Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Proteomic analysis of primary colon cancer-associated fibroblasts using the SELDI-ProteinChip platform*

Zhan-huai WANG[§1], Ke-feng DING[§2], Jie-kai YU[1], Xiao-hui ZHAI[1], Shu-qin RUAN[1], Shan-wei WANG[1], Yong-liang ZHU[3], Shu ZHENG[1], Su-zhan ZHANG[†‡1]

([1]*Cancer Institute (Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education, Key Laboratory of Molecular Biology in Medical Sciences, Zhejiang Province, China), the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([2]*Department of Oncology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

([3]*Department of Gastroenterology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, China*)

[†]E-mail: Suzhan.zhang@hotmail.com

Received Aug. 17, 2011; Revision accepted Nov. 30, 2011; Crosschecked Dec. 14, 2011

Abstract: Objective: Cancer-associated fibroblasts (CAFs) are one of the hallmarks of the cancer microenvironment. Recent evidence has indicated that CAFs are more competent in enhancing cancer cell growth and migration than normal fibroblasts. However, the unique protein expression of CAFs has not been fully elucidated. This study aims to investigate the characterizations of colon CAFs by comparing the differential protein expression between CAFs and normal fibroblasts. Methods: Primary fibroblasts were isolated from surgical specimen of human colon cancer and matched normal colonic tissue. Purity of the cell population was verified through immunostain analysis. Total cell lysates and conditioned media from each group of cells were extracted, and protein expression analysis was conducted using the surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) ProteinChip platform. Results: Most primary cells showed typical fibroblast-like features after two weeks. Increased proportion of α-smooth muscle actin-positive myofibroblasts was detected within the CAFs in four of the six pairs of primary cells. Fibroblast activation protein was weakly expressed in most cells without differences. Using SELDI-TOF-MS ProteinChip platform, four protein peaks mass over charge ratio (*m*/*z*) 1142, 3011, 4035, and 4945 were detected in the total cell lysates, and two protein peaks *m*/*z* 1368 and 1389 were detected in the conditioned media. The potential candidate proteins found in the Swiss-Prot database include morphogenetic neuropeptides, FMRFamide-related peptides, insulin-like growth factor II, thymosin β-4-like protein 3, and tight junction-associated protein 1. Conclusions: Using the SELDI-ProteinChip platform, differential protein expressions were identified in colon CAFs compared with normal colonic stromal fibroblasts. The complex proteomic alternations in colon CAFs may play important roles related to the colon cancer microenvironment.

Key words: Colon cancer, Cancer microenvironment, Cancer-associated fibroblasts, Proteomics, Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS)
doi:10.1631/jzus.B1100266 **Document code:** A **CLC number:** R735.3[+]5

1 Introduction

Cancer invasion depends on many cellular and molecular changes, not only in epithelial cells, but also in surrounding stromal cells. The interaction between cancer cells and their stromal microenvironment is crucial for cancer development and progression. Changes in the cancer microenvironment

‡ Corresponding author
§ The two authors contributed equally to this work

* Project supported by the National Natural Science Foundation of China (Nos. 81000892, 81071801, and 30801341) and the Research Fund for the Doctoral Program of Higher Education of China (No. 200803351107)

during cancer progression involve angiogenesis, lymphogenesis, macrophage infiltration, release of cytokines/growth factors and proteolytic enzymes, and alterations in the extracellular matrix (Almholt and Johnsen, 2003). The putative cancer stroma consists of different cellular elements, such as inflammatory cells, endothelial cells, and fibroblasts. One of the hallmarks of these elements is cancer-associated fibroblasts (CAFs). CAFs can be propagated in vitro for extended periods without changing their phenotypes; hence, a number of studies have been conducted to investigate their phenotype characteristics, tumor-enhancing functions, and distinctive gene expression profiles (Allinen *et al*., 2004; Orimo *et al*., 2005; Bauer e*t al*., 2010).

However, gene and protein expression levels cannot be easily correlated because proteins could exist in different functional states through post-translational modification. Therefore, gaining an overall view of CAFs at the protein level is important. However, to date, few studies have investigated the protein expression that can reflect differences between CAFs and their counterpart, normal fibroblasts (NFs).

Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) is a proteomic technique particularly powerful for analyzing complex biologic samples. Using chromatographic surfaces to retain proteins and peptides on ProteinChip arrays based on their physicochemical properties, the platform performs direct analysis via TOF-MS. Peak patterns are formed via MS, which represent protein expression profiles. The location and intensity of every peak in the pattern reflect the mass over charge ratio (m/z) and abundance of the corresponding protein (Chen *et al*., 2004; Kumar *et al*., 2008). This high-throughput technique allows the screening of low molecular weight proteins compared with other traditional methods, such as 2D gel electrophoresis. Combined with bioinformatic approaches, SELDI-TOF-MS is valuable in establishing protein expression profiles and in discovering new biomarkers with high sensitivity and specificity (Chen *et al*., 2004; Melle *et al*., 2006; Xu *et al*., 2006; Enkelmann *et al*., 2011).

