Pathol. Oncol. Res. (2012) 18:371–376
DOI 10.1007/s12253-011-9454-z

RESEARCH

Increase of α-SMA$^+$ and CK$^+$ Cells as an Early Sign of Epithelial-Mesenchymal Transition during Colorectal Carcinogenesis

Gábor Valcz · Ferenc Sipos · Tibor Krenács · Jeannette Molnár · Árpád V. Patai · Katalin Leiszter · Kinga Tóth · Barna Wichmann · Béla Molnár · Zsolt Tulassay

Received: 26 April 2011 / Accepted: 11 August 2011 / Published online: 13 September 2011

Abstract Our aim was to examine cell transition events by detecting the frequency of intrapithelial α-smooth muscle actin (SMA)$^+$/cytokeratin (CK)$^+$ cells during colorectal adenoma–carcinoma sequence, in relation to E-cadherin expression. Our further aim was to determine the proliferative activity of intraepithelial α-SMA$^+$ cells. Histologically healthy, adenoma, and colorectal cancer (CRC) biopsy samples were taken during routine colonoscopy and were included into tissue microarrays (TMAs). Slides immunostained for Ki-67, α-SMA, E-cadherin and pan-cytokeratin were digitalized and analyzed by using a digital microscope software. The proportion of α-SMA$^+$/CK$^+$ cells was significantly higher in CRC samples (3.34±1.01%) compared to healthy (1.94±0.69%) or adenoma (1.62±0.78%) samples (p <0.01). E-cadherin expression negatively correlated with the number of α-SMA$^+$ cells. The majority of intraepithelial α-SMA$^+$ cells were in the proliferative phase. During tumor progression, the appearance of dot-like α-SMA staining in CK positive cells may indicate the initial phase of the epithelial-to-mesenchymal transition (EMT). The high proportion of intraepithelial α-SMA$^+$ proliferating cells may refer to their increased plasticity compared to differentiated cells. The negative correlation between E-cadherin and intraepithelial α-SMA expression suggests that EMT is facilitated by a loss of epithelial cell contact.

Keywords Epithelial–myofibroblast transition · Adenoma–carcinoma sequence · Cytokeratin · Alpha–smooth muscle actin

G. Valcz (✉) · F. Sipos · Á. V. Patai · K. Leiszter · K. Tóth · B. Wichmann
Cell Analysis Laboratory, 2nd Department of Internal Medicine, Semmelweis University,
Budapest, Hungary
e-mail: valczg@yahoo.com

T. Krenács
1st Department of Pathology and Experimental Cancer Research, Semmelweis University,
Budapest, Hungary

J. Molnár
National Institute of Food and Nutrition Science,
Budapest, Hungary

B. Molnár · Z. Tulassay
Molecular Medicine Research Unit,
Hungarian Academy of Sciences,
Budapest, Hungary

Introduction

The colonic epithelial monolayer has several distinct functions, such as the absorption of water and drugs, ion transport and it is a barrier against luminal pathogens, as well [1–3]. The polarized cells of this continuous sheet are connected through different adhesion structures (i.e. adherens-, tight-, gap junctions, and desmosomes) [4]. The epithelial cells desert from the epithelial layer and may be a source of stromal mesenchymal cells by a series of phenotypic changes, called epithelial-to-mesenchymal transition (EMT). This and its reverse process (mesenchymal-to-epithelial transition/MET) are well characterized by the change of cell-cell adhesion, cell shape, cell migration properties and protein expression. Although both EMT and MET are physiological processes in embryogenesis, they may also appear in different stages of tumor development (e.g. initiation and metastasis formation) [5–10]. EMT is a change from apico-basolateral to front-rear polarity, reduced expression of epithelial markers (e.g. cytokeratin, MUC1, claudin, desmoplakin, entactin, laminin), acquisition of motility, a

spindle-cell shape, and the expression of mesenchymal markers such as N-cadherin, FSP1, vimentin and α-SMA [5, 6, 11–13]. A special form of EMT is the epithelial-to-myofibroblast transition (EMyT), a multistep process involving the loss of adhesive proteins (i.e. epithelial (E)-cadherin zonula occludens protein/ZO), actin-skeleton remodeling and myogenic reprogramming (α-smooth muscle actin/SMA expression) [14–16]. The disruption of cell-cell junctions can induce major α-SMA transcription factors, i.e. serum response factor (SRF) via the Rho/ROK pathway [17]. EMyT originated cells are capable to enter the "reactive" tumor stroma, where, as carcinoma associated fibroblasts (CAFs) they may influence the proliferative properties of cancer cells/cancer stem cells [18]. Furthermore, by their actin-based motility they can migrate to the metastatic place and enter the epithelial layer via MET/MyET (invasion-metastasis cascade) [4].

