International Journal of Cancer

Novel signatures of cancer-associated fibroblasts

Benedek Bozóky[1], Andrii Savchenko[1], Péter Csermely[2], Tamás Korcsmáros[2,3], Zoltán Dúl[2,3], Fredrik Pontén[4], László Székely[1*] and George Klein[1*]

[1] Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Stockholm, Sweden
[2] Department of Medical Chemistry, Faculty of Medicine, Semmelweis University, Budapest, Hungary
[3] Department of Genetics, Eötvös Loránd University, Budapest, Hungary
[4] Department of Immunology, Genetics and Pathology, Rudbecklaboratoriet, Uppsala, Sweden

Increasing evidence indicates the importance of the tumor microenvironment, in particular cancer-associated fibroblasts, in cancer development and progression. In our study, we developed a novel, visually based method to identify new immunohistochemical signatures of these fibroblasts. The method employed a protein list based on 759 protein products of genes identified by RNA profiling from our previous study, comparing fibroblasts with differential growth-modulating effect on human cancers cells, and their first neighbors in the human protein interactome. These 2,654 proteins were analyzed in the Human Protein Atlas online database by comparing their immunohistochemical expression patterns in normal *versus* tumor-associated fibroblasts. Twelve new proteins differentially expressed in cancer-associated fibroblasts were identified (DLG1, BHLHE40, ROCK2, RAB31, AZI2, PKM2, ARHGAP31, ARHGAP26, ITCH, EGLN1, RNF19A and PLOD2), four of them can be connected to the Rho kinase signaling pathway. They were further analyzed in several additional tumor stromata and revealed that the majority showed congruence among the different tumors. Many of them were also positive in normal myofibroblast-like cells. The new signatures can be useful in immunohistochemical analysis of different tumor stromata and may also give us an insight into the pathways activated in them in their true *in vivo* context. The method itself could be used for other similar analysis to identify proteins expressed in other cell types in tumors and their surrounding microenvironment.

Recent advances have highlighted the importance of the tumor microenvironment and its interaction with cancer cells including the influence of cancer-associated fibroblasts (CAFs).[1] The normal microenvironment is believed to play a restrictive role, capable of nipping incipient or disseminated cancer cells in the bud. CAFs have often lost this capacity and may even stimulate tumor growth.[2–4] Using the *in vitro* neighbor suppression system described by Stoker *et al.*,[5] we could confirm that normal and CAFs differ in their inhibitory effect on tumor cell proliferation. We have also found that normal fibroblasts from the same patient may differ, depending on their site of origin.[6] Based on this, the RNA expression profiles of fibroblasts with low and high inhibitory capacity against an established line of prostatic carcinoma cells were compared and 1,033 differently expressed genes were identified.[7] A further analysis was made where first neighbor interactors of the encoded proteins were found.

The Human Protein Atlas is an online database (www.proteinatlas.com) of immunostained tissue samples. It provides data on the protein expression patterns of various cell types in both cancerous and normal tissues.[8,9] In contrast to *in vitro* models, the Human Protein Atlas provides information about the protein expression of cells in their true *in vivo* environment. Using the Human Protein Atlas, we examined the reactivity of antibodies directed against proteins and their first neighbor interactors found by the RNA profiling. We found 12 previously unknown signatures preferentially expressed in CAFs.

Material and Methods

Analyzed genes

In a previous study, gene expression was analyzed in two isogenic pairs of inhibitory and noninhibitory fibroblasts. The *in vitro* pair consisted of inhibitory and less inhibitory subclones of the telomerase immortalized, BjhTERT foreskin fibroblast line separated by morphology. The *ex vivo* pair consisted of inhibitory (skin) and noninhibitory (hernia) fibroblasts, derived from the same donor.[7] Genes consistently

Key words: tumor microenvironment, cancer associated fibroblasts, Rho kinase, myofibroblasts
Additional Supporting Information may be found in the online version of this article.
L.S. and G.K. contributed equally to this work
Grant sponsor: The Knut and Alice Wallenberg foundation, The Swedish Cancer Society and Vetenskapsrådet, The Cancer Research Institute in New York, The Concern Foundation in Los Angeles; **Grant sponsor:** The Hungarian National Science Foundation; **Grand number:** OTKA-K83314; **Grant sponsor:** The EU; **Grand number:** TÀMOP-4.2.2/B-10/1-2010-0013
DOI: 10.1002/ijc.28035
History: Received 21 Aug 2012; Accepted 2 Jan 2013; Online 15 Jan 2013
Correspondence to: Benedek Bozóky, Department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet, Smedvägen 4, Tullinge 146 36, Stockholm, Sweden, Fax: +46-8-330498, E-mail: benedek.bozoky@gmail.com

What's new?