The present study focuses on CAFs in colon cancer by establishing primary fibroblast cultures from surgical specimens of patients with colon cancers and deals with the common phenotypical features of CAFs in vitro. The protein profiles of both total cell lysate and conditioned media extracted from CAFs and NFs were compared via SELDI-TOF-MS to better understand the specific features of CAFs at the protein level.

2 Materials and methods

2.1 Materials

Iscove's modified Dulbecco medium (IMDM) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Collagenase Type I and hyaluronidase were purchased from Sigma (St. Louis, MO, USA). The cell strainer was purchased from BD Biosciences (San Diego, CA). The antibodies used, including anti-Pan cytokeratin (ab78478), anti-desmin (ab15200), anti-α-smooth muscle actin (α-SMA, ab15734), and anti-fibroblast activation protein (FAP) (ab28244), were all from Abcam (HK, China); Dako EnVision system and anti-vimentin (clone V9) were from Dako (Glostrup, Denmark). The protease inhibitor cocktail was purchased from Thermo (Rockford, IL).

2.2 Patients and tissue specimens

Fresh surgical specimens were obtained with informed consent from patients who underwent surgical resection for colon cancer. The study was approved by the Research Ethics Board at Zhejiang University. The cancer-associated regions selected were minimally necrotic regions of the tumor mass. The normal colonic mucosa selected from the same specimen was at least 5 cm distal to the outer margin of the cancer mass. The specimens were determined through cross-examination at the time of the surgical excision and confirmed via subsequent histological analysis. The tumor characteristics of the patients are listed in Table 1. The World Health Organization (WHO) standard grading system containing four categories was used: well differentiated, moderately differentiated, poorly differentiated, and undifferentiated (Aaltonen *et al*., 2000).

2.3 Isolation of human colon fibroblasts and cell culture

To obtain the CAFs, the fresh specimens were immediately minced into 1-mm^3 sizes and extensively washed in 100 mmol/L ice-cold phosphate buffered

Table 1 Characteristics of patients and patient tumours

Patient No.	Sex	Age (year)	Tumour site	TNM	Stromal cell culture
1	Female	48	Sigmoid	T3N2M0	CAF-1, NF-1
2	Female	69	Left colon	T3N0M0	CAF-2, NF-2
3	Male	61	Sigmoid	T3N0M0	CAF-3, NF-3
4	Female	88	Right colon	T3N0M0	CAF-4, NF-4
5	Female	59	Sigmoid	T3N0M1	CAF-5, NF-5
6	Male	53	Left colon	T3N1M0	CAF-6, NF-6

saline (PBS) with 5% antibiotic-antimycotic solution (50000 U/ml penicillin, 125 μg/ml amphotericin B, and 50000 μg/ml streptomycin). Subsequently, the solution was placed in IMDM, 1% FBS, 1 mg/ml collagenase Type I, and 1 mg/ml hyaluronidase, and then incubated for 2 h at 37 °C. The solution was vigorously shaken every 10 min to encourage dissociation. Mucosa strips were dissected from the submucosa to isolate the normal colonic fibroblasts; the colonic smooth muscle and serosa layers were discarded. Using the technique previously described by Brenmoehl *et al*. (2009), the mucosa strips were denuded of epithelial cells through sequential treatments with 2 mmol/L ethylenediaminetetraacetic acid (EDTA) followed by enzyme digestion. The dissociated cells were filtered through a 40-μm cell strainer, centrifuged, and resuspended in IMDM, 10% FBS, and 1% antibiotic-antimycotic solution. The cells were then plated into 10-cm petri dishes. Cell viability was detected via trypan blue dye exclusion assay. All the experiments were performed in cells between passages 5 and 10.

2.4 Morphologic features and immunostaining of primary cultured fibroblasts

The morphologic features and sizes of the colon CAFs and NFs were assessed as cells passaged for five population doublings. For the immunostain experiments, the cells were cultured overnight on chamber slides, fixed with 4% paraformaldehyde, and then permeabilized with 0.25% Triton X-100 for the detection of vimentin, pan cytokeratin, desmin, α-SMA, and FAP. To quantify the percentage of α-SMA-positive cells, the positive cell numbers relative to the total cell numbers (>100 counted cells) were evaluated in 10 independent fields from three different wells of each fibroblast type. Paraffin sections prepared from human colon tissues were used as the control.

2.5 Preparation of total cell lysates and conditioned media

For total cell lysate extraction, the primary cells of both CAF and NF groups were harvested after PBS washing and then resuspended in lysis buffer composed of 7 mol/L urea, 2 mol/L thiourea, 100 mol/L dithiothreitol, 4% 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate, and protease inhibitor cocktail. After 60 min lysis on ice, the total cell lysates were obtained through centrifugation at 20000×*g* for 15 min to remove insoluble debris. For secreted protein analysis, both CAF and NF groups were grown in standard culture media (IMDM with 10% FBS) until they reached a confluency state of approximately 80%, washed three times with PBS, and then incubated in conditioned media (serum-free IMDM) for 24 h. The conditioned media were collected, cooled on ice, centrifuged at 200×*g* for 10 min to remove floating cells and cellular debris, and then subjected to sterile filtration (pore size: 0.22 mm). The conditioned media were dialyzed, concentrated through lyophilization, and then reconstituted in a buffer. The protein concentration was measured using a BioRad DC protein assay kit (BioRad, Hercules, CA) and adjusted to 3.5 mg/ml (total cell lysate) and 1.5 mg/ml (conditioned media) for expression analysis.