The adenoma-carcinoma sequence (ACS) is the most common pathway of colorectal carcinoma (CRC) formation [19]. However, the characterization of transition events in different histological stages of the ACS has not yet been determined. We characterized transition events by detecting the frequency of α-SMA/cytokeratin (CK) double-positive cells within the epithelium at different stages of the ACS in relation to the intraepithelial E-cadherin expression. We also determined the proliferative activity of intraepithelial α-SMA$^+$ cells.

Materials and Methods

Histologically healthy (only longitudinally oriented) (n=8), adenoma (n=8) and CRC (n=8) biopsy samples were taken during routine colonoscopy and included into tissue microarrays (TMAs) with 1 mm diameter cores. The samples were fixed in formaldehyde and embedded in paraffin and 4 μm thick tissue sections were cut. After deparaffination and rehydration, antigen retrieval was performed in TRIS-EDTA buffer (pH 9.0) using a microwave oven (900 W/10 minutes, 340 W/40 minutes). Then the samples were incubated with anti-Ki-67 (RM 9106-SO, clone:sp6, LabVision, performed with Alexa Fluor 546) monoclonal and FITC labeled anti–cytokeratin (CK3-6H5) (Miltényi Biotec, Bergisch Gladbach, Germany, 1:1 solution) polyclonal antibodies at room temperature for 60 minutes. Depending on the parallel staining, anti-α-SMA (Dako, California, USA 1:1 solution) was performed with Alexa Fluor 546 (α-SMA/CK costaining) and AMCA (AMCA-conjugated anti-mouse IgG H + L, 715-155-15, Jackson ImmunoResearch Laboratories,West Growe, Philadelphia, USA) (α-SMA/Ki-67 costaining). In case of E-cadherin immunohistochemistry, we blocked the endogenous peroxidase with 0.5% hydrogen peroxide and methanol mixture (30 minutes, room temperature), than antigen retrieval (TRIS-EDTA buffer pH 9) performed in a microwave oven (at 900 W for 10 minutes and at 370 W for 40 minutes) were carried out on dewaxed TMA samples. Non-specific blocking with 1% bovine serum albumin was applied. Immunohistochemical detection of E-cadherin was carried out in a humidified chamber using an anti-E-cadherin monoclonal antibody (ECH-6, 1:1 dilution, 60 minutes, Histopathology). Histols Detectation System (Rabbit and Mouse, Histopathology) and diaminobenzidine-hydrogen peroxidase-chromogen substrate system (Cytomation Liquid DAB + Substrate Chromogen System, K3468, Dako) were used for signal conversion. In case of some slides, high-resolution confocal scanning laser microscopy (BIO-RAD MRC 1024, Bio-Rad Laboratories, Philadelphia, USA) was used. Then the slides were digitally archived using a Pannoramic Scan instrument (software version 1.11.25.0, 3DHistech, Budapest, Hungary), and the immunosignals of the digital slides were analyzed using a digital microscope software (Pannoramic Viewer, v. 1,11,43,0, 3DHistech, Budapest, Hungary). 1000–1200 epithelial cells (depending on the size of the biopsy) were counted. Using a built-in modem called Marker Counter the exact cell number was determined. One-way ANOVA test was used for data analysis.

