Relocation of CLIC1 Promotes Tumor Cell Invasion and Colonization of Fibrin
Lisa A. Gurski1, Lynn M. Knowles1, Per H. Basse2, Jodi K. Maranchie1,2, Simon C. Watkins2,3, and Jan Pilch1,2,4

Abstract
Chloride intracellular channel 1 (CLIC1) has been shown to be upregulated in various malignancies but its exact function remains unclear. Here, it is revealed that CLIC1 is critical for the stability of invadopodia in endothelial and tumor cells embedded in a 3-dimensional (3D) matrix of fibrin. Invadopodia stability was associated with the capacity of CLIC1 to induce stress fiber and fibronectin matrix formation following its β3 integrin (ITGB3)-mediated recruitment into invadopodia. This pathway, in turn, was relevant for fibrin colonization as well as slug (SNAI2) expression and correlated with a significant role of CLIC1 in metastasis in vivo. Mechanistically, a reduction of myosin light chain kinase (MYLK) in CLIC1-depleted as well as β3 integrin-depleted cells suggests an important role of CLIC1 for integrin-mediated actomyosin dynamics in cells embedded in fibrin. Overall, these results indicate that CLIC1 is an important contributor to tumor invasion, metastasis, and angiogenesis.

Implications: This study uncovers an important new function of CLIC1 in the regulation of cell–extracellular matrix interactions and ability of tumor cells to metastasize to distant organs. Mol Cancer Res; 13(2); 1–8. ©2014 AACR.

Introduction
The central function of the clotting cascade is to sustain hemostasis; however, clotting factors also make important contributions to inflammation, wound healing, angiogenesis, and cancer (1–4). In the course of the clotting process, soluble fibrinogen is converted into insoluble fibrin that provides a provisional adhesive matrix for inflammatory, endothelial, and tumor cells (5). In the case of tumor metastasis, a key function of fibrin is to protect circulating tumor cells from the cytotoxic activity of natural killer cells (4). However, adhesion of tumor cells to 3-dimensional (3D) matrices of fibrin or fibrin–fibronectin also directly affects critical prometastatic functions such as invasion, survival, and colony formation (6, 7). In addition, 3D fibrin has been shown to provide a permissive environment for the maintenance and proliferation of tumor-initiating cells (8). Together, these data underscore that a thorough understanding of the adhesive interactions between tumor cells and blood clot could lead to novel strategies to inhibit tumor metastasis.

Fibrin and fibrinogen are specifically recognized by β3 integrins and it appears that the expression of activated integrin αvβ3 is required for the interaction of tumor cells with clotted plasma (6, 7). Moreover, paralleling its positive effects on invadopodia and colony formation in clot-embedded tumor cells, integrin αvβ3 has been shown to support tumor metastasis in a mechanism that depends on the generation of fibrin–fibronectin complexes (7). Fibronectin is critical for αvβ3-mediated functions in clot-embedded tumor cells in at least two ways: upstream of integrins as an activating stimulus for αvβ3 and downstream as an αvβ3 ligand that provides the necessary matrix rigidity for tumor cells to generate stress fibers, form colonies, and induce epithelial–mesenchymal transition (EMT), e.g., through expression of the EMT master regulator, Slug (6, 7). Interestingly, stress fiber formation is also associated with αvβ3-dependent maintenance and proliferation of tumor-initiating cells in fibrin, suggesting that mechanotransduction mediated by integrin αvβ3 is an important stimulus for expression of metastasis-related genes in clot-embedded tumor cells (8).

In search of factors that mediate the interaction of tumor cells with clotted plasma, we became interested in chloride intracellular channel 1 (CLIC1), which functions as an internalizing receptor for the clot-binding peptide CLT1 on endothelial cells (9). CLIC1 is overexpressed in the tumor vasculature and frequently upregulated in patients with cancers originating from the breast, lung, and liver (10–12). Increasing concentrations of CLIC1 have also been detected in the serum of patients with aggressive ovarian cancer, suggesting that CLIC1 could be useful as a tumor marker (13). CLIC1 has been shown to induce invasion and proliferation of tumor as well as endothelial cells, but the mechanism behind this function is not clear (14, 15). We previously observed that ligation of integrin αvβ3 leads to redistribution of CLIC1 into lamellipodia of endothelial cells, suggesting that CLIC1 membrane recruitment is relevant for cell spreading (9). On the basis of the established role of
integrin αvβ3 for clot invasion and the apparent cooperation between αvβ3 and CLIC1, we aimed to analyze the role of CLIC1 for invadopodia formation in fibrin-embedded endothelial and tumor cells.