2.6 SELDI protein profiling

Up to 200 μl sodium acetate was added to each spot of the bioprocessor (Ciphergen Biosystems) that contains the ProteinChip arrays. The bioprocessor was sealed and agitated on a platform shaker for 5 min at 4 °C; the process was repeated. The chips were removed from the bioprocess, and diluted samples (5 μl) were applied to each spot of the protein arrays. Each sample was added to three different spots. The chips were plugged into a wet box at room temperature for an hour. The excess of the mixtures was

discarded. The chip arrays were placed in the bioprocessor; the chips were then washed thrice with 200 μl sodium acetate and twice with deionized water. Finally, the chips were removed from the bioprocessor and air-dried. Prior to SELDI-TOF-MS analysis, 1 μl semi-saturated solution of the scintillation proximity assay in 50% cerium (IV) ammonium nitrate and 0.5% trifluoroacetic acid was applied twice onto each spot, and the chips were again air-dried. The chips were analyzed using a PBS-II plus mass spectrometer reader (Ciphergen Biosystems). Data were obtained by averaging 140 laser shots with an intensity of 170, a detector sensitivity of 5, a high mass of 100000 Da, and an optimized range of 1000 to 20000 Da. Mass accuracy was calibrated using the all-in-one peptide molecular mass standard (Ciphergen Biosystems).

2.7 Data analysis

Data of every spectrum were collected and analyzed using the ProteinChip Software version 3.0 (Ciphergen Biosystems) with integrated Biomarker Wizard software (Ciphergen Biosystems) as previously described (Ge *et al*., 2008). The baselines were subtracted, and normalization was performed according to the software instructions. Biomarker Wizard was used to identify the corresponding peaks in the spectra. Significant differences in the peak intensity of particular proteins between each group were calculated through the software using the Mann-Whitney non-parametrical test and then further examined via paired Student's *t*-test or paired Wilcoxon test if the data did not follow normal distribution. $P<0.05$ was considered to indicate statistical significance.

Based on the adjustment of the isoelectric point ranging from 4 to 14 and the specific mass provided by the ProteinChip software with a variation of 0.3% in the mass range, the possible protein candidates were searched using the protein database TagIdent provided by ExPASy molecular biology server.

3 Results

3.1 Isolation of primary fibroblastic population from human colon tissue

Fibroblasts were extracted from six human colon carcinoma tissues obtained from surgical resection. The tumor masses were dissociated, and various cell types were separated to obtain populations of CAFs. The matched NFs were isolated from the normal colonic mucosa of the same six patients. The cultures reached confluency after two weeks and were stored in −80 °C at cell passages 2 to 3. The cultured primary cells showed typical fibroblast-like features, with spindle-like shapes and elongated projections (Fig. 1).

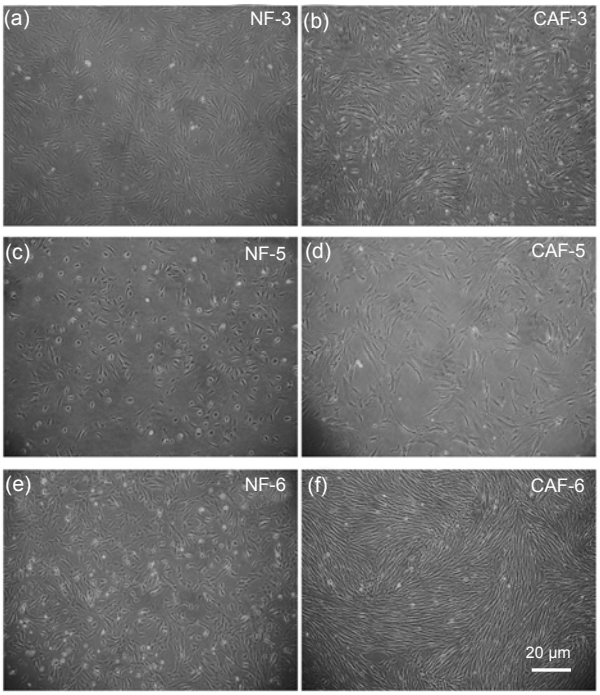

Fig. 1 Morphological features
Representative microscopic fields show the cancer-associated fibroblasts CAF-3 (b), CAF-5 (d), and CAF-6 (f), and their counterpart normal fibroblasts NF-3 (a), NF-5 (c), and NF-6 (e)

3.2 Characteristics of CAFs and NFs

In cell passage 5, the purities of the various fibroblast populations were verified through immunostaining. The population expressed the fibroblastic marker vimentin (Fig. 2a) but lacked the epithelial cell marker cytokeratin (Fig. 2b) and the smooth muscle cell marker desmin (Fig. 2c). A small fraction in both groups of cells expressed the myofibroblast marker α-SMA (Fig. 2e). An enhanced expression of α-SMA in CAFs in four out of the six pairs was observed. In patient 2, the expression of α-SMA was more enhanced in NFs compared with CAFs, whereas the expression was equal in patient 5 (Table 2). FAP, another myofibroblast marker, was weakly expressed