Results

We found only a small number (1.69–3.34%) of intraepithelial α-SMA$^+$/CK$^+$ cells showing different levels of myofibroblastic dedifferentiation. The high percentage of *de novo* α-SMA expressing epithelial cells showed only weak, single (Fig. 1a, b, c) or multiple (Fig. 1d, e) dot-like α-SMA expression in close proximity to the nucleus. In several cases, the localization of α-SMA reached to the nucleus (Fig. 1a, b, c). High resolution confocal scanning laser microscopy proved that these α-SMA$^+$ foci were deep nuclear invaginations of cytoplasmic reaction (Fig. 1c). Epithelial cells with dot-like α-SMA expression showed a cuboid cell shape with apico-basolateral polarity. Rarely, α-SMA expression was detected in the cytoplasma (weak or strong diffuse). These cells contained a large round- or oval-shaped nucleus (Fig. 2a, b). In some cases migrating α-SMA$^+$/CK$^+$ cells from the epithelial layer were observed, that could represent transition process (Fig. 1e). The localization and range of α-SMA expression was similar using both α-SMA/Ki-67 and α-SMA/CK double fluorescent staining. The proportion of α-SMA$^+$/CK$^+$ cells was significantly higher in CRC samples (3.34±1.01%) compared to healthy (1.94±0.69%) or adenoma (1.62±0.78%) samples (Fig. 3). The difference between normal and CRC and between adenoma and CRC samples was significant (p=0.013 and p=0.0026, respectively). A high percentage of intraepithelial α-SMA$^+$ cells were in the proliferative phase (healthy 87.7±10.7%, adenoma 81.4±7% and CRC

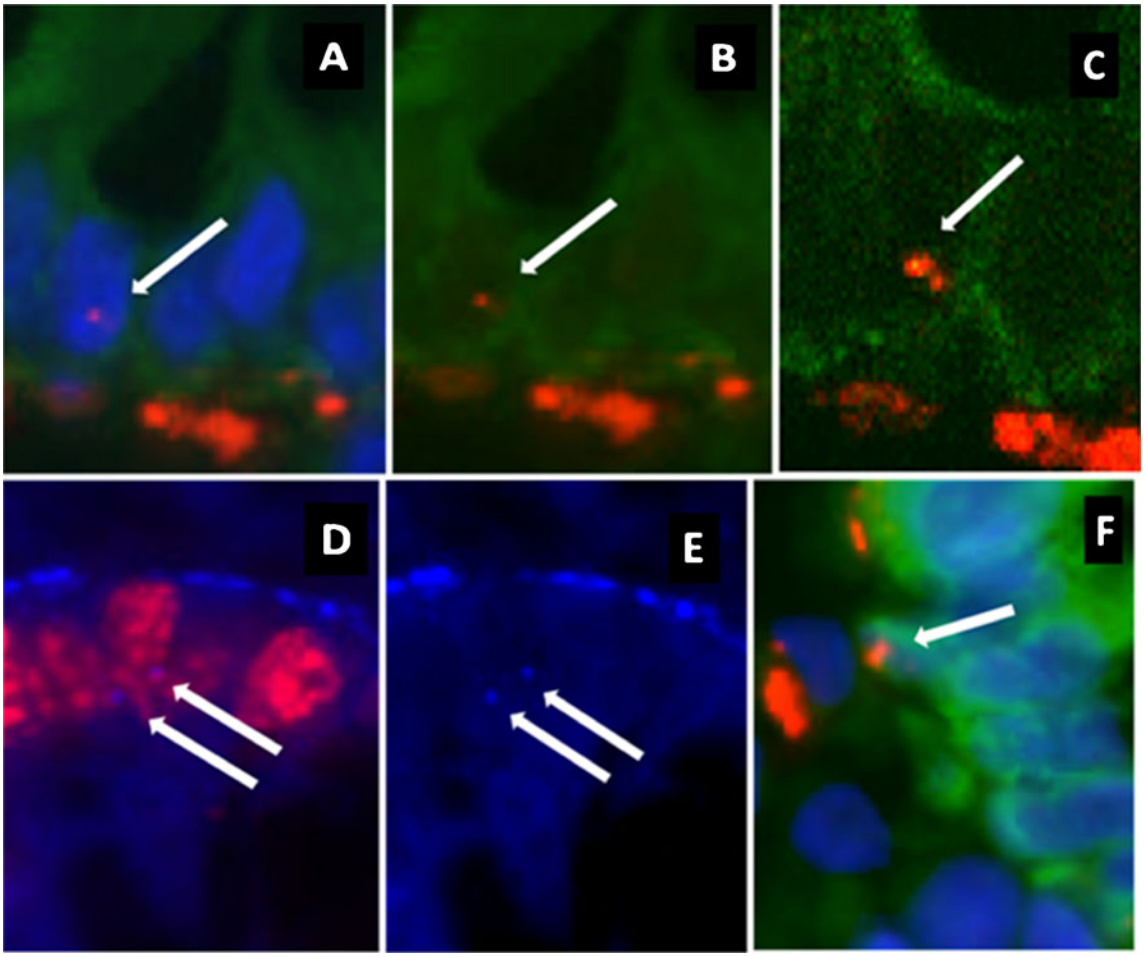

Fig. 1 Detection of different α-SMA expression (*white arrows*) with fluorescent staining. The majority of cells showed single- or multi dot-like α-SMA expression in close proximity to the nucleus or in the nuclear domain (**a**, **b**, **c**, **f**: α-SMA (*red*)/CK (*green*) and **d**, **e**: α-SMA (*blue*)/Ki-67 (*red*) fluorescent staining)

