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Differential Expression of a Chloride Intracellular Channel Gene, CLIC4, in Transforming Growth Factor-β1-Mediated Conversion of Fibroblasts to Myofibroblasts

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Conversion of fibroblasts into myofibroblasts as mediated by transforming growth factor-β1 (TGF-β1) is the most prominent stromal reaction to a number of epithelial lesions including breast cancer. To identify genes which are regulated during this process, the mRNA profiles from primary breast fibroblasts treated with or without TGF-β1 were analyzed by differential display. Ninety-five differentially expressed transcripts were PCR-cloned and sequenced, and 28 clones were selected for verification in a hybridization array. By use of gene-specific sequence tags, nine differentially expressed genes were identified. One of the clones, identified as CLIC4, a member of the CLIC family of chloride channels, was up-regulated more than 16 times in myofibroblasts and was therefore chosen for further analysis. Using RT-PCR, comparison with CLIC1, CLIC2, CLIC3, and CLIC5 demonstrated that CLIC4 was unique by being up-regulated by TGF-β1 in myofibroblasts. Immunohistochemistry showed a hitherto unknown, distinctive pattern of CLIC4 expression in breast stroma. Whereas normal breast fibroblasts were devoid of CLIC4 protein expression, myofibroblasts of breast carcinomas were strongly CLIC4-positive. The functional significance of CLIC4 was analyzed in MEF/3T3 fibroblasts by conditional expression using the tetracycline-repressive gene regulation system. In a migration assay, we found that CLIC4 inhibited cell motility by 27%. These results suggest that CLIC4 is differentially regulated in fibroblasts and that its expression contributes to a collective stationary myofibroblast phenotype. ***(Am J Pathol 2002, 161:471–480)***

The fibroblast is the most abundant cell type in normal connective tissues and plays a central role in synthesis, degradation, and remodeling of the extracellular matrix in health and disease. The majority of fibroblasts demonstrate the ability to convert into α-smooth muscle (α-sm) actin-containing myofibroblasts in response to specific stimuli.[1–3]

Whereas myofibroblasts are only transiently present in normal wound healing, continuous conversion of fibroblasts into myofibroblasts followed by myofibroblast proliferation are chronic processes in numerous fibrotic lesions including the desmoplastic response to invading breast carcinoma.[4–6]

We have previously demonstrated that expression of α-sm actin in breast myofibroblasts is triggered by transforming growth factor (TGF)-β1,[7] and TGF-β1 is now considered as a key molecule and a master switch for the general fibrotic program.[8] Beyond the level of cytoskeletal modulation, little is known about which genes are regulated during the induction of myofibroblast differentiation. The aim of the present study was to identify such genes. Using fibroblasts from normal primary breast biopsies isolated under chemically defined conditions, which support the *in situ* G_0 phenotype, differential display-reverse transcription PCR (DD-RT-PCR) was used to unravel transcriptional changes after TGF-β1 stimulation. Our data indicate that independently of cell cycle, TGF-β1 regulates transcription factors, cytoskeletal proteins, extracellular matrix molecules, cytokines and receptors, and transport proteins, including a member of the chloride intracellular channel (CLIC) gene family, CLIC4. The human genes of the CLIC protein family so far includes five members: CLIC1/NCC27, CLIC2/XAP121, CLIC3, CLIC4, and CLIC5,[9–14] whose expression have been described in various tissues. It has been a matter of debate if the proteins are subunits of a chloride channel

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complex rather than channels themselves.[12] It has, however, recently been demonstrated that members of the CLIC gene family indeed function as chloride transporters[15,16] but the functional significance of CLIC expression *per se* has hitherto not been addressed. In this work, we show that a possible function of CLIC4 in breast myofibroblasts is to retard cell motility.

Materials and Methods

Cell Culture and RNA Isolation

Normal breast biopsies ($n = 3$) were cut and rotated for 24 hours in serum-free medium, Dulbecco's modified Eagle's medium–Ham's F12 (DME-F12) supplemented with 2 mmol/L glutamine (Sigma-Aldrich, Vallensbæk Strand, Denmark) and 50 μg gentamicin/ml (Garamycin, Schering, Kenilworth, NJ) and 900 IU collagenase/ml (CLS III, Worthington Biochemical Corporation, purchased from Medinova, Hellerup, Denmark). The fibroblasts were isolated by differential centrifugation of the collagenase digest and plated in DME-F12 in Primaria T-25 flasks (Falcon 3813, Becton Dickinson, Albertslund, Denmark) as previously described.[7] Within the first week of cultivation, the cultures were stimulated with 100 pg of TGF-β1/ml (T-1654, Sigma-Aldrich) or vehicle for 6 days[7] before RNA extraction. RNA was extracted with TRIZOL Reagent (Gibco BRL, Life Technologies, Tåstrup, Denmark) according to the manufacturer's instructions. The yield was 7 to 18 μg of total RNA per subconfluent T-25 flask.