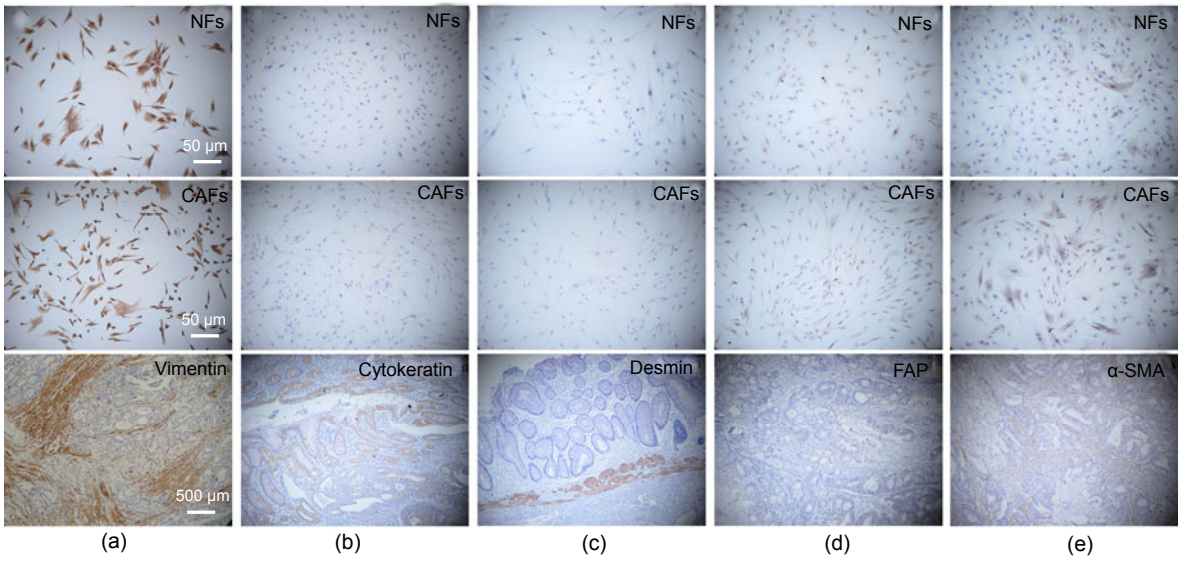

Fig. 2 Immunostaining characteristics of primary fibroblastic cells

All the primary fibroblastic cells strongly expressed fibroblastic marker vimentin (a) but were negative for epithelial cell marker cytokeratin (b) and smooth muscle cell marker desmin (c). Both CAFs and NFs weakly expressed FAP (d), but CAFs showed enhanced expression of α-SMA (e) compared with NFs. Paraffin sections prepared from human colon tissues were used as the control

Table 2 Quantify of α-SMA-positive cell number

Patient No.	α-SMA-positive cells in cultures (%)	
	CAFs	NFs
1	25.90	14.70
2	4.06	21.20
3	7.53	5.16
4	15.30	10.70
5	4.63	4.87
6	19.00	6.40

in most primary fibroblasts. No obvious difference was observed in the expression of FAP between CAFs and NFs (Fig. 2d).

3.3 Protein profiling of total cell lysates and conditioned media

After cluster analysis through Biomarker Wizard (Ciphergen Inc., Fremont, CA; ProteinChip software versions 3.0), 398 peaks of the total cell lysates and 547 peaks of the conditioned media were detected for discriminating CAFs and NFs. The peaks were between *m*/*z* 1000 and 30000. Peaks with *m*/*z*<1000 were mainly ion noise from the matrix; thus, they were excluded. The peaks were further examined using paired Student's *t*-test or paired Wilcoxon test if the data did not follow normal distribution. Four protein peaks *m*/*z* 1142, 3011, 4035, and 4945 in the total cell lysates and two protein peaks *m*/*z* 1368 and 1389 in the conditioned media distinguished CAFs from NFs (P<0.05, Fig. 3). Table 3 presents the descriptive statistics of the six peaks. The spectra in peak view and gel view of the protein peaks are shown in Fig. 4.

Table 3 Descriptive statistics of candidate protein (CM10) peaks in total cell lysates and conditioned media

Solution	Peak *m*/*z*	Relative peak intensity*		*P* value
		NFs	CAFs	
Total cell lysates	1142	15.1±3.76	11.2±2.70	0.040
	3011	5.68±0.92	9.74±3.56	0.020
	4035	3.72±0.64	4.71±1.46	0.026
	4945	5.97±2.48	9.24±1.94	0.028
Conditioned media	1368	8.27±2.70	4.99±1.27	0.046
	1389	3.08±1.68	1.03±0.91	0.016

