

There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ, and invasive breast lesions

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Aims: Smooth muscle actin (SMA) positive myofibroblasts have been implicated in tumour invasion; however, acquisition of SMA is not limited to peritumorous fibroblasts and other changes in fibroblasts may be more specifically related to the malignant environment. CD34 is a sialomucin expressed by normal breast fibroblasts but lost in invasive carcinomas. The aim of this study was to establish the relation between CD34 and SMA expression in breast fibroblasts and to analyse whether loss of CD34 is specific for invasive disease.

Methods: Immunohistochemistry for CD34 and SMA was performed on 135 cases including 10 normal, 10 fibroadenomas, 40 infiltrating ductal carcinomas, 55 cases of ductal carcinoma in situ (DCIS), and 20 radial scar/complex sclerosing lesions. The relation between staining pattern and histopathological features was recorded as positive, negative, or reduced.

Results: Fibroblasts around all normal duct-lobule units and those showing epithelial hyperplasia were CD34 positive and mainly SMA negative. In fibroadenomas, fibroblasts retained CD34 but acquired SMA expression. In contrast, fibroblasts around invasive carcinoma were CD34 positive and SMA negative. In DCIS, loss of CD34 was significantly more frequent in high grade tumours than in low or intermediate grade ones ($p < 0.001$). The acquisition of SMA was seen more frequently than the loss of CD34, particularly in non-high grade DCIS. In all radial scars, fibroblasts were SMA positive but CD34 negative, and a similar pattern was seen in stromal cells in areas of fibrosis following core biopsy.

Conclusions: These results show that SMA positive myofibroblasts exhibit variable expression of CD34, indicating that these markers are not coordinately controlled. Loss of CD34 is strongly related to the malignant phenotype, in both invasive and preinvasive disease, but is not entirely specific because radial scar fibroblasts and fibroblasts in reactive fibrosis exhibit a similar phenotype. The functional relevance of altered CD34 expression is unclear but the very focal changes implicate local signalling mechanisms probably of epithelial origin.

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There is increasing evidence to indicate that the tumour microenvironment exerts a major modulatory effect on epithelial tumours and that the stroma makes an important contribution to the process of tumour progression.^{1–4} In vitro studies have demonstrated for many tumours that tumour cell–fibroblast interactions enhance tumour cell invasion,^{5–7} and in vitro and tissue studies indicate that this may be mediated at least in part through the stimulation of proteolytic enzymes,^{8–11} with the fibroblasts being the major source of many of these enzymes, such as the matrix metalloproteinases (MMPs).^{12–15} The suggestion that stromal changes may be important in tumour progression is also reflected in the identification of loss of heterozygosity in the stromal compartment of tumours, some of which may be expected to result in functional changes.¹⁴ In several systems, the stroma has been shown to have a profound effect on normal and tumour cell behaviour,^{15–17} and it has been suggested that stroma may play a dominant role in regulating breast epithelial cell function.¹⁸

The stroma around invasive breast tumours is known to differ from normal breast, with alterations in stromal protein synthesis¹⁹ and expression of MMP.^{20–21} Many of these features are attributed to activated fibroblasts, termed myofibroblasts, reflecting their acquisition of α smooth muscle actin (α SMA) expression. However, myofibroblastic differentiation is not specific for malignancy, being characteristic of stromal cells in healing wounds^{22–23} and also being seen in benign lesions of the breast.²⁴ Furthermore, the term myofibroblast implies a

homogeneous cell population, but there is increasing recognition of the heterogeneity of fibroblast populations,^{25–27} and it is therefore highly likely that not all myofibroblasts are the same, and there may be other aspects of the fibroblast phenotype that more closely reflect their functional differentiation.