Differential Display and Sequencing

Before RT-PCR and DD, total RNA samples were treated with DNase I (18068–015, Gibco BRL) to remove any possible DNA contamination. DD-RT-PCR was performed using the HIEROGLYPH mRNA profile kit (Genomyx Corporation, Foster City, CA) which includes 12 oligo(dT) anchored T7 3′ primers (5′-ACGACTCACTATAGGGCTTTTTTTTTTTXX-3′) and 20 arbitrary M13r 5′ primers (5′-ACAATTTCACACAGGA(10X)-3′) which in combination cover up to 95% of the entire mRNA pool. For reverse transcription, 2 μl of total RNA (0.1 μg/μl) was mixed with 2 μl of anchored primer (2 μmol/L), and incubated at 65°C for 5 minutes in a thermal cycler with a heated lid (PTC−100, MJ Research), and cooled on ice. 16 μl of a core mix containing a final concentration of 1X SuperScript II RT buffer (18064–14, Gibco BRL), 25 μmol/L dNTP mix (Boehringer Mannheim purchased from Ercopharm Roche, Hvidovre, Denmark), 10 mmol/L DTT (Gibco BRL), 1 unit/μl RNasin (N2511, Promega, purchased from Bie & Berntsen, Rødovre, Denmark) and 2 units/μl SuperScript II RT enzyme (Gibco BRL) was added per tube, and RT was run in the thermal cycler at 25°C for 10 minutes, 42°C for 60 minutes, 70°C for 15 minutes, followed by hold at 4°C. In each experiment two control samples without RT enzyme were included. The following DD-PCR was carried out in duplicate. For each sample, 2 μl of the arbitrary primer (2 μmol/L) was mixed with 2 μl RT mix, and a PCR core mix was prepared for each anchored primer containing a final concentration of 1X PCR buffer (15 mmol/L $MgCl_2$), 20 μmol/L dNTP mix, 0.2 μmol/L anchored primer, 0.05 unit/μl Tag DNA polymerase (Boehringer Mannheim), and 0.125 μCi/μl [α-^{33}P]dATP (AH9904, Amersham Pharmacia Biotech, Hørsholm, Denmark). 16 μl PCR core mix was added per tube, and DD-PCR was performed at 95°C for 2 minutes, 4 cycles at 92°C for 15 seconds, 46°C for 30 seconds, 72°C for 2 minutes, 25 cycles at 92°C for 15 seconds, 60°C for 30 seconds, 72°C for 2 minutes, followed by 7 minutes at 72°C and hold at 4°C. Samples were mixed with Stop Solution (US70724, USB Corporation, purchased from Amersham), heat denatured and loaded on a 6% denaturing polyacrylamide gel (HR-1000, Genomyx), and run in a GenomyxLR programmable DNA sequencer apparatus (Genomyx) at 40°C, 800 V, 100 W for 16 hours. After washing and drying, the gel was exposed overnight to a Kodak Biomax MR film (Kodak, Glostrup, Denmark). Differentially expressed bands were cut out, reamplified with the Expand High Fidelity PCR System (Boehringer Mannheim) and purified with QIAquick Gel Extraction Kit (Struers KEBO Lab, Albertslund, Denmark) before automatic sequencing in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Nærum, Denmark) using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit. The cDNA fragments were sequenced with M13 (−48) 24-mer reverse sequencing primer resulting in sequence information corresponding to the 3′ end of the mRNA.

Nucleotide sequences were used to search the National Center for Biotechnology Information database with the use of the BLAST program.[17] Multiple sequence alignment was performed with the Clustal W multiple alignment program (v. 1.7) and shaded with Boxshade 3.2.1.

Hybridization Array

The sequences derived from the selected DD bands were used to design specific 5′ primers of 20–23 bases for use with the appropriate 3′-anchor primers allowing PCR-reamplification of homogeneous gene tags for the verification of expression patterns by hybridization analysis.[18] Bands, which were not differentially expressed, and subsequently identified as KIAA1007 protein mRNA, were treated as described above and included as a control. After PCR, the products were run on an agarose gel, purified with QIAquick Gel Extraction Kit (Qiagen, Struers KEBO Lab) and spotted onto nylon membranes (Zeta-Probe GT, BioRad Laboratories, Copenhagen, Denmark) in duplicate by using a 96-well slot blot manifold (Bio-Dot, BioRad Laboratories). Following denaturation, each gene tag was applied in a dilution series of three (1, 1/5, 1/25); the starting amount for each tag was determined empirically.

Five μg total RNA from activated and non-activated fibroblasts were used as template for the generation of ^{32}P-labeled cDNA. Reverse transcription was performed with Superscript Preamplification System (Gibco BRL)

Table 1. Primers Specific for CLIC and Control Genes Used for RT-PCR

Primer	Sequence	T_A	Cycles	Amplicon size
CLIC1 FW	ACACAGCTGGGCTGGACATA	54°	38	190
CLIC1 RV	AACTTCCTCTGAGAGACACCTTCA			
CLIC2 FW	CCTCACCTGAGTCCCAAGTACAA	59°	29	374
CLIC2 RV	TCTTCACGGGCATAGGCATT			
CLIC3 FW	GGACGGCGACAGGCTCAC	59°	29	186
CLIC3 RV	AGGATCTCGGCGCTGTGC			
CLIC4 FW	AGCAGAAGCAGCAGCAG	56°	27	987
CLIC4 RV	TATACCTTGTCTATCCTTGATCCTA			
CLIC5 FW	GGAGATTGACGCCAACACTT	54°	33	199
CLIC5 RV	ACGGGCATAGGCGTTCTT			
KIAA1007 FW	GCCACATCCTTGGGGTCTTC	58°	26	293
KIAA1007 RV	CCAAAGGGCTGGGAAAGTCA			
GAPDH FW	GAAGGTGAAGGTCGGAGT	54°	25	226
GAPDH RV	GAAGATGGTGATGGGATTTC			

using oligo(dT) primers and dNTP(÷C) mix, in the presence of 50 μCi of $\alpha[^{32}P]$dCTP (NEN, Dupharma, Kastrup, Denmark). Unincorporated nucleotides were removed with QIAquick Nucleotide Removal Kit (Qiagen, Struers Kebo Lab) Ten million cpm of each labeled cDNA preparation was hybridized to the duplicate membranes in 10 ml of hybridization solution (50% formamide, 5X SSPE, 2X Denhardt's solution, 0.1% SDS) overnight at 42°C. After washing (once in 1X SSC/0.1% SDS for 30 minutes at room temperature, and three times in 0.2X SSC/0.1% SDS for 30 minutes at 65°C), the membranes were exposed to x-ray film (BioMax MS & TranScreen HE, Kodak) for 1 to 3 days. The developed films were scanned on a SHARP JX-330/FSU and the signal intensities were measured using Gel-Pro Analyzer 3.0 (Media Cybernetics, Silver Spring, MD).

Reverse Transcription PCR

Two μg of total RNA from fibroblasts with or without serum or TGF-β1 stimulation were DNase treated (DNase I Amp Grade, Gibco BRL) and used as template for first strand synthesis with oligo(dT) primers (SuperScript Preamplification System, Gibco BRL) in a 30 μl volume.

