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Review

Cancer Research

Visualizing Epithelial–Mesenchymal Transition Using the Chromobody Technology

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Abstract

The epithelial–mesenchymal transition (EMT) is a complex cellular program involved in the progression of epithelial cancers to a metastatic stage. Along this process, epithelial traits are repressed in favor of a motile mesenchymal phenotype. A detailed characterization and monitoring of EMT-related processes is required for the design of screening strategies needed to develop novel antimetastatic therapies. Overexpression of the canonical EMT biomarker vimentin correlates with increased tumor growth and invasiveness, as well as with reduced patient survival across various epithelial cancers. Moreover, recent findings have demonstrated an active role of vimentin in regulating and reorganizing the cellular architecture toward a migratory and invasive phenotype. However, current studies suffer from a lack of appropriate methods to trace the induction and dynamics of vimentin in cell-based assays. Recently, we have reported a novel intrabody (chromobody)–based approach to study the spatiotemporal organization of endogenous vimentin upon induction of EMT by high-content imaging. In this review, we discuss the relevance of the chromobody technology with regard to the visualization of EMT-related processes in living systems. *Cancer Res; 76(19); 5592–6. ©2016 AACR.*

Introduction

The epithelial–mesenchymal transition (EMT) is a reversible cellular reprogramming process involved in embryogenesis, wound healing, and cancer progression. Triggered by cytokines and/or growth factors, which induce the activation of transcriptional repressors (EMT inducers), epithelial cells undergo a change in signaling programs and gene expression. This results in a loss of epithelial markers, such as E-cadherin, occludin, or cytokeratin, while mesenchymal markers, including N-cadherin or vimentin, gain in expression. Consequently, these cells lose their adhesions and apical–basal polarity and reorganize their cytoskeleton toward a mesenchymal phenotype, characterized by a high migratory and invasive potential (reviewed in ref. 1). A growing number of studies indicate that EMT not only correlates with but can be even causative for metastasis formation, drug resistance, and the development of circulating tumor cells (CTC) and cancer stem cells. Thus, new EMT-targeting therapeutics are emerging to prevent the dissemination of epithelial cancers (reviewed in ref. 2). In this context, a detailed understanding of EMT-related processes is required for the design of screening strategies to develop antimetastatic therapies.

To identify cellular targets or chemical modulators of EMT, different experimental approaches based on (i) analysis of gene expression (3, 4), (ii) high-content imaging of altered functional and/or morphologic phenotypes (5, 6), and/or (iii) gene reporter assays (7, 8) have been described and adapted to screening campaigns. Strikingly, only the gene reporter–based screens consider the relevance of vimentin as one of the most prominent EMT biomarkers. This is remarkable as detection of vimentin at mRNA and protein level is widely established in both basic EMT research and the diagnosis of metastatic stages of epithelial cancers. Thus, it might be reasonable to expand current EMT screening settings toward readouts determining the expression and localization of vimentin.

Vimentin's Impact on a Migratory EMT Phenotype

Elevated levels of vimentin have been reported across various epithelial tumors and often correlate with an increase in metastasis, reduced patient survival, and poor prognosis (9–11). Moreover, vimentin expression does not only correlate with but is functionally involved in the migration and invasion of metastasizing cancer cells. It has been shown that ectopic expression of vimentin in epithelial-like breast cancer cells is sufficient to induce EMT-related features, including an elongated cell shape and increased cell motility (12). This is in accordance with recent findings describing an impact of vimentin on the formation and morphology of membrane protrusions at the leading edge of migrating cells (13–15). Together with cytoskeletal actin networks, vimentin is required for elongation and stabilization of invadopodia, which facilitates the migration of invasive cells through the extracellular environment (15). Further evidence for the interplay between actin stress fibers and vimentin has been provided by Jui and colleagues, demonstrating that vimentin filaments associate with actin transverse arcs to control their retrograde flow and thereby the width of the lamellum (13). Moreover, vimentin regulates the organization of lamellipodia by forming a gradient of different assembly