Materials and Methods

Three-dimensional cell culture

Human umbilical venous endothelial cells (HUVEC) were purchased from Lonza, human 786-6 kidney cancer and HT1080 fibrosarcoma cells were purchased from the ATCC. Primary human tumor cells were isolated from kidney tumors of 2 patients with metastatic and 2 patients with localized RCC as previously described (6). The cells were designated as M1/2 (metastatic) and L1/2 (localized) accordingly. M1 was derived from a female (70–79 years) with metastasis to the lung (pT3bN0M1), while M2 was derived from a male (50–59 years) with metastasis to the lung, liver, and lymph node (pT3bN2Mx). The tumor stage of L1 (male, 40–49 years) and L2 (male, 40–49 years) was in each case pT1aNxMx. Cells were cultured at 37°C under a humidified, 5% CO2 atmosphere according to the manufacturers’ specifications. For 3D culture, cells were mixed with 2 mg/mL fibrinogen (Enzyme Research Laboratories, Inc.) in the presence of 2 mM/L CaCl2 and 25 μg/mL FXIII (Enzyme Research Laboratories) to generate fibrin gels as previously described (6). Clotting was induced with 2.5 U/mL thrombin (Sigma), and 15 µL suspensions were pipetted onto tissue culture plates and inverted at room temperature for 10 minutes to solidify. Embedded cells were incubated with media supplemented with FBS at 37°C under a humidified, 5% CO2 atmosphere.

Invadopodia analysis

Clots were analyzed for invadopodia formation 24 hours after embedding at designated areas by phase contrast microscopy (Nikon Eclipse TS100; ×10 magnification). Invadopodia formation was classified as complete (i.e., elongated or stellate shape) or incomplete (i.e., round shape with or without rudimentary invadopodia) and calculated for completely spread cells as a percentage of the total cell number of a microscopy field. Colonization was measured by counting the total number of clot-embedded cells per microscopy field after 48 hours. In addition, we analyzed invadopodia formation over time by live cell imaging. To this end, fibrin-embedded 786-0 cells were transferred to a BioStation IM (Nikon) prequillified at 37°C, 5% CO2. Phase contrast images were captured every 10 minutes for 2 hours and then every 30 minutes for the next 22 hours to track invadopodia. Images and videos were prepared using the BioStation IM software. Using these images, Imagej software was used to measure the length of each invadopodium in each of five fields and an average was calculated for selected time points. Using the videos, the lifetime of each invadopodium was determined by setting the time points at which an invadopodium first formed and then disappeared. Average invadopodia lifetime was calculated from videos of five optical fields.

Confocal microscopy

Fibrin-embedded cells were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and incubated with anti-fibronectin (Millipore), anti–CLIC1 (Abcam), anti–β3 integrin (Abcam), anti-pMLC (Cell Signaling Technology), or isotype control, followed by incubation with Alexa Fluor 488- or 546-conjugated secondary antibody (Invitrogen). To visualize the cytoskeleton and nuclei, cells were stained with Alexa Fluor 546–phalloidin (Invitrogen) and Draq5 (eBioscience), respectively. The myosin light chain kinase (MLCK) inhibitor ML-7 was added at 10 μmol/L where indicated. Fibrin-embedded cells were analyzed using a confocal microscope (Leica TCS SL) and images were processed with Adobe Photoshop. To quantify fibronectin matrix formation, fibrin-embedded cells were stained for fibronectin as described except that the cells were not permeabilized. Confocal images (×20) were analyzed for the percentage area of fibronectin staining using Imagej software. Imaged was also used to assess CLIC1 expression in invadopodia and the cell periphery, which were identified using F-actin staining. To this end, we measured the average CLIC1 fluorescence intensities in the cell periphery/invadopodia compared with the cell interior (without the nucleus) and calculated the ratio between the two compartments.

