

Expression of Fibronectin Isoforms in Human Breast Tissue: Production of Extra Domain A⁺/Extra Domain B⁺ by Cancer Cells and Extra Domain A⁺ by Stromal Cells

Ei-ichi Matsumoto,^{1,2} Toshimichi Yoshida,¹ Yoshifumi Kawarada² and Teruyo Sakakura^{1,3}

¹Department of Pathology and ²First Department of Surgery, Mie University School of Medicine, 2-174 Edobashi, Tsu, Mie 514-8507

The expression of fibronectin (FN) isoforms including extra domain A (EDA) and extra domain B (EDB) segments, was investigated in 36 invasive ductal carcinomas and 13 benign tumors of human breast tissues by *in situ* hybridization using probes specific to alternative splicing sites. Signals for the constant region of FN mRNA in cancer cells were found in 53% of the invasive ductal carcinomas. The EDA⁺ and EDB⁺ mRNA signals were found in 47% and 33%, respectively. Stromal cells expressing FN, EDA⁺ and EDB⁺ mRNA signals were present in 100%, 69% and 14% of cases, respectively. Expression of FN mRNAs by cancer cells was most frequent in intraductal lesions or large cancer nests, and that by stromal cells was associated with desmoplastic areas. In representative cases, proportions of FN mRNA-positive cancer cells expressing EDA and EDB segments were 45% and 39%, respectively, signals for both being frequently found in the same cells. EDA⁺ and EDB⁺ mRNA were labeled in 25% and 6% of the FN mRNA-positive stromal cells, a large proportion thus being EDA⁺/EDB⁻ FN. In conclusion, the splicing pattern of FN pre-mRNA is dependent on the cell type and histology of breast cancer tissues. The observed lack of expression in fibroadenomas and other benign conditions suggests a link with tumor progression.

Key words: Fibronectin — EDA — EDB — Breast cancer

Fibronectin (FN) is an adhesive extracellular matrix glycoprotein, which is involved in many kinds of cellular events including proliferation, migration and differentiation. The cellular FN molecule has three segments, which can be alternatively spliced at the mRNA level: EDA (extra domain A), EDB (extra domain B) and IIICS (type III homology connecting segment) regions. The splicing patterns of these segments are developmentally regulated and are tissue- or cell-type specific,^{1–5} expression being very limited in normal adult tissues.

Many recent studies have demonstrated that FN is up-regulated in cancer tissues, with reappearance of alternatively spliced isoforms.^{6–9} In breast cancers, immunohistochemical studies using specific monoclonal antibodies have demonstrated that FN isoforms, including EDA (EDA⁺ FN) and EDB (EDB⁺ FN) can be detected in cancer stroma.^{10–12} In contrast, it has been considered that the effect of the splicing may be quantitative rather than qualitative,¹³ and the functional significance of molecular differences has yet to be clarified in detail. However, recent

studies have demonstrated that EDA⁺ FN exhibits a high affinity for integrin,¹⁴ and EDB⁺ isoform induces tyrosine phosphorylation of focal adhesion proteins.¹⁵

To establish the possible roles of different FN isoforms in cancer progression, it is important to clarify the cellular sources of these isoforms. Therefore, we examined mRNA expression of EDA and EDB, and well as total FN isoforms, by *in situ* hybridization (ISH) using cRNA probes specific to the constant region and alternatively spliced sites, in benign and malignant human breast lesions.

MATERIALS AND METHODS

Tissues A total of 49 human breast tumors (36 invasive ductal carcinomas and 13 benign tumors) were investigated. All tissues were surgically resected, and immediately fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, at 4°C overnight. After having been rinsed in PB, they were dehydrated through a graded ethanol series and xylene, embedded in paraffin, sectioned at 4 μm, and placed on silane-coated glass slides (Dako Japan, Kyoto).

Histological classification was made using sections stained with hematoxylin and eosin. To examine the relationship of expression of FN isoforms to morphological

³ To whom all correspondence should be addressed.