“It has been suggested that there is an inverse relation between CD34 expression and myofibroblastic differentiation”

CD34 is a transmembrane, highly glycosylated protein expressed by haemopoietic stem/progenitor cells (HPSCs),²⁸ endothelial cells,²⁹ and mesenchymal cells at several different sites, including breast.³⁰ On endothelial cells, CD34 acts as a ligand for L-selectin,³¹ although this is not the case in HPSCs, where its function remains unclear. The CD34 positive fibroblast in many organs is thought to represent an uncommitted cell capable of multidirectional mesenchymal differentiation,³² and it has been suggested that there is an

Abbreviations: ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; HPSC, haemopoietic stem/progenitor cell; HUT, usual type epithelial hyperplasia; IDC, infiltrating ductal carcinoma; LCIS, lobular carcinoma in situ; MMP, matrix metalloproteinase; SMA, smooth muscle actin

Table 1 Summary of the pattern of expression of CD34 and α smooth muscle antigen (α SMA) in fibroblasts in relation to histopathological features

Histology	CD34			α SMA			Total no. duct/lobular units
	+	-	+/-	+	-	+/-	
Normal	301	0	0	0	278	23	301
HUT	88	0	0	0	79	9	88
DCIS: low	28	21	11	33	16	11	60
DCIS: intermediate	22	29	26	52	3	22	77
DCIS: high	48	311	39	362	0	36	398
LCIS	12	0	0	0	12	0	12
Invasive carcinoma	0	40	0	40	0	0	40
Microinvasion	0	6	0	6	0	0	6
Radial scar	0	21	3	5	4	15	24
Fibroadenoma	10	0	0	10	0	0	10
ADH	3	0	3	0	6	0	6
Apocrine cyst	11	0	0	0	11	0	11
Postbiopsy fibrosis	0	4	0	4	0	0	4

For normal breast, ductal carcinoma in situ (DCIS), lobular carcinoma in situ (LCIS), atypical ductal hyperplasia (ADH), and hyperplasia of usual type (HUT), figures relate to individual duct-lobular units. For fibroadenomas, radial scars, postbiopsy fibrosis, and invasive carcinomas, figures relate to case numbers.

inverse relation between CD34 expression and myofibroblastic differentiation.²⁴ Whereas the normal mammary stroma comprises CD34 positive spindle cells, CD34 expression is lost on the fibroblasts surrounding invasive carcinomas,³³ although it

is retained by endothelial cells, making it a useful marker for studies of angiogenesis.³⁴

The aim of our study was to examine the relation between CD34 and α SMA expression in breast fibroblasts, and to