88±2.4%) on the basis of Ki-67 expression. In healthy transversal samples, α-SMA$^+$/Ki-67$^+$ cells were increased in the basal (proliferative, Ki-67$^+$) region compared to the apical region. E-cadherin expression showed a progressive

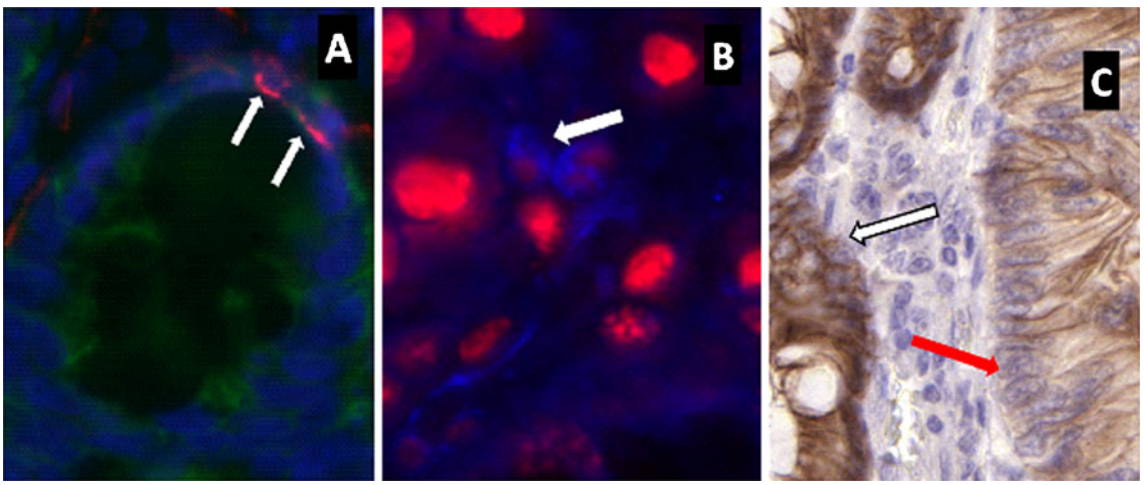

Fig. 2 **a**, **b**: Intrapithelial cells (*white arrow*) with strong cytoplasmic α-SMA expression (**a**: α-SMA (*red*)/CK (*green*), **b**: α-SMA (*blue*)/Ki67 (*red*) fluorescent staining), **c:** Different E-cadherin expression between the tumorous (*red arrow*) and adjacent healthy crypts (*white arrow*)