Cancer-associated fibroblasts (CAFs) in the tumor microenvironment influence the growth and progression of malignant disease. This study describes 12 previously unknown CAF immunohistochemical signatures, each with a unique expression pattern in the microenvironment of basal cell carcinomas. The signatures correlated with the myofibroblastic phenotype, and the majority of signatures were expressed at increased levels in different tumor stromata when compared with the normal tissues. Four signatures were linked to Rho kinase signaling. The findings suggest that the method could be useful for gaining insight into mechanisms of CAF activation and activity.

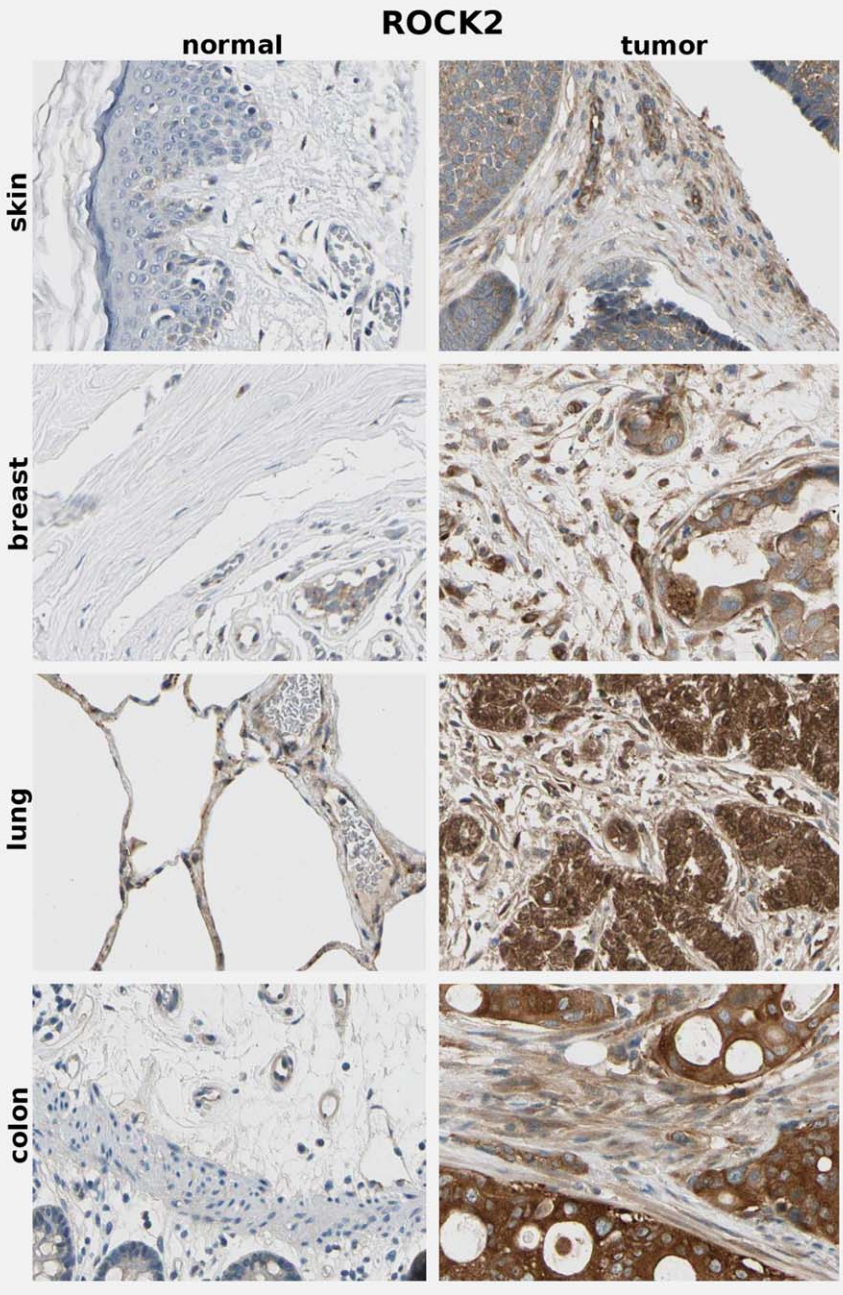

Figure 1. ROCK2 expression compared between several normal and tumorous tissues. Left column shows normal and the right column shows tumor tissues with its associated stroma. Each row represents a different tissue. Images were obtained from the Human Protein Atlas. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

upregulated in the inhibitory fibroblasts and genes upregulated in the noninhibitory fibroblasts were selected. Altogether 1,033 such genes were found with around half in each category.

Searching the UniProt resource for the protein products of the 1,033 differently expressed genes, we found 762 UniProt Accession numbers for 759 proteins (including a triplicate annotation) that we used for further analysis.[10] To identify the first neighbor interactors of these proteins, we used high-throughput and small-scale protein–protein interaction data from BioGRID and Human Protein Reference Database resources, respectively.[11,12] In total, 1,892 proteins were then found as possible direct interactors to the coding genes of the original, differentially expressed list. The proteins from UniProt resource and the possible direct interactors of these (total, 2,654) were selected for the image analysis (http://netbiol.elte.hu/karolinska/).