E-mail: sakakura@doc.medic.mie-u.ac.jp

Abbreviations: FN, fibronectin; EDA, extra domain A; EDB, extra domain B; ISH, *in situ* hybridization; PB, phosphate buffer; DIG, digoxigenin; TN-C, tenascin-C.

features, we classified the lesions according to the histological patterns of their cancer nests (large or small) and stroma (desmoplastic or not desmoplastic). Cases in the 'large cancer nests' group had more than 50% of their cancer nests, including intraductal lesions, over 200 μm in diameter. The 'desmoplastic' cases had more than 50% desmoplastic stroma.

Preparation of digoxigenin (DIG)-labeled cRNA probes and ISH Human FN cDNAs were generous gifts from Dr. K. Sekiguchi (Osaka). cDNA inserts were subcloned in pGEM-3Z or pGEM-4Z (Promega Corporation, Madison, WI). The inserts for the constant region were: (1) 8th type I repeat-4th type III repeat, a 1069 bp *Sall*-*Pst*I insert, (2) 6th-9th type III repeats without EDB, a 919 bp *Pst*I-*Bam*HI insert. Inserts of 270 bp and 273 bp were used for the EDA and EDB segments, respectively. DIG-labeled cRNAs and ISH were prepared using the previously described methods.^{16,17} cRNA probes for tenascin-C (TN-C) were generated as described elsewhere.^{17,18} Hybridization signals were detected using alkaline-phosphatase-conjugated anti-DIG antibodies and visualized by incubation in nitroretazolium blue/5-bromo-4-chloro-3-indolyl phosphate solution. The sections were then counterstained with 0.1% nuclear fast red in 5% aluminum sulfate solution. Tumors showing multifocal to diffuse distributions having more than 10 positive cells per $\times 40$ objective field were evaluated as positive.

Quantification of proportions of FN mRNA-positive cells labeled with EDA and EDB segments To examine the proportions of EDA- and EDB-labeled cells, 9 cases showing strong labeling of cancer cells by FN mRNA were chosen from the 'large cancer nest' group for detailed analysis. In ten high-power fields for each case, FN mRNA-positive cancer cells were counted, and then the cells labeled with EDA and EDB probes were counted in the same fields in serial sections. For analysis of expression by stromal cells, 13 cases were chosen from the 'desmoplastic' group. The data are presented as total numbers of EDA⁺ or EDB⁺ mRNA-positive cells/total numbers of FN mRNA-positive cells $\times 100$ (%).

RESULTS

Breast cancers Expression of FN mRNAs was observed in both cancer and stromal cells, and the ISH results for FN, EDA⁺ FN and EDB⁺ FN are summarized in Table I. Signals for the constant region of FN mRNA in cancer cells were found in 53% of cases, and for EDA⁺ and EDB⁺ mRNAs in 47% and 33%, respectively. Stromal cells expressing FN, EDA⁺ and EDB⁺ mRNA signals in cancer tissue were present in 100%, 69% and 14%, respectively.

Expression of FN mRNAs by cancer cells was most frequent in intraductal lesions and large cancer nests with

papillary projections and tubular formations. Positive cells were often found at the margins of a cancer nest in contact with mesenchymal elements (Fig. 1A). Some of these cells could be shown to be positive for EDA and/or EDB (Fig. 1, B and C). Expression of the EDA⁺ mRNA by stromal cells was often associated with an active desmoplastic reaction (Fig. 1E). In peripheral areas of the cancer tissues, EDA⁺ mRNA was frequently observed. In hyalinized stroma in the tumor center, EDA⁺ and EDB⁺ mRNA signals were completely lacking, but some cells were labeled by the constant region probe detecting all FN isoforms.

Cancers were classified according to the size of the cancer nests and extent of the desmoplastic reaction. In large cancer nests, mRNA signals for FN were found in 68% and for EDA⁺ and EDB⁺ in 64% and 45%. The figures for intraductal lesion were especially high at 100%, 91% and 58%, respectively. Small cancer nests were less likely to include positive cells (Table I). Expression of EDA⁺ FN mRNA was found in 94% and 47%, respectively, of cases with or without a desmoplastic reaction. Expression of EDB⁺ mRNA by the stromal cells was rare (Fig. 1F).