* Values are expressed as mean±SD

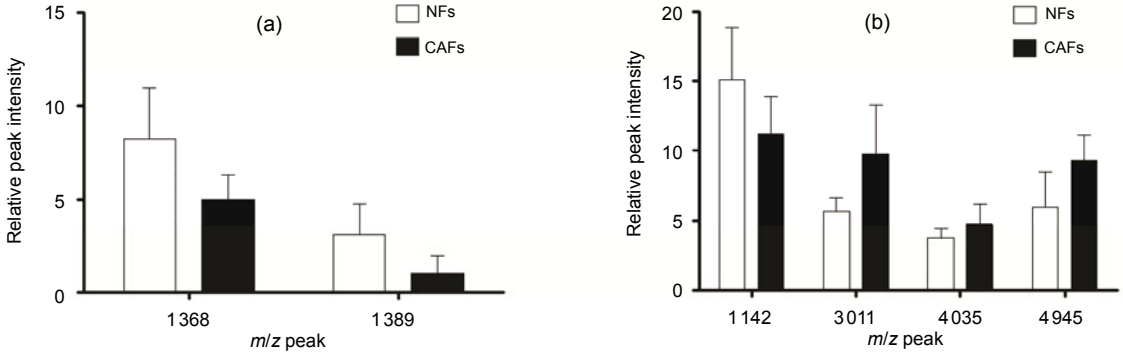

Fig. 3 Differences in protein expression between CAFs and NFs using SELDI-ProteinChip platform

(a) In conditioned media, protein peaks *m*/*z* 1368 and 1389 were decreased in CAFs compared with NFs, with $P<0.05$; (b) In the total cell lysates, protein peaks were sorted by *m*/*z*, with $P<0.05$, among which protein peaks *m*/*z* 3011, 4035, and 4945 were increased in CAFs and protein peak *m*/*z* 1142 was decreased in CAFs compared with NFs