A volume of 1 μl from this cDNA served as template for the subsequent PCR amplifications, using primers specific for CLIC1, CLIC2, CLIC3, CLIC4, CLIC5, KIAA1007 protein mRNA, and GAPDH (Table 1), or primers specific for metallothionein (FW: GTGGGCTGTGCCAAGTGT and RV: GGTCACGGTCAGGGTTGT) and WBSCR9 (FW: TGGACGTGCAAGAGTACTGG and RV: TCCCACAGCATATTTGGTCA) using 56°C as annealing temperature (T_A) and 28 and 35 PCR cycles, respectively.

For each reaction amplification was performed with denaturation at 94°C for 1 minute, annealing at the specified T_A (Table 1) for 1 minute, and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 7 minutes.

Each reaction was performed in a 50 μl volume using Expand High Fidelity PCR System (Roche Molecular Biochemicals) with 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, and 200 nmol/L of forward and reverse primers. For RT-PCR on total RNA from CLIC4-transfected MEF/3T3 fibroblasts (see below), mouse-specific primers (α-sm actin, FW: ACTGGGACGACATGGAAAAG and RV: AGAGGCATAGAGGGACAGCA, T_A: 56°C, 26 cycles; GAPDH, FW: CACTCTTCCACCTTCGATGC and RV: CGAGTTGGGATAGGGCCTC, T_A: 57°C, 25 cycles) were used. Control amplification was performed on RNA samples not subjected to reverse transcription to verify that no contaminating genomic DNA was present (data not shown). The PCR products were analyzed by electrophoresis in 1.5% agarose gels.

Cloning and Transfection

The coding region of CLIC4 was inserted into pRevTRE (Clontech, BD Biosciences, Brøndby, Denmark) and stably transfected into MEF/3T3 Tet-Off (Clontech) using the RetroMax retroviral transfection assay as described by the manufacturer (Imgenex, San Diego, CA).

MEF/3T3 Tet-Off cells were cultured in DMEM 11885 containing 10% Tet System Approved Fetal Bovine Serum (Clontech) supplemented with 2 mmol/L L-glutamine and 100 μg/ml G418 (Gibco BRL, purchased from Invitrogen, Tåstrup, Denmark). Clones containing the pRevTRE-CLIC4 vector were selected by adding 400 μg/ml hygromycin B (Gibco BRL).

The resulting pRevTRE-CLIC4 containing cell line was cultured using the culture medium described above, with the addition of 10 ng/ml of the tetracycline derivative, doxycycline (Sigma-Aldrich, purchased from Gerner Jensen, Charlottenlund, Denmark), and CLIC4 expression was induced by removing doxycycline from the culture medium.

Immunohistochemistry

Cryosections (8 μm) of normal or neoplastic breast (diagnosed and kindly provided by Dr. Fritz Rank, Rigshospitalet) were fixed in methanol for 5 minutes at − 20°C, blocked in 10% normal goat serum in phosphate-buffered saline, and incubated in the primary sequence for 2 hours with affinity-purified antiserum AP1058 raised against recombinant CLIC4/huH1(1:5;[13]) washed in 10% normal goat serum, and incubated for 30 minutes in the second sequence with FITC-conjugated goat anti-rabbit

IgG (1:25, 4050–02, Southern Biotechnology Associates, Birmingham, AL). For double staining fluorescence of CLIC4 and α-sm actin, an affinity-purified antiserum raised against purified GST-p64H1 (1:100, CUMC29, kindly provided by Dr. C-H Sung, Weill Medical College of Cornell University, New York, NY[12]) and monoclonal anti-α-sm actin (clone 1A4, A-2547, 1:1000; Sigma-Aldrich) were used in the primary sequence followed by FITC-conjugated goat anti-rabbit IgG (1:25, 4050–02) and Texas Red-labeled goat anti-mouse IgG2a (1:50, 1080–07, Southern Biotechnology Associates) in the second sequence. Preimmune serum was included as a control for CUMC29. After rinsing, the sections were mounted in Fluoromount-G (Southern Biotechnology Associates) containing 2.5 mg/ml freshly prepared *n*-propyl gallate (Sigma-Aldrich). Images were obtained using a Zeiss LSM 510 laser scanning microscope (Carl Zeiss, Jena GmbH).

CLIC4-MEF/3T3 fibroblasts were fixed in methanol, incubated with AP1058 (1:5) or anti-α-smooth muscle actin (1:1000; 1A4, Sigma-Aldrich), and peroxidase immunocytochemistry was performed essentially as previously described[19] with the inclusion of mouse anti-rabbit antibodies (M737, Dako, Glostrup, Denmark) between the primary and secondary antibody for AP1058. Affinity-purified antiserum AP95 raised against a β-galactosidase p64 fusion protein, which recognizes native bovine p64, but not p64 homologues in human or mouse tissue[13,20] was included as a control.

Migration Assay

Migratory activity was assessed using 8 μm pore size polyethylene terephthalate (PET) filter inserts in a 12-well companion plate (catalog nos. 3182 and 3503, Falcon, Becton Dickinson). 1.5 ml of culture medium with or without doxycycline was added to the wells, the filter inserts were placed in the wells and the culture plate was placed in the incubator, while the cells were prepared. Subconfluent cultures of CLIC4-MEF/3T3 or MEF/3T3 Tet-Off fibroblasts (control without CLIC4 insert) with or without 3 days of exposure to 10 ng/ml of doxycycline were trypsinized and counted. 2×10^5 were pelleted by centrifugation ($125 \times g$ for 5 minutes), resuspended in 0.5 ml of medium with or without doxycycline, and plated on the filters. After 6 hours of incubation, the cells on the upper side of the filter were removed with a cotton swab, and the filter inserts were transferred to a well containing a cell stain solution (part no. 20294 of catalog no. ECM502, Chemicon International, Inc. purchased from AHdiagnostics, Aarhus, Denmark), stained for 20 minutes, rinsed in H_2O, and dried. The average number of migrated cells/field was counted using an ocular grid at 20× magnification. The relative percentage of migrated cells is given as the average of four sets of experiments ± SEM.

To study migration at the individual cell level, time lapse video microscopy was used as previously described[21] in three sets of paired measurements ± SD. Significance was tested by Student's *t*-test.