In addition, we examined the proportions of the cells expressing EDA and EDB segments with respect to the cells labeled with the constant region probe for FN isoform. In representative cases with large cancer nests, EDA⁺ and EDB⁺ mRNAs were expressed in 45% and 39% of the FN mRNA-positive cancer cells, respectively, both being (EDA⁺/EDB⁺ FN) frequently labeled in the same cells. In stromal cells of desmoplastic areas, the EDA⁺ and EDB⁺ mRNAs were labeled in 25% and 6% of the FN mRNA-positive stromal cells, respectively.

Benign lesions Expression of FN isoforms in benign lesions of the human breast was studied using 8 fibroadenomas, 3 cases of fibrocystic disease and 2 intraductal papillomas (Table II). In the fibroadenomas, FN mRNA

Table I. mRNA Expression of FN Isoforms in Breast Cancer Tissues

	No.	FN (%)	EDA (%)	EDB (%)
Cancer cells				
Overall	36	19 (53)	17 (47)	12 (33)
Large cancer nests	22	15 (68)	14 (64)	10 (45)
Intraductal lesions	12	12 (100)	11 (91)	7 (58)
Small cancer nests	14	4 (29)	3 (21)	2 (11)
Stromal cells				
Overall	36	36 (100)	25 (69)	5 (14)
Desmoplastic lesion				
$\geq 50\%$	17	17 (100)	16 (94)	3 (18)
$< 50\%$	19	19 (100)	9 (47)	2 (11)