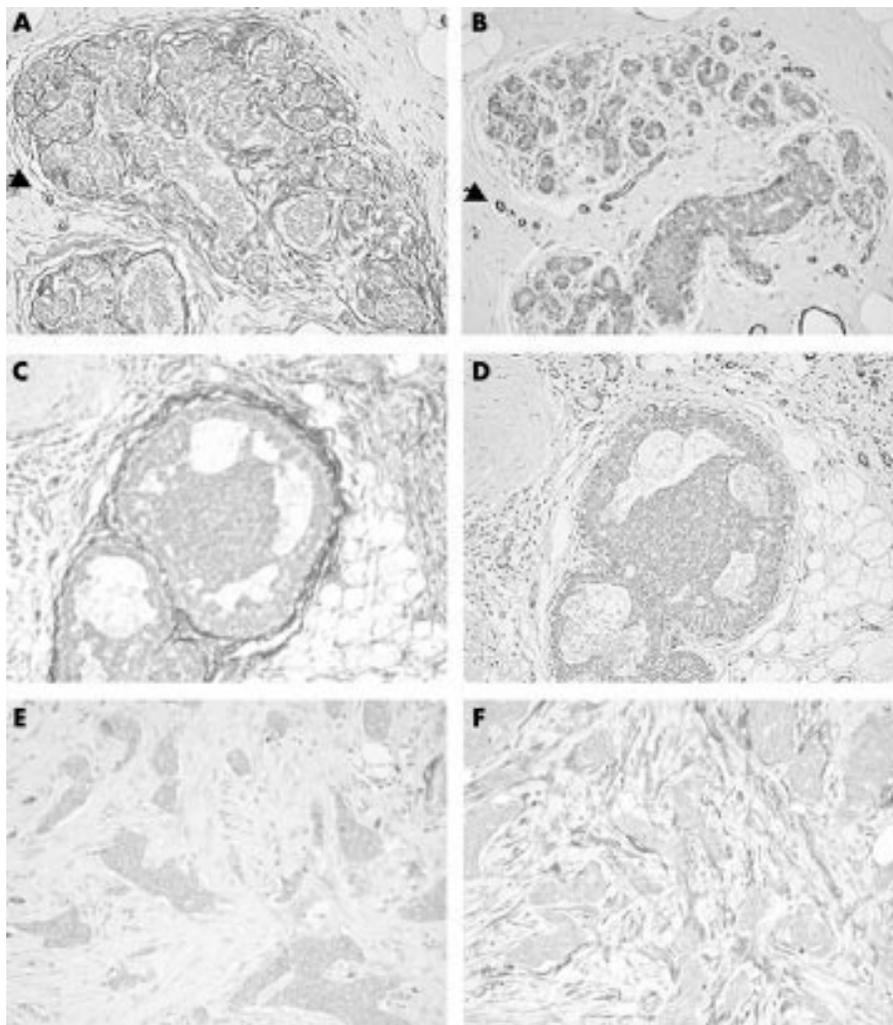


Figure 1 Fibroblasts are CD34 positive around (A) normal breast ducts and lobules and (C) hyperplasia of usual type, but are negative for α smooth muscle actin (α SMA) (B, D). Small calibre blood vessels are also CD34 positive (arrowhead in A and B). In invasive carcinomas, peritumorous fibroblasts are uniformly (E) CD34 negative and (F) α SMA positive.

