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MIGRATION STIMULATING FACTOR (MSF). ITS ROLE IN THE TUMOUR MICROENVIRONMENT.

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Dedication: This chapter is dedicated to the memory of Professor Seth L Schor. He is remembered for his humour, kindness, enthusiasm and love of Science. His belief in data above dogma made the research on MSF possible. He is a main contributor to this chapter's contents.

ABSTRACT

Migration Stimulating Factor (MSF) is a 70kDa truncated isoform of fibronectin (FN); its mRNA is generated from the FN gene by an unusual two-stage processing. Unlike full-length FN, MSF is not a matrix molecule but a soluble protein which displays cytokine-like activities not displayed by any other FN isoform due to steric hindrance. There are two isoforms of MSF; these are referred to as MSF+aa and MSF-aa, while the term MSF is used to include both.

MSF was first identified as a motogen secreted by foetal and cancer-associated fibroblasts in tissue culture. It is also produced by sprouting (angiogenic) endothelial cells, tumour cells and activated macrophages. Keratinocytes and resting endothelial cells secrete inhibitors of MSF that have been identified as NGAL and IGFBP-7, respectively. MSF+aa and MSF-aa show distinct functionality in that only MSF+aa is inhibited by NGAL.

MSF is present in 70-80% of all tumours examined, expressed by the tumour cells as well as by fibroblasts, endothelial cells and macrophages in the tumour microenvironment (TME). High MSF expression is associated with tumour progression and poor prognosis in all tumours examined, including breast carcinomas, non-small cell lung cancer (NSCLC) salivary gland tumours (SGT) and oral squamous cell carcinomas (OSCC). Epithelial and stromal MSF carry independent prognostic value. MSF is also expressed systemically in cancer patients, being detected in serum and produced by fibroblast from distal uninvolved skin. MSF-aa is the main isoform associated with cancer, whereas MSF+aa may be expressed by both normal and malignant tissues.

The expression of MSF is not invariant, it may be switched on and off in a reversible manner which requires precise interactions between soluble factors present in the TME and the extracellular matrix in contact with the cells. MSF expression in fibroblasts may be switched-on by a transient exposure to several molecules, including TGFβ1 and MSF itself, indicating an auto-inductive capacity.

Acting by both paracrine and autocrine mechanisms, MSF stimulates cell migration/invasion, induces angiogenesis and cell differentiation and alters the matrix and cellular composition of the TME. MSF is also a survival factor for sprouting endothelial cells. IGD tri- and tetra-

peptides mimic the motogenic and angiogenic activities of MSF, with both molecules inhibiting AKT activity and requiring αvβ3 functionality. MSF is active at unprecedently low concentrations in a manner which is target cell-specific. Thus, different bioactive motifs and extracellular matrix requirements apply to fibroblasts, endothelial cells and tumour cells. Unlike other motogenic and angiogenic factors, MSF does not affect cell proliferation but it stimulates tumour growth through its angiogenic effect and downstream mechanisms.

The epithelial-stromal pattern of expression and range of bioactivities displayed puts MSF in the unique position of potentially promoting tumour progression from both the "seed" and the "soil" perspective.

Key words: Migration Stimulating Factor (MSF); Fibronectin isoforms; Onco-foetal protein; Cancer associated fibroblasts (CAF); Sprouting (angiogenic) endothelial cells; Tumour cells; Macrophages; IGD peptides; TGFβ1; NGAL; IGFBP-7; Cell migration; Angiogenesis; Prognostic factor; Tumour microenvironment (TME).

INTRODUCTION

Migration Stimulating Factor (MSF) is a 70kDa truncated isoform of fibronectin (FN). FN is a major component of the extracellular matrix; unlike full-length FN, MSF is not a matrix molecule but a soluble onco-foetal protein which displays cytokine-like activities at unprecedently low (femtomolar) concentrations. In this chapter we summarise published and unpublished data under the following headings:

1. Background. Initial identification of MSF.

The extracellular matrix was commonly considered to be an inert scaffolding in the 1980s. The concept that the extracellular matrix could modulate gene expression and cellular response was not generally accepted, in spite of clear evidence from a few pioneering researchers and previous developmental studies. The discovery of MSF was a serendipitous result of considering the limitations of studying cell invasion in vitro and the importance of the extracellular matrix in determining cell behaviour. In this section we introduce the migration assay used to assess MSF bioactivity and the discovery of two fibroblast phenotypes (foetal and adult) which differ in their migratory behaviour and the secretion of MSF. According to these criteria, aberrant (foetal-like, MSF-producing) fibroblasts are present not only in tumours but in distant uninvolved skin of cancer patients.

2. Molecular structure of MSF. Function-neutralising and identification antibodies.

Two isoforms of MSF have been cloned. These are referred to as MSF+aa and MSF-aa while the term MSF or total MSF is used to include both. MSF isoforms are described, highlighting their bioactive motifs, and comparing their structure to that of FN. In our laboratory, three types of antibodies have been used to identify and characterise MSF protein. These include: (i) MSF-specific identification antibodies that recognise both MSF+aa and MSF-aa, (ii) MSF-aa –specific identification antibodies and (iii) MSF-function-neutralising antibodies.

3. MSF expression. Diagnostic and prognostic relevance.

MSF is secreted by tumour and stromal cells in tissue culture. Endothelial cells can display two distinct and reversible phenotypes: resting and angiogenic. Angiogenic endothelial cells secrete MSF whereas resting endothelial cells, as well as keratinocytes, secrete an inhibitor of MSF. MSF inhibitors produced by keratinocytes and endothelial cells have been identified as

NGAL and IGFBP-7, respectively. The expression of MSF is not invariant, it may be switched on and off in a reversible manner that requires precise interactions between soluble factors present in the microenvironment and the extracellular matrix in contact with the cells. MSF expression in fibroblasts may be switched on by a transient exposure to several molecules, including MSF itself, indicating an auto-inductive capacity.

The expression of MSF in excised tissues mirrors the expression by cells in tissue culture and shows an association with tumour development. Immunostaining indicates that MSF-aa is the main isoform present in tumours. High MSF expression in oral and breast carcinomas is associated with poor patient survival. In breast tumours, stromal MSF and epithelial MSF appear to be regulated differently and carry different prognostic significance.

4. MSF bioactivities, modulation and mode of action

MSF is an unusually potent effector molecule. Acting through both paracrine and autocrine signalling, it alters the TME by inducing cell migration and angiogenesis and by triggering changes in the extracellular matrix composition and in metabolic pathways. MSF motogenic activity is matrix-dependent in a way that is specific to different cell types. The amino acid motifs mediating MSF motogenic activity are also cell-specific, being different for fibroblasts, endothelial cells and tumour cells. Small IGD peptides mimic the motogenic and angiogenic activities of MSF, with both molecules requiring $\alpha v\beta 3$ functionality. MSF+aa and MSF-aa show the same motogenic activity, but only MSF+aa is inhibited by NGAL. Therefore the manifestation of MSF bioactivity depends on the type of isoform and possible inhibitors present. Functional analyses indicate that both MSF isoforms may be present in serum and are secreted by tumour cells. MSF inhibits AKT phosphorylation and activity and activates Cdc42 and NFκB.

5. Conclusions and future work

A large number of motogenic and angiogenic factors have been described. MSF shows unique characteristics, not shared by other factors or by other isoforms of FN. Much remains to be investigated, particularly in relation to (a) MSF auto-activation, (b) the systemic expression of MSF, (c) MSF isoforms, their interactions with other molecules in the TME and value as tumour biomarkers, (d) MSF bioactivities and potential as a target for anti-tumour therapy.

1. BACKGROUND. INITIAL IDENTIFICATION OF MSF.

1a. Relevance of the extracellular matrix used in migration assays.

MSF was identified in the 1980s as an oncofoetal motogenic factor produced by fibroblasts and expressed locally and systemically in cancer patients. The development of a migration/invasion assay, using 3-dimensional (3D) gels of native collagen, played a decisive role in the discovery of MSF. In order to study tumour cell invasion in tissue culture, S.L. Schor endeavoured to provide the cells with a more physiologically relevant substratum than the frequently used plastic or denatured collagen (gelatin). Accordingly, he developed a novel assay for quantifying cell attachment, migration and proliferation within 3D gels of native type I collagen fibres [Schor 1980a, 1980b, Schor et al, 1982, 1985a].

This approach has demonstrated that very different results could be obtained using native collagen rather than denatured collagen or plastic substrata [Schor et al 1980, 1996, 1999, 2003, 2006; Gibson et al 1982; Ellis et al 1999]. The first controversial finding to emerge was that cells adhered to the surface of the native collagen gel in the absence of serum or FN. It was widely accepted at that time that FN was required to mediate cell adhesion to collagen. However, Schor and collaborators demonstrated that this is not the case when using native collagen, as opposed to its denatured counterpart [Schor and Court 1979, Schor1980b, Schor et al 1981a, 1981b]. Following attachment, certain cell types (e.g. melanoma cells, fibroblasts) invade the underlying 3D-collagen gel, and this takes place in the absence of collagenolytic activity [Schor 1980a**,** Schor et al 1981c, 1985a; Schor and Schor 1986]. The percentage of cells that migrate into the 3D gels can then be quantified microscopically (Fig 1) [Schor 1980a, Schor et al, 1982]. A variation of this assay, called the “sandwich assay”, allows the assessment of chemotaxis and chemokinesis in 3D gels. In the sandwich assay,

PDGF-AB and TGFβ3 stimulate fibroblast migration under both chemotactic and chemokinetic conditions whereas TGFβ1 and TGFβ2 are completely devoid of motogenic activity. These results are consistent with the differential bioactivities of PDGF-AB and TGFβ3, compared to TGFβ1 and TGFβ2, in animal models of wound healing. In contrast, all four molecules induced chemotaxis and chemokinesis in the transmembrane assay, using membranes coated with either native or denatured type I collagen [Schor et al 2006]. In spite of initial scepticism, the importance of tissue-level factors in regulating the functionality of soluble factors is now fully accepted. As it is the case with TGFβ isoforms, the response of the cells to soluble factors is modulated by the composition, topological organisation and physical presentation of the extracellular matrix in contact with the cells [Bissell and Barcellos-Hoff 1987; Schor 1994; Schor et al 2006; Keatch et al 2002, 2012].