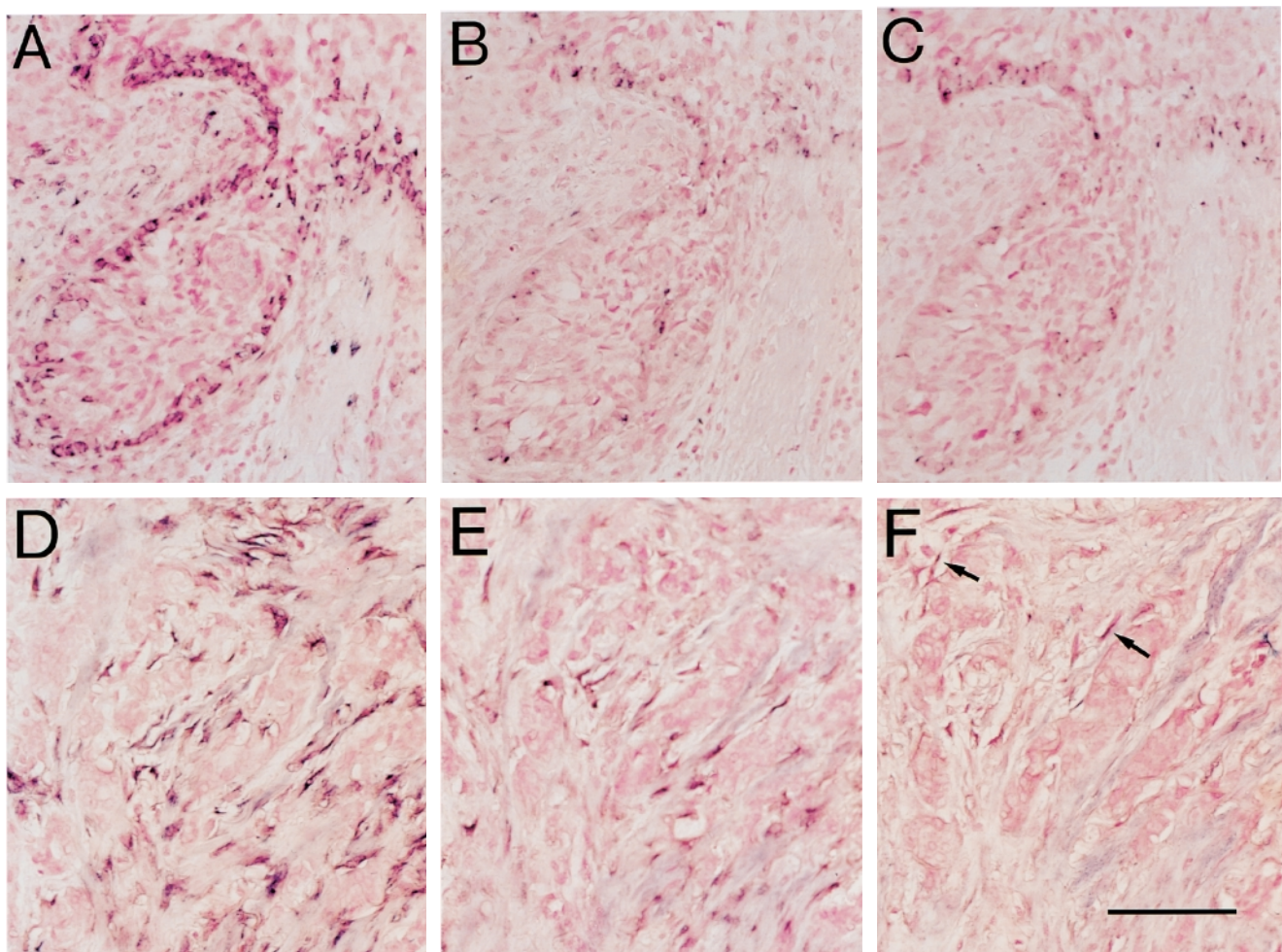


Fig. 1. Expression of FN and FN isoforms in breast cancer. *In situ* hybridization using the constant region cRNA probe for all FN (A, D), and those specific for the EDA segment (B, E) and the EDB segment (C, F). Positive cancer cells are present at the margin of a large cancer nest in contact with the mesenchymal elements (A, B, C). Expression of all FN and EDA⁺ FN mRNAs by stromal cells is displayed by desmoplastic stroma (D, E), but EDB⁺ FN mRNA is rare (F). Bar=100 μ m.

Table II. mRNA Expression of FN Isoforms in Epithelial Cells and Stromal Cells in Benign Human Breast Tissues

	No.	Expression in:					
		Epithelial cells			Stromal cells		
		FN (%)	EDA (%)	EDB (%)	FN (%)	EDA (%)	EDB (%)
Fibroadenoma	8	0 (0)	0 (0)	0 (0)	4 (50)	0 (0)	0 (0)
Fibrocystic disease	3	0 (0)	0 (0)	0 (0)	1 (33)	1 (33)	0 (0)
Intraductal papilloma	2	1 (50)	0 (0)	0 (0)	2 (100)	2 (100)	0 (0)

was observed in the myxoid stroma, but EDA⁺ or EDB⁺ mRNA was not detected. In the fibrocystic disease, EDA⁺ mRNA was found in stromal cells in florid adenoses. In

the intraductal papillomas, myoepithelial cells between the epithelial cells and the basement membrane showed expression of EDA⁺ FN, as did the stromal cells in the