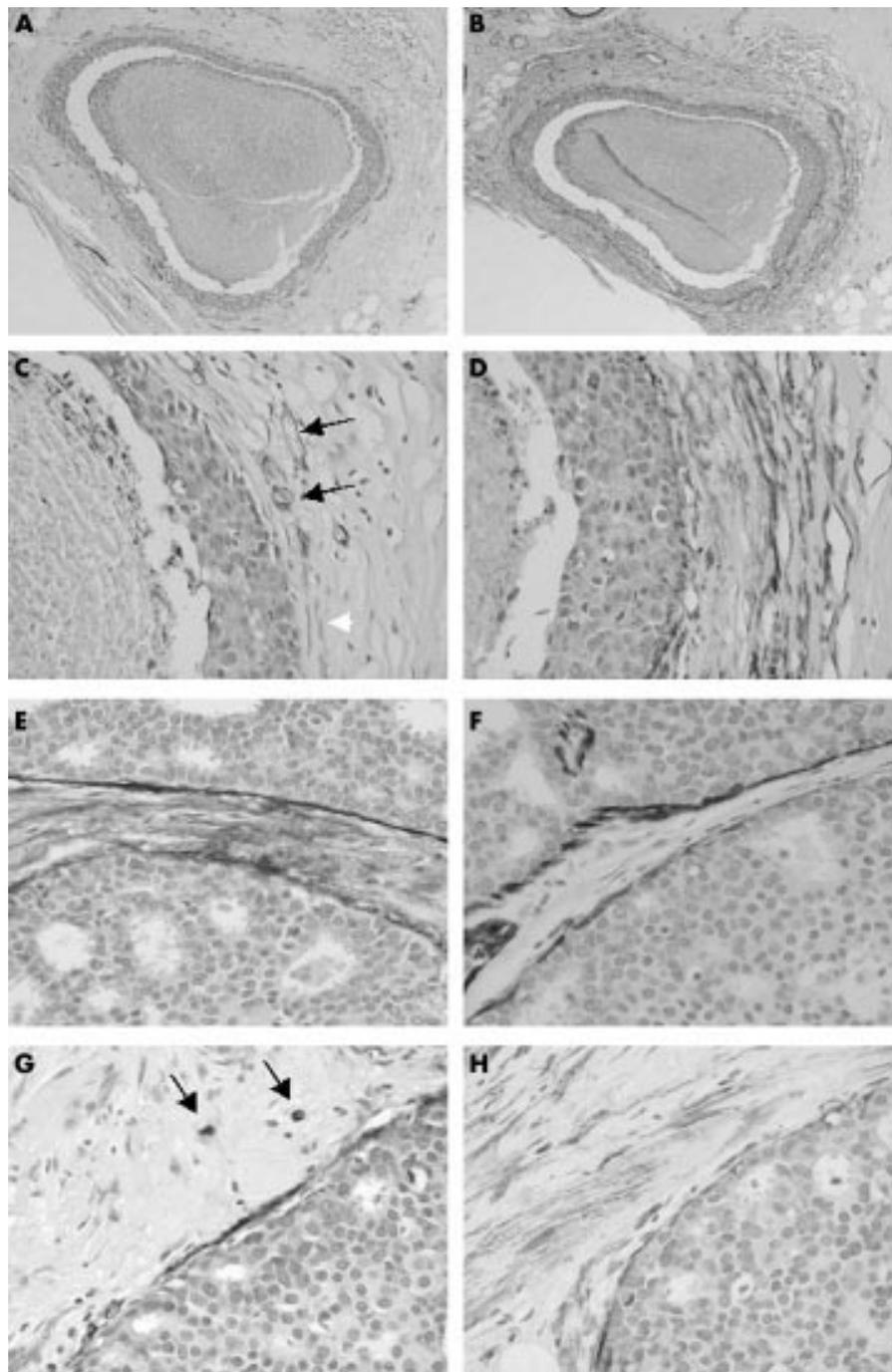


Figure 2 In ducts displaying features of high grade DCIS, 78% were associated with (A, C) CD34 negative periductal fibroblasts and (B, D) the acquisition of α smooth muscle actin (α SMA). (C) Black arrows indicate retained CD34 on small blood vessels in contrast to the fibroblasts (white arrowhead). In low grade DCIS, (E) 47% of duct-lobular units retained CD34 on periductal fibroblasts with (F) 27% remaining α SMA negative; however, 35% of low grade DCIS ducts showed a similar expression pattern to high grade DCIS, with (G) loss of CD34 (arrows indicate CD34 positive blood vessels) and (H) gain of α SMA.

investigate whether the loss of CD34 expression is a more sensitive or specific marker of malignancy in the breast than α SMA expression, with particular emphasis on expression patterns in preinvasive and at risk lesions.

MATERIALS AND METHODS

Immunohistochemistry for CD34 and α SMA was performed on serial sections taken from normal breast (n = 10), fibroadenoma (n = 10), infiltrating ductal carcinoma (n = 40), ductal carcinoma in situ (n = 55), and radial scar/complex sclerosing lesions (n = 20). A standard avidin

biotin complex technique, without prior antigen retrieval, was used incorporating 10 minutes' incubation in 60% H_2O_2 to block endogenous peroxidase. The primary antibodies were diluted in Dako antibody diluent (Qbend-10; Dako, Ely, Cambridgeshire, UK) at 1/100 for CD34 and 1/600 for α SMA. Staining of blood vessels was used as an internal positive control for CD34 and of normal myoepithelial cells for α SMA. For each case, serial sections were also stained with haematoxylin and eosin and in a proportion of cases serial sections were stained with anti-CD31 (Dako) as a further marker of endothelial cells.