1b. Distinct fibroblast migratory phenotypes.

Using the 3D collagen gel assay, it was observed that the extent of migration of foetal and adult skin fibroblasts into this matrix is differentially affected by both cell type and cell density, indicating the existence of two distinct (foetal / adult) migratory phenotypes. When plated at high density, foetal skin fibroblasts migrate into the 3D collagen gel matrix to a significantly greater extent than do their adult counterparts, and this difference in behaviour results from the secretion by foetal fibroblasts of a soluble "migration stimulating factor" or MSF. For these experiments, the medium in which the cells were grown (conditioned medium) was harvested and added to confluent fibroblast cultures. Adult fibroblasts cultured in the presence of foetal fibroblast conditioned medium display an elevated level of migration, comparable to that of the foetal cells. On the other hand, the migration of foetal fibroblasts is not affected by the presence of conditioned medium from the adult cells (Fig 1).

Therefore, adult skin fibroblasts from healthy individuals do not produce MSF but respond to exogenous MSF. This responsiveness provided the basis of a sensitive bioassay for assessing MSF production by cells in tissue culture and monitoring the purification of MSF from bioactive conditioned media.

Subsequent work with tissue specimens demonstrated that fibroblasts explanted from breast tumours are often foetal-like in terms of their migratory phenotype and production of MSF. It was assumed at that point that fibroblasts obtained from distant uninvolved skin (i.e. an apparently normal adult cell population) would represent a good control for the tumour-derived fibroblasts. However, most unexpectedly, paired forearm skin fibroblasts from the same breast cancer patients are also foetal-like regarding their migratory phenotype and production of MSF, thereby indicating the systemic presence of aberrant stromal cells in these individuals. Furthermore, foetal-like fibroblasts were also isolated from a number of their healthy first-degree relatives [Durning et al, 1984; Schor et al, 1985b, 1985c,1986,1988a, 1988b, Haggie et al, 1987] (Fig 2). This systemic abnormality is not unique to patients with breast cancer, as MSF-producing fibroblasts were also obtained from approximately 50% of both tumour and uninvolved distal skin of individuals with a variety of common cancers, including melanoma, soft tissue sarcoma and carcinomas of the colon, lung and prostate [Schor 1995]. In all of these studies, only 10-12% of age- and sex-matched healthy controls were found to have foetal-like skin fibroblasts, as defined by their migratory phenotype and the presence of bioactive MSF in their conditioned media.

MSF expression in the adult is not exclusively associated with cancer: MSF is transiently expressed during wound healing and is constitutively present in certain locations, particularly in the oral mucosa, a tissue that displays a foetal-like mode of healing [Picardo et al 1992;

Irwin et al 1994] (Fig 2). MSF-producing fibroblasts were also found in explants from normal breast tissue; however, there was a significant difference between tumour and non-tumour patients regarding the phenotype of the intra-lobular fibroblasts [Schor et al 1994] (Fig 2).

MSF purified from the conditioned media of foetal and cancer patients fibroblasts was shown to be a very potent protein, stimulating both the migration of adult fibroblasts and hyaluronic acid (HA) synthesis, with half maximal activity at 0.01 to 0.1pg/ml [Schor et al 1988a, 1989; Grey et al 1989]. The protocol developed to purify MSF has been applied to identify MSF in biological fluids and in conditioned medium from different cell lines. This, and the development of a function-neutralising polyclonal antibody, complemented the migration assay for the initial identification of MSF in cell cultures. Statistical analysis revealed a strong association between the expression of a foetal-like migratory phenotype and the production of MSF by the cells ($p < 0.00001$) [Irwin et al 1994].

Fibroblast heterogeneity and the presence of aberrant fibroblasts in the tumour stroma have long been recognised and are the subject of many current studies. However, the finding of systemic aberrant fibroblasts in sporadic cancer patients was (and remains) difficult to reconcile with the predominant “epithelio-centric” models of cancer progression. Instead, Schor and collaborators proposed alternative hypotheses and advocated the importance of the microenvironment and the role of cell-cell and cell-matrix interactions at the “tissue level”, concepts now fully accepted within “epigenetics”, but not encouraged in the reductionist ethos of the time [Schor et al 1987,1991; Schor and Schor 1987a, 1987b, 1997, 2011; Schor 1995].

2. MOLECULAR STRUCTURE OF MSF. FUNCTION-NEUTRALISING AND IDENTIFICATION ANTIBODIES.

2a. The structure of MSF. Comparison with fibronectin.

Initial amino acid sequencing of purified MSF (from fibroblast conditioned medium) indicated that it was a 70kDa protein exhibiting sequence homology with the gelatin-binding domain (Gel-BD) of fibronectin (FN). MSF and Gel-BD did not appear to be the same molecule, as they displayed very different activities regarding their effects on the migration of subconfluent adult skin fibroblasts. However, Gel-BD and MSF showed identical activities regarding the stimulation of migration and hyluronic acid (HA) synthesis on confluent adult fibroblasts [Grey et al 1989, Schor 1995, Schor et al 1996]. This was unexpected and controversial at the time, as Gel-BD had been described as an inactive domain of FN [Hynes, 1990]. The gene for MSF proved difficult to clone, in part as a result of the exceedingly small quantities produced by foetal and cancer patient fibroblasts, the homology with the very abundant FN and the reliance on a labour intensive bioassay to monitor its purification. MSF was eventually cloned and shown to be a 70kDa truncated isoform of FN [Schor et al 2003].

Fibronectins are modular glycoprotein consisting of the following functional domains: Hep 1/Fib-1 (N-terminal low affinity binding to heparin and fibrin), Gel-BD (binding to gelatin/collagen), Cell-BD (RGD-mediated binding to integrins), Hep-2 (high affinity heparin binding) and Fib-2 (C-terminal fibrin binding site). Each functional domain is composed of a different number of three possible homology modules, called type I, II and III (Fig 3). Approximately 20 “full-length” FN isoforms are produced by alternative splicing, involving the inclusion or exclusion of two particular type III modules (EDA and EDB) and a more

complex splicing repertoire at the IIICS region. All such isoforms consist of two peptide chains (250-280kDa each) covalently linked by disulfide bonds at their respective C-termini. In the FN gene, all type I and II homology modules are coded by correspondingly named single exons; in contrast, all type III modules are coded by two consecutive exons (designated "a" and "b"), with the exception of the individual exons coding for the "extra domain" type III modules (EDA and EDB) which are found in those isoforms displaying an oncofoetal expression profile [Hynes, 1990].

MSF mRNA is generated from the FN gene by an unusual two-stage processing mechanism. In the first stage, an MSF-specific primary transcript is generated from the FN gene by read-through of intron 12, separating exons III-1a and III-1b. This is followed by intra-intronic cleavage to produce a 5.9 kb MSF pre-message [Kay et al, 2005]. The pre-message remains sequestered within the nucleus, where it is rapidly degraded. Under standard tissue culture conditions, this pre-message is produced by cells that express MSF protein as well as by cells which do not. In cells which do express MSF, the second stage takes place whereby the intron-derived 3' UTR of the pre-message is cleaved a second time to produce a 2.1 kb mature MSF message. This has a significantly shorter (195bp) intron-derived 3' sequence containing a 30bp in-frame coding sequence (immediately contiguous with exon III-1a), followed by a 165bp 3'-UTR containing several in-frame stop codons and a cleavage / polyadenylation signal. The mature message is rapidly exported to the cytoplasm for translation [Schor et al, 2003; Kay et al, 2005].

Therefore, human MSF is identical to the N terminus of full-length FN, up to and including the amino acid sequence coded by exon III-1a, with the addition of an MSF-unique (intron-coded) 10 amino acid C-terminus [Schor et al, 2003; Kay et al, 2005] (Fig 3).

FN is a major component of the extracellular matrix. Unlike FN, MSF is a monomer and is not a matrix molecule but a soluble factor which exhibits a range of cytokine-like bioactivities, including the stimulation of cell migration. As discussed in section 4, the motogenic activity of MSF is mediated by the IGD motifs, present in modules I3, I5, I7 and I9 of the Gel-BD domain and, in some cases, by the HEEGH motif present in module I8 [Schor et al, 1999, 2003; Houard et al, 2005; Millard et al, 2007] (Fig 3).

Two isoforms of human MSF have been cloned by our group (Fig 4). Both contain the same unique 10 amino acid C-terminus as well as the same bioactive IGD and HEEGH amino acid motifs. The two isoforms differ solely in terms of a 45bp deletion in exon II-1 and are consequently referred to as MSF+aa and MSF-aa to indicate the retention or deletion of a 15 amino acid sequence in module II-1 (Figs 3 and 4). The terms MSF or total MSF will be employed to denote both isoforms. The generation of MSF+aa and MSF-aa message presumably occurs by the same mechanism, with the addition of the splicing out of a 45bp sequence in exon II-1.

MSF is significantly less abundant and more unstable than FN. A comparison of MSF with full-length FN in different MSF-producing fibroblast lines demonstrated that MSF mRNA is approximately 1000-fold less abundant than full-length FN transcripts. Also, MSF mRNA decreased over 30 times more rapidly than full-length FN transcripts. MSF mRNA displayed a biphasic decay pattern, with initial levels decreasing by half in approximately 45 min. In contrast, full-length FN transcripts took more than 24 hours to fall to half their initial concentration [Schor et al 2003].

Murine MSF (mMSF) has also been cloned. mMSF is homologous to its human counterpart: it consists of the N-terminus of mouse FN, up to and including exon III-1a and terminates in a unique 3'coding sequence (12-mer) derived from the intron separating exons III-1a and III-1b. However, mMSF' 12-mer unique sequence and human MSF's 10-mer unique sequence are not homologous [Kay et al, unpublished].

MSF is not the only truncated isoform of FN. Another form, named FN2, has been found in fish and mammals. Unlike MSF, whose exonic content ends at exon III-1a, FN2 terminates at III-3b and contains a unique 20-amino-acid C-terminal tail that is different from the C-terminus of FN in that it lacks the two cysteines that are usually involved in the formation of inter-chain disulfide bonds. Recombinant FN2 promotes the attachment and spreading of fish embryo cells in culture [Zhao et al 2001; Liu et al 2003]. The inclusion of the first three type III domains of FN (present in FN2 as well as in all full length FN isoforms) makes these molecules very different from MSF regarding their bioactivities [Vakonakis et al 2009].