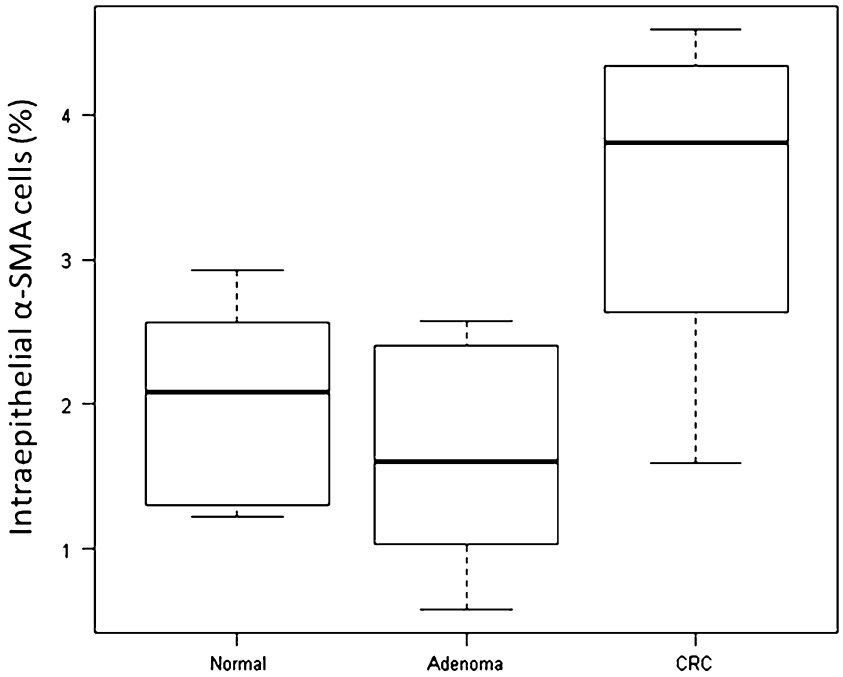

Fig. 3 The percentage of intraepithelial α-SMA$^+$/CK$^+$ cells in CRC significantly exceeds those either of normal or adenoma samples ($p<0.01$)

decrease during ACS (Fig. 4). In healthy samples, predominantly strong membrane and moderate cytoplasma staining (typical scoring value: +2) was detected, both of which were moderately decreased in adenoma samples (typical scoring value: 1 or 0). In most CRC samples we found only weak and fragmented membrane staining (typical scoring value: −2) (Fig. 2c).

Discussion

In this study, we examined the frequency of transition events using α-SMA/CK co-staining during the ACS. We found a significantly increased number of intraepithelial double positive cells in CRC samples compared to adenoma and healthy samples. The majority of α-SMA$^+$/

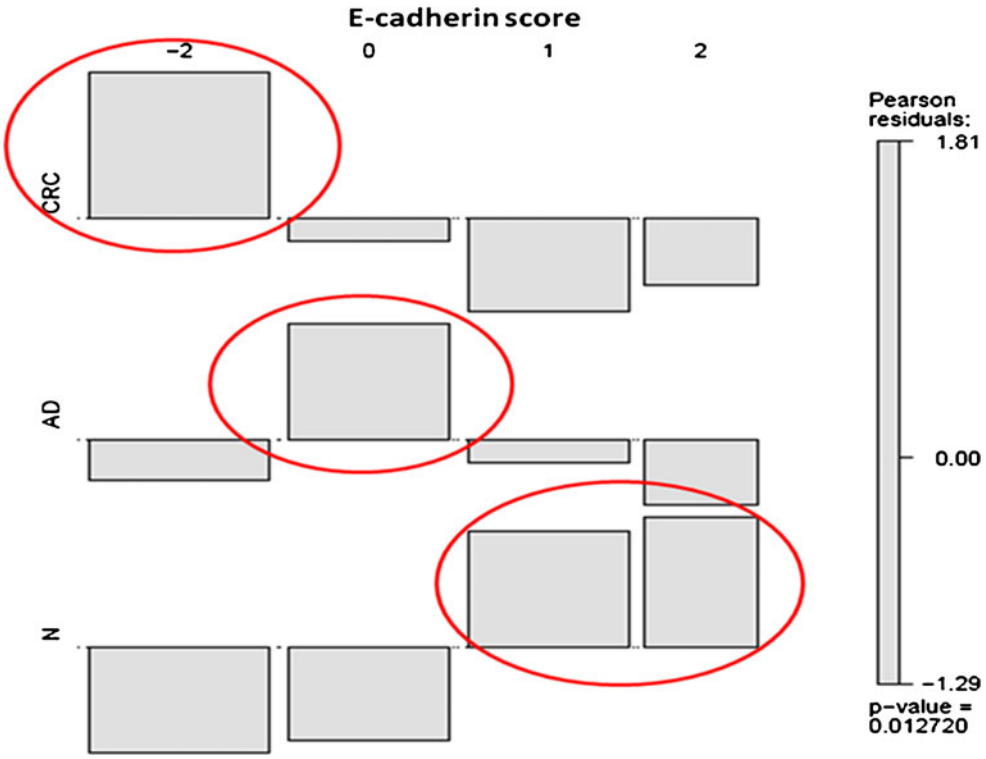

Fig. 4 Change in E-cadherin expression during ACS. CRC (colorectal carcinoma): typical scoring value: −2, AD (adenoma): typical scoring value: +1, N (normal/healthy): typical scoring value: +2 and +1