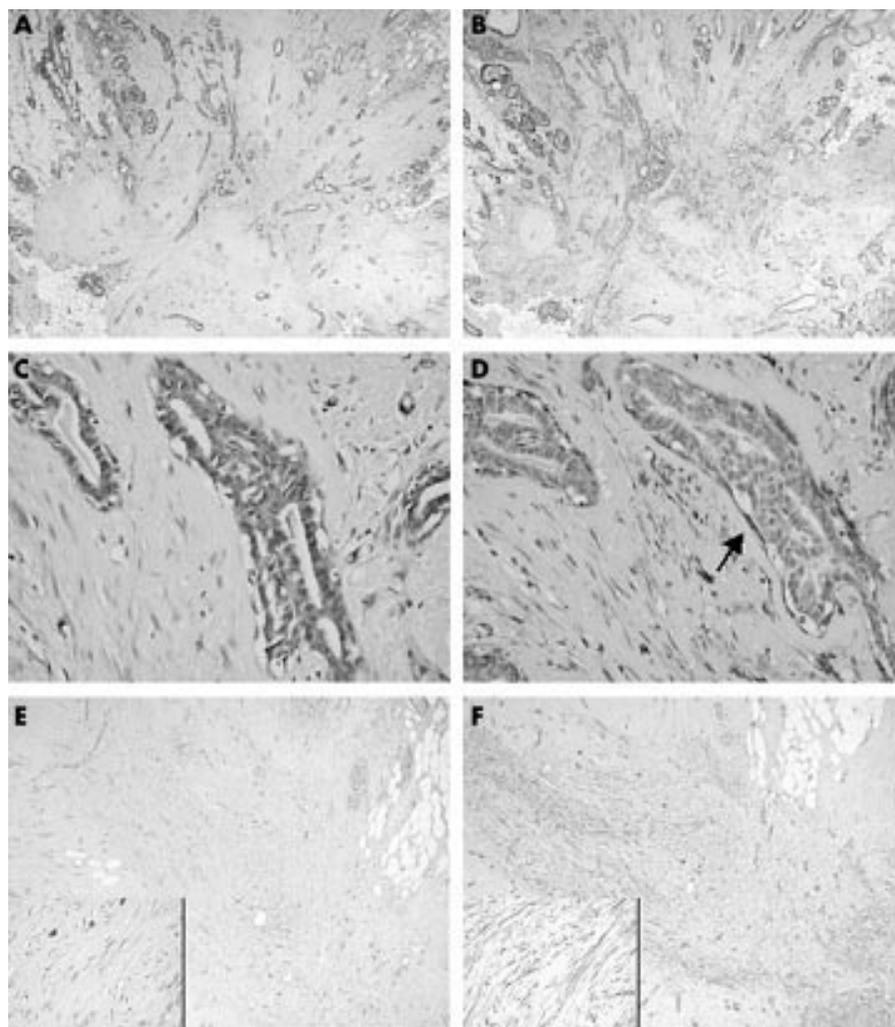


Figure 3 The predominant pattern in radial scars was (A, C) loss of CD34 expression associated with (B, C) acquisition of α smooth muscle actin (α SMA) expression. Strong α SMA expression was also evident around the glandular structures in relation to myoepithelial cells (arrow in D). In four radial scars, reactive fibrosis relating to previous core biopsy was present, and in these areas the spindle cells were uniformly (E) CD34 negative and (F) α SMA positive. Inserts in (E) and (F) are high power magnifications ($\times 400$) of the central area of fibrosis.

Fibroblast reactivity for CD34 and α SMA was recorded as positive, negative, and—where there was focal loss—as reduced. Because many of the sections contained ducts and lobules showing a range of histopathological features, the relation between staining pattern and pathology was interpreted for each duct–lobular unit. Thus, in addition to 40 infiltrating ductal carcinomas (IDCs) and 10 fibroadenomas, 301 normal duct–lobular units were scored, 535 ducts with ductal carcinoma in situ (DCIS; 60 low grade, 77 intermediate, and 398 high grade), six cases showing microinvasion, 88 duct–lobular units exhibiting usual type epithelial hyperplasia (HUT), six with atypical ductal hyperplasia (ADH), 12 lobular carcinoma in situ (LCIS), 11 apocrine cysts, and 24 radial scars, four of which contained areas of reactive fibrosis following preoperative core biopsy (table 1).