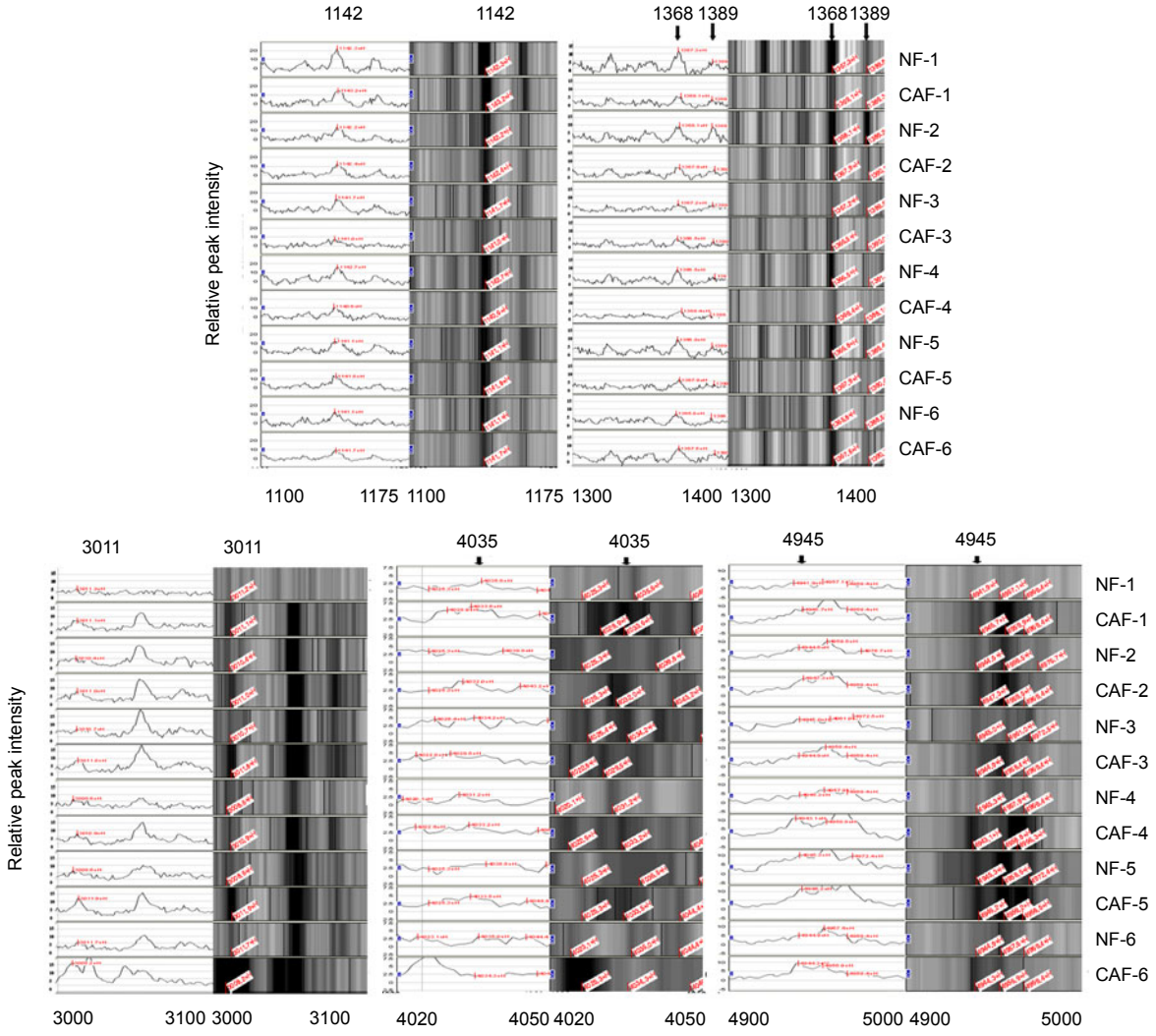

Fig. 4 Spectra in peak view and gel view

The protein peaks corresponding to *m*/*z* 1142, 1368, and 1389 were weakly expressed in the CAFs, but highly expressed in the NFs. The protein peaks corresponding to *m*/*z* 3011, 4035, and 4945 were highly expressed in the CAFs, but weekly expressed in the NFs

3.4 Database search for potential protein candidates

The Swiss-Prot database was searched using the TagIdent tool, along with the pI and mass information, to obtain matching proteins. Six pieces of matching information were obtained from the database. These potential candidate proteins include morphogenetic neuropeptide, FMRFamide-related peptides, insulin-like growth factor II, thymosin β-4-like protein 3, and tight junction-associated protein 1. Therefore, protein peaks with *m*/*z* 1142, 1368, 4035, and 4945 have greater potential to identify colon cancer-associated stroma in future research (Table 4).

Table 4 Swiss-Prot database searching results

Protein peak *m*/*z*	Meaningful matching information in Swiss-Prot database			Comments
	Protein name	M_w	pI	
1142	Morphogenetic neuropeptide chain: 1–11	1142	8.75	An autocrine growth factor
1368	FMRFamide-related peptides chain: 66–76	1369	9.47	Morphine modulating peptides, wide-ranging physiologic effects
4035	Insulin-like growth factor II chain: 93–126	4030	8.42	Possesses growth-promoting activity
4945	Thymosin β-4-like protein 3 chain: 2–44	4932	5.03	Plays an important role in the organization of the cytoskeleton, inhibits actin polymerization
	Tight junction-associated protein 1 chain: 1–43	4959	8.16	Cell junction, Golgi apparatus, tight junction

Searching was conducted using TagIdent tool with mass and the pI (4.0–14.0) information for CM10

4 Discussion

Primary stromal cell cultures isolated from colon cancer might in part represent the corresponding cancer microenvironment, thus providing a useful complement to the current cellular biochemistry and therapeutic research in colon cancer. Previous studies have investigated colon CAFs by establishing CAFs in primary fibroblast cultures from metastatic colon cancer in the liver (Nakagawa *et al*., 2004; Mueller *et al*., 2007). However, few studies have exploited the characteristics of CAFs in primary colon cancer.

In the present study, an optimized method was used to isolate and culture the paired stromal fibroblasts derived from the human colon cancer and from the adjacent normal tissue in the same specimen. To avoid bias due to individual differences, all experiments were performed by comparing paired CAFs and corresponding NFs. High serum media suitable for fibroblast growth were used. With the growth advantage of fibroblasts, most tumor cells and colonic epithelial cells diminished around cell passages 2 and 3. To avoid cross-contamination of smooth muscle cells in the NF cultures, the colonic mucosa was dissected from the submucosa, and the smooth muscle layers were discarded. Individual differences among the different specimens due to age, stage, and status resulted in different cultivating characteristics. Generally, the primary CAFs have an advantage in proliferation rate. Morphologically, all established stromal cultures grew as a monolayer of cells with the same fibroblast-like appearance. The CAFs were slightly more slender than the NFs, but without significant differences (Fig. 1). The features of these cells were further characterized with the fibroblastic marker vimentin, epithelial cell marker cytokeratin, smooth muscle cell marker desmin, putative myofibroblast marker α-SMA, and FAP. Vimentin, a common mesenchymal cytoskeletal marker, was highly expressed in all primary cells (Fig. 2a), whereas cytokeratin was negative in most cells (Fig. 2b). This result indicates that vimentin is expressed in cells of mesenchymal origin. Very few cytokeratin-positive cells were detected, which may be due to epithelial-mesenchymal transition. Monoclonal desmin antibody was used as the smooth muscle cell marker. The negative expression of desmin in most primary cells indicated minimal contamination of the smooth muscle cells (Fig. 2c).

Myofibroblasts, a prominent component of CAFs, distinguish CAFs from NFs. α-SMA, a protein marker of myogenic fibroblast differentiation, is a common marker of myofibroblasts (Bhowmick *et al*., 2004; Orimo *et al*., 2005). In the present study, monoclone α-SMA and FAP antibodies were used in the immunostain analysis to check for possible

presence of myofibroblast cells (Fig. 2e). An increased proportion of α-SMA-positive cells was found in four of the six pairs; this result indicates that the isolated CAFs contain a high proportion of myofibroblasts (Table 2). This high expression of α-SMA in cancer stromal cells is in agreement with a previous study (Orimo *et al*., 2005). However, a larger sample analysis is required for statistical analysis. FAP is a good myofibroblast marker in immunohistochemical analyses; it was mainly expressed in the invasive colon cancer stroma, but negative in the normal colonic mucosa (Henriksson *et al*., 2011). In the present study, FAP was weakly expressed in most primary fibroblasts (Fig. 2d); the different expressions of FAP between CAFs and NFs were not observed.