2b. Antibodies used to identify and characterise MSF protein.

Following the cloning of MSF, various cell and molecular biology techniques have been used by our group and others in order to identify MSF mRNA and protein in biological samples and to characterise MSF functionality in different systems [Schor et al 2003; Houard et al 2005; Jones et al 2007; Yoshino et al 2007; Hu et al 2009; Solinas et al 2010; Aljorani et al 2011; Perrier et al 2012;Carito et al 2012; Deng et al 2013]. We have shown that two different isoforms of MSF can be found in the TME and that MSF may be present in biological samples in a non-bioactive form (sections 3 and 4). In different studies, the presence of MSF has been determined by in-situ hybridisation, RT-PCR, immunohistochemistry (IHC) and biochemical methods. In our laboratory, the presence of bioactive MSF has been defined by the expression of its motogenic activity on target cells

attached to native collagen substrata (either collagen gel or transmembrane assay), in combination with other methods, such as (i) abolition of the motogenic activity with MSF-function-neutralising antibodies, (ii) removal of the activity by immunoprecipitation or affinity chromatography to MSF-identification antibodies, (iii) separation of MSF from other molecules by gel filtration chromatography and SDS-PAGE [Grey et al 1989; Schor et al 2003, 2012; Jones et al 2007].

Three types of antibodies have proved most valuable for these studies:
(i) MSF-specific identification antibodies that recognise the unique C-terminal sequence of MSF+aa and MSF-aa; that is, total MSF (antibody code VSI).
(ii) Identification antibodies that recognise MSF-aa but not MSF+aa (code TYN); and
(iii) MSF-function-neutralising antibodies (code pepQ) that recognise MSF+aa, MSF-aa and Gel-BD (Figs 3, 4). The peptides used as antigens to raise these antibodies and an overview of the results obtained are shown in Table 1. The antibodies were fully characterised by standard biochemical techniques, IHC and their ability to abrogate or remove MSF bioactivity [Schor et al 2003, 2012, 2020]. As expected, different antibodies were useful for certain techniques and not for others. Some examples of the results obtained are shown in Figs 5 and 6 and in section 3.

3. MSF EXPRESSION. DIAGNOSTIC AND PROGNOSTIC RELEVANCE.

3a. Presence of MSF and MSF inhibitors in tissue culture.

The expression of MSF in cell culture studies points to an association with angiogenesis and cancer. Besides its expression by tumour-associated fibroblasts (section 1, Fig 2), MSF is produced by tumour cell lines (Table 2), activated macrophages [Solinas et al 2010] and sprouting endothelial cells [Schor and Schor 2010].

MSF mRNA and/or protein has been detected in 15/18 tumour cell lines examined, including lines derived from breast, lung, skin, colon, salivary gland and oral mucosa tumours (Table 2). Breast carcinoma MCF7 is one of the three lines that do not express MSF [Houard et al 2005, Table 2]. However, MSF is strongly expressed when these cells are transfected with dominant-negative p53 [Table 2].

Endothelial cells may display two reversible phenotypes in culture: cobblestone and sprouting. These phenotypes differ in many characteristics, including their morphology, synthesis of extracellular matrix components and, as discussed below, the expression of bioactive MSF. The transition from a cobblestone to a sprouting phenotype has long been used as an in vitro model of angiogenesis [Schor et al 1983; Schor and Schor, 1986, 1988, 2010; Schor and Canfield, 1993; Canfield et al 1986, 1990; Schor et al 2001, 2005].

Angiogenesis is required for tumour growth and metastasis in experimental animal models. This formation of new blood vessels takes place through a series of complex events summarised as follows: (i) In response to an angiogenic factor, the “resting” endothelial cells lining the lumen of a mature microvessel become activated and migrate through the vascular

basement membrane into the surrounding 3D tissue stroma. (ii) Once in this new environment, the endothelial cells adopt a spindle-shaped "sprouting" morphology. (iii) The sprouting cells migrate towards the source of the angiogenic factor; they aggregate in a head-to-tail fashion, forming multicellular sprouts which eventually become a new (angiogenic) blood vessel in functional continuity with the parental (mature) blood vessel. Angiogenesis is regulated by interdependent interactions between endothelial and peri-endothelial stromal cells, mediated by both soluble factors and extracellular matrix molecules (Fig 7).

Cell activation and sprouting cell aggregation may be modelled in vitro by plating vascular endothelial cells on the surface of a 3D collagen gel. Under standard culture conditions, these cells proliferate to form a homogeneous monolayer of "resting" or "cobblestone" cells reminiscent of the resting cell monolayer lining the mature vessel lumen. In the absence of exogenous angiogenic factors, the resting cell monolayer is stable for prolonged periods in culture. Addition of an angiogenic factor results in the induction of a second layer of cells displaying a spindle-shaped "sprouting" or "angiogenic" morphology which form a network of multicellular aggregates under the resting cell monolayer. These cells migrate through the extracellular matrix deposited by the resting cells and into the underlying 3D collagen gel. A homogenous culture of sprouting cells (in the absence of a resting monolayer) may be formed by plating the cells directly within a 3D collagen gel, rather than on its surface. In this case the cells spontaneously and uniformly adopt the sprouting cell morphology and proceed to form multicellular aggregates in the absence of exogenous angiogenic factors (Figure 7D). These observations raise the possibility that spouting cells within a 3D matrix may express an endogenous angiogenic factor. That is in fact the case: Sprouting cells in culture express MSF as indicated by the presence of MSF bioactivity in their conditioned medium (Fig 8) and by immunostaining (Fig 9). In contrast, conditioned medium from the resting "cobblestone"

cells contains an inhibitor of MSF (endothelial MSFI) (Fig 8) [Schor and Schor, 1986, 1988, 2010].

The appearance of sprouting cells underneath a resting monolayer may be induced by the addition of serum or diverse angiogenic factors (e.g. MSF, FGF2, PDGF, VEGF) to the culture medium. Irrespective of the factor(s) used, incubation with MSF-function-neutralising antibodies (Mab pepQ) results in the apoptosis of the sprouting cells, without affecting the resting cell monolayer (Fig 9). Homogeneous cultures of sprouting cells (formed by plating the cells within a 3D gel in the absence of exogenous angiogenic factors) also undergo apoptosis when incubated with Mab pepQ (not shown). These results indicate that MSF is both an angiogenic factor and also a survival factor for angiogenic endothelial cells.

Normal early passage keratinocytes, as well as an immortalised keratinocyte cell line (HaCaT) also form a monolayer of cobblestone appearance. Like the cobblestone endothelial cells, keratinocytes produce an inhibitor of MSF (epithelial MSFI). Endothelial MSFI and epithelial MSFI were initially identified by their identical inhibitory activity in the 3D collagen assay (Fig 8). A protocol was devised to purify MSFI from serum-free conditioned medium collected from confluent monolayers of epithelial (HaCaT) and endothelial cells. Every step of the purification was monitored by the assessment of MSFI bioactivity in the 3D collagen migration assay using rhMSF-stimulated fibroblast migration as the positive control (Fig 8). Following the same protocol for their purification, endothelial MSFI and epithelial MSFI show certain similar characteristics, however, they are not the same molecule. Epithelial MSFI has been identified as neutrophil gelatinase-associated lipocalin (NGAL / lipocalin 2). Endothelial cells do not produce NGAL; their MSFI has been identified as

insulin growth factor-binding protein-7 (IGFBP-7 / IGFBP-rP1) [Jones et al 2007; unpublished].

NGAL and IGFBP-7 are secreted pleiotropic molecules, widely distributed in tissues and implicated in a number of physiological and pathological processes. They are present in tumours but their role and prognostic value is not clear, as contradictory results indicating opposing roles (tumour promoter and tumour suppressor) have been published for both NGAL [Fernandez et al 2005; Feng et al 2016; Bauvois et al 2018; Guo et al 2020] and IGFBP-7 [Burger et al 2005; Sato et al 2015; Li et al 2019; Jin et al 2020].

3b. Modulation of MSF expression

The expression of MSF is not invariant; MSF may be switched on and off in cell culture, usually in a reversible way. For example, a transition from a cobblestone to a sprouting endothelial phenotype, accompanied by the on/off expression of MSF and MSFI, can be implemented multiple times simply by changing cell attachment from a 2D to a 3D environment and vice-versa.

Using a co-culture of human blood monocytes with conditioned media from different cancer cell lines, Solinas et al [2010] found that monocytes were induced to express MSF. M-CSF, IL-4, and especially TGFβ strongly promoted MSF production, whereas LPS, TNF-a, and IFN-g did not. MSF expression was specifically associated with M2 macrophages, as its expression was significantly downregulated in M2 cells that had been reverted to M1 (by treatment with LPS or IFN-g). Conversely, MSF was strongly upregulated in M1 cells skewed toward the M2 phenotype by IL-4.

In the case of foetal skin fibroblasts, a proportion of cells (both cloned and uncloned) which show a foetal migratory behaviour and secrete bioactive MSF in early passages, switch off MSF production upon passage in culture, and this occurs many passages before the cells become senescent [Schor and Schor 1987a].

Normal adult skin fibroblasts, which do not typically produce MSF, switch-on MSF expression following a brief transient exposure to TGFβ1 when cultured on certain matrix substrata (such as denatured type I collagen or fibrin, so called "wounded" substrata) [Kay et al 2005; Schor et al, 2012]. These activated fibroblasts maintain MSF expression for the entire duration of their in vitro lifespan in the absence of TGFβ1. However, they can be persistently de-activated (switching-off MSF expression) by a second transient exposure to TGFβ1, provided that the cells are growing on a "healthy" matrix of native type I collagen (Fig 10). This matrix-dependent "on-off" switch may be repeated numerous times and strictly requires the concerted action of TGF-β1 and the appropriate matrix. For example, exposure of previously activated fibroblasts to TGF-β1 will not switch-off MSF expression if the cells are growing on a "wounded" matrix.