Gene silencing

HUVEC were grown for 24 hours before transfection with 25 nmol/L CLIC1 (Dharmacon On-TARGETplus SMARTpool L-009350-00) or nontargeting control (Dharmacon On-TARGETplus D-001810-10) siRNA. SMARTpool siRNA contains a pool of four siRNA sequences directed against the target gene. Cells were transfected in Opti-MEM medium (Invitrogen) using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. After 6 hours, cells were placed in normal culture medium and grown for an additional 42 hours. To achieve stable knockdown of CLIC1 and β3 integrin, we transduced 786-0 and HT1080 cells with five lentiviral shRNA vectors compared with a scrambled control shRNA (University of Pittsburgh Cancer Institute Vector Core Facility, Pittsburgh, PA). For our experiments, we used the two clones with the highest knockdown efficiency for each target. Subsequently, cells were treated with virus suspension containing 8 μg/mL polybrene for 19 hours at 32°C, 5% CO2. Then, after a recovery phase of 24 hours in complete medium at 37°C, cells were placed under puromycin selection for 2 weeks. Target knockdown was confirmed by Western blot analysis and the two most efficiently knocked down clones were selected for further experimentation (CLIC1-2: CCGGGCGCTTGGCCAAAGTTACACATCCTCGGATGTGTAACTTTGGCAACAGGTTTT; CLIC1-3: CCGGGCGCTTGGCCAAAGTTACACATCCTCGGATGTGTAACTTTGGCAACAGGTTTT TTG; CLIC1-4: CCGGGCGCTTGGCCAAAGTTACACATCCTCGGATGTGTAACTTTGGCAACAGGTTTT TTG; β3-3: CCGGGCGCTTGGCCAAAGTTACACATCCTCGGATGTGTAACTTTGGCAACAGGTTTT TTG; β3-5: CCGGGCGCTTGGCCAAAGTTACACATCCTCGGATGTGTAACTTTGGCAACAGGTTTT TTG).

Western blot analysis

Cell pellets were lysed by adding 2× SDS sample buffer. Proteins were separated by SDS-PAGE, transferred onto nitrocellulose, and stained with 0.05% Ponceau S (Sigma) to ensure equivalent protein loading. Immunoblots were blocked with 5% bovine serum albumin and probed overnight at 4°C with anti-CLIC1 (Abcam), anti-fibronectin (Millipore), anti-Slug (Cell Signaling Technology), anti-MLCK (Sigma), anti–phospho-MLCK (Life Technologies), anti-β3 (BD Biosciences), and anti–β–Actin (Sigma) antibodies followed by incubation with peroxidase-conjugated anti-mouse or rabbit IgG antibody (Bio-Rad). Finally, the
blots were incubated with enhanced chemiluminescence (PerkinElmer) to visualize antibody binding.

**Experimental metastasis**

To induce metastasis, $5 \times 10^5$ HT1080 cells were injected into the tail vein (i.v.) of female, 6- to 8-week-old athymic nude mice (Charles Rivers). Three weeks after tumor cell injection, lungs were isolated and fixed in Bouin solution (Sigma). To assess tumor multiplicity, tumor nodules were counted on the surface of lungs using a stereo microscope (Zeiss Stemi 2000-C).

**Statistical analysis**

Significance was determined using the Student two-tailed t tests or one-way ANOVA followed by the posthoc Tukey multiple comparisons test (GraphPad Prism 5) with $P < 0.05$ considered significant. Error bars show mean ± SEM.