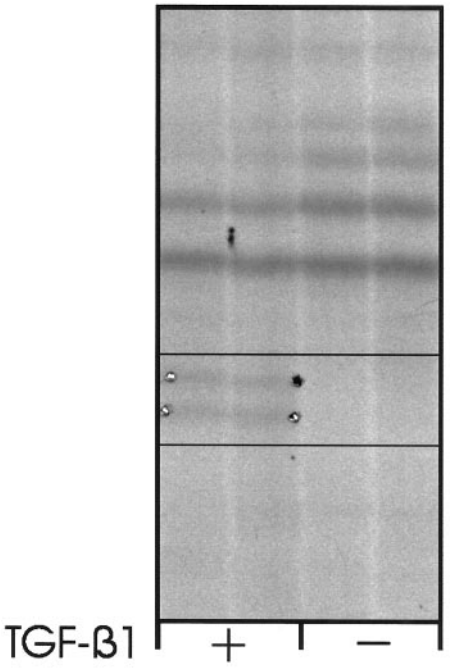

Figure 1. Long-range differential display of TGF-β1-activated fibroblasts. A DD gel demonstrating TGF-β1-mediated expression of two ~1 kb transcripts (**box**) that were eventually identified as representing CLIC4.

Results

Identification of Differentially Expressed Genes in Myofibroblasts

To identify TGF-β1-specific changes in gene expression during conversion of fibroblasts to myofibroblasts, total RNA was extracted from three sets of primary breast fibroblasts, which were either untreated or stimulated for 6 days with TGF-β1.[7] DD-RT-PCR was performed using the HIEROGLYPH mRNA profile kit (Figure 1). Ninety-five differentially expressed transcripts were isolated, reamplified, purified and automatically sequenced. Seventy-seven successfully sequenced nucleotide sequences were used to search the NCBI BLASTN database. Thirty-five of the transcripts represented known genes (Table 2), and 42 represented unknown genes of which 17 resembled PAC/BAC clones (not shown). The major part of the known genes (11/35) represented enzymes, of which nine were up-regulated in myofibroblasts. Three of the up-regulated messages were identified as transport molecules, including a member of the CLIC family of chloride channel proteins. Twenty-eight of the 95 transcripts that were differentially expressed in fibroblasts from at least two of the three biopsies were selected for further verification by hybridization array using gene-specific primers.[18] Of these, nine turned out to be truly differentially expressed (Figure 2). Normal breast biopsies are limited in supply and experiments requiring larger quantities of cells are further hampered by the difficulty of expanding the cell population without extensive selection. We and others have previously demonstrated that fetal calf serum and TGF-β1 both readily convert fibroblasts to myofibroblasts.[7,22] Therefore, to obtain sufficient RNA for verifica-

Table 2. Identified Known Genes Regulated by TGF-β1 in Normal Breast Fibroblasts

Regulated genes	Status in TGF-β1-activated cells
Enzymes	
HPK/GCK-like kinase	↑
Human 68kD PIP5 KI α1	↑
Human Krit 1	↑
Lactate dehydrogenase	↓
NADH4	↑
N-terminal acetyl transferase ard1 subunit	↓
PAPS synthetase 2	↑
PI kinase	↑
Putative helicase	↑
SnoN2	↑
SOD-1	↓
Transcription factors	
Cyclin I	↓
EF-1α	↑
TIF-1	↓
Transport proteins	
Metallothionein	↑
High-density lipoprotein-binding protein	↑
Putative Cl^- channel	↑
ECM modulators	
Collagen X	↑
Fibronectin	↑
Lysyl oxidase	↓
TIMP3	↑
Cytokines and receptors	
Eotaxin	↓
HGF-like activator	↑
KGF	↓
Lipocortin/annexin	↑
TGF-β3R	↓
Structure/motility proteins	
Ankyrin-like protein	↓
Cornichon-like mRNA	↓
Myosin I heavy chain	↑
Myosin class I myh-1c	↓
Others	
GW128 mRNA	↑
TGF-β1-induced mRNA	↑
KIAA0551 protein mRNA	↑
KIAA1334 protein mRNA	↑
WBSCR 9 mRNA	↓

tion in the present study, we routinely used RNA from serum-generated myofibroblasts, which may explain why the expression pattern of metallothionein and WBSCR9 was opposite of that observed in DD of the TGF-β1-generated myofibroblasts. Thus, that TGF-β1 indeed up-regulates metallothionein was further confirmed by RT-PCR (data not shown), and others have shown that >24 hours of exposure of fibroblasts to FCS as used here leads to down-regulation of metallothionein.[23] In contrast, regulation of WBSCR9 by TGF-β1 could not be confirmed by RT-PCR (data not shown). The transcripts showing the highest ratios of differential expression in the hybridization array were the TGF-β3 receptor, down-regulated 24.8-fold, and the CLIC message, up-regulated 16.8-fold (Figure 2). TGF-β3R has been shown by others[24] to be regulated in serum-stimulated fibroblasts. Therefore, the CLIC transcript was chosen for further analysis.

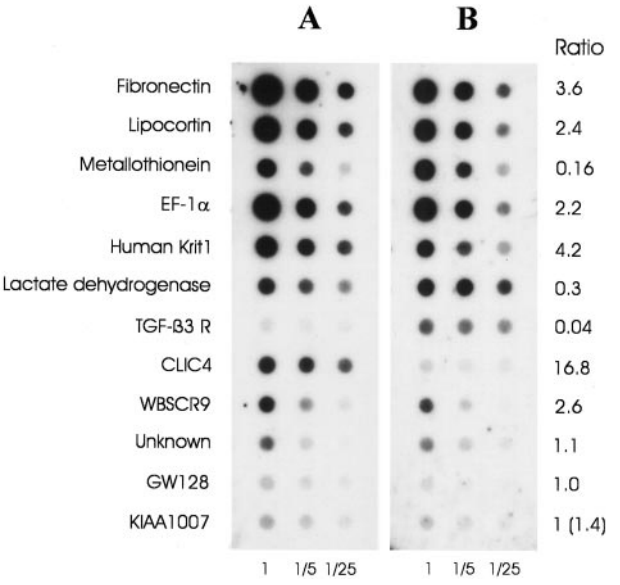

Figure 2. Verification of differential expression by hybridization analysis. Gene tags generated by PCR using gene-specific primers were spotted onto a nylon membrane in a dilution series of 1, 1/5, and 1/25 and hybridized with ^{32}P-labeled cDNA from serum-stimulated fibroblasts (**A**) or non-activated fibroblasts (**B**). The ratios of differential expression (Ratio) was determined by measuring the respective signal intensities. A non-differentially expressed transcript, representing KIAA1007 protein, was used as reference (ratio measured: 1.4, set as 1.0, and the other ratios adjusted accordingly).