TGFβ1 is not the only activator of MSF expression in normal adult skin fibroblasts. A transient exposure of these cells to 5-azacytidine (5-azaC, a pharmacological agent inducing changes in gene expression by CpG island demethylation), results in a persistent switch-on of MSF expression [Schor et al 2012]. As is the case with MCF-7 carcinoma cells (Table 2), transfection of adult skin fibroblasts with dominant-negative p53 also results in the persistent switch-on of MSF expression. It is of particular interest that MSF expression can also be switched-on by a transient exposure to either MSF itself or MSF-related bioactives (Gel-BD,

IGDS peptide, see section 4) in an apparent process of auto-activation [Schor et al 1996, 1999; unpublished]. Irrespective of the activator, induced expression of MSF in fibroblasts is reversible and may be switched off by a subsequent exposure of the activated cells to TGF-β1 when the cells are grown on a native collagen substratum [Schor et al 2012].

Foetal fibroblasts and fibroblasts from cancer patients express MSF constitutively. MSF expression by these cells may also be persistently switched-off by a transient exposure to TGFβ1 when the cells are growing on a native type I collagen substratum [Schor et al, 2012].

Tumour cells can be induced to upregulate MSF expression by cytotoxic/carcinogenic agents. Yoshino et al [2007] reported that exposure of a bronchioloalveolar carcinoma cell line (A549) to the tobacco carcinogen benzo[a]pyrene results in the induction of MSF expression. Using RNA microarray analysis, they found changes in 5491 genes as a result of the treatment; the increase in MSF expression was ranked number 1 (38.9- fold) among these. Furthermore, MSF overexpression was permanent, still ranked number 1 (50.1-fold) eight weeks after the retrieval of benzo[a]pyrene from the cultures.

3c. Presence of MSF in tissues ex vivo. Diagnostic and prognostic value.

The presence of MSF in excised human tissues confirms the results obtained in tissue culture. MSF is strongly expressed in the majority of tumours examined to date, including tumours of the breast, skin, lung, salivary gland, oral mucosa, colon, oesophagus, prostate and brain. By comparison, MSF expression is low or negligible in their normal tissue counterparts [Schor et al, 2003; Houard et al 2005; Hu et al, 2009; Aljorani et al 2011, Perrier et al 2012; Deng et al 2013; unpublished]. MSF is constitutively expressed in foetal skin and in certain oral tissues.

For example, MSF is present in the papillary region of the gingival mucosa and in the tongue, but not in the buccal mucosa. In histologically normal salivary glands adjacent to tumours, MSF is expressed only in the excretory and striated ducts [Irwin et al 1994; Schor et al 2003; Aljorani et al 2011; unpublished].

MSF is not expressed in the majority of adult skin from healthy donors but it is transiently expressed during wound healing (Fig 11) [Schor et al, 2003, 2009, unpublished]. As indicated in Fig 6 and Table 1, we have developed two types of MSF-specific identification antibodies: VSI antibodies recognise total MSF (MSF+aa and MSF-aa isoforms) whereas TYN antibodies only recognise MSF-aa. A comparison of immunostaining with both antibodies showed that MSF is not present in the majority of adult skin tissues from healthy individuals, whereas it is frequently present in the normal skin obtained from uninvolved sites of cancer patients. When present, MSF+aa is the only isoform expressed in adult skin. During wound healing, MSF+aa is strongly expressed by day 7 in both epithelial and stromal cells (Fig 11) whereas it is no longer present by day 21, when the wound is fully healed (not shown) [Schor et al, 2003, 2009].

In tumours, MSF mRNA and protein are expressed by the tumour cells and by cells of the TME, including fibroblasts, microvascular endothelial cells, macrophages and, occasionally, other cells of the inflammatory infiltrate [Schor et al, 2003;Houard et al 2005; Hu et al, 2009;Solinas et al 2010;Aljorani et al 2011, Perrier et al 2012; Deng et al 2013]. Different patterns of MSF expression have been observed, including homogenous expression by the different cell populations throughout the tumour, focal expression in specific “hotspots” and, rarely, diffuse or local expression by only the tumour cells. A close association between MSF mRNA and protein expression has been noted using in-situ hybridisation or RT-PCR

and IHC in skin and breast tissues [Schor et al 2003; Houard et al 2005]. However, using RT-PCR and IHC, no association was found in non-small cell lung cancer (NSCLC) [Deng et al 2013].

Comparisons between normal tissues, benign and malignant tumours indicates that MSF expression is associated with cancer development. MSF mRNA and protein levels were markedly higher in NSCLC than in matched adjacent tumour-free tissue. Additionally, the level of MSF protein expression in NSCLC samples was significantly higher in stage III and IV than in stage I and II [Deng et al 2013], indicating that MSF is a biomarker of tumour progression in these tumours.

In breast tissues, MSF mRNA and protein expression is negligible in normal breast and significantly higher in breast tumours [Schor et al 2003, Houard et al 2005, Perrier 2012]. Furthermore, we have observed a step-wise increase in MSF expression associated with disease progression in these tissues. Cell culture studies had initially indicated that intra-lobular fibroblasts isolated from histologically normal breast adjacent to a tumour (NB-T) expressed MSF, whereas such fibroblasts isolated from normal breast tissue from reduction mammoplasties (NB) did not [Schor et al, 1994; Schor and Schor 2001] (Fig 2). This finding has been confirmed in a semi-quantitative IHC study assessing MSF expression in archival breast tissues [Perrier et al 2012]. Although NB and NB-T are both histologically normal, they differ regarding MSF expression, which is significantly higher in the latter. This difference may reflect the occurrence of “field cancerisation”, here defined as the presence of functionally aberrant cells in the absence of overt histological abnormalities. MSF expression is similar in benign tumours (B) and NB-T and is highest in malignant tumours (T) (Fig 12). When assessed separately, epithelial and stromal MSF indices in both

histologically normal tissues (NB, NB-T) were significantly and directly correlated; in contrast, such an association was not observed in tumours (B, T). These data indicate that epithelial and stromal MSF may be differently regulated and may carry different diagnostic or prognostic value [Perrier et al 2012].

MSF is also a biomarker of salivary gland tumour (SGT) progression. SGTs are a morphologically and clinically diverse group which present a challenge in terms of diagnosis and clinical management. We have reported that MSF expression increased significantly in a step-wise fashion from normal salivary gland to benign and malignant tumours (Fig 12) [Aljorani et al 2011]. More detailed analyses revealed a significant difference between malignant and benign tumours in all the cellular compartments examined (tumour cells, fibroblasts, blood vessels and inflammatory cells). In the histologically normal salivary gland tissue, some MSF expression was present in the ductal epithelium, whereas the blood vessels and serous cells were negative. Normal salivary gland tissue from healthy adults was not available in this study; therefore it is not possible to know whether MSF expression by the normal appearing salivary gland (adjacent to tumours) represents field cancerisation [Aljorani et al 2011].

A comparison of immunostaining with VSI and TYN antibodies in breast and oral tumours shows variable patterns of staining. Both MSF+aa and MSF-aa isoforms can be present in these tumours, but MSF-aa appears to be the main isoform produced by tumour and tumour-associated stromal cells (Figure 13, Table 3). Therefore, our results (Figs 11 and 13) suggest that MSF-aa is a tumour-associated biomarker, whereas MSF+aa may be expressed by both normal and malignant tissues. Nevertheless the presence of both isoforms may have some functional significance. For example, in a small retrospective study with oral squamous cell

carcinomas (OSCC) we found that high MSF expression was significantly associated with shorter survival times. That was the case for both total MSF and MSF-aa; however analysis of the survival curves showed that the level of significance was stronger when both isoforms were assessed (Fig 14).

A retrospective study with breast carcinomas suggests that MSF expressed by tumour cells may play a different role than MSF expressed by the cells of the TME. In this study, the expression of MSF-aa was assessed separately in the carcinoma cells and in the stroma by image analysis. With a follow-up of 10 years, the survival curves showed that high MSF-aa expression in the stromal compartment was significantly associated with shorter survival times. MSF-aa expression by the carcinoma cells followed the same trend, but did not reach statistical significance (Table 4).

4. MSF BIOACTIVITIES, MODULATION AND MODE OF ACTION

MSF is an unusually potent effector molecule. It alters the TME by inducing cell migration, angiogenesis and changes in the extracellular matrix composition and metabolic pathways. Most of the available information about MSF bioactivities comes from tissue culture studies concerned with its motogenic activity.

4a. Motogenic activity. Permissive and non-permissive matrices

Both exogenous and endogenous MSF stimulate cell migration/invasion in all normal and tumour cell lines tested. Normal cells examined include fibroblasts, human and bovine vascular cells (endothelial cells, pericytes), keratinocytes and monocytes [Schor et al 2003; Schor and Schor 2010; Solinas et al 2010]. Exogenous MSF stimulates the migration of tumour cells which are either non-MSF-producers (Table 2) or produce MSF in moderate amounts (Fig 15) [Schor et al 2003; Houard et al 2005; Schor and Schor 2010; Solinas et al 2010; Aljorani et al 2011; Carito et al 2012; unpublished]. The migration of high MSF-producing tumour cells (e.g. MDA-MB435; HaCaT-MET) is not increased further by exogenous MSF but is inhibited by MSF-function-neutralising Mab pepQ [Schor et al 2020]. Likewise, the migration of fibroblasts and tumour cells is significantly increased by transfecting the cells with MSF [Houard et al 2005; Carito et al 2012; Deng et al 2013].

Dose-response to exogenous MSF and the extent of stimulation depend on the cell type and assay conditions (Fig 15). The dose-response of fibroblasts, endothelial cells, pericytes and keratinocytes follows a bi-phasic (bell shape) curve, with maximal stimulation at 10-100pg/ml in the 3D collagen gel assay and at higher concentrations (100pg/ml to 10ng/ml) in the transmembrane assay. In contrast, the stimulation of tumour cell migration reaches a plateau at approximately 10ng/ml. In the case of fibroblasts, migration through native type I collagen is 2-3-fold higher in the 3D collagen gel than in the transmembrane assay. The response of endothelial cells, pericytes and fibroblasts is matrix-dependant, so that matrix macromolecules may be described as permissive or non-permissive for the migration-stimulating effects of MSF and related compounds (Schor et al 1996, 1999, 2003, Schor and Schor 2010). Permissive matrices include native type I collagen, type IV collagen, matrigel, fibrin, cellular FN and plasma FN. Non-permissive matrices may be different for different cell types. For example, denatured collagen (gelatin) is permissive for endothelial and

tumour cells, but not for fibroblasts or pericytes (Fig 15) [Schor and Schor 2010]. We have investigated the role of thrombospondin-1 (TSP-1) and hyluronic acid (HA) matrices by using these molecules mixed with native type I collagen in various proportions. TSP-1 and HA rendered the collagenous matrices non-permissive for the motogenic effects of MSF, even at the lowest concentration tested (10%), which had no effect on cell attachment. In parallel experiments, the same collagenous matrices containing TSP-1 and HA were permissive for the motogenic effect of PDGF and serum. It is of interest that both TSP-1 and HA had different effects when presented to the cells in a soluble form [Schor et al 1989; Ellis et al 1992; Schor, 1994; Motegi et al 2002, 2008; unpublished].