To further investigate the protein profile, SELDI-MS analyses of total cell lysates and conditioned media taken from CAFs and NFs were used. A range of peaks across CM10 surfaces increased (*m*/*z* 3011, 4035, and 4945) or decreased (*m*/*z* 1142, 1368, and 1389) in the CAFs compared with their counterpart NFs (Table 3 and Fig. 3). However, no direct relationship was found in the protein profiles of the total cell lysates and conditioned media.

The Swiss-Prot database was searched using the TagIdent tool with pI and mass information to obtain matching information for each protein peak (Table 4). The search results suggest that morphogenetic neuropeptide is the candidate for protein mass 1142 Da, decreased in CAFs (Fig. 4). Morphogenetic neuropeptide is an autocrine growth factor found in neural cells, mammalian intestine, and hypothalamus; it is also involved in cell cycle, cell division, and mitosis. FMRFamide-related peptides are the candidates for protein mass 1368 Da, decreased in CAFs (Fig. 4). They have wide-ranging physiologic effects, including the modulation of morphine-induced analgesia, elevation of arterial blood pressure, and increased somatostatin secretion from the pancreas. For protein mass 4035 Da, increased in CAFs (Fig. 4), the candidate protein is insulin-like growth factor II (4030 Da). Insulin-like growth factors possess growth-promoting activity. In vitro, they are potent mitogens for cultured cells. For protein mass 4945 Da, increased in CAFs (Fig. 4), the database provided two candidate proteins. One is thymosin β-4-like protein (4932 Da), which plays an important role in the organization of the cytoskeleton; the expression of thymosin β-4 promotes tumorigenicity and metastatic potential and induces drug resistance of malignant cells. The second candidate is tight junction-associated protein 1 (4959 Da), which is related to cell junction, Golgi apparatus, and tight junction. Tight junction-associated protein modulates cell proliferation and is useful in the diagnosis of soft tissue sarcoma (Facchetti *et al*., 2007; Jayagopal *et al*., 2011). Other protein masses, including 1389 and 3011 Da, were searched across the database; however, no meaningful matching proteins were found, indicating that the functions of these putative uncharacterized proteins are unknown.

CAFs are heterogeneous and possess complex mechanisms in facilitating tumor development, which express different sets of protein markers. α-SMA, FAP, S100A4, platelet derived growth factor receptor-β, and chondroitin sulfate proteoglycan are markers of CAFs, but none of them can exclusively reflect the characteristics of CAFs (Sugimoto *et al*., 2006). The primarily cultured CAFs and NFs in the present study are a heterogeneous population, which to some extent, share similar features. The possible protein peaks detected in the present study include peptides or small proteins with low abundance, which can hardly be detected using traditional methods, but are essential for associated research. Further verification and function investigation are planned. Although the SELDI-ProteinChip platform is good for protein profiling research, it does not allow direct purification and functional identification, and still lacks resolution for the detection of higher molecular weight proteins. Most expected growth factors or matrix proteins have larger molecular weights beyond the limits of SELDI detection.

5 Conclusions

A cell culture method was developed for the isolation and characterization of colon CAFs and their normal counterpart colonic fibroblasts. The current study is the first to reveal complex proteomic changes using the SELDI-ProteinChip platform in distinguishing colon CAFs from colonic NFs. These proteomic alternations may play important roles in the interaction between cancer cells and cancer stroma. The current study provided better understanding of

the complex colon cancer microenvironment. Further studies on altered protein in colon cancer progression are currently in progress.

Acknowledgements

We are very grateful to Prof. Xun HU, Prof. Rong-zhen XU, Dr. Wei-ting GE, and Dr. Jia-wei ZHANG in Cancer Institute, School of Medicine, Zhejiang University for their assistance and suggestions.

References

Aaltonen, L.A., Hamilton, S.R., World Health Organization and International Agency for Research on Cancer, 2000. Pathology and Genetics of Tumours of the Digestive System. IARC Press, Lyon; Oxford University Press (Distributor), Oxford.

Allinen, M., Beroukhim, R., Cai, L., Brennan, C., Lahti-Domenici, J., Huang, H., Porter, D., Hu, M., Chin, L., Richardson, A., *et al*., 2004. Molecular characterization of the tumor microenvironment in breast cancer. *Cancer Cell*, **6**(1):17-32. [doi:10.1016/j.ccr.2004.06.010]

Almholt, K., Johnsen, M., 2003. Stromal cell involvement in cancer. *Recent Results Cancer Res.*, **162**:31-42. [doi:10.1007/978-3-642-59349-9_3]

Bauer, M., Su, G., Casper, C., He, R., Rehrauer, W., Friedl, A., 2010. Heterogeneity of gene expression in stromal fibroblasts of human breast carcinomas and normal breast. *Oncogene*, **29**(12):1732-1740. [doi:10.1038/onc.2009.463]