The relation between the staining pattern and the different histological features was compared using the χ^2 test, and $p < 0.05$ was considered significant.

RESULTS

In the normal breast, both intralobular and extralobular fibroblasts exhibited strong uniform staining for CD34 and were α SMA negative. This pattern was maintained in the presence of other pathology, including adjacent carcinoma; thus, in all 301 normal duct–lobular units fibroblasts were CD34 positive

and, with the exception of 23 cases, α SMA negative (fig 1A,B). Similarly, fibroblasts around glands showing HUT, LCIS, or apocrine cyst formation were CD34 positive/ α SMA negative (fig 1C,D), with the exception of focal α SMA positivity around nine of 79 ducts with epithelial hyperplasia. Fibroadenomas showed a homogeneous pattern of staining, with fibroblasts being strongly CD34 positive and α SMA positive. In contrast, all IDCs and foci of microinvasion exhibited consistent loss of CD34 expression on fibroblasts, but acquisition of α SMA (fig 1E,F). Discrete staining for CD34 was evident on small calibre blood vessels within the stroma, confirmed by their expression of the vascular antigen CD31.

The most variable pattern of expression was seen on fibroblasts surrounding ducts with DCIS; however, the loss of CD34 expression was significantly more frequent in high nuclear grade compared with low or intermediate grade samples ($p < 0.001$). In high grade DCIS, fibroblasts were CD34 negative in 78% cases compared with 29 of 77 (33%) intermediate and 21 of 60 (35%) cases of low grade DCIS (table 1; fig 2). In some cases, partial loss of CD34 was evident around a duct. The incidence of reduced staining was again variable between the grades; however, only 12% of cases of high grade DCIS retained normal CD34 expression compared with 22 of 77 (29%) intermediate grade and 28 of 60 (47%) low grade cases. Staining of serial sections of 20 of these cases with CD31

demonstrated clearly the pattern of small blood vessel formation around the ducts, and these vessels were easily distinguishable from the fibroblast population. In most cases, loss of CD34 expression was accompanied by the acquisition of α SMA; however, this relation was not absolute, with gain of α SMA being more frequent than loss of CD34 (table 1), and only 27% of periductal fibroblasts in low grade DCIS were α SMA negative, compared with 47% retaining expression of CD34. Again, a mixed pattern of staining was evident in relation to ADH, with a proportion of CD34 negative fibroblasts in half the cases; however, in none of the six cases was there evidence of α SMA staining.

In the radial scars, most fibroblasts were CD34 negative/ α SMA positive (fig 3), although some cases displayed CD34 positive/ α SMA positive fibroblasts at the periphery of the lesion. The pattern was similar in those radial scars admixed with DCIS (n = 4) and those not associated with malignant change (n = 20). Interestingly, in four cases, areas of reactive fibrosis were evident from preoperative core biopsy, and in these sites the fibroblasts were uniformly CD34 negative/ α SMA positive (fig 3).

DISCUSSION

CD34 is a transmembrane glycoprotein that is thought to be involved in the modulation of cell adhesion and signal transduction,²⁸ and is expressed by mesenchymal cells at several sites, including the normal mammary stroma.^{30–33} Loss of CD34 by mesenchymal cells has been described in several situations where there is malignant transformation of the mesenchymal population. Malignant phyllodes tumours of the breast exhibit lower levels of CD34 expression than benign phyllodes or fibroadenomas,^{24–33} and CD34 is lost in sarcomas arising within CD34 positive dermatofibrosarcoma protuberans.³⁶ Loss of fibroblast CD34 has also been described in non-neoplastic fibroblasts around epithelial tumours, such as basal cell carcinoma³⁷ and colorectal carcinoma.³⁸

“Loss of CD34 may be related to invasive potential”