**Results**

**CLIC1 supports invadopodia formation in fibrin in vitro and metastasis in vivo**

We previously identified CLIC1 as a target for the antiangiogenic peptide CLT1 (9). This study implicated CLIC1 in regulating adhesive interactions between integrin αβ3 and fibronectin, two adhesion proteins involved in the invasion of blood clot (6, 7). To determine whether CLIC1 itself promotes clot invasion, we embedded CLIC1-depleted 786-0 kidney cancer and HT1080 fibrosarcoma cells in a 3D matrix of fibrin and scored clots for invadopodia-positive cells by phase contrast microscopy. Knockdown with CLIC1 (shCLIC1) compared with scrambled shRNA (shSCR) led to a marked decrease of CLIC1 protein expression in two independently isolated 786-0 and HT1080 clones, which translated into significant inhibition of invadopodia as well as colony formation, but had no effect on cell death in fibrin-embedded tumor cells (Fig. 1A–C and Supplementary Fig. S1). Additionally, invadopodia formation was markedly reduced, when endothelial cells were treated with siRNA against CLIC1 (Fig. 1A and D and Supplementary Fig. S1). Treatment with IAA94, on the other hand, had no effect, suggesting that the role of CLIC1 in invadopodia formation is independent of its function as an ion channel. To determine whether CLIC1 is relevant for metastasis, we injected shCLIC1- and shSCR-HT1080 cells into the tail vein of athymic nude mice and analyzed lungs for tumor multiplicity 3 weeks later (Fig. 1E). Paralleling our *in vitro* results, we found that knockdown of CLIC1 significantly reduced experimental lung metastasis, suggesting that CLIC1-mediated functions are necessary for efficient tumor cell seeding in the lungs. Together, these results show that fibrin-embedded tumor and endothelial cells depend on CLIC1 for invadopodia and colony formation *in vitro* and lung metastasis *in vivo*.

**CLIC1 is upregulated in invadopodia of fibrin-embedded tumor and endothelial cells**

To determine the function of CLIC1 for invadopodia formation, we analyzed the subcellular localization of CLIC1 in fibrin-embedded HUVEC and 786-0 cells by confocal microscopy. This study revealed that CLIC1 expression was concentrated around nascent invadopodia first in a punctate pattern (after 1 hour) and then in form of a solid, cortical ring (after 2 hours; Fig. 2A). From a largely cortical localization, CLIC1 shifted toward invadopodia, which developed in 786-0 within 1 hour and in HUVEC within 24 hours of embedding (Fig. 2A and B). After 24 hours, CLIC1 was almost exclusively localized in mature invadopodia of both HUVEC and 786-0, where it partially colocalized with β3 integrin (Fig. 2B). Conversely, we found that recruitment of CLIC1 toward the cell membrane or into invadopodia was largely impaired in β3-depleted 786-0 cells (shβ3) even though CLIC1 was expressed at similar protein levels (Fig. 2C and Supplementary Fig. S2). To determine the clinical relevance of CLIC1 expression and localization, we stained primary tumor cells from patients after embedding in fibrin with anti-CLIC1. Using confocal microscopy, we found a strong colocalization of CLIC1 for invadopodia in RCC cells from 2 patients with metastasis (Fig. 3). This was in sharp contrast to primary RCC cells from 2 patients without metastasis, which lacked the pronounced expression of CLIC1 at the cell membrane. These
results show that the localization of CLIC1 in invadopodia correlates with expression of β3 integrin. Moreover, they suggest that CLIC1 is highly enriched in invadopodia of metastatic tumor cells.

CLIC1 promotes tumor cell spreading through effects on myosin-light chain kinase

To establish a detailed account of the role of CLIC1 for tumor cell spreading, we performed live cell imaging with fibrin-embedded 786-0 cells. Video microscopy revealed that both 786-0-shCLIC1 and the control 786-0-shSCR cells were very similar in the early phase (0–8 hours), which is characterized by the formation of dynamic, rapidly extending and retracting invadopodia (Fig. 4A and B and Supplementary Movies S1 and S2). Then, after 8 hours, control cells began to stabilize and further lengthen their invadopodia as a prerequisite for spreading. Invadopodia in CLIC1 knockdown cells, in contrast, did not gain much length after 8 hours and, instead, destabilized at an increasing rate leaving membrane fragments behind. Interestingly, a similar effect was achieved when we inhibited cell spreading with the myosin-light chain kinase (MLCK) inhibitor ML7. Although ML7 had no effect on the initial invadopodia outgrowth (i.e., within the first 24 hours), the inability of cells to generate stress fibers correlated with significantly reduced invadopodia stability and colony formation, indicating that MLCK generates important signals for proliferation in 3D fibrin (Fig. 5A and B). To follow up on these results, we analyzed CLIC1-dependent actomyosin dynamics by staining 48-hour fibrin-embedded 786-0 cells with an antibody against phosphorylated myosin light chain (MLC) as well as phalloidin. Using confocal microscopy, we found that CLIC1 knockdown significantly impaired the capacity of fibrin-embedded tumor cells to activate MLC and generate stress fibers (Fig. 5C and D). Further upstream, we detected a marked reduction of phosphorylated and total MLCK in 786-0-shCLIC1 while RhoA activity remained unaffected (Fig. 5E and Supplementary Fig. S3A). Interestingly, MLCK was also reduced in 786-0-shβ3, suggesting that CLIC1 regulates MLC activity downstream of integrin αvβ3 (Fig. 5E). Together, our results indicate that CLIC1 promotes tumor cell spreading through effects on MLCK.
CLIC1 and MLCK are necessary for fibronectin matrix formation in 3D fibrin