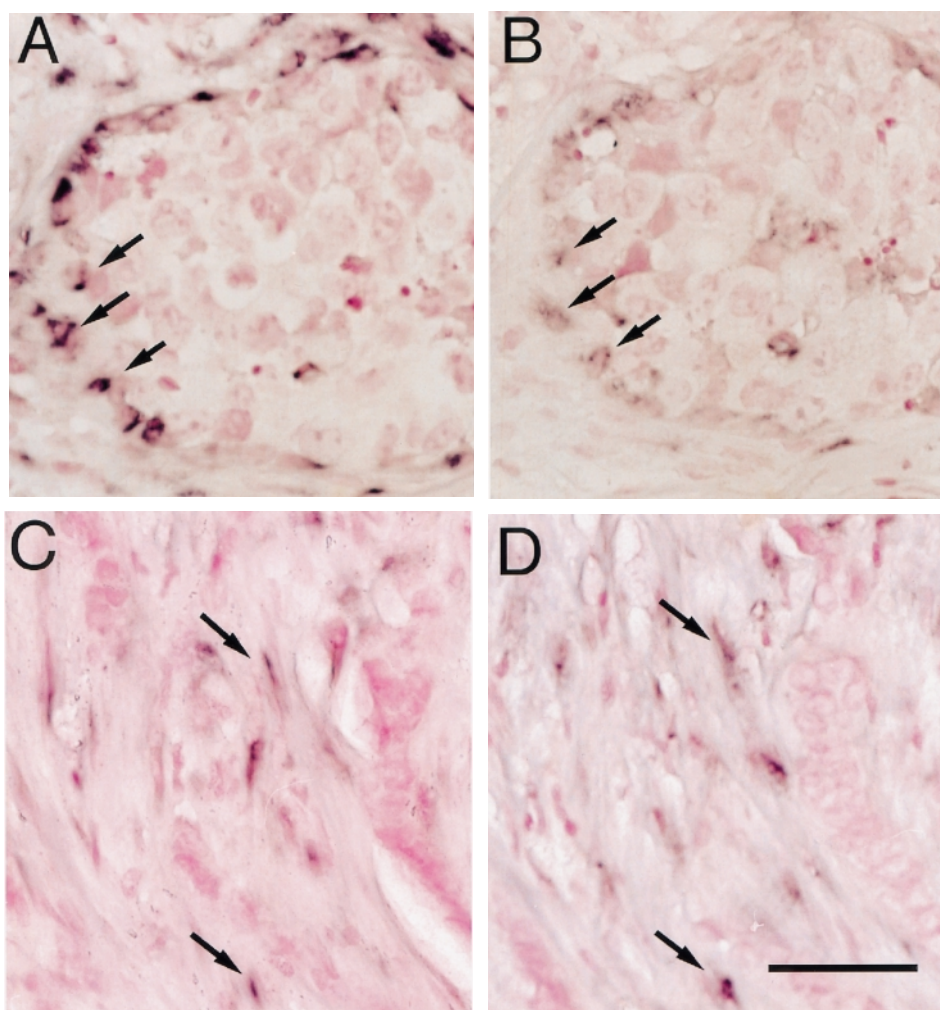


Fig. 2. Co-expression of EDA⁺ FN and TN-C in breast cancers. *In situ* hybridization using cRNA probes for the EDA segment (A, C) and TN-C (B, D). In serial sections, co-expression of EDA⁺ FN and TN-C mRNA is apparent in cancer cells (arrows in A, B) and in stromal cells (arrows in C, D). Bar=50 μ m.

interstitial tissues surrounding the duct. EDB⁺ FN proved negative in all the benign lesions.

Co-expression of EDA⁺ FN and TN-C We have previously reported that TN-C and FN are co-expressed by cancer and stromal cells.¹⁷⁾ Cancer cells at the margin of intraductal lesions and stromal cells in desmoplastic lesions were here also found to co-express EDA⁺ FN and TN-C mRNA (Fig. 2).

DISCUSSION

Previous immunohistochemical studies demonstrated expression of FN isoforms in various breast lesions. In intraductal carcinoma, immunostaining of EDA⁺ FN was

observed diffusely but moderately in the stroma, being enhanced as periductal rims around tumor-containing ducts, while EDB⁺ FN was limited to the periductal regions. The interstitial matrix of infiltrating ductal carcinomas showed diffuse, homogenous and intense labeling for EDA⁺ FN, whereas EDB⁺ FN was absent.¹⁰⁻¹²⁾ The present results demonstrate that FN expression is up-regulated, with reappearance of both EDA⁺ and EDB⁺ isoforms in breast cancer cells and EDA⁺ in stromal elements. Thus, cancer cells produce and secrete EDA⁺/EDB⁺ FN isoforms densely in the periductal zone of intraductal lesions and around large nests. In desmoplastic lesions, stromal fibroblasts produce EDA⁺ FN, that could cause a homogenous deposition of EDA⁺ FN in cancer

stroma, but EDB⁺ FN are rarely produced. Positive staining for EDB⁺ FN was earlier observed around vessels of tumor stroma, suggesting an involvement of EDB⁺ FN in angiogenesis.^{12, 19)} However, we could not find any signals of EDB⁺ mRNA in vascular cells, though the vessels were sometimes labeled by the probe for total FN isoforms (not shown). The discrepancy suggests that expression of the mRNA by vascular cells may be very much lower than that by cancer cells, and the turnover rate of the proteins may be slower in the perivascular tissues. EDB⁺ FN was not expressed in benign breast tumors, as demonstrated in previous immunohistochemical studies.