CK$^+$ cells showed only low, dot-like α-SMA positivity in close proximity to the nucleus and/or nuclear domain which suggests that these cells were in the initial phase of EMT. In agreement with Storch et al. [20], we observed α-SMA$^+$ foci in the cytoplasm as intranuclear invaginations. The change in cell shape by α-SMA bundles and the related mechanical force may contribute to an altered gene expression via nuclear transcription regulators (e.g. nuclear matrix protein/NMP-1,-2) [21]. According to this hypothesis, not only intercellular forces can cause mechanotransduction [17, 22, 23], but endogenously generated mechanical tension is also converted into biochemical signals. Consequently, we think that α-SMA$^+$ bundles serve as a "messenger" component of the mechano-transition system, and they may influence further α-SMA expression by activating the MAP kinase p38 as an important regulator of the α-SMA promoter.

The increased appearance of a fragmented migratory apparatus (i.e. dot-like α-SMA pattern) in the majority of epithelial cells during tumor progression suggests EMT does not require high cell motility. Presumably, mechanical force by the adjacent epithelial cells is more important in the desertion of transited cells from the epithelium, and the later phase (i.e. stromal migration) requires independent cell motility. In five cases intraepithelial cells showed a strong cytoplasmatic α-SMA expression, but these cells might have originated from MET.

The majority (approximately 83%) of intraepithelial α-SMA$^+$ cells were also positive to Ki-67 in all histological stages. This is similar to the results of Ng et al. that showed 60–80% of α-SMA$^+$ tubular epithelial cells in the proliferative phase (i.e. PCNA$^+$) in nephrectomized rats [24]. During tumor formation, the joint appearance of proliferation, α-SMA expression and E-cadherin repression may be explained by the increased inhibition of the GSK-3β protein via oncogenic pathways (i.e. PI3K/Akt, MAPK, Wnt), which causes nuclear accumulation of Snail and β-catenin [25]. The intranuclear localized Snail can suppress E-cadherin expression via recognizing E-box motifs in its promoter region [26]. Intranuclear β-catenin can influence proliferation and EMT as well [27].

Although, we observed a moderate repression of E-cadherin in adenomas compared to normal samples, it was not followed by an increased number of intraepithelial transited cells. This result may be in connection with the similar expression pattern of active transforming growth factor (TGF)-β_1 (as main regulator of MET) during ACS [28]. CRC samples showed increased disruption of cell junctions which can cause the contact–dependent overexpression of the main SMA-inducing transcription factor: serum response factor (SRF) via the Rho/ROK pathway [29, 30].

In some CRC samples, E-cadherin repression was not obvious. This can be well explained by a different DNA microsatellite stability status. Pino et al. observed endogenous TGF-β_1 induced EMT in microsatellite stable (MSS) colon cancer cell lines (SW480 and HT29) but not in microsatellite unstable (MSI) cell lines (DLD1 and HCT116) in vitro [31].

In summary, cell phenotype transition may be the key events in the development of colorectal carcinoma via an altered cell composition of the cancer stem cell niche and they are also fundamental in metastasis formation. Although, several details of transition events have been revealed already, the complete evaluation of these complex processes will require further study.