4b. Lack of mitogenic activity while promoting tumour growth

In our laboratory, the possible effects of MSF on cell proliferation have been assessed for most of the cell lines examined for migration. Unlike other motogenic factors, MSF and MSF-related bioactives do not affect cell proliferation or saturation cell density [Schor et al 1988b, 2020; Deng et al 2013]. Nevertheless MSF significantly increases tumour growth by stimulating angiogenesis [Deng et al 2013] and driving glycolytic metabolism in the TME [Carito et al, 2012].

4c. Bioactive motifs in MSF

The motogenic activity of MSF on fibroblasts resides in its gelatin-binding domain (Gel-BD, Fig 3). Full-length FN and its RGDS-containing cell binding domain (Cell-BD) have been shown to stimulate fibroblast migration in the transmembrane assay, using filters coated with denatured collagen. Under those conditions, Gel-BD and MSF are not active, as denatured collagen is not a permissive matrix for fibroblasts (Fig 15). However, using native collagen as substratum, Gel-BD (like MSF) is a potent stimulator of cell migration, whereas FN, Cell-BD and all other domains of FN (including the Hep1-Fib1 domain, also present in MSF) are inactive [Schor et al, 1996]. Interestingly, a recombinant N-terminal FN fragment (100kDa), including Gel-BD and the first three type-III domains of FN, also lacks motogenic activity, apparently due to intramolecular interactions [Vakonakis et al 2009]. This fragment matches closely a truncated form of FN identified in zebrafish, amphibians, birds, and mammals [Zhao et al, 2001; Liu et al 2003].

The IGD (iso-gly-asp) tripeptide amino acid motif is a highly conserved feature of FN type I modules. MSF contains four such motifs, located in modules I3, I5, I7 and I9. The last two are in the Gel-BD domain. Synthetic IGD peptides (e.g. IGD, IGDS, IGDQ, IGDT) stimulate fibroblast migration with the same matrix-dependence characteristics as Gel-BD and MSF. The scrambled SDGI peptide and the well-characterised RGDS peptide, present in the Cell-BD of FN (Fig 3) were devoid of motogenic activity on native collagen (Schor et al 1999). It should be noted that RGD peptides do not stimulate cell migration when presented to cells in soluble form, but rather inhibit the promotion of cell adhesion and migration (on denatured collagen) mediated by the RGD motif contained within the Cell-BD. The fact that soluble IGD peptides mimic the activity of Gel-BD and MSF was therefore unexpected. We have examined the ability of engineered hydrogels, in conjunction with tethered amino acid motifs, to support cell adhesion and proliferation. Whilst the classical adhesion motif, RGD, supports cell attachment, the motogenic IGD motif does not [Keatch et al 2002].

4d. Cell heterogeneity regarding MSF bioactive motifs

In vitro mutagenesis studies indicate that the stimulation of fibroblast migration by MSF is mediated by the two IGD motifs present in modules I7 and I9. Mutation of IGD motifs in I7 and I9 is sufficient to completely abolish the motogenic response of the cells, whereas mutation of IGD in modules in I3 and I5 do not affect MSF's motogenic activity on fibroblasts. However, the IGD sequences in I3 and I5 are also capable of exhibiting motogenic activity when present within fragments of MSF. Nevertheless, modules I3 and I5 differ from I7 and I9 regarding their requirement of vitronectin for their motogenic activity to be manifest. These results suggest that both MSF and FN contain cryptic bioactive fragments and steric hindrance determines the motogenic activity of these molecules [Schor et al 2003; Millard et al 2007; Vakonakis et al 2009; Ellis et al 2010b].

A second bioactive motif has been identified in MSF. Houard et al [2005] found that the putative zinc-binding motif HEEGH, located in module I8, is required for MSF's fibronectin-proteinase activity and motogenic activity in the breast tumour cell line MCF-7. Mutagenesis of the two histidine residues to phenylalanine (FEEGF) abolished the FN-proteinase activity of MSF and reduced its motogenic activity, suggesting that MSF induces MCF-7 cell migration through both FN-proteinase- dependent and independent mechanisms [Houard et al 2005].

These results suggest that the motogenic activity of MSF is mediated by different motifs on different target cells. This is in fact the case: Mutations in modules I7 and I9 are sufficient to abolish MSF bioactivity on fibroblasts, but not on endothelial cells. In these cells, mutations in I7, I9 and HEEGH partly abolished MSF motogenic activity but full inhibition required the mutation of IGD in all four modules (I3,I5,I7,I9) and the mutation of HEEGH in I8. In contrast the migration of three oral tumour cell lines examined required the integrity of all the motifs investigated and was abolished by mutation of any one of them (Fig 16). It is possible that different mechanisms may apply to other tumour cell lines.

4e. Motogenic activity of MSF isoforms. Interactions with MSFIs

MSF+aa and MSF-aa display identical motogenic activities on target fibroblasts, endothelial cells and tumour cells. Both isoforms require the presence of native type I collagen for fibroblast response, but not for the response of endothelial or tumour cells. However, their interaction with NGAL demonstrates distinct functionality. As discussed in section 3, inhibitors to MSF secreted by keratinocytes and endothelial cells have been identified as NGAL and IGFBP-7, respectively. NGAL inhibits cell migration stimulated by MSF+aa to baseline levels, but has no effect on MSF-aa stimulated migration (Fig 17) [Jones et al 2007; unpublished]. IGFBP-7 inhibits the motogenic effect of both MSF+aa and MSF-aa in the 3D collagen gel, but not in the transmembrane assay. Function neutralising Mab pepQ abolished the activities of both MSF isoforms (Fig 17).

4f. Evidence of distinct MSF isoforms present in serum and tumour cell lines

We have previously reported that MSF bioactivity is present in the serum of 23/26 breast cancer patients compared to only 2/20 of healthy age-matched controls, a value consistent with the incidence of MSF-secreting skin fibroblasts originally detected in these individuals [Picardo et al, 1991]. Recent studies have confirmed and expanded these results. Serum samples were initially fractionated by size-exclusion chromatography. MSF motogenic activity was assessed and found to be located in those fractions corresponding to approximately 70kDa in most cancer patients and rarely in controls. In total, MSF bioactivity has been found in the serum of 48/53 breast cancer patients and 3/30 of matched controls. However, ELISA analysis of the fractionated serum revealed immunoreactive MSF,

coinciding with the 70KDa fractions, in both control and cancer patient sera (Fig 18; unpublished). Further analysis of the fractions containing motogenic activity involved separation by SDS-PAGE. The gels were then sliced, eluted and further analysed as previously described [Jones et al 2007] (Fig 18). Under non-reducing conditions, bioactive MSF was found in the serum of cancer patients, but not in serum from controls. However, under reducing conditions both patient and control sera contain bioactive MSF as well as an inhibitor of MSF (serum MSFI). Functional studies indicated that serum MSFI inhibited the motogenic activity of MSF isolated from control serum as well as MSF+aa. On the other hand, serum MSFI did not affect the motogenic activity of MSF isolated from patient serum or MSF-aa.

Serum MSFI has been identified as NGAL. Recombinant human NGAL exhibited the same effects as serum MSFI. That is: MSF isolated from control sera was (like MSF+aa) inhibited by rhNGAL whereas MSF isolated from cancer patient sera was (like MSF-aa) not inhibited by rhNGAL. The motogenic activity of both control-MSF and cancer patient-MSF was abolished by function neutralising Mab pepQ (Fig 19). These results suggest that MSF+aa is in fact present in serum of control non-cancer patients but is not able to manifest its motogenic bioactivity as a consequence of its association with NGAL. In contrast, MSF-aa is present in the serum of cancer patients and NGAL, also present, does not affect its bioactivity.

The motogenic activity of MSF secreted by tumour cells lines was also completely abolished by Mab pepQ whereas the inhibition by rhNGAL varied from 100% inhibition (i.e. reversal to baseline migration) in the case of A549 cells to no effect in the case of HaCaT-MET cells. MSF secreted by most tumour cells was partly inhibited (50-80%) by rhNGAL (Fig 19; unpublished), suggesting that it consists of a mixture of MSF+aa and MSF-aa.

4g. Angiogenic activity

In tissue culture, MSF induces endothelial cell activation, as manifest by the formation of a sprouting (angiogenic) phenotype. MSF also appears to function as a survival factor for the sprouting cells (Figs 7-9). In vivo, MSF and IGDS induced angiogenesis in several animal models including the chick embryo yolk sac membrane, subcutaneous implants in rats, mice and pigs and wound healing in diabetic mice [Schor et al 2005; Schor and Schor 2010;

unpublished]. In the yolk sac assay, both MSF and IGDS stimulate angiogenesis over a broad range of concentrations (0.5-500ng/application). The angiogenic response to MSF (but not to IGDS at the concentrations tested), followed a clear bi-phasic dose-response, similar to the motogenic effect in tissue culture (Fig 20). Hu et al [2009] employed a functional antibody library-based proteomic screen to identify proteins that participate in angiogenesis and might be used as therapeutic targets for tumour-related angiogenesis. Using this method, they identified MSF as a critical angiogenic factor driving oesophageal cancer progression. Treatment with antibody to MSF significantly suppressed tumour growth in mice through inhibition of angiogenesis.

4h. Integrin binding and downstream signalling

Cellular response to MSF and IGD peptides requires maintenance of integrin αvβ3 functionality: The motogenic activity of MSF/IGD on fibroblasts, endothelial and oral tumour cells is abrogated by an antibody to αvβ3. This activity is also inhibited by small molecule inhibitors of the PI3 kinase/AKT signal transduction pathway. MSF inhibits AKT phosphorylation and activity in human fibroblasts and endothelial cells. In fibroblasts, treatment with either MSF or the AKT inhibitor Akti-1/2, stimulated migration into 3D collagen gels to a similar extent. This is in contrast with the effects of most motogenic / angiogenic factors, which stimulate both the phosphorylation of AKT and its activity [Schor et al 1999; Ellis et al 2010a].