Actin–myosin interactions are a prerequisite for fibronectin fibril formation (16). Therefore, we decided to assess the effect of CLIC1 on fibrin matrix assembly. Staining for fibronectin and F-actin showed that fibrin-embedded 786-0-shSCR control cells begin to develop invadopodia within 15 minutes after embedding in fibrin and that this process was accompanied by the relocation of intracellular fibronectin toward the cortical cytoskeleton adjacent to the nascent invadopodia (Fig. 6A). Between 1 and 4 hours, fibronectin colocalized increasingly with invadopodia, while by 48 hours, a mature extracellular matrix was visible in areas where invadopodia have retracted. Notably, this process of fibronectin assembly was impaired in shCLIC1-transformed as well as tumor cells treated with the MLCK inhibitor ML-7 resulting in a significantly diminished fibronectin matrix after 48 hours of embedding in fibrin (Fig. 6A and B and Supplementary Fig. S3B). We previously showed that fibronectin matrix assembly is necessary to maintain expression of the EMT master regulator Slug (6). Paralleling these data, we detected reduced Slug expression in 786-0-shCLIC1 cells as well as in 786-0 cells treated with ML-7 (Fig. 6C). Together, these results indicate that both CLIC1 and MLCK are necessary for fibronectin matrix assembly and expression of the EMT master regulator Slug.

Discussion

We previously identified CLIC1 as an internalizing receptor for the antiangiogenic peptide CLT1 on proliferating endothelial cells (9). Here, we demonstrate that CLIC1 supports invadopodia stability and stress fiber formation in clot-embedded cells through effects on MLCK. This function was accompanied by relocation of CLIC1 into invadopodia, which, in turn, depended on the expression of β3 integrin. Through its effects on actomyosin, CLIC1 supports fibronectin matrix assembly as well as Slug expression and, therefore, mediates critical tumor cell functions such as fibrin invasion, colony formation, and lung metastasis.

CLIC1 was originally identified as a nuclear chloride channel protein (NCC27) in a macrophage cell line, but since its discovery CLIC1 expression has been detected in many different tissues (17, 18). Despite its ubiquitous expression, CLIC1 has regularly been shown to be upregulated in patients with malignant tumors of the brain, liver, lung, ovaries, and gastrointestinal tract and in many of these malignancies, CLIC1 expression correlates with aggressive disease and poor outcome (12, 19–22). In addition, CLIC1 has been shown to be overexpressed in the tumor vasculature, indicating that CLIC1 plays an important role in the different aspects of tumor growth and metastasis (10). In agreement with this, CLIC1 was found to be involved in cell migration, invasion, and proliferation but its function in these processes remains largely elusive (15, 23, 24). We previously demonstrated that tumor metastasis to the lungs correlates with the ability of tumor cells to generate invadopodia in clotted plasma and that this mechanism depends on integrin αvβ3 and fibronectin (6, 7, 25). Here, we established that CLIC1 cooperates with integrin αvβ3 and fibronectin to support invadopodia and colony formation in fibrin in vitro and that this function correlates with the capacity of CLIC1 to promote lung metastasis in vivo.