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states: High concentrations of mature filaments are mainly located at the tail and at perinuclear regions of migrating cells, whereas nonfilamentous vimentin particles are enriched within membrane protrusions at the leading edge. This is in line with the observation that filamentous vimentin at the cell periphery inhibits the formation of lamellipodia (14). The assembly states of vimentin strongly depend on posttranslational modifications, in particular phosphorylation (reviewed in ref. 16). Generally, Ser/Thr phosphorylation promotes disassembly of mature filaments, while dephosphorylation allows reassembly of vimentin (16). Thus, it is not surprising that kinases previously described to be involved in cell migration and EMT (e.g., PKC, PAK, Akt; refs. 1, 17) also phosphorylate vimentin (18–20).

Despite the steadily growing insight in the molecular and mechanistic role of vimentin in cell migration and invasion, the potential of vimentin as a screening marker for EMT has not been fully explored. Common approaches to analyze vimentin at the protein level include antibody-based detection, for example, in Western blots or immunofluorescence (14, 21, 22). Adaption of these methods to screening campaigns is limited by their low-throughput character and technical complexity. For live-cell analysis, fluorescent fusions of vimentin are employed (12–14). However, as mentioned earlier, ectopic expression of vimentin alters the cell morphology as well as the organization of endogenous vimentin, nudging the cells toward a migratory phenotype (12). Thus, any method that interferes with endogenous vimentin levels is inappropriate for screening of EMT modulators. Circumventing the restrictions of current technologies, we developed a novel approach based on the chromobody technology that allows the visualization of endogenous vimentin in living cells without perturbing its expression level or the cellular phenotype.

Vimentin-Specific Chromobodies Visualize EMT in Living Cells

Single-domain antibody fragments (V_HHs, nanobodies) from camelids are a versatile alternative to conventional antibodies (23). Owing to their small size, robustness, and structural simplicity, nanobodies can be selected to target and trace antigens in living cells (reviewed in ref. 24). Thus, a new class of cellular biosensors (termed chromobodies) has been established by genetically fusing these binding molecules to fluorescent proteins (Fig. 1A). Upon intracellular expression, chromobodies, unlike conventional antibodies, do not require cell fixation/permeabilization. Therefore, their application is not restricted to endpoint readouts and less prone to artefacts. Although ectopically expressed fluorescent fusion proteins are widely applied to study the localization and the mobility of proteins in living cells, these constructs may fail to localize properly due to saturation of the targeting machinery, hampered complex formation, or restricted posttranslational modification (reviewed in ref. 25). Hence, chromobodies as novel intracellular affinity reagents provide a straightforward approach to overcome current limitations of related techniques, as they allow tracking of endogenous proteins in their native surroundings in real time.

Since their first description in 2006 (26), chromobodies directed against numerous antigens, including cyokeratin-8, laminA/C, PCNA, actin, β_2-AR, β-catenin, γH2AX, and PARP-1, have been developed and applied in various cellular models as well as in whole organisms (26–32).

Recently, we have developed chromobodies recognizing vimentin. To this end, we have followed an immunization strategy using recombinant vimentin, thus identifying eight unique vimentin-specific nanobodies from an alpaca-derived V_HH-gene library by phage display. Two nanobodies, VB3-NB and VB6-NB, were selected, as they specifically detected endogenous vimentin in biochemical applications, such as Western blot analysis, immunofluorescence, and immunoprecipitation (33). For intracellular imaging of vimentin, the nanobodies were converted into the chromobody expression constructs VB3-CB and VB6-CB by genetically fusing their binding moieties to GFP. Mammalian cells transiently expressing these chromobodies show characteristic vimentin filaments upon induction with TGFβ. In contrast to GFP–vimentin, expression of the chromobodies does not result in unspecific aggregation nor induces morphologic changes. This might be due to a transient intracellular binding of both chromobodies, as shown by fluorescence recovery after photobleaching (33).

