workshop on pseudoxanthoma elasticum: molecular biology and pathology of the elastic fibers. Jefferson Medical College, Philadelphia, Pennsylvania, June 10, 1992. J Invest Dermatol 1992;99:660–663.
36. Lebwohl M, Neldner K, Pope FM, *et al*. Classification of pseudoxanthoma elasticum: report of a consensus conference. J Am Acad Dermatol 1994;30:103–107.
37. Langmann T, Mauerer R, Schmitz G. Human ATP-binding cassette transporter TaqMan low-density array: analysis of macrophage differentiation and foam cell formation. Clin Chem 2006;52:310–313.
38. Vandesompele J, De Preter K, Pattyn F, *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002;3:research0034.1–0034.11.
39. Bodzioch M, Orso E, Klucken J, *et al*. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat Genet 1999;22:347–351.
40. Lawn RM, Wade DP, Garvin MR, *et al*. The Tangier disease gene product ABC1 controls the cellular apolipoprotein-mediated lipid removal pathway. J Clin Invest 1999;104:R25–R31.
41. Brousseau ME, Bodzioch M, Schaefer EJ, *et al*. Common variants in the gene encoding ATP-binding cassette transporter 1 in men with low HDL cholesterol levels and coronary heart disease. Atherosclerosis 2001;154:607–611.
42. Wenzel JJ, Piehler A, Kaminski WE. ABC A-subclass proteins: gatekeepers of cellular phospho- and sphingolipid transport. Front Biosci 2007;12:3177–3193.
43. Frikke-Schmidt R, Nordestgaard BG, Jensen GB, *et al*. Genetic variation in ABC transporter A1 contributes to HDL cholesterol in the general population. J Clin Invest 2004;114:1343–1353.
44. Wang J, Near S, Young K, *et al*. ABCC6 gene polymorphism associated with variation in plasma lipoproteins. J Hum Genet 2001;46:699–705.
45. Nagata K, Yamamoto A, Ban N, *et al*. Human ABCA3, a product of a responsible gene for abca3 for fatal surfactant deficiency in newborns, exhibits unique ATP hydrolysis activity and generates intracellular multilamellar vesicles. Biochem Biophys Res Commun 2004;324:262–268.
46. Denis M, Bissonnette R, Haidar B, *et al*. Expression, regulation, and activity of ABCA1 in human cell lines. Mol Genet Metab 2003;78:265–274.
47. Goldstein JL, Brown MS. Regulation of the mevalonate pathway. Nature 1990;343:425–430.
48. Borst P, van de Wetering K, Schlingemann R. Does the absence of ABCC6 (Multidrug Resistance Protein 6) in patients with Pseudoxanthoma elasticum prevent the liver from providing sufficient vitamin K to the periphery? Cell Cycle 2008;7:1575–1579.
49. Lu H, Klaassen C. Gender differences in mRNA expression of ATP-binding cassette efflux and bile acid transporters in kidney, liver, and intestine of 5/6 nephrectomized rats. Drug Metab Dispos 2008;36:16–23.
50. Maher JM, Dieter MZ, Aleksunes LM, *et al*. Oxidative and electrophilic stress induces multidrug resistance-associated protein transporters via the nuclear factor-E2-related factor-2 transcriptional pathway. Hepatology 2007;46:1597–1610.
51. Borst P, de Wolf C, van de Wetering K. Multidrug resistance-associated proteins 3, 4, and 5. Pflugers Arch 2007;453:661–673.
52. Wielinga PR, van der Heijden I, Reid G, *et al*. Characterization of the MRP4- and MRP5-mediated transport of cyclic nucleotides from intact cells. J Biol Chem 2003;278:17664–17671.
53. Bryan J, Munoz A, Zhang X, *et al*. ABCC8 and ABCC9: ABC transporters that regulate K+ channels. Pflugers Arch 2007;453:703–718.
54. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 2001;25:402–408.