A recent biochemical study demonstrated that insertion of the EDA segment into FN molecules enhances the integrin-binding affinity, promoting cell adhesion. In addition, cell migration is stimulated.¹⁴⁾ In the light of these biochemical properties and expression patterns of FN containing EDA, it could be considered that FN isoforms stimulate migration activity of cancer cells, associated with intraductal extension of cancer cells and invasion from the ducts or nests. Motility of the stromal cells may also be enhanced by EDA⁺ FN deposited in cancer stroma, followed by remodeling of the stroma. In addition, this isoform could promote cancer cell invasion in the stroma. We recently reported a high rate of expression of TN-C mRNA in breast cancer, and as confirmed here, this may be linked to expression of EDA⁺ FN. Since TN-C also enhances migration,^{20, 21)} these proteins may act

synergistically. Another biochemical study reported that, leading to tyrosine phosphorylation of focal adhesion proteins, EDB domain induces cell signaling events by a mechanism different from that mediated by integrins through RGD and the synergistic sequences of FN type III8-9-10.¹⁵⁾ The expression of the EDB⁺ isoform *in vivo* is more restricted than that of the EDA⁺ isoform.^{2, 10)} This ISH study demonstrated that cancer cells are a major cellular source of FN containing EDB. The EDB⁺ isoform possibly acts on cancer cells in an autocrine manner. Thus, the preferential production of this isoform by cancer cells, but not in benign tumors, and the EDB-dependent signaling could have special significance in tumor malignancy.

In conclusion, the splicing pattern of FN pre-mRNA is cell type-specific, and histology-dependent in breast cancer tissues. FN isoforms containing EDA and/or EDB and their production by cancer and stromal cells might play important roles in cancer progression.

ACKNOWLEDGMENTS

We thank Ms. Satsuki Naota for her expert technical assistance. This research was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture, Japan, and from the Mie Medical Research Foundation.

(Received November 17, 1998/Revised December 24, 1998/Accepted December 29, 1998)

REFERENCES

- 1) Carnemolla, B., Balza, E., Siri, A., Zardi, I., Nicotra, M. R., Bigotti, A. and Natali, P. G. A tumor-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. *J. Cell Biol.*, **108**, 1139–1148 (1989).
- 2) ffrenchi-Constant, C. and Hynes, R. O. Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. *Development*, **106**, 375–388 (1989).
- 3) Oyama, F., Murata, Y., Sukanuma, N., Kimura, T., Titani, K. and Sekiguchi, K. Patterns of alternative splicing of fibronectin pre-mRNA in human adult and fetal tissues. *Biochemistry*, **28**, 1428–1434 (1989).
- 4) Pagani, F., Zagato, L., Vergani, C., Casari, G., Sidoli, A. and Baralle, F. E. Tissue specific splicing pattern of fibronectin messenger RNA precursor during development and aging in rat. *J. Cell Biol.*, **113**, 1223–1229 (1991).
- 5) Lantinen, L., Vartio, T. and Virtanen, I. Cellular fibronectins are differentially expressed in human fetal and adult kidney. *Lab. Invest.*, **64**, 492–498 (1991).
- 6) Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K. and Sekiguchi, K. Coordinate oncogene developmental modulation of alternative splicing of fibronectin pre-messenger RNA at ED-A, ED-B, and CS1 regions in human liver tumors. *Cancer Res.*, **53**, 2005–2011 (1993).
- 7) Natali, P. G., Nicotra, M. R., Filippo, F. Di. and Bigotti, A. Expression of fibronectin, fibronectin isoforms and integrin receptors in melanocytic lesions. *Br. J. Cancer*, **71**, 1243–1247 (1995).
- 8) Pujuguet, P., Hammann, A., Moutet, M., Samuel, J. L., Martin, F. and Martin, M. Expression of fibronectin ED-A⁺ and ED-B⁺ isoforms by human and experimental colorectal cancer: contribution of cancer cells and tumor-associated myofibroblasts. *Am. J. Pathol.*, **148**, 579–592 (1996).
- 9) Menzin, A. W., de Mola, J. R. L., Bilker, W. B., Wheeler, J. E., Rubin, S. C. and Feinberg, R. F. Identification of oncofetal fibronectin in patients with advanced epithelial ovarian cancer. *Cancer*, **82**, 152–158 (1998).
- 10) Gould, V. E., Koukoulis, G. K. and Virtanen, I. Extracellular matrix proteins and their receptors in the normal, hyperplastic and neoplastic breast. *Cell Differ. Dev.*, **32**, 409–416 (1990).
- 11) Koukoulis, G. K., Howedy, M., Korhonen, I., Virtanen, I. and Gould, V. E. Distribution of tenascin, cellular fibronectins and integrins in the normal, hyperplastic and neoplastic breast. *J. Submicrosc. Cytol. Pathol.*, **25**, 285–295 (1993).