Image analysis

The protein expression of the selected genes was analyzed in the Human Protein Atlas online database using a tailored

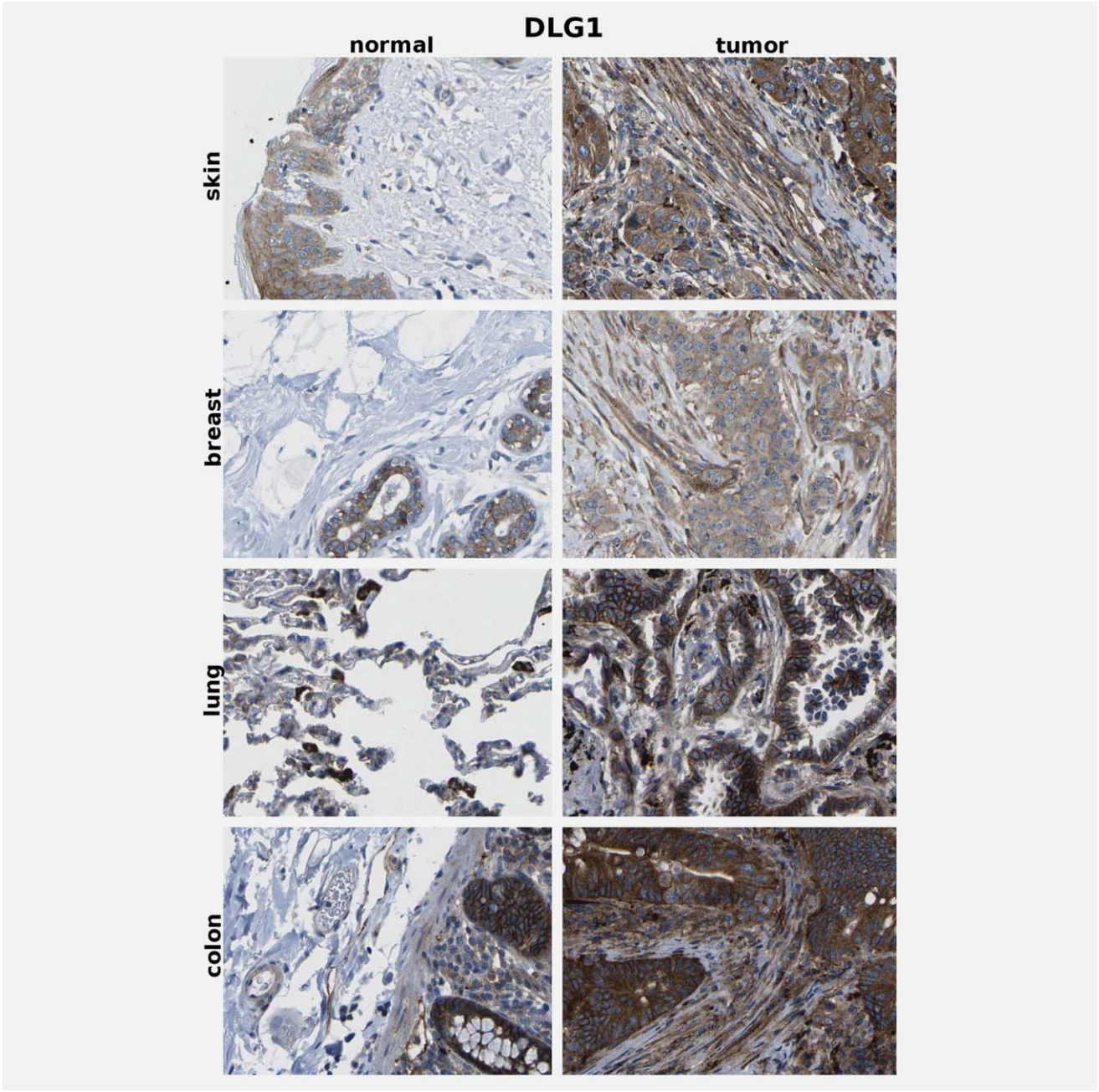

Figure 2. DLG1 expression compared between several normal and tumorous tissues. Left column shows normal and the right column shows tumor tissues with its associated stroma. Each row represents a different tissue. Images were obtained from the Human Protein Atlas. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

software (Atlas Grabber) developed by our team. The software was designed to use a list of our selected proteins and fetched the corresponding images from the Human Protein Atlas online database website. By displaying the normal and matching cancerous tissue images side by side for a particular protein, it significantly assisted and speeded up the analysis.

We looked for differentially expressed proteins in the stroma of basal cell carcinoma, compared to normal skin fibroblasts. Genes strongly expressed in the tumor's microenvironment but not in normal skin were defined as CAF signatures. An additional selection was made based on the regularity of expression in multiple tumor samples.

The expression of the signatures was also compared in fibroblasts in the vicinity of squamous cell carcinoma, breast cancer, colorectal cancer and lung cancer. The percentage of positive CAF samples was plotted for each specific protein, for the five different tumor types. To examine the relationship of these signatures to the myofibroblastic phenotype, their expression was also analyzed in several normal tissue fibroblast and myofibroblastic cells.