Our study has shown that fibroblast CD34 expression is consistently lost in invasive breast carcinomas that include microinvasion, and in a high proportion of cases of DCIS, particularly high grade lesions, which are thought to be more likely to progress to invasion.³⁹ Loss of expression is also seen in a proportion of ADH, but not around glandular structures showing LCIS. This is of interest because ADH and DCIS are regarded as premalignant lesions, whereas LCIS is considered to confer an increased risk of developing carcinoma, but the risk relates to the development of carcinoma in either breast, not to the site of the LCIS.⁴⁰ This raises the possibility that loss of CD34 may be related to invasive potential. The change in CD34 expression is very localised, with loss around a duct containing DCIS, but retained expression around adjacent normal breast glands, and this strongly implicates epithelial–mesenchymal interactions in the control of expression, as has been suggested previously in the case of phyllodes tumours.³³ What determines the loss of CD34 is of interest because not all DCIS cases show loss, and this may point to different functional states of the neoplastic epithelium, which are important in determining their invasive potential. However, whereas it has previously been suggested that the loss of CD34 is specific to malignancy,⁴¹ we show that this is not the case. Although most benign lesions, including HUT, retain CD34 expression, loss of CD34 was a consistent finding in the fibroblasts associated with areas of fibrosis following core biopsy and in radial scars; in this last case, whether they were associated with malignancy or not. Although on the surface this makes CD34 less valuable as a diagnostic marker of malignancy—particularly in sclerotic lesions—it raises interesting questions as to the state of differentiation of fibroblasts

Take home messages

- Smooth muscle actin positive myofibroblasts express CD34 to varying degrees, indicating that these markers are not coordinately controlled
- The loss of CD34 is strongly related to the malignant phenotype, in both invasive and preinvasive disease, but is not entirely specific because radial scar fibroblasts and fibroblasts in reactive fibrosis exhibit a similar phenotype
- The functional relevance of altered CD34 expression is unclear but the very focal changes implicate local signalling mechanisms, probably epithelial in origin
- In vitro studies should be undertaken to establish the role of CD34 in the breast fibroblast

in different breast lesions, and the potential relevance of this in terms of fibroblast function. Some studies suggest that radial scars are associated with an increased risk of development of malignancy,⁴² and several other studies have shown similar alteration in the stroma of radial scars as seen in invasive carcinomas, such as increased hyaluronic acid⁴³ and increased expression of ED-A fibronectin and vascular endothelial growth factor.⁴⁴ The importance of loss of expression of CD34 on stromal cells in reactive fibrosis is unclear, although it could possibly be related to the terminal differentiation of these cells.

“It is essential that the changes in fibroblast phenotype associated with malignancy are carefully dissected, not only to validate their use as possible diagnostic markers, but also to establish their potential as therapeutic targets”

The importance of changes in fibroblast function in promoting tumour progression is increasingly recognised and such a tumour promoting effect is frequently attributed to the activation of fibroblasts to α SMA positive myofibroblasts. However, our study clearly demonstrates that acquisition of the myofibroblast phenotype is not indicative of malignancy in the breast, and it also demonstrates that not all myofibroblasts have the same phenotype, some being CD34 positive, as in fibroadenomas, and others being CD34 negative, as in invasive and many in situ lesions. The potential for the stromal response to tumours becoming a target for treatment is increasing, with clinical trials in place for fibroblast derived factors such as MMP⁴⁵ and Tenascin.⁴⁶ However, it is essential that the changes in fibroblast phenotype associated with malignancy are carefully dissected, not only to validate their use as possible diagnostic markers, but also to establish their potential as therapeutic targets.

The functional relevance of fibroblast CD34 expression and its loss is not clear, although it may represent a change from a multipotent mesenchymal cell to a committed cell type. However, the variable association between CD34 and α SMA expression demonstrates that not all myofibroblasts are the same, and that the loss of CD34 appears to be more closely related, although clearly not specific, to malignancy than the acquisition of α SMA. This suggests that α SMA may not be the most important marker of fibroblast function, and in vitro studies are now indicated to establish the role of CD34 in the breast fibroblast.

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