- 12) Kaczmarek, J., Castellani, P., Nicolo, G., Spina, B., Allemanni, G. and Zardi, L. Distribution of oncofetal fibronectin isoforms in normal, hyperplastic and neoplastic human breast tissues. *Int. J. Cancer*, **58**, 11–16 (1994).
- 13) Frenchi-Constant, C. Alternative splicing of fibronectin—many different proteins but few different functions. *Exp. Cell Res.*, **221**, 261–271 (1995).
- 14) Manabe, R., Oh-e, N., Maeda, T., Fukuda, T. and Sekiguchi, K. Modulation of cell adhesive activity of fibronectin by the alternatively spliced EDA segment. *J. Cell Biol.*, **139**, 295–307 (1997).
- 15) Chen, W. and Culp, L. A. Adhesion to fibronectin's EDB domain induces tyrosine phosphorylation of focal adhesion proteins in Balb/c 3T3 cells. *Clin. Exp. Metastasis*, **16**, 30–42 (1998).
- 16) Ishihara, A., Yoshida, T., Tamaki, H. and Sakakura, T. Tenascin expression in cancer cells and stroma of human breast cancer and its prognostic significance. *Clin. Cancer Res.*, **1**, 1035–1041 (1995).
- 17) Yoshida, T., Matsumoto, Ei., Hanamura, N., Kalembeiyi, I., Katsuta, K., Ishihara, A. and Sakakura, T. Co-expression of tenascin and fibronectin in epithelial and stromal cells of benign lesions and ductal carcinomas in the human breast. *J. Pathol.*, **182**, 421–428 (1997).
- 18) Hanamura, N., Yoshida, T., Matsumoto, Ei., Kawarada, Y. and Sakakura, T. Expression of fibronectin and tenascin-C mRNA by myofibroblasts, vascular cells and epithelial cells in human colon adenomas and carcinomas. *Int. J. Cancer*, **73**, 10–15 (1997).
- 19) Castellani, P., Viale, G., Dorcaratto, A., Nicolo, G., Kaczmarek, J., Querze, G. and Zardi, L. The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int. J. Cancer*, **59**, 612–618 (1994).
- 20) Tucker, R. P., Brunso-Bechtold, J. K., Jenrath, D. A., Khan, N. A., Poss, P. M. and Sweatt, A. J. Cellular origins of tenascin in the developing nervous system. *Perspect. Dev. Neurobiol.*, **2**, 89–99 (1994).
- 21) Chung, C. Y., Murphy-Ullrich, J. E. and Erickson, H. P. Mitogenesis, cell migration, and loss of focal adhesions induced by tenascin-C interacting with its cell surface receptor, annexin II. *Mol. Biol. Cell*, **7**, 883–892 (1996).