Results

CAF signatures

Out of the original list of 759 Uniprot-identified protein products of 1,033 differentially expressed genes and their first neighbors in the human interactome (proteins in total, 2,654), 1,876 had available expression profiles in the Human Protein Atlas. Twelve proteins were identified out of these

Table 1. List of identified CAF immunohistochemical signatures in basal cell carcinoma

PROTEIN name and description	Antibody	Available info
ARHGAP26—Rho GTpase-activating protein 26	HPA035107	Encodes a GTPase-activating protein that binds to focal adhesion kinases and mediates the activity of the GTP-binding proteins RhoA and Cdc42.[13]
ARHGAP31—Rho GTPase-activating protein 31	HPA036380	Functions as a GTPase-activating protein (GAP) for RAC1 and CDC42. It is required for cell spreading, polarized lamellipodia formation and cell migration.[14,15] The Rho GTPases control many aspects of cell behavior, such as the organization of the cytoskeleton, cell migration, cell–cell and cell–matrix adhesion, cell-cycle progression, gene expression and cell polarity.[16–18]
DLG1—disks, large homolog 1	CAB016307	It has been shown to be important in stabilizing Net1, a Rho guanine nucleotide exchange factor specific for the RhoA subfamily of small G proteins, and therefore its ability to stimulate RhoA activation in cells.[19]
ROCK2—Rho-associated, coiled-coil containing protein kinase 2	HPA007459	Its activation was shown to elevate tissue stiffness *via* increased collagen. The same study also suggested that tumor number growth and progression were increased by ROCK activation, whereas ROCK blockade was inhibitory.[20]
EGLN1—EGL nine homolog 1 or hypoxia-inducible factor prolyl hydroxylase 2	HPA022129	Is a cellular oxygen sensor that catalyzes, under normoxic conditions, the post-translational formation of 4-hydroxyproline in hypoxia-inducible factor (HIF) α proteins.[21,22]
ITCH—ITCHY E3 ubiquitin protein ligase homolog	HPA021126	It has been shown to facilitate complex formation between TGF-β receptor and Smad2 and to enhance TGF-β-induced transcription.[23] It has also been found to mediate the ubiquitination and sorting of the G protein-coupled receptor CXCR4.[24]
RNF19A—ring finger protein 19A or dorfin	CAB011455	is a ubiquitin ligase.[25]
PKM2—pyruvate kinase, muscle	CAB019421	Is one of the several Pyruvate kinase isoenzymes functioning as rate-limiting enzyme during glycolysis. PKM2 is predominantly found in fetal and in tumor cells in contrast to PKM1 found in normal adult cells.[26–28] It has also been shown that fibroblasts overexpressing PKM1 and PKM2 increase the mitochondrial activity of adjacent breast cancer cells and promote their growth.[29]
AZI2—5-azacytidine induced protein 2	HPA035258	Activates serine/threonine-protein kinase TBK1 and facilitates its oligomerization. It also enhances the phosphorylation of a NF-κB subunit, promotes TBK1-induced as well as TNF-α- or PMA-induced activation of NF-κB, and participates in IFN-β promoter activation *via* TICAM1.[30,31]
BHLHE40—basic helix–loop–helix family, member e40	HPA028921	It has been shown to be differentially expressed in several malignancies.[32,33] It has also various roles in cell proliferation, apoptosis, differentiation, carcinogenesis, cellular metabolism, circadian rhythms, immune regulation and functions as a hypoxia inducible gene as well.[34–38]
PLOD2—procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	CAB025898	Is involved in fibrotic processes and tissue remodeling and catalyzes the hydroxylation of lysyl residues as a post-translational event in collagen biosynthesis. Hypoxia may stimulate the expression of PLOD *via* the HIF-1 pathway.[39–41]
RAB31—member RAS oncogene family	HPA019717	Is a small GTP-binding protein of the RAB family, which plays an essential role in vesicle and granule targeting.