The Putative Chloride Channel Gene Was Identified as CLIC4

When searched against the BLAST database our cDNA clone of approximately 1 kb (represented by two differentially expressed cDNAs in Figure 1) showed the highest similarity to Homo sapiens mRNA, cDNA DKFZp566G223; and an intracellular chloride channel p64H1 mRNA (GenBank accession numbers AL117424.1 and AF109196.1, respectively). The first cloned intracellular chloride channel, p64, was originally purified from bovine kidney microsomes and has been proposed to function as a chloride channel.[25,26] A p64 homologue, described as CLIC4/huHI and p64H1(H), respectively, by two independent groups has recently been described in human tissue.[12,13] The DKFZ clone has been submitted as part of the cDNA sequencing consortium of the German Genome Project, and has not been characterized further.

The clone we have isolated is almost identical to DKFZp566G223 in the nucleotide sequence except for one position (A/G at position 488), where it resembles CLIC4/huH1 (data not shown). The predicted translated sequence is identical to that of DKFZp566G223, which differs from the reported sequences of both CLIC4 and p64H1(H) at positions 133 and 134 (RP *versus* SA) (Figure 3). This difference is entirely due to an alternate reading of the sequence through a small GC cluster (Figure 3). Critical review of the old sequencing gels used to generate the original CLIC4 sequence, and the observation that the current predicted amino acid sequence at these positions is conserved among bovine, rat, and mouse CLIC4, lead us to conclude that this isolated cloned sequence does indeed represent human CLIC4

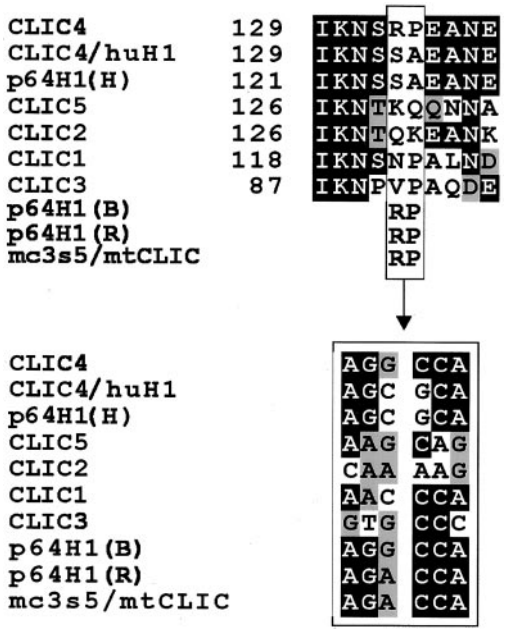

Figure 3. Comparison of CLIC sequences. Comparison of the deduced amino acid sequence of the cloned chloride channel (CLIC4, this study) with the sequences of the human (CLIC4/huH1[13] and p64H1 (H)[12]) bovine (p64H1 (B)[12]) rat (p64H1 (R)[32]) and mouse (mc3s5/mtCLIC[33]) p64 homologues reveals an inconsistency with the reported human CLIC4 amino acid composition at position 133–134 (RP instead of SA; **box**). The difference is localized within a region of heterogeneity among CLIC species. The RP composition is found also in the bovine, rat, and mouse homologues, although the underlying nucleotide sequences are different (**lower box**). The gene is therefore identified as CLIC4 with an RP composition at position 133–134.

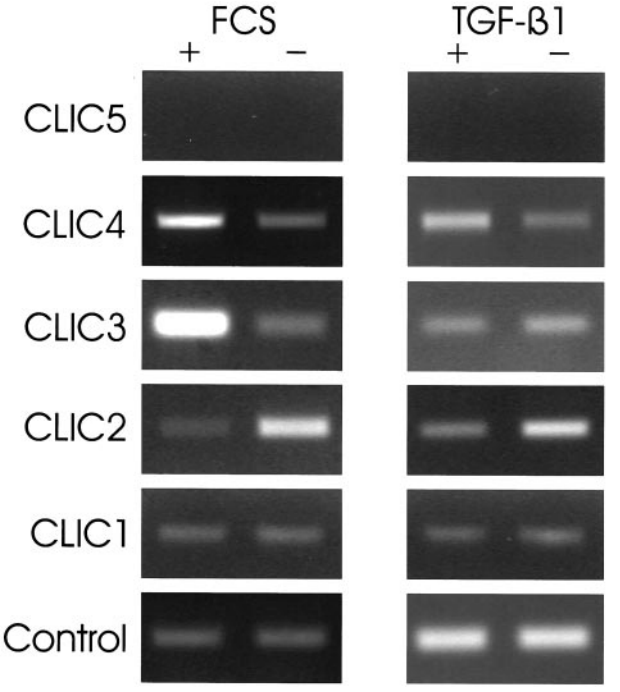

Figure 4. The CLIC family gene expression profile distinguishes TGF-β1- and serum-activated fibroblasts. Detection of CLIC-specific messengers by RT-PCR on total RNA derived from primary cultures of explanted normal breast fibroblasts with (+) or without (−) stimulation with 20% fetal calf serum (FCS) for 4 days or 100 pg/ml TGF-β1 (TGF-β1) for 6 days. KIAA1007 protein mRNA served as internal control (Control).

and that the correct nucleotide sequence through these two codons is AGG CCA with the correct amino acid sequence of RP (Figure 3).