References

1. Schultheiss G, Diener M (1998) K+ and Cl- conductances in the distal colon of the rat. Gen Pharmacol 31:337–342
2. Lu L, Walker WA (2001) Pathologic and physiologic interactions of bacteria with thegastrointestinal epithelium. Am J Clin Nutr 73:1124–1130
3. Edmonds CJ (1984) Absorption and secretion of fluid and electrolytes by the rectum. Scand J Gastroenterol Suppl 93:79–87
4. Hollier BG, Evans K, Mani SA (2009) The epithelial-to-mesenchymal transition and cancer stem cells: a coalition against cancer therapies. J Mammary Gland Biol Neoplasia 14:29–43
5. Thiery JP, Acloque H, Huang RY et al (2009) Epithelial-mesenchymal transitions in development and disease. Cell 139:871–890
6. Kalluri R, Weinberg RA (2009) The basics of epithelial-mesenchymal transition. J Clin Invest 119:1420–1428
7. Kalluri R (2009) EMT: when epithelial cells decide to become mesenchymal-like cells. J Clin Invest 119:1417–1419
8. Hugo H, Ackland ML, Blick T et al (2007) Epithelial–mesenchymal and mesenchymal–epithelial transitions in carcinoma progression. J Cell Physiol 213:374–383
9. Radisky DC (2005) Epithelial-mesenchymal transition. J Cell Sci 118:4325–4326
10. Boyer B, Vallés AM, Edme N (2000) Induction and regulation of epithelial-mesenchymal transitions. Biochem Pharmacol 60:1091–1099
11. Zavadil J, Bottinger EP (2005) TGF-β and epithelial-to-mesenchymal transitions. Oncogene 24:5764–5774
12. Lorusso G, Rüegg C (2008) The tumor microenvironment and its contribution to tumor evolution toward metastasis. Histochem Cell Biol 130:1091–1103
13. Miyazono K (2009) Transforming growth factor-beta signaling in epithelial-mesenchymal transition and progression of cancer. Proc Jpn Acad Ser B Phys Biol Sci 85:314–323
14. Masszi A, Speight P, Charbonney E et al (2010) Fate-determining mechanisms in epithelial-myofibroblast transition: major inhibitory role for Smad3. J Cell Biol 188:383–399
15. Masszi A, Fan L, Rosivall L, McCulloch CA et al (2004) Integrity of cell-cell contacts is a critical regulator of TGF-beta 1-induced epithelial-to-myofibroblast transition: role for beta-catenin. Am J Pathol 165:1955–1967
16. Reichert M, Müller T, Hunziker W (2000) The PDZ domains of zonula occludens-1 induce an epithelial to mesenchymal transition of Madin-Darby canine kidney I cells. Evidence for a role of beta-catenin/Tcf/Lef signaling. J Biol Chem 275:9492–9500
17. Wang J, Zohar R, McCulloch CA (2006) Multiple roles of alpha-smooth muscle actin in mechanotransduction. Exp Cell Res 312:205–214
18. Li H, Fan X, Houghton J (2007) Tumor microenvironment: the role of the tumor stroma in cancer. J Cell Biochem 101:805–815

19. Jass JR (2007) Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 50:113–130
20. Storch KN, Taatjes DJ, Bouffard NA et al (2007) Alpha smooth muscle actin distribution in cytoplasm and nuclear invaginations of connective tissue fibroblasts. Histochem Cell Biol 127:523–530
21. Thomas CH, Collier JH, Sfeir CS et al (2002) Engineering gene expression and protein synthesis by modulation of nuclear shape. Proc Natl Acad Sci USA 99:1972–1977
22. Wang J, Chen H, Seth A et al (2003) Mechanical force regulation of myofibroblast differentiation in cardiac fibroblasts. Am J Physiol Heart Circ Physiol 285:1871–1881
23. Gomez EW, Chen QK, Gjorevski N et al (2010) Tissue geometry patterns epithelial-mesenchymal transition via intercellular mechanotransduction. J Cell Biochem 110:44–51
24. Ng YY, Huang TP, Yang WC et al (1998) Tubular epithelial-myofibroblast transdifferentiation in progressive tubulointerstitial fibrosis in 5/6 nephrectomized rats. Kidney Int 54:864–876
25. Zhou BP, Deng J, Xia W et al (2004) Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. Nat Cell Biol 6:931–940
26. Batlle E, Sancho E, Franci C et al (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol 2:84–89
27. Brabletz T, Hlubek F, Spaderna S et al (2005) Invasion and metastasis in colorectal cancer: epithelial-mesenchymal transition, mesenchymal-epithelial transition, stem cells and beta-catenin. Cells Tissues Organs 179:56–65
28. Hawinkels LJ, Verspaget HW, van der Reijden JJ et al (2009) Active TGF-beta1 correlates with myofibroblasts and malignancy in the colorectal adenoma-carcinoma sequence. Cancer Sci 100:663–670
29. Fan L, Sebe A, Péterfi Z et al (2007) Cell contact-dependent regulation of epithelial-myofibroblast transition via the rho-rho kinase-phospho-myosin pathway. Mol Biol Cell 18:1083–1097
30. Masszi A, Di Ciano C, Sirokmány G et al (2003) Central role for Rho in TGF-beta1-induced alpha-smooth muscle actin expression during epithelial-mesenchymal transition. Am J Physiol Renal Physiol 284:911–924
31. Pino MS, Kikuchi H, Zeng M et al (2010) Epithelial to mesenchymal transition is impaired in colon cancer cells with microsatellite instability. Gastroenterology 138:1406–1417