Carito et al [2012] generated an immortalized fibroblast cell line that recombinantly overexpresses MSF. Overexpression of MSF is sufficient to confer myofibroblastic differentiation. MSF activates Cdc42 and the inflammation-associated transcription factor NFκB, resulting in the onset of autophagy/mitophagy, thereby driving glycolytic metabolism (L-lactate production) in the TME. As a result, MSF transfected fibroblasts significantly increased tumour growth, by up to 4-fold.

4i. Remodelling the microenvironment

Besides altering the glycolytic metabolism [Carito et al, 2012], MSF can also change the composition of the extracellular matrix in the TME by its FN-protease activity [Houard et al 2005] and by the stimulation of HA synthesis.

The synthesis of HA by fibroblasts is associated with the level of endogenous MSF and the migratory phenotype of the cells. Exogenous MSF stimulates adult skin fibroblasts to synthesise high molecular weight HA, which is secreted in a soluble form in the culture medium. MSF-stimulated migration is suppressed by hyaluronidase [Chen et al 1989; Schor et al 1989; Ellis et al 1992].

5. CONCLUSIONS AND FUTURE WORK.

MSF is a truncated onco-foetal isoform of FN that shows unique characteristics and activities not present in any other FN variant. MSF is present in 70-80% of all tumours examined, being expressed by the tumour cells as well as by fibroblasts, endothelial cells and macrophages in the TME. Acting as a cytokine, by both paracrine and autocrine mechanisms, MSF alters the matrix and cellular composition of the microenvironment, promotes cell mobility/invasion, induces angiogenesis and cell differentiation. Unlike other motogenic and angiogenic factors, MSF does not affect cell proliferation but it stimulates tumour growth through its angiogenic effect and downstream mechanisms. It is therefore not surprising that MSF expression in tumours is associated with disease progression and lower survival rates. Practically every other finding about MSF has been unexpected. MSF has been studied by very few groups outside our own [Houard et al 2005; Yoshino et al 2007; Hu et al, 2009; Solinas et al 2010; Carito et al 2012; Deng et al 2013]. These groups have contributed most valuable information about MSF bioactivities on different target cells and experimental systems. However, much remains to be investigated about the role of MSF in the TME and its effects on the different cells and cellular interactions in this environment. A few of the pending questions are related to the results discussed below.

5a. Cell motility and MSF auto-activation.

Cell motility is an essential component of normal development, inflammation, tissue repair, angiogenesis, and tumour invasion. New concepts for the understanding of cell motility have emerged from the use of different assays and from the separation of cell motility into individual components. A most unexpected observation is that cell migration induced by diverse motogenic factors (MSF, IGD peptides, PDGF-AB, TGF-β1, EGF, TGF-α) may be resolved into two stages: (i) cell "activation" by a transient exposure to the motogen, and (ii) the subsequent "manifestation" of an enhanced migratory phenotype in the absence of motogen. The cell activation and manifestation stages for each motogen may be mediated by distinct matrix-dependent mechanisms and may involve different signal transduction pathways [Schor et al 1996, 1999, 2012; Ellis et al 2007; Jones et al 2007]. The temporal separation of cell motility into activation and manifestation stages has important implications for our understanding of the complex interactions taking place during tumour invasion, since the effects of a given factor on a particular cell type can persist long after a transient

exposure. MSF expression is induced by MSF and other factors but the precise mechanisms are not understood.

5b. Systemic expression of MSF. Relevance to tumour development.

MSF differs from other tumour markers in that it is expressed by both tumour and stromal cells, it exerts a direct effect on these same cells and it is systemically expressed in approximately 50% of cancer patients and their disease-free relatives. This epithelial-stromal pattern of expression in tumours puts MSF in the unique position of potentially promoting tumour progression from both the "seed" and the "soil" perspectives. We initially suggested that: (i) the presence of persistent foetal-like (MSF-producing) fibroblasts in the adult may lead to the development of an epithelial tumour by altering homeostatic epithelial-mesenchymal interactions, and (ii) perturbation of such interactions could enhance susceptibility to cancer development and accelerate cancer progression [Schor et al, 1987; Schor and Schor, 1987a,b, 1997; Schor, 1995]. These concepts are now widely accepted and many research projects are concerned with the role and therapeutic potential of the TME and cancer-associated fibroblasts (CAFs) [Gascard and Tlsty, 2016; Olumi et al 1999; Mishra et al 2019; Sahai et al 2020, Prazeres et al, 2020]. The origin of CAFs is not entirely clear. There is evidence that they may be initiated by genetic lesions and epigenetic mechanisms, either independently of lesions in the epithelial cells [Moinfar et al,2000] or preceding them [Schor and Schor, 2001, 2011].

CAFs include heterogeneous cell populations. Some CAFs are MSF-producers, but MSF-producing fibroblasts are not necessarily CAFs, being present in distant sites as well as in 10-12% of healthy controls.

MSF production is not the only aberrant foetal-like characteristic observed in fibroblasts from distant sites [Schor and Schor 2011]. Regarding their origin, we have speculated that (i) distinct subpopulations of foetal-like fibroblasts pre-exist in the healthy adult, (ii) the foetal-like characteristics of these cells do not result from an acquired mutation, but reflect epigenetically regulated changes in gene expression analogous to those which occur during embryonic development and (iii) their relative numbers may be increased, both locally and systemically, as a consequence of clonal expansion and/or epigenetic induction in response to both internal and environmental cues [Schor and Schor, 1987; Schor et al, 1987, 2011]. The possible role of such aberrant systemic fibroblasts remains difficult to conceptualise. At one end of the spectrum is the notion that these fibroblasts make no direct contribution to cancer

pathogenesis, but provide a biomarker of exposure to factors (endogenous and/or environmental) which induced similar changes to stromal and epithelial cells. At the other extreme, it is also possible that these cells do indeed precede and make a direct contribution to cancer pathogenesis, possibly by generating bioactive soluble factors (such as MSF) or by providing a receptive microenvironment (e.g. hyluronan-rich) to support the development of a primary tumour or the seeding of metastatic tumour cells. Further studies are required to discriminate between these possibilities.

5c. Expression of MSF isoforms and modulators in development and in tumours. Interactions and value as tumour biomarkers

MSF expression can be switched on and off by precise interactions between environmental factors (including the presence of other soluble molecules) and the nature of the extracellular matrix in contact with the cells. Likewise, the cellular response to MSF is dependent upon the extracellular matrix, the isoform of MSF expressed and the presence or absence of other soluble factors such as TGFβ1, NGAL and IGFBP-7. TGFβ1 may promote or inhibit MSF expression whereas NGAL and IGFBP-7 are inhibitors of MSF activity.

MSF-aa only differs from MSF+aa by a 15 amino acid deletion; they were both cloned from an immortalised foetal fibroblast cell line and both contain the same unique sequence that is encoded by an intronic DNA sequence downstream of FN exon III-1a. Their presence in serum, tumour sections and cells in tissue culture is shown by their differential immunoreactivity and interaction with NGAL. Further studies are needed to quantify MSF mRNA and protein in these samples, to determine how MSF isoforms are produced and to understand their interaction with their inhibitors and activators.

MSF is a tumour biomarker. The diagnostic and prognostic value of MSF may be enhanced by the combined analysis of individual MSF isoforms and MSF inhibitors.

Further studies are also needed to understand the expression and role of MSF in development. In a preliminary study using IHC, MSF was found to be expressed in a number of tissues during murine development, including skin, spinal cord, brain, skeletal muscle, heart and gut (unpublished). Only foetal human skin has been examined.

5d. MSF bioactivities and possible target for anti-tumour therapy

The full extent of MSF expression and bioactivities in the TME remains to be elucidated. For example, we have observed MSF expression in the central nervous system during murine development (preliminary data); considering the presence of sensory nerves in the TME and their effect on cancer progression [Prazeres et al 2020], it will be interesting to investigate the possible role of MSF on such nerves. Based on our current knowledge, the diversity of MSF-producer cells, target cells and bioactivities suggest that MSF may drive tumour progression by different mechanisms, including the induction of angiogenesis. Angiogenesis may be induced by many factors and is controlled by multi-component regulatory networks. This intrinsic redundancy will make it difficult to inhibit tumour-induced angiogenesis by focussing on only one candidate angiogenic factor. Hu et al [2009] found that MSF is a critical angiogenic factor in oesophageal cancer and treatment with antibody to MSF suppressed tumour growth through inhibition of angiogenesis. We have found that sprouting endothelial cells can be induced by many angiogenic factors in tissue culture, but irrespective of the stimulus, sprouting cells secrete MSF, which is critical for their survival. MSF therefore appears to provide a convergent anti-angiogenesis target. Angiogenesis is essential for tumour growth and metastasis in experimental tumours and certain types of human tumours. However, it may not be required for tumours developing in highly vascularised tissues like the breast or the lung [Pazouki et al 1997; Chandrachud et al 1997; Pendelton et al 1998; Schor et al 1998c,d]. Carito et al [2012] found that fibroblasts overexpressing MSF promoted tumour growth through glycolysis, independently of angiogenesis. In all systems tested, MSF increases cell motility, which is essential for tumour dissemination. MSF activity may be neutralised with antibodies or by other means [Schor et al 1999; Hu et al 2009; Ellis et al 2010a; Carito et al 2012]. The ability to switch off MSF expression in tissue culture [Schor et al 2011] points to another potential therapeutic strategy. That is, it may be possible to switch off MSF expression in tumours by genetic or pharmacologic intervention.

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TABLES

TABLE 1

Peptide used as antigen	Ab code	Reactivity of Abs
VSIPPRNLGY 10 mer, MSF-unique C-terminus	VSI	Mab and Pab recognise MSF+aa and MSF-aa. Do not recognise FN, Gel-BD or Hep 1/Fib-1 domains.
TNEGVMYRIGDQWDKQHDMGH 21-mer, IGD-containing peptide in module I-7	pepQ	Mab recognise MSF+aa, MSF-aa and Gel-BD. Do not recognise FN.
TYNDRTDSTTSNY 13 mer, present in MSF-aa, II-1. In MSF+aa these amino acids are adjacent to the sequence deleted in MSF-aa (6 before and 7 after)	TYN	Mab and Pab recognise MSF-aa. Do not recognise MSF+aa, FN or Gel-BD

Table 1. Overview of the antibodies raised and results obtained.