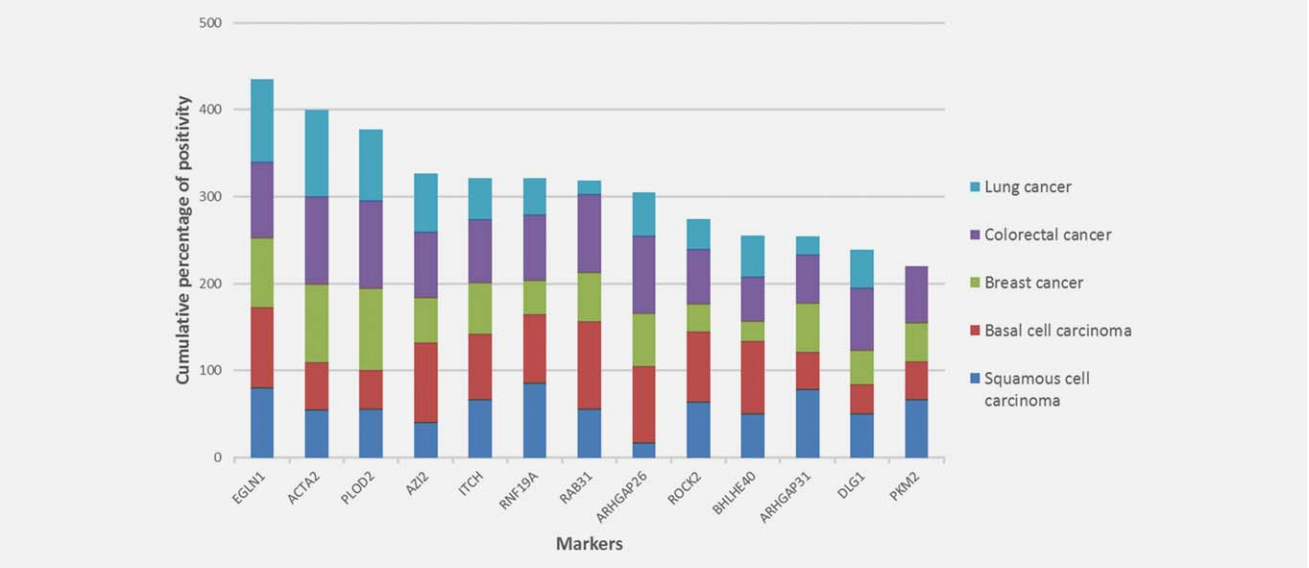

Figure 3. Expression of the identified CAF signatures in fibroblasts associated with different tumors. The percentage of positive samples for each tumor type was measured for each signature and ranked in order of cumulative percentage. Smooth muscle alpha actin (ACTA2), the currently main CAF marker, was also included in the figure to show its comparison to the novel signatures. This figure shows that some signatures such as EGLN1 and PLOD2 were similarly expressed, whereas others, such as PKM2 and ARHGAP26, can show considerable difference between tumor types. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 2. Expression of the identified signatures in different normal fibroblasts. The table illustrates the expression patterns of the identified CAF signatures in different normal tissue fibroblasts. Most of them were expressed (+) in myofibroblastic cells (bone marrow fibroblasts, mesangial cells and subepithelial small intestinal fibroblasts) but were mostly negative (−) in normal fibroblasts (peritubular fibroblasts, intra and interlobular breast fibroblasts). +/− indicates uncertain results.

	Bone marrow	Kidney		Small intestine villi	Breast	
	Fibroblast	Mesangial myofibroblast	Peritubular fibroblast	Subepithelial fibroblast	Intralobular fibroblast	Interlobular fibroblast
ARHGAP26	+	+	−	+	−	−
ARHGAP31	+/−	+	−	+/−	−	−
RNF19A	+	+/−	−	+	−	−
EGLN1	+	+	−	+	−	−
ITCH	+	+/−	−	+	+/−	−
PKM2	−	−	−	+	−	−
AZI2	+	+	−	+	+	−
DLG1	−	+	−	+	−	−
PLOD2	−	+	−	+/−	−	−
BHLHE40	−	−	−	+	−	−
ROCK2	−	−	−	−	−	−
RAB31	−	−	−	+	−	−

that were expressed in the CAF of basal cell carcinoma but not in normal skin fibroblasts (Figs. 1 and 2, Table 1 and Supporting Information Figs. 1–10). Figure 3 shows the expression patterns of the signatures in additional tumors and tissues. It demonstrates that the expression patterns for each protein varied between the different tumors. For example, ARHGAP26 was positive in most of the basal cell carcinoma tissue samples, but the expression could be seen only in a few of the squamous cell carcinoma samples. Smooth muscle alpha actin (ACTA2), the current main myofibroblast marker, was also included in the figure to show its comparison to the novel signatures.

Comparison to the myofibroblastic phenotype

To examine the connection between the CAF phenotype and the myofibroblastic phenotype, the expression patterns of the novel signatures were also checked in several myofibroblast-like cells in normal tissues (Table 2). The findings

demonstrate that most of them were similarly expressed in myofibroblast-like cells (bone marrow fibroblast, renal mesangial cell, subepithelial intestinal villous myofibroblasts), whereas they were not expressed in normal tissue fibroblasts (renal peritubular fibroblasts, intra- and interlobular breast fibroblasts). A notable exception was ROCK2 that seemed to be expressed only in CAFs.

Discussion

By using RNA profiling data[7] and human protein interactome neighbor analysis combined with the Human Protein Atlas database, a novel, visually based method was developed to identify differentially expressed proteins between normal fibroblasts and CAFs (Figs. 1 and 2, Supporting Information Figs. 1–10). The method resulted in the identification of 12 such proteins (Table 1). These can provide new biomarkers for immunohistochemical analysis of tumor stromata and may also offer insights into the pathways that are active in the tumor microenvironment. As the method includes the analysis of real tumor samples, the expression patterns identified are in the context of their true *in vivo* settings. Several of the identified signatures can also be expressed in cells of epithelial origin (Figs. 1 and 2; Supporting Information Figs. 1–9). This is, however, not directly relevant for the work that focuses on the expression patterns in fibroblasts alone.