The CLIC Family Gene Expression Profile Distinguishes TGF-β1- and Serum-Activated Fibroblasts

It is generally accepted that myofibroblasts may be generated by either serum or TGF-β1 stimulation and exhibit essentially the same phenotype.[7,22,27,28] However, the hybridization analysis in the present study revealed that one of the genes, metallothionein, displayed the opposite expression pattern in serum-stimulated fibroblasts as compared to TGF-β1-stimulated fibroblasts. We therefore speculated that the CLIC gene expression profile might serve to uncover different programs of myofibroblast differentiation. Since the CLIC protein family includes five human members: CLIC1/NCC27, CLIC2/XAP121, CLIC3, CLIC4, and CLIC5 of high level of similarity,[9–14] we hypothesized that their expression profile in fibroblasts/myofibroblasts may resolve the underlying differentiation program. We therefore broadened our analysis to include the expression of the different CLICs in primary breast fibroblasts and myofibroblasts. KIA1007 protein mRNA and GAPDH (not shown) served as internal controls and gave similar results. Whereas CLIC4 was up-regulated in serum-stimulated fibroblasts as expected, CLIC2 displayed the opposite pattern and was down-regulated under the same conditions (Figure 4). The expression pattern of CLIC3 resembled that of CLIC4, whereas CLIC1 was not differentially expressed, and the CLIC5 transcript could not be detected at all (Figure 4). Moreover, the regulating factor in (myo)fibroblasts was narrowed down to TGF-β1 for CLIC4 and CLIC2, whereas CLIC3 apparently is regulated by other factors in serum (Figure 4). Thus, CLIC4 is the only CLIC which is up-regulated by TGF-β1. Moreover, the CLIC gene expression profile delineates three levels of fibroblast differentiation: non-activated fibroblasts with a low expression of CLIC4 and a high expression of CLIC2; TGF-β1-stimulated fibroblasts with an increased expression of CLIC4, and low expression of CLIC2; and serum-stimulated fibroblasts with high expression of CLIC4, a low expression of CLIC2 and a very high expression of CLIC3.

CLIC4 Protein is Expressed in Breast Carcinoma Myofibroblasts and Contributes to Their Stationary Phenotype

We next investigated by immunohistochemistry whether the observed induction of CLIC4 on TGF-β1- or serum-mediated conversion of fibroblasts to myofibroblasts in culture reflected CLIC4 expression in stromal myofibroblasts *in situ*. Staining of breast tissue with the affinity-purified antibody, AP1058[13] showed that whereas normal breast stroma as well as normal, non-involved stroma peripheral to the neoplastic lesion in carcinomas (not

shown, $n = 4$) were devoid of CLIC4, a subset of stromal α-sm actin-positive myofibroblasts in 9 of 10 breast carcinomas expressed the protein (Figure 5). Co-localization of CLIC4 and α-sm actin in myofibroblasts was further confirmed by double staining with another affinity-purified antibody, CUMC29[12] (Figure 5C).

Finally, we analyzed the possible functional significance of CLIC4 expression. Previously, chloride currents have been shown to be obligatory for migration of glioma cells[29] and expression of a Ca^{2+}-activated chloride channel, CLCA2, has been shown to reduce migration of breast carcinoma cell lines.[30] Also, we have shown previously that myofibroblasts exhibit reduced motility in culture.[21] Therefore, a migration-regulating function of CLIC4, although a member of a completely different chloride channel family, was investigated. For this purpose we used mouse MEF/3T3 cells and conditional expression using the tetracycline-repressive gene regulation system, in which exogenous CLIC4 expression was switched off by addition of a tetracycline derivative. The concurrent protein expression was confirmed by immunoperoxidase staining (Figure 6A). To measure migratory activity of CLIC4-on and CLIC4-off fibroblasts, equal numbers of cells were plated on a porous filter, and the number of cells, which had migrated through the filter over a 6 hour period was counted. As shown in Figure 6B, CLIC4 expression significantly inhibited migration by 27% (73 ± 5%) as compared to the control (100 ± 1%). No significant difference in migratory activity was induced by doxycycline in MEF/3T3 Tet-Off fibroblasts without CLIC4 insert (100 ± 0% *versus* 96.3 ± 6.0%, $n = 3$). Finally, also at the individual cell level CLIC4-on fibroblasts exhibited reduced migration as compared to CLIC4-off fibroblasts (7.9 ± 2.0 μm/h and 11.7 ± 0.6 μm/h, respectively, $P < 0.05$). Since we have previously shown that filamentous α-sm actin inhibits cell motility,[21] we speculated that the mechanism by which CLIC4 reduces cell motility might involve α-sm actin induction. However, induction of CLIC4 did not affect α-sm actin expression neither at the messenger nor at the protein level (Figure 7).

Discussion

In many of the common carcinomas such as those of the breast, colon, stomach, and pancreas, the stroma comprises the majority of the tumor mass. One of the most prominent features of tumor stroma is the appearance of α-smooth muscle actin-positive myofibroblasts. We have previously shown that in the breast, the predominant contribution to myofibroblast generation comes from resident fibroblasts, and we and others have identified TGF-β1 as the key molecular switch in myofibroblast generation.[6,7,22,27,31] However, whereas the significance of TGF-β1 in fibrotic processes is well recognized[8] its role in gene regulation during myofibroblast conversion is not fully understood. To approach this issue, we combined a previously described chemically defined model system for conversion of fibroblasts to myofibroblasts[7] with a DD-RT-PCR protocol to identify differentially expressed transcripts.

The main outcome of our study is the hitherto unknown differential expression of CLIC4 in myofibroblasts and fibroblasts. CLIC4 is a member of a family of closely related proteins, which all show high homology to the C-terminal half of p64. p64 was originally discovered by purification of chloride channel activity from bovine kidney microsomes.[20] More recently, a rat p64 homologue, p64H1, was cloned and suggested to be associated with intracellular chloride transport.[32] Subsequently the human homologue, described as CLIC4/huH1 or p64H1 (H)[12,26] and a mouse homologue, mc3s5/mtCLIC,[33] have been identified. The deduced amino acid sequence of the differentially expressed transcript identified in the present study differs from both CLIC4/huH1 and p64H1 (H) at positions 133 and 134 (RP *versus* SA; ie, AGG CCA *versus* AGC GCA). However, since this difference appears in a GC region, which can be difficult to resolve by manual sequencing, and since the RP composition is also found in the bovine, rat, and mouse homologues, we find it likely that the correct amino acid composition of human CLIC4 indeed is RP. This RP composition clearly distinguishes CLIC4 from the other CLICs, and the underlying nucleotide sequence apparently signifies species specificity (Figure 3).