The peptides indicated were used as antigens to raise monoclonal (Mab) and polyclonal (Pab) antibodies; these were then tested for reactivity with MSF+aa, MSF-aa, full-length fibronectin (FN) and the FN domains Gel-BD and Hep 1/Fib-1, which are also present in MSF (Fig 3).

TABLE 2

Cell line - tumour of origin	Presence of MSF		Reference
	mRNA	**protein**	
MDA-MB435-breast	yes	yes	1
MDA-MB231-breast	yes	yes	1, 2
BT549-breast	yes	yes	2
Hs578T-breast	yes	yes	2
A549-lung	yes	yes	1, 3
HaCaT-MAL-skin	yes	yes	1
HaCaT-MET-skin	yes	yes	1
HSG-salivary gland	nd	yes	1
TYS-salivary gland	nd	yes	1
HSY-salivary duct	nd	yes	1
BHY-oral SCC	nd	yes	1
HNt-oral SCC	nd	yes	1
B88-oral SCC	nd	yes	1
SAS-HI-oral SCC	nd	yes	1
T47D-breast	NO	NO	2
T47D-breast	yes (low)	yes	1
MCF7-breast	NO	NO	1, 2
MCF7-p53	yes	yes	1
HT49-colon	NO	NO	1
PANC1-pancreas	nd	NO	1

Table 2. MSF expression by tumour cell lines in culture.

The presence of MSF mRNA was determined by RT-PCR or in-situ hybridisation. MSF protein was detected by IHC and/or by the expression of bioactive MSF [Houard et al 2005; Solinas et al 2010; Deng et al 2013;Schor et al, 2003 2012, 2020, unpublished]. In our laboratory, the presence of bioactive MSF was confirmed by neutralisation with Mab pepQ (MSF-function-neutralising antibodies) and by removal of the motogenic activity by affinity chromatography to MSF-identification antibodies (VSI) [Jones et al 2007; Schor et al, 2003 2012, unpublished]. An agreement between mRNA and bioactive protein expression was

found in all tumour lines examined. Results from different laboratories agreed regarding MDA-MB231, A549 and MCF7 cell lines, but not T47D.

(1) Results from our laboratory [Aljorani et al 2011, unpublished] (2) Houard et al 2005
(3) Deng et al 2013 SCC: squamous cell carcinoma; nd: not done

TABLE 3

Staining with Ab		**Comparison of MSF grades (VSI v TYN)**	**Pattern (no.) and specimens showing the pattern (n/79)**
VSI	**TYN**		
+ve	**+ve**	The same grades with both	no.1 (53)
+ve	**+ve**	Lower grades with TYN	no.2 (3)
+ve	**-ve**	No staining (grade 0) with TYN	no.3 (12)
-ve	**-ve**	No staining (grade 0) with both	no.4 (11)

Table 3. Expression of MSF isoforms in breast tumours.
Serial sections of archival breast carcinomas (n=79) were stained with Mab VSI and Mab TYN. Duplicate sections for each specimen and antibody were assessed by 2-3 independent observers and the final results were obtained by consensus. MSF staining was graded by comparison to calibration slides as negative (grade 0), weak (grade 1), moderate (grade 2) or strong (grade 3). The results are summarised in the Table as positive (**+ve;** grades 1, 2 and 3) or negative (**-ve;** grade 0). Mab VSI recognises total MSF (MSF+aa and MSF-aa isoforms) whereas Mab TYN only recognises MSF-aa, as indicated in Fig 6 and Table 1. Examples of the different staining patterns are shown in Fig 13. Our interpretation of the results obtained is as follows:
Pattern no.1: MSF-aa is likely to be the only isoform present in 67% of the tumours.
Pattern no.2: both MSF+aa and MSF-aa are present in 4% of the tumours.
Pattern no.3: MSF+aa is the only isoform present in 15% of the tumours.
Pattern no.4: Neither MSF+aa or MSF-aa are expressed in 14% of the tumours.

TABLE 4

		Comparison of survival curves (p value)	
MSF index / division for survival analysis	**survival analysed**	**MSF in the tumour stroma**	**MSF in the carcinoma cells**
% area stained / quartiles	Overall	0.01**	0.09*
% area stained / median	Overall	0.02**	0.13
final score (% area x intensity / median	Overall	0.06*	0.25
final score (% area x intensity / median	Disease-free	0.005**	0.81

Table 4. Analysis of of breast cancer patient survival according to MSF expression. Differential prognostic value of MSF-aa expression in the tumour stromal cells. Archival breast carcinoma specimens were obtained from a cohort of 71 patients with a follow- up of 10 years. Histological sections were stained with MSF-aa-identification Ab Mab TYN. The level of staining was quantified by image analysis in the carcinoma cells and in the tumour stroma by three MSF indices: (i) % area stained, (ii) intensity of the staining and (iii) final score, a combination of area x intensity. The values obtained were divided either in quartiles or by the median to analyse patient survival. Kaplan-Meier survival curves were compared by Log-rank and Gehan-Breslow-Wilcoxon tests (p values shown).

In the tumour stroma, high MSF-aa expression was associated with poor survival. The results were statistically significant for the percentage of area stained, but not for the intensity (not shown). MSF in the carcinoma cells followed the same trend but it did not reach statistical significance.

** statistical significant at 95% level of confidence. *near significant values (90% level).

FIGURE LEGENDS

Fig 1. Collagen gel migration assay. Detection of migration stimulating activity in the conditioned medium of foetal fibroblasts.

Foetal and adult skin fibroblasts were plated onto the surface of 3D collagen gels at high density, with or without conditioned medium as indicated in the graph. After 4 days incubation, the number of cells on the gel surface and within the 3D gels were counted in 10 fields of duplicate cultures [Schor 1980a]. The number of cells that migrated into the 3D gel is expressed as % of the total cells present (mean ± SD).

F: Foetal skin fibroblasts. **F+aCM**: Foetal fibroblasts with conditioned medium from adult fibroblasts. **A**: Adult skin fibroblasts. **A+fCM**: Adult fibroblasts with conditioned medium from foetal fibroblasts.

Fig 2. Production of MSF by different fibroblast lines in tissue culture.

Fibroblasts, explanted from the different tissues indicated, were initially classified as MSF-producers or non-producers by their migratory phenotype (foetal or adult) and the presence of MSF activity in their conditioned medium [Schor et al, 1988a]. Conditioned medium was obtained from confluent cultures between passages 8-15. Following detection of motogenic activity, the presence of MSF protein in the conditioned medium was confirmed in different cell lines by various means, including abrogation of the.motogenic activity by an MSF-function neutralising antibody, removal of the motogenic activity by affinity to anti-MSF identification antibody and, in some cases, fractionation of the conditioned medium by gel filtration chromatography and further purification of MSF [Gray et al 1989; Jones et al 2007; Schor et al 2003, 2011]. RT-PCR was used to detect MSF mRNA in some cell lines [Schor et al 2003; Kay et al 2005]. The number of fibroblast lines assessed for each type of tissue or experimental group is shown within brackets. The different experimental groups are:

F (n=37): Foetal skin fibroblast. **A** (n=52): Adult skin fibroblasts. ***Ts** (n=55): Fibroblasts derived from malignant breast tumours. Patients had no family history of breast cancer (sporadic cancer). **SkpTs** (n=55): Normal skin from the same patients with sporadic breast cancer (paired to *Ts). **Tf** (n=18): Normal skin from patients with familial breast cancer. **CmTf** (n=12): Normal skin from control donors matched to Tf in age and gender. **Rf** (n=15): Normal skin from first degree relatives of patients with familial breast cancer. **CmRf** (n=12): Normal skin from control donors matched to Rf in age and gender. **Bs** (n=11): Normal skin

from patients with sporadic benign breast disease (no family history of breast cancer). **Bf** (n=9): Normal skin from patients with benign breast disease and family history of breast cancer. ***G** (n=12): Gingival fibroblasts from the papillary tips. **SkpG** (n=10): Forearm skin fibroblasts from the same donors (paired to *G). ***NB-T** (n=13): Intralobular fibroblasts from histologically normal breast tissue adjacent to carcinoma. **SkpNB-T** (n=7): Normal skin from the same breast cancer patients (paired to *NB-T). ***NB** (n=10): Intralobular fibroblasts from histologically normal breast tissue from reduction mammoplasties or adjacent to fibroadenomas. **SkpNB** (n=5): Normal skin from the same non-cancer patients (paired to *NB).
*asterisk indicates fibroblasts derived from tissues other than skin. All the rest originated from skin explants. Arrows denote a direct comparison of fibroblasts from histologically normal breast tissue.

Fig 3. The structure of fibronectin (FN) and MSF. FN is a modular glycoprotein consisting of the following domains: Hep 1/Fib-1 (N-terminal low affinity binding to heparin and fibrin), Gel-BD (binding to gelatin/collagen), Cell-BD (RGD-mediated binding to integrins), Hep-2 (high affinity heparin binding) and Fib-2 (C-terminal fibrin binding site). Each functional domain is composed of three possible homology modules, called type I, II and III. MSF is identical to the N terminus of FN, up to and including the amino acid sequence coded by exon III-1a, with the addition of an MSF-unique (intron-coded) 10 amino acid C-terminus [Schor et al, 2003; Kay et al, 2005]. Two isoforms of MSF have been cloned. These differ solely in terms of a 45bp deletion in exon II-1 and are consequently referred to as MSF+aa and MSF-aa to indicate the retention or deletion of a 15 amino acid sequence in module II-1.

Fig 4. MSF+aa and MSF-aa protein sequences.
The sequences highlighted are: MSF-unique decamer shown in red (sequence present in MSF but not found in FN). MSF 15 amino acid region present in MSF+aa and absent in MSF-aa in blue. IGD and HEEGH motifs in purple and underlined.