Our data indicate that CLIC1 controls invadopodia stability and cell spreading by regulating MLCK, which is known to mediate critical biologic functions such as cell division and motility (26, 27). In addition, MLCK has important effects on vesicular transport as well as cell contractility, which is required for the assembly of single fibronectin molecules into
dependent stimulation of the RhoA also been shown to contribute to MLC activity via syndecan-cyctic function of macrophages, which appears to depend on (32). To this end, CLIC1 plays an important role for the phagocyte membrane-spanning oligomer with chloride channel function characteristics of a glutathion transferase or as an insoluble, distinct forms, namely as a soluble monomer featuring structural

ity of tumor cells to spread and generate a
teration and metastasis in this system depended on the capac-

However, this mechanism did not seem to play a role in

However, it is important to note that CLIC4 has a number of additional functions, which do not seem to depend on anion currents, including the stabilization of phospho-smad2/3 as well as the p65 subunit of NFKb (35, 36). The interaction of CLIC4 with NF-kb in endothelial cells is significant for pulmonary hypertension as it leads to activation of hypoxia-inducible factor-1α (HIF1α) and induction of an overall mesenchymal phenotype with invadopodia and stress fibers (36). Another CLIC family member, CLIC3, in turn contributes to cell invasion through recycling of MT1-MMP and integrin α5β1 to the cell surface, suggesting that stabilizing proteins involved in cell–extracellular matrix interactions is a common theme of CLIC family members (37, 38). CLIC1 fits neatly into this scheme as it functions to maintain MLCK expression, either directly or indirectly.

We have previously shown that interaction of a tumor homing peptide CLT1 with CLIC1 and CLIC3 can cause endocytosis of fibronectin–CLT1 complexes (9, 39). A prerequisite of CLIC1-mediated internalization was the ligation of integrin αvβ3 with fibronectin, which caused CLIC1 cell surface expression (9). CLIC3-mediated internalization on the other hand depended on ligation of integrin α5β1 (39). Paralleling these data, our current study indicates that the process of relocating CLIC1 into invadopodia depends on β3 integrin. Interestingly, CLIC1 translocation into the cell membrane can be induced by NADPH oxidase, which, in turn, has been shown to be activated by the Rho GTPase Rac following integrin ligation (40, 41). Another key function of Rac is to mediate F-actin formation and, thus, to provide membrane binding sites for CLIC1 in invadopodia (42, 43). Notably, binding of CLIC1 to F-actin has been shown to inhibit CLIC1 chloride conductance, which is in agreement with our result that the CLIC1 channel blocker IAA94 had no effect on invadopodia formation (42).

On the basis of our results, we propose a model where β3 integrin–mediated recruitment of CLIC1 into invadopodia promotes expression of MLCK in clotted-embayed tumor and, presumably, endothelial cells. The resulting contraction of the actin cytoskeleton is necessary for the generation of stress fibers as well as fibronectin fibrils in fibrin-embedded tumor cells and this, in turn, is a prerequisite for focal adhesion-dependent growth signaling (30, 44). Our previous results show that fibro-nectin expression plays an important role in maintaining expression of the EMT transcription factor Slug, which, in turn, promotes fibrin invasion and lung metastasis (6). In line with the concept that CLIC1 acts through stress fiber and fibronectin matrix formation, we show now that clot colonization, Slug expression, and lung metastasis are impaired in CLIC1 knockdown cells. Moreover, antagonizing MLCK function with the MLCK inhibitor ML-7 also impairs invadopodia stability, fibro-nectin matrix assembly, and Slug expression, strongly suggesting that the role of CLIC1 in clot colonization and Slug expression is a direct result of CLIC1’s effect on MLCK. Together, this study indicates an important function of CLIC1 in the regulation of cell–extracellular matrix interactions, which have direct effects on the ability of circulating tumor cells to colonize clot in vitro and to metastasize to distant organs in vivo. Thus, strategies to inhibit CLIC1 could be useful for the treatment of aggressive cancer.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L.A. Gurski, J. Pilch
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.A. Gurski, L.M. Knowles, P.H. Basse, J. Pilch
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.A. Gurski, L.M. Knowles, J. Pilch
Writing, review, and/or revision of the manuscript: L.A. Gurski, L.M. Knowles, P.H. Basse, J.K. Maranchie, J. Pilch
Study supervision: J. Pilch

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