In general, little is known about the regulation of CLIC proteins. We show here that TGF-β1, which is also responsible for activation of the myofibroblast phenotype, up-regulates CLIC4 but not other CLICs. Moreover, our data suggest that CLIC4 expression is a feature of myofibroblasts *in situ* as well. As opposed to normal breast stroma, which is devoid of CLIC4 expression, CLIC4 is localized to α-smooth muscle actin-positive myofibroblasts in the stroma of invasive breast carcinomas. Whether CLIC4 expression in other tissues is also contributed by stromal cells remains to be elucidated. It has been shown recently by Berryman and Bretscher,[14] that in placenta, CLIC4 is localized to the trophoblast epithelium as well as "cells associated with blood vessels." The latter may, however, represent myofibroblasts as has been described by others.[34]

Chloride channels in general are involved in several crucial cell processes regulating cell volume, membrane potential, transepithelial transport, signal transduction, and acidification of organelles, and evidence is accumulating that members of the CLIC family represent voltage-gated channel proteins. It has been a matter of speculation whether p64/CLICs act as ion channels themselves or form part of an ion complex. However, recent data on bacterial expression of CLIC1 and CLIC4 demonstrate that both proteins are capable of forming novel, chloride-selective channels in the absence of other subunits or proteins.[15,16]

Hitherto, there have been two reports in the literature on differential expression of CLIC. Thus, exposure of a human histiocytic lymphoma cell line to phorbol 12-myristate 13-acetate or interleukins up-regulates CLIC1, and the regulation was found to be associated with the maturational state of the cell.[9] Likewise, expression of mc3s5/mtCLIC is up-regulated during keratinocyte mat-

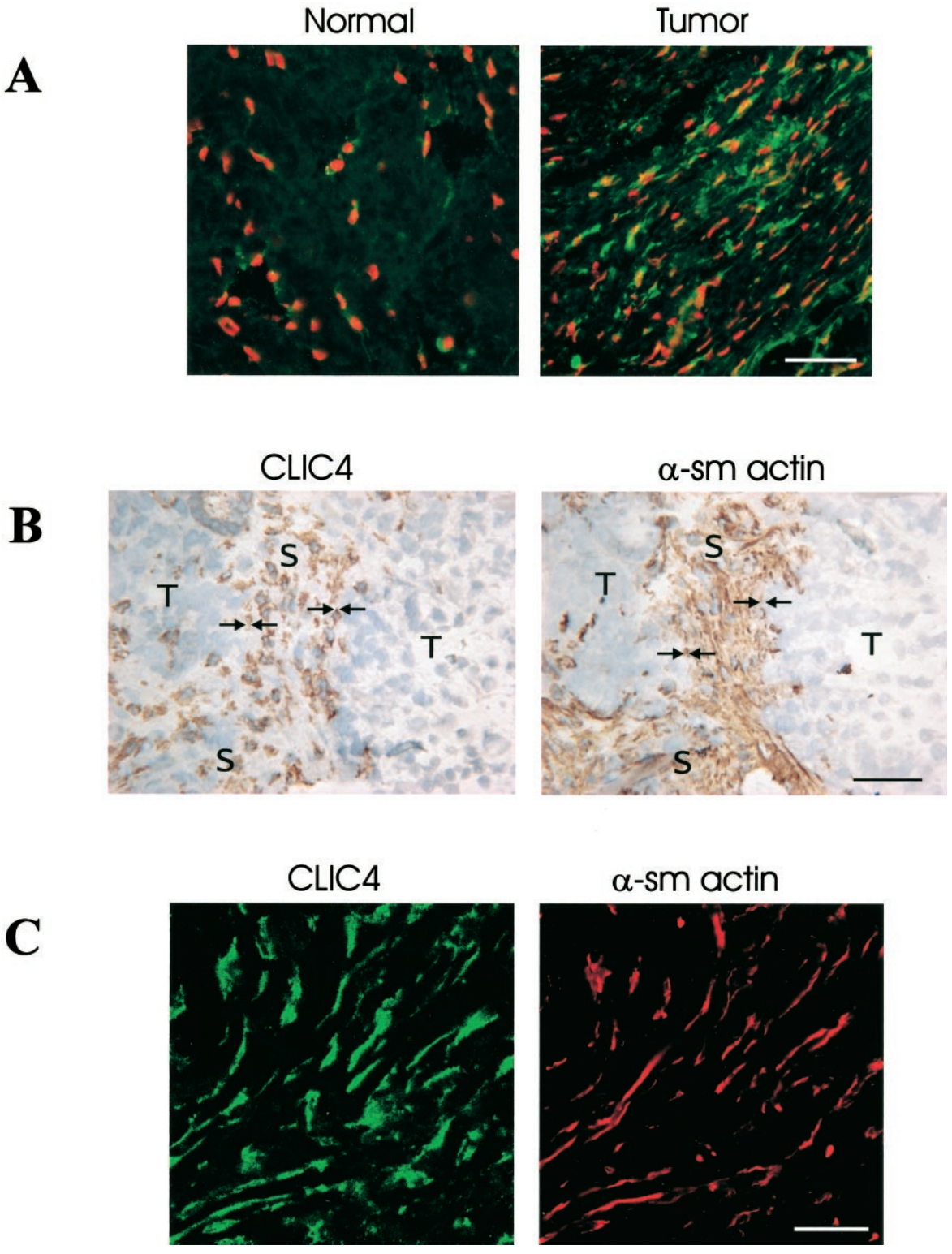

Figure 5. Immunohistochemical analysis co-localizes CLIC4 with α-sm actin-positive myofibroblasts in breast carcinomas. **A:** Whereas normal breast stroma (normal) is devoid of CLIC4 protein expression, tumor stroma (tumor) of invasive breast carcinomas expresses the protein as detected with AP1058 in the primary sequence and FITC-conjugated IgG in the secondary sequence. Nuclei are counterstained with propidium iodide. **B:** CLIC4 expression (CLIC4) in the stroma (S) of carcinomas is localized to α-sm muscle actin-positive myofibroblasts (α-sm actin) adjacent to invading tumor cells (T) as detected by immunoperoxidase staining of consecutive sections. **Arrows** indicate epithelial-stromal interface. **C:** Co-localization of CLIC4 and α-sm actin in myofibroblasts is further confirmed by double-labeling fluorescence using CUMC29 and 1A4 in the primary sequence and FITC-conjugated IgG and Texas Red-conjugated IgG2a in the second sequence. Scale bars, 50 μm.