By stable genomic integration of the VB6-CB construct in A549 lung cancer cells, a well-established cell line in the field of EMT (6, 22), we have generated a cellular model that allows visualization of endogenous vimentin in real time (Fig. 1A). Employing continuous time-lapse imaging for 96 hours, we have validated the applicability of the A549_VB6-CB cells for long-term experiments. At the beginning of the time series, EMT was induced by the addition of TGFβ, which was removed after 48 hours to enable a reversion of the process. This approach, which marked the debut in real-time analysis of endogenous vimentin, allowed the visualization of EMT-associated increase in vimentin expression as well as its relocalization from the perinuclear region toward the entire cytoplasm (33). As both the expression and the binding of the chromobody might interfere with the localization and/or function of its target molecule, any cellular chromobody system requires careful evaluation. Notably, we did not detect any significant alterations regarding cell viability, morphology, migration, invasion, or gene expression of other EMT markers between the chromobody cell line (A549_VB6-CB) and A549 wild-type cells.

Besides imaging of vimentin on a single-cell level, we focused on high-content imaging of the A549_VB6-CB cell system to identify phenotypic changes of vimentin in a statistically relevant number of cells. For quantification, we developed a chromobody-based readout that encompasses a precise segmentation of vimentin fiber segments normalized to the number of cells (Fig. 1B). In a proof-of-principle study, we applied this readout to quantify EMT-related alterations of vimentin upon stimulation with TGFβ as well as upon treatment with the vimentin-modifying and EMT-affecting compound withaferin A (WFA). We were able to determine time- and dose-dependent effects on vimentin for both treatments. In contrast to the continuous increase in vimentin fiber segments following treatment with TGFβ for 24 hours, a WFA-mediated reduction in vimentin fibers reached its maximum at an earlier stage (12 hours) and was almost completely absent toward the end of the analysis (24 hours). This finding illustrates how the chromobody technology could be implemented into cellular EMT-screening campaigns with the advantage to quantify even transient compound effects that would simply be overlooked by currently available endpoint assays.