Four of the listed genes, ARHGAP26, ARHGAP31, DLG1 and ROCK2, are linked to Rho kinase signaling (Table 1). This pathway is believed to be responsible for regulating actin cytoskeleton, cell adhesion and cell migration and is therefore also partially responsible for the myofibroblastic phenotype seen in CAFs (Table 2). This pathway also includes alpha smooth muscle actin, generally used as a CAF marker.[42] Rho kinase signaling has been linked to increased tissue stiffness, which in turn significantly contributes to tumor cell survival, proliferation and progression.[43]

Analysis of the interaction network neighborhood of the proposed CAF signatures (Fig. 4) showed a highly connected protein network. However, none of the CAF signatures is directly connected to each other. Interestingly, the CAF signatures and their first neighbors form denser components (*i.e.*, protein mod-

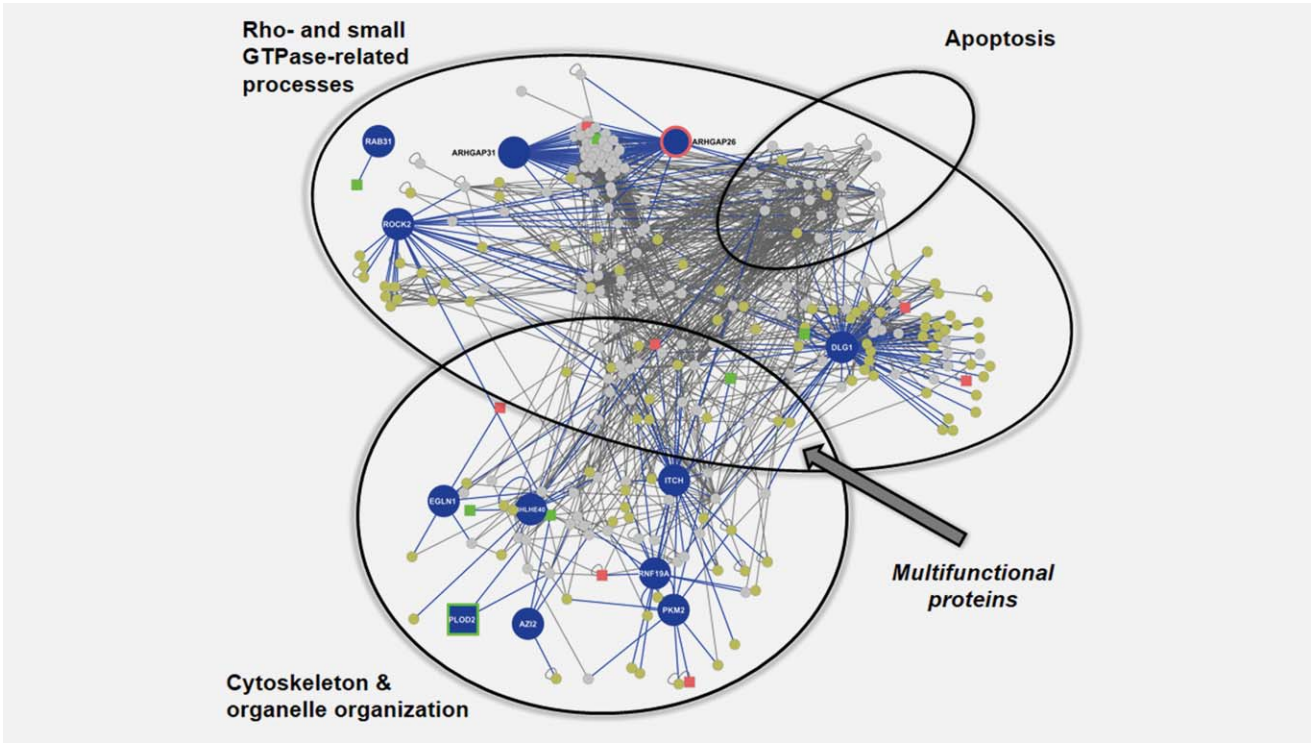

Figure 4. The interaction network neighborhood of the identified CAF signatures. This figure shows the protein–protein interaction subnetwork of the identified CAF signatures (highlighted with dark blue) and their first neighbors. The interactions of the CAF signatures are shown with blue edges and other interactions with gray edges. Those neighbors whose transcripts were found consistently upregulated in inhibitory or in noninhibitory fibroblasts are marked with red and green rectangles, respectively. Those proteins that are both neighbors of these differentially expressed proteins and the CAF signatures are marked with gray circles, whereas those proteins that are only the neighbors of the CAF signatures are marked with light brown circles. Note that PLOD2 has been identified as a CAF signature without any interactions but upregulated in noninhibitory fibroblasts, whereas ARHGAP26, which was upregulated in inhibitory fibroblasts, was also found as first neighbor of other differentially expressed proteins, and was also identified as a CAF signature. The network has been functionally analyzed based on Gene Ontology Biological Processes. The characteristic cellular functions are shown for each protein group. Note that the CAF signature RAB31 has no connection to the network. For details, see the main text. This figure was created with Cytoscape[44] using a spring-embedded network layout followed by manual adjustment. The functional analysis was carried out with GOTermFinder.[45] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ules or groups). Functional analyzes of these groups showed three major processes in the network: (*i*) Rho- and GTPase-related processes, (*ii*) Apoptosis, (*iii*) Cytoskeleton and organelle organization. In addition, many proteins have multiple functions; thus, they are localized in the overlaps of the protein groups. Note the slight overlap between the Rho- and GTPase-related and the Apoptosis groups. The Apoptosis group, formed by 26 proteins, is special as it is the only group that does not contain any CAF signature, but members of this group are highly connected to the CAF signatures and other CAF-neighbor proteins.

Conclusions

In conclusion, the novel method to compare Human Protein Atlas Images employed here proved to be useful in identifying 12 genes as novel signatures of CAFs. This type of analysis can also prove to be useful to compare other *ex vivo*, isolated system findings about tumor–host interactions to real *in vivo* expression patterns.

Acknowledgement

T.K. is a Janos Bolyai Research Scholar of the Hungarian Academy of Sciences.