Bhowmick, N.A., Neilson, E.G., Moses, H.L., 2004. Stromal fibroblasts in cancer initiation and progression. *Nature*, **432**(7015):332-337. [doi:10.1038/nature03096]

Brenmoehl, J., Miller, S.N., Hofmann, C., Vogl, D., Falk, W., Schölmerich, J., Rogler, G., 2009. Transforming growth factor-β 1 induces intestinal myofibroblast differentiation and modulates their migration. *World J. Gastroenterol.*, **15**(12):1431-1442. [doi:10.3748/wjg.15.1431]

Chen, Y.D., Zheng, S., Yu, J.K., Hu, X., 2004. Artificial neural networks analysis of surface-enhanced laser desorption/ionization mass spectra of serum protein pattern distinguishes colorectal cancer from healthy population. *Clin. Cancer Res.*, **10**(24):8380-8385. [doi:10.1158/1078-0432.CCR-1162-03]

Enkelmann, A., Heinzelmann, J., von Eggeling, F., Walter, M., Berndt, A., Wunderlich, H., Junker, K., 2011. Specific protein and miRNA patterns characterise tumour-associated fibroblasts in bladder cancer. *J. Cancer Res. Clin. Oncol.*, **137**(5):751-759. [doi:10.1007/s00432-010-0932-6]

Facchetti, F., Lonardi, S., Gentili, F., Bercich, L., Falchetti, M., Tardanico, R., Baronchelli, C., Lucini, L., Santin, A., Murer, B., 2007. Claudin 4 identifies a wide spectrum of epithelial neoplasms and represents a very useful marker for carcinoma versus mesothelioma diagnosis in pleural and peritoneal biopsies and effusions. *Virchows Arch.*, **451**(3):669-680. [doi:10.1007/s00428-007-0448-x]

Ge, Z., Zhu, Y.L., Zhong, X., Yu, J.K., Zheng, S., 2008. Discovering differential protein expression caused by CagA-induced ERK pathway activation in AGS cells using the SELDI-ProteinChip platform. *World J. Gastroenterol.*, **14**(4):554-562. [doi:10.3748/wjg.14.554]

Henriksson, M.L., Edin, S., Dahlin, A.M., Oldenborg, P.A., Oberg, A., van Guelpen, B., Rutegard, J., Stenling, R., Palmqvist, R., 2011. Colorectal cancer cells activate adjacent fibroblasts resulting in FGF1/FGFR3 signaling and increased invasion. *Am. J. Pathol.*, **178**(3):1387-1394. [doi:10.1016/j.ajpath.2010.12.008]

Jayagopal, A., Yang, J.L., Haselton, F.R., Chang, M.S., 2011. Tight junction-associated signaling pathways modulate cell proliferation in uveal melanoma. *Invest. Ophthalmol. Vis. Sci.*, **52**(1):588-593. [doi:10.1167/iovs.10-5746]

Kumar, N., Maurya, P., Gammell, P., Dowling, P., Clynes, M., Meleady, P., 2008. Proteomic profiling of secreted proteins from CHO cells using surface-enhanced laser desorption ionization time-of-flight mass spectrometry. *Biotechnol. Prog.*, **24**(1):273-278. [doi:10.1021/bp070244o]

Melle, C., Ernst, G., Schimmel, B., Bleul, A., Mothes, H., Kaufmann, R., Settmacher, U., von Eggeling, F., 2006. Different expression of calgizzarin (S100A11) in normal colonic epithelium, adenoma and colorectal carcinoma. *Int. J. Oncol.*, **28**(1):195-200.

Mueller, L., Goumas, F.A., Affeldt, M., Sandtner, S., Gehling, U.M., Brilloff, S., Walter, J., Karnatz, N., Lamszus, K., Rogiers, X., *et al*., 2007. Stromal fibroblasts in colorectal liver metastases originate from resident fibroblasts and generate an inflammatory microenvironment. *Am. J. Pathol.*, **171**(5):1608-1618. [doi:10.2353/ajpath.2007.060661]

Nakagawa, H., Liyanarachchi, S., Davuluri, R.V., Auer, H., Martin, E.W.Jr., de la Chapelle, A., Frankel, W.L., 2004. Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. *Oncogene*, **23**(44):7366-7367. [doi:10.1038/sj.onc.1208013]

Orimo, A., Gupta, P.B., Sgroi, D.C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., Carey, V.J., Richardson, A.L., Weinberg, R.A., 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*, **121**(3):335-348. [doi:10.1016/j.cell.2005.02.034]

Sugimoto, H., Mundel, T.M., Kieran, M.W., Kalluri, R., 2006. Identification of fibroblast heterogeneity in the tumor microenvironment. *Cancer Biol. Ther.*, **5**(12):1640-1646. [doi:10.4161/cbt.5.12.3354]

Xu, W.H., Chen, Y.D., Hu, Y., Yu, J.K., Wu, X.G., Jiang, T.J., Zheng, S., Zhang, S.Z., 2006. Preoperatively molecular staging with CM10 ProteinChip and SELDI-TOF-MS for colorectal cancer patients. *J. Zhejiang Univ.-Sci. B*, **7**(3):235-240. [doi:10.1631/jzus.2006.B0235]