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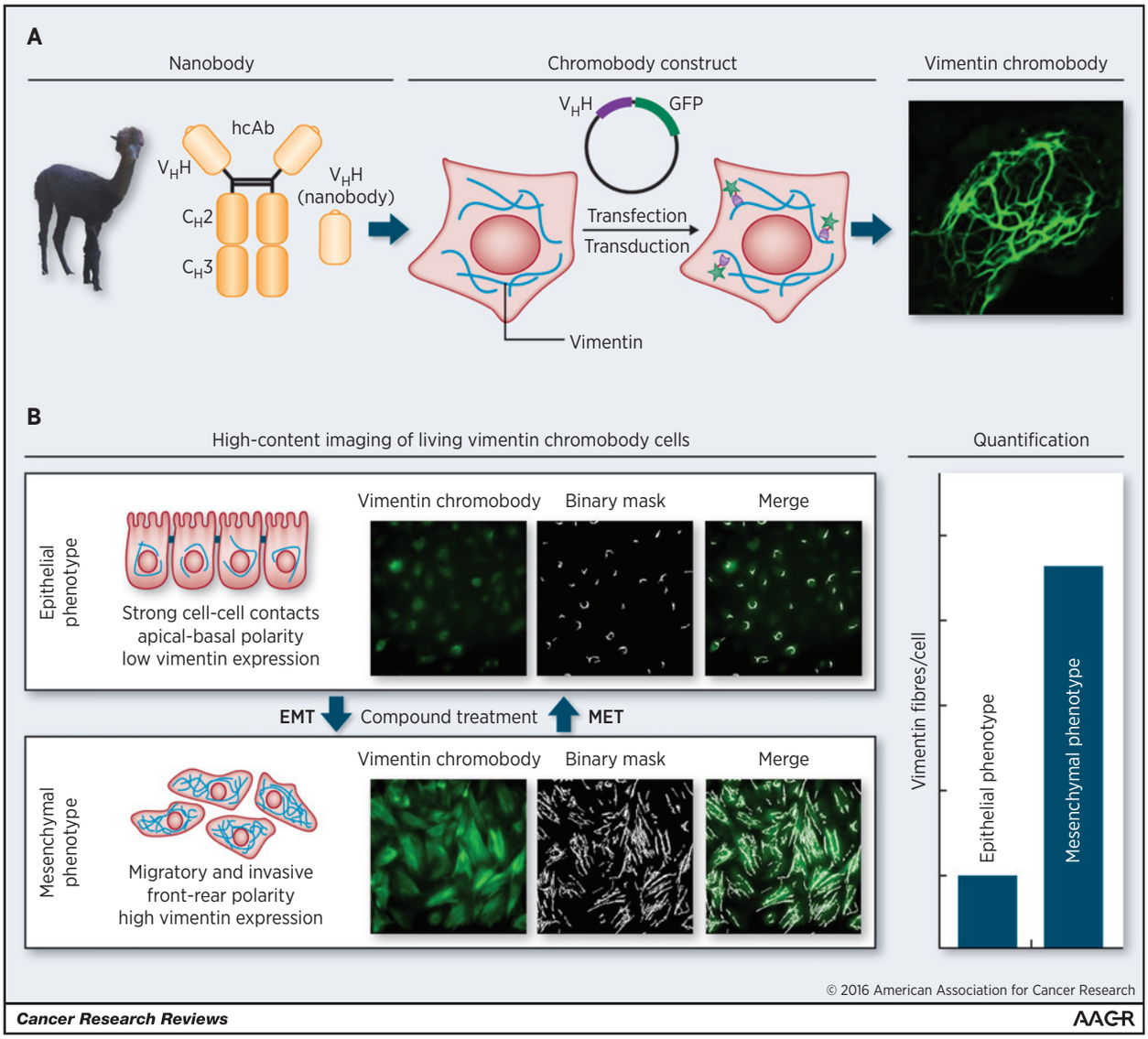

Figure 1.

Schematic illustration of the vimentin chromobody-based high-content imaging system. **A,** a vimentin-specific nanobody was selected from a phage-display library consisting of variable domains (V_HHs) of heavy-chain antibodies (hcAbs) of alpacas. Genetic fusion of the nanobody to GFP resulted in the vimentin chromobody construct. Intracellular expression of the chromobody allows the visualization of endogenous vimentin in living cells. **B,** high-content imaging of EMT in living cells stably expressing the vimentin chromobody. Cells are cultured in multi-well plates and treated with EMT-modulating compounds. Time lapse–derived images are subjected to software-assisted analysis. By means of an algorithm detecting filamentous structures visualized by the chromobody, a binary mask of vimentin fibers is generated. On the basis of this, mask fibers are quantified and normalized to the number of nuclei. Consequently, a low number of vimentin fibers per cell corresponds to an epithelial phenotype, while a high number of fibers indicates a more mesenchymal phenotype.

Perspectives and Outlook

Further applications of the vimentin chromobody in single cells

In addition to its use in EMT screening, the vimentin chromobody could be a valuable tool to extent current methods to study vimentin and its functions in the cytoskeletal reorganization of migratory cells. In particular, in cells with low basal expression levels of vimentin, visualization of the chromobody signal might enable a (re)evaluation of our knowledge on vimentin dynamics in real time, which is currently almost exclusively derived from ectopically expressed or microinjected fluorescent vimentin fusions. As the exact epitope of the chromobody is not yet known, one has to consider a potential interference of the chromobody with, for example, posttranslational modification or protein–protein interactions. However, binding of the chromobody has been mapped to the rod domain, which is known to be less posttranslationally modified compared with the head and tail

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domain of vimentin. Moreover, in contrast to a covalent modification, the chromobody binds vimentin only transiently and thus might be less prone to interfere with the above mentioned processes.