References

1. Allen M, Louise Jones J. Jekyll and Hyde: the role of the microenvironment on the progression of cancer. *J Pathol* 2011;223:162–76.
2. Elkabets M, Gifford AM, Scheel C, et al. Human tumors instigate granulin-expressing hematopoietic cells that promote malignancy by activating stromal fibroblasts in mice. *Histopathology* 2011;121:784–99.
3. Franco OE, Shaw AK, Strand DW, et al. Cancer associated fibroblasts in cancer pathogenesis. *Semin Cell Dev Biol* 2010;21:33–9.
4. Campbell I, Qiu W, Haviv I. Genetic changes in tumour microenvironments. *J Pathol* 2011;223:450–8.
5. Stoker MG, Shearer M, O'Neill C. Growth inhibition of polyoma-transformed cells by contact with static normal fibroblasts. *J Cell Sci* 1966;1:297–310.
6. Flaberg E, Markasz L, Petranyi G, et al. High-throughput live-cell imaging reveals differential inhibition of tumor cell proliferation by human fibroblasts. *Int J Cancer* 2011;128:2793–802.
7. Flaberg E, Guven H, Savchenko A, et al. The architecture of fibroblast monolayers of different origin differentially influences tumor cell growth. *Int J Cancer* 2012;131:2274–83.
8. Uhlén M, Björling E, Agaton C, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. *Mol Cell Proteomics* 2005;4:1920–32.
9. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. *Nat Biotechnol* 2010;28:1248–50.
10. Boutet E, Lieberherr D, Tognolli M, et al. UniProtKB/Swiss-Prot: The Manually Annotated Section of the UniProt KnowledgeBase. *Methods Mol Biol* 2007;406:89–112.
11. Keshava Prasad TS, Goel R, Kandasamy K, et al. Human Protein Reference Database—2009 update. *Nucleic Acids Res* 2009;37: D767–72.
12. Stark C, Breitkreutz B-J, Chatr-Aryamontri A, et al. The BioGRID Interaction Database: 2011 update. *Nucleic Acids Res* 2011;39:D698–704.
13. Hildebrand JD, Taylor JM, Parsons JT, et al. An SH3 domain-containing GTPase-activating protein for Rho and Cdc42 associates with focal adhesion kinase. *Mol Cell Biol* 1996;16:3169–78.
14. Itoh RE, Kurokawa K, Ohba Y, et al. Activation of Rac and Cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol Cell Biol* 2002;22:6582–91.
15. Tcherkezian J, Triki I, Stenne R, et al. The human orthologue of CdGAP is a phosphoprotein and a GTPase-activating protein for Cdc42 and Rac1 but not RhoA. *Biol Cell* 2006;98:445–56.
16. Burridge K, Wennerberg K. Rho and Rac take center stage. *Cell* 2004;116:167–79.
17. Hall A. Rho GTPases and the control of cell behaviour. *Biochem Soc Trans* 2005;33:891–5.
18. Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling networks. *Genes Dev* 1997;11:2295–322.
19. Carr HS, Cai C, Keinänen K, et al. Interaction of the RhoA exchange factor Net1 with discs large homolog 1 protects it from proteasome-mediated degradation and potentiates Net1 activity. *J Biol Chem* 2009;284:24269–80.
20. Samuel MS, Lopez JI, McGhee EJ, et al. Actomyosin-mediated cellular tension drives increased tissue stiffness and β-catenin activation to induce epidermal hyperplasia and tumor growth. *Cancer Cell* 2011;19:776–91.
21. Epstein ACR, Gleadle JM, Mcneill LA, et al. *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 2001;107:43–54.
22. Ivan M, Haberberger T, Gervasi DC, et al. Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. *Proc Natl Acad Sci USA* 2002;99:13459–64.
23. Bai Y, Yang C, Hu K, et al. Itch E3 Ligase-mediated regulation of TGF-beta signaling by modulating Smad2 phosphorylation. *Mol Cell* 2004;15:825–31.
24. Marchese A, Raiborg C, Santini F, et al. The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell* 2003;5:709–22.
25. Niwa J, Ishigaki S, Doyu M, et al. A novel centrosomal ring-finger protein, dorfin, mediates ubiquitin ligase activity. *Biochem Biophys Res Commun* 2001;281:706–13.