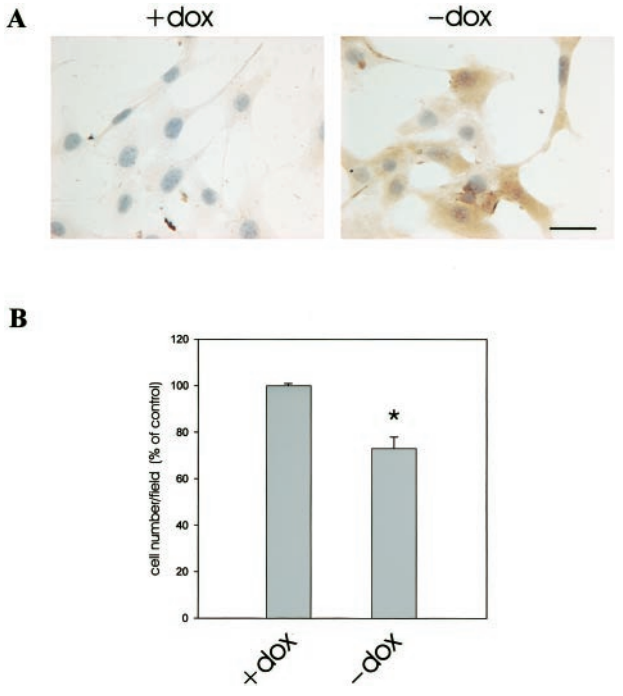

Figure 6. Conditional expression of CLIC4 in MEF/3T3 fibroblasts reduces cell motility. **A:** Whereas cells cultured with doxycycline in the culture medium (+dox) do not express CLIC4 protein, CLIC4-transfected cells (−dox) exhibit a prominent protein expression as detected by AP1058 and immunoperoxidase staining. **B:** Migratory activity of CLIC4-on and CLIC4-off fibroblasts was assessed by plating equal numbers of cells on a porous filter, and the number of cells, which had migrated through the filter over a 6-hour period was counted. The number of migrating cells/field without CLIC4 was set as 100%. CLIC4 expression (−dox) significantly inhibited migration by 27% ($n = 4$; ± SEM; **asterisk** indicates significance, $P < 0.01$) as compared to the control (+dox).

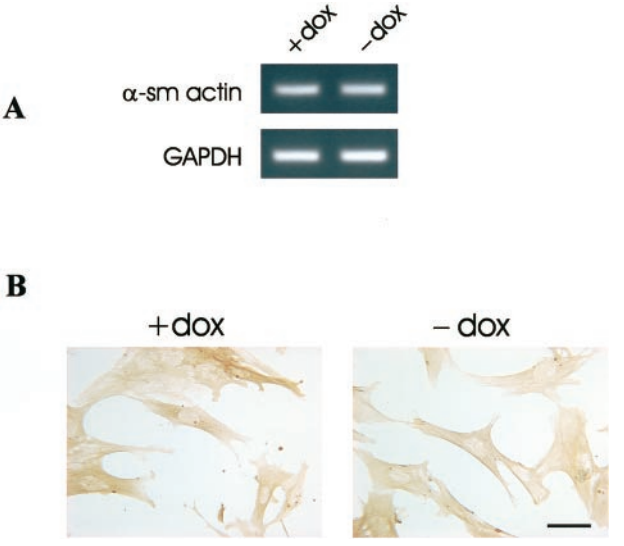

Figure 7. Reduced motility in CLIC4-transfected cells is not mediated by induction of α-sm actin. **A:** Equal levels of α-sm actin messengers are detected by RT-PCR on total RNA from CLIC4-off (+dox) and CLIC4-on (−dox) fibroblasts. GAPDH is used as internal control. **B:** Likewise, no difference in α-sm actin protein expression in CLIC4-off (+dox) and CLIC4-on (−dox) fibroblasts is detected by immunoperoxidase staining. Scale bar, 50 μm.

uration and following treatment with tumor necrosis factor-α.[33] The present study shows that human CLIC4 is significantly higher expressed in myofibroblasts versus fibroblasts. Collectively, these findings suggest a role for CLIC4 in cell differentiation. The present study is the first to demonstrate that a function of CLIC4 is to inhibit cell motility. The cellular mechanism responsible for this remains to be elucidated, but it has been suggested by others that members of the CLIC family, including CLIC4, are associated with the actin cytoskeleton.[14,35] We have shown previously that the cytoskeletal remodeling accompanying myofibroblast differentiation, ie, induction of filamentous α-sm actin, inhibits cell motility,[21] and the presence of larger focal contacts in α-sm actin-positive cells may promote the adhesiveness of these cells.[21,36] The present study suggests that simultaneous induction of CLIC4 may further support a stationary phenotype of myofibroblasts. The hierarchy of events in the myofibroblast differentiation program has not been established, but CLIC4 expression *per se* does not trigger increased α-sm actin expression, and the two proteins may, thus, act independently. A most recent study has shown that rat CLIC4 binds directly to dynamin I in a complex containing β-actin, tubulin and 14–3-3.[35] Whereas CLIC4 did not bind directly to F-actin, it may bind to non-polymerized G-actin,[35] and as such may reduce the pool of G-actin available for polymerization.[21,37]

Other ion channels including a Ca^{2+}-activated chloride channel, CLCA2, have been associated with inhibition of breast cancer growth and metastasis.[30,38] Thus, when reintroduced into CLCA2-negative breast carcinoma cell lines, CLCA2 inhibits migration and reduces tumorigenicity and invasion of human breast carcinoma cells, and as such may act as a tumor suppressor in breast cancer.[30] These studies have, however, focused mainly on the epithelium, and the functional implications of chloride channel expression in breast stroma has yet to be established.

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