While the vimentin network can be traced by the chromobody signal with conventional fluorescence microscopes, the structural organization of individual vimentin fibers can only be visualized with super-resolution microscopy technologies, such as photoactivated localization microscopy, stochastic optical reconstruction microscopy, or stimulated emission depletion. In our study, we have demonstrated that the vimentin nanobodies can be labeled with organic dyes and applied to direct imaging of cellular structures without the need of a secondary antibody (33). Considering recent achievements in resolving other structural components, such as tubulin with fluorescently labeled nanobodies (34), we propose that the vimentin nanobodies are suited to resolve vimentin organization on a nanometer scale.

Vimentin chromobody in 3D models and *in vivo*

It is widely accepted that 2D cell culture models reflect the complexity of cancerous tissue *in vivo* only to a limited extent. For example, they omit the contribution of the extracellular matrix, into which cells invade during EMT. To simulate the *in vivo* situation more properly, novel 3D approaches to study EMT have been reported (5, 7, 22, 35). A 3D invasion model was developed that specifically addresses the link between EMT and invasion into stromal collagen: 3D spheroids of various breast cancer cell lines were established in a Matrigel, mimicking the basement membrane. The detection of individual cells migrating into a surrounding collagen environment shows the occurrence of an EMT-induced invasive phenotype (35). Another 3D model, described by Aref and colleagues, employed a microfluidic system utilizing spheroids of A549 cells in coculture with endothelial HUVEC cells in a 3D hydrogel scaffold. Notably, the authors described significant differences in drug response regarding cell proliferation and migration between their 2D and 3D models as well as between mono- and coculture (22). Acknowledging current developments in the field, we started to transfer our vimentin chromobody cell system into cellular 3D models. We propose that the chromobody signal allows a spatial and temporal resolution of distinct areas within living spheroids in which cells undergo EMT. Furthermore, in 3D coculture models, the chromobody signal would enable selective visualization of vimentin exclusively in the cell population of interest.

Even though 3D models are physiologically more relevant than 2D models, for downstream validation of potential drug candidates, animal studies are indispensable. As most screening campaigns on EMT are based on *in vitro* assays, the validity of these data for the *in vivo* situation has been under intense debate (36). This may be explained by the challenge to differentiate between cells that underwent cancer-induced EMT (e.g., CTCs) and mesenchymal-like cells of other origins (e.g., fibroblasts). However, a growing number of studies indicate that data derived from *in vitro* EMT studies can be indeed translated into an *in vivo* setting (37, 38). In this context, Bonnomet and colleagues developed a mouse model in which vimentin-negative MDA-MB-468 cells were xenografted into mice. By determining vimentin expression, they identified regions of increased EMT at the border of primary heterogeneous xenografts. In addition, the analysis of CTCs derived from these tumors revealed the transcription of high levels of vimentin and EMT-inducing transcription factors, suggesting that spontaneous EMT events promote intravasation and metastatic dissemination *in vivo* (38).

On the basis of these findings, it is reasonable to apply the vimentin chromobody for *in vivo* studies. Xenograft models of human tumors expressing the vimentin chromobody would provide the unique possibility to directly trace vimentin-expressing cells that undergo EMT in an animal model. Recently, several chromobodies, as well as other intrabodies, have been successfully introduced into whole organisms (e.g., zebrafish, mice) for live animal imaging (28, 39). Hence, we propose that tracing of endogenous vimentin by applying the chromobody technology might facilitate live experiments and histologic studies regarding EMT processes, the formation of CTCs, and metastatic dissemination *in vivo*.

Disclosure of Potential Conflicts of Interest

U. Rothbauer has ownership interest (including patents) in and is a consultant/advisory board member for ChromoTek GmbH. No potential conflicts of interest were disclosed by the other authors.

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