26. Hathurusinghe HR, Goonetilleke KS, Siriwardena AK. Current status of tumor M2 pyruvate kinase (tumor M2-PK) as a biomarker of gastrointestinal malignancy. *Ann Surg Oncol* 2007;14: 2714–20.
27. Christofk HR, Vander Heiden MG, Harris MH, et al. The M2 splice isoform of pyruvate kinase is important for cancer metabolism and tumour growth. *Nature* 2008;452:230–3.
28. Steták A, Veress R, Ovádi J, et al. Nuclear translocation of the tumor marker pyruvate kinase M2 induces programmed cell death nuclear translocation of the tumor marker pyruvate kinase. 2007;67:1602–8.
29. Chiavarina B, Whitaker-menezes D, Martinez-outschoorn UE, et al. Pyruvate kinase expression (PKM1 and PKM2) in cancer-associated fibroblasts drives stromal nutrient production and tumor growth. *Cancer Biol Ther* 2012;12:1101–13.
30. Fujita F, Taniguchi Y, Kato T, et al. Identification of NAP1, a Regulatory Subunit of IκB kinase-related kinases that potentiates NF-κB signaling. *Mol Cell Biol* 2003;23:7780–93.
31. Sasai M, Oshiumi H, Matsumoto M, et al. NF kappa B—activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated IFN regulatory factor 3 activation. *J Immunol* 2005;174:27–30.
32. Li Y, Zhang H, Xie M, et al. Abundant expression of Dec1/stra13/sharp2 in colon carcinoma: its antagonizing role in serum deprivation-induced apoptosis and selective inhibition of procaspase activation. *Biochem J* 2002;367:413–22.
33. Chakrabarti J, Turley H, Campo L, et al. The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumour grade in human breast cancers. *Br J Cancer* 2004;91:954–8.
34. Guillaumond F, Lacoche S, Dulong S, et al. Altered Stra13 and Dec2 circadian gene expression in hypoxic cells. *Biochem Biophys Res Commun* 2008;369:1184–9.
35. Noshiro M, Usui E, Kawamoto T, et al. Liver X receptors (LXRalpha and LXRbeta) are potent regulators for hepatic Dec1 expression. *Genes Cells* 2009;14:29–40.
36. Sato F, Bhawal UK, Kawamoto T, et al. Basic-helix-loop-helix (bHLH) transcription factor DEC2 negatively regulates vascular endothelial growth factor expression. *Genes Cells* 2008;13:131–44.
37. Lennon DP, Edmison JM, Caplan AI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. *J Cell Physiol* 2001;187:345–55.
38. Yun Z, Maecker HL, Johnson RS, et al. Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. *Dev Cell* 2002;2:331–41.
39. Ruotsalainen H, Vanhatupa S, Tampio M, et al. Complete genomic structure of mouse lysyl hydroxylase 2 and lysyl hydroxylase 3/collagen glucosyltransferase. *Matrix Biol* 2001;20:137–46.
40. van der Slot AJ, Zuurmond A-M, Bardoel AFJ, et al. Identification of PLOD2 as telopeptide lysyl

hydroxylase, an important enzyme in fibrosis. *J Biol Chem* 2003;278:40967–72.

41. Noda T, Yamamoto H, Takemasa I, et al. PLOD2 induced under hypoxia is a novel prognostic factor for hepatocellular carcinoma after curative resection. *Liver Int* 2012;32:110–8.
42. Zhao X-H, Laschinger C, Arora P, et al. Force activates smooth muscle alpha-actin promoter activity through the Rho signaling pathway. *J Cell Sci* 2007;120:1801–9.
43. Egeblad M, Rasch MG, Weaver VM. Dynamic interplay between the collagen scaffold and tumor evolution. *Curr Opin Cell Biol* 2010;22:697–706.
44. Smoot ME, Ono K, Ruscheinski J, et al. Cytoscape 2.8: new features for data integration and network visualization. *Bioinformatics (Oxford, England)* 2011;27:431–2.
45. Boyle EI, Weng S, Gollub J, et al. GO::TermFinder—open source software for accessing Gene Ontology information and finding significantly enriched Gene Ontology terms associated with a list of genes. *Bioinformatics (Oxford, England)* 2004;20:3710–5.