Fig 5. Sandwich ELISA for total MSF. Lack of cross-reactivity with full-length fibronectin (FN), Gel-BD and Hep1/Fib1 domains.
The protocol to detect total MSF involved the use of MSF-function-neutralising antibodies (code pepQ) and MSF-specific identification antibodies (code VSI) (Table 1). Standard sandwich-ELISA methods were followed [Schor et al 2020]. In summary: (i) the ELISA plates were coated with "capture antibody" Mab pepQ (10µg/ml);(ii) the antigen was added in serial dilutions from 1µg/ml; (iii) the plates were then incubated with "sandwich antibody" rabbit Pab VSI (1:1000); (iv) the plates were finally incubated with visualisation Pab goat-anti rabbit (HRP conjugate) and the OD was determined.
The graph shows the standard curve generated with rhMSF+aa; the same curve was obtained with rhMSF-aa (not shown). The sensitivity of the assay was 5-10ng/ml. No cross-reactivity was apparent with full-length FN, or with the Gel-BD and Hep1/Fib1 domains, both of which are present in MSF and FN.

Fig 6. Western blot showing the specific identification of MSF-aa with TYN antibody.
rhMSF+aa and rhMSF-aa were loaded into 10% SDS PAGE gels under reducing conditions and immunoblotted with either TYN or VSI antibodies as previously described [Schor et al 2003, 2020; Jones et al 2007]. Antibody details are shown in Table 1. The same amount of protein (150ng) was loaded per track, as indicated. Both proteins were recognised by VSI antibody but only MSF-aa was recognised by TYN antibody.

Fig 7. Endothelial cells may display two reversible phenotypes: resting and sprouting.
A. Sketch of angiogenesis in vivo. Arrows indicate the regulation of angiogenesis by complex interdependent interactions between different cells, soluble factors and the extracellular matrix. **B**. Endothelial cells plated on a 2D surface (such as plastic dishes or the surface of a 3D collagen gels) form a monolayer of "resting" or "cobblestone" appearance. **C**. Addition of an exogenous angiogenic factor results in the formation of "sprouting" cells underneath the resting monolayer. **D**. Endothelial cells plated within a 3D gel matrix spontaneously adopt a sprouting cell phenotype in the absence of exogenous angiogenic factors.

Fig 8. Sprouting endothelia cells secrete MSF whereas resting endothelial cells and keratinocytes secrete an inhibitor of MSF (MSFI) into the culture medium. Serum free conditioned medium (CM) was collected from sprouting endothelia cells (CM-Spr), resting endothelial cells (CM-Rest) and keratinocytes (CM-K). The migration of adult skin fibroblasts in the presence of the indicated CM and control unconditioned medium (control) was tested in the 3D collagen migration assay (Fig 1) in the presence or absence of rhMSF (100pg/ml).

Without MSF. In control cultures, a small percentage of cells migrated into the gels in the absence of MSF. Cell migration was significantly stimulated by CM from the sprouting cells and this effect could be abolished by MSF-function neutralising Ab Mab pepQ (not shown). Migration was not affected by CM from resting endothelial cells or keratinocytes.

With MSF. MSF stimulated cell migration in the absence of CM (control). CM from the sprouting cells did not affect this elevated level of migration, whereas CM from resting endothelial cells and keratinocytes inhibited MSF-stimulated migration to baseline levels. The same results were obtained with CM from **v**arious lines of endothelial cells and keratinocytes.

Fig 9. Differential expression of MSF in resting and sprouting endothelial cells. MSF function-neutralising antibodies induce the selective death of sprouting cells.

A, B. Human endothelial cells were plated on and within 3D collagen gels [Schor, Schor and Allen 1983; Schor et al 2001]. The gels were fixed, paraffin-embedded and histological sections were stained with MSF-specific antibody Mab VSI (section 3). **A.** Cells were plated on the surface of the gel only. The resting (cobblestone) endothelial monolayer (arrow) on the surface of the gel shows negative staining with MSF antibody. **B**. Cells were plated both on the surface and within the 3D collagen gel. The resting endothelial monolayer on the surface of the gel (arrow) still shows negative staining, whereas positive MSF staining is apparent in the cells plated within the gel matrix (sprouting cells).

C, D, E. Endothelial cultures containing resting and sprouting cells were incubated with MSF function-neutralising antibody Mab pepQ (section 3). **C**. Monolayer of resting endothelial cells. **D**. A network of sprouting cells is present underneath the resting monolayer. **D**. Selective death of the sprouting cells by Mab pepQ. The same results were obtained with homogenous cultures of sprouting cells (within a 3D gel).

Fig 10. Bi-stable switch regulating MSF expression.

Histological sections of collagen gels containing fibroblasts were stained with MSF-identification Ab Mab VSI (Table 1).

A. Normal adult skin fibroblasts do not express MSF.

B. The same cells are "activated" and express MSF following **treatment 1.** Activated cells can remain as MSF-producers for their entire lifespan. Foetal fibroblasts and fibroblasts derived from cancer patients produce MSF constitutively. All MSF-producing fibroblasts, including activated cells, stain positive with Mab VSI and secrete MSF in their conditioned medium. MSF expression by these fibroblasts can be switched off by **treatment 2** and the cells can remain as non-MSF-producers for their entire lifespan. This on/off switch in MSF expression may be repeated multiple times between MSF-producer and non-producer cells.

Treatment 1: Cells adherent to a "wounded" matrix (such as fibrin, denatured type I collagen or plastic) are incubated for 18-24 hours with one of the following: (a) TGFβ1, (b) 5-azacytidine, (c) IGD peptide, (d) Gel-BD, (e) MSF [Schor et al 1996, 1999, 2003; Kay et al 2005; unpublished].

Treatment 2: Cells adherent to a ''healthy tissue'' matrix, such as native type I collagen are incubated with TGFβ1 for 18-24 hours [Schor et al 2003; Kay et al 2005]

Fig 11. Expression of MSF in normal adult skin and in healing wounds.

Skin biopsies were taken from healthy donors (controls, day 0) and a second biopsy that included the healing wound was taken at various intervals later (3, 7, 14 or 21 days after the first biopsy). Serial sections were immunostained with two antibodies :

(1) Mab VSI, that recognises MSF+aa and MSF-aa (Total MSF): Figs **A, C, E**.

(2) Mab TYN, that recognises only MSF-aa: Figs **B, D, F** (see Table 1, Fig 6).

A, B: Control, day 0; donor 1. **A**: Some weak staining. **B**: negative.

C, D: Control, day 0; donor 2. **C**: Diffuse staining in the epithelium. **D**: negative.

E, F: Healing wound, day 7; donor 3. **E**: Strong MSF expression in epithelial and stromal cells. **F**: negative.

NOTE: The majority of control biopsies did not show MSF staining. However, to compare the two antibodies, biopsies were selected from two donors that showed some staining. We conclude that MSF is rarely present in normal healthy adult skin. When present, it is the

MSF+aa isoform. During wound healing MSF+aa is temporarily upregulated and MSF-aa is still not expressed.

Fig 12. Assessment of MSF expression in tumours and normal tissues.
Histological sections of paraffin-embedded archival specimens were stained with MSF-identification Ab Mab VSI (Table 1). Duplicate sections of each specimen were assessed by 2-4 independent observers and final results were obtained by consensus. MSF staining was graded by comparison to calibration slides as negative (grade 0), weak (grade 1), moderate (grade 2) or strong (grade 3). Results are presented as the percentage of specimens showing the indicated MSF grade.
A. Breast tissues. **NB** (n=19): Histologically normal breast from reduction mammoplasties. **NB-T** (n=18): Histologically normal breast adjacent to carcinomas. **B** (n=8): Benign breast tumours. **T** (n=23): Malignant breast carcinomas. MSF expression increased in a step-wise manner from NB to T with significant differences between all groups (p=0.02-0.001) except between NB-T and B, which were not significantly different. The percentage of specimens showing moderate or strong MSF expression (MSF grades 2-3) was 0% (NB), 45% (NB-T), 50% (B), and 78% (T) [Perrier et al 2012].
B. Salivary gland tissues. **NSG** (n=16): Histologically normal salivary gland adjacent to tumours. B (n=7): Benign salivary gland tumours. **M** (n=27): Malignant salivary gland tumours. MSF expression increased significantly in a step-wise fashion from NSG to B and T (p= 0.04-0.0001). Moderate and strong positive specimens were grouped together to achieve consensus by 4 independent observers. These represented 6%, 33% and 74% of the normal, benign and malignant specimens, respectively [Aljorani et al 2011].

Fig 13. Expression of MSF in breast and oral tumours
Serial sections of archival tumour specimens were immunostained with two antibodies:
(1) Mab VSI, that recognises MSF+aa and MSF-aa (Total MSF): Figs **A, C, E, G**.
(2) Mab TYN, that recognises only MSF-aa: Figs **B, D, F, H**. (see Table 1, Fig 6):
A, B, C, D, E, F. Three breast carcinoma specimens showing: (A, B) strong similar expression of total MSF and MSF-aa; (**C, D**) negative staining for both. (**E, F**) weaker staining for MSF-aa than for total MSF.

G, H. Oral squamous cell carcinoma showing slightly weaker staining for MSF-aa than for total MSF.

These results indicate that MSF-aa is either the main or the only isoform in some specimens (A,B) and that both MSF+aa and MSF-aa are present in other specimens (E,F and G,H). See further details in Table 3.

Fig 14. Prognostic value of MSF expression in oral squamous cell carcinoma (OSCC). Archival specimens of OSCC were obtained from 45 patients with a follow-up of 5 years. Serial histological sections were stained with two MSF identification Abs: Mab VSI recognises total MSF (MSF+aa and MSF-aa) and Mab TYN recognises MSF-aa. The level of staining was semi-quantified in the invasive tumour front (ITF) by consensus of 3-4 observers. Kaplan-Meier survival curves were compared by Log-rank and Gehan-Breslow-Wilcoxon tests. With both Abs, high expression of MSF (grades 2/3) was significantly associated with lower survival rate (p values shown in the graph).

Fig 15. Motogenic effect of MSF on fibroblasts, endothelial cells and tumour cells.
A. Dose response and quantitative differences due to the migration assay. Adult skin fibroblasts, endothelial cells and tumour cells were incubated with MSF at the concentrations indicated (from 1pg/ml to 1µg/ml, in serum-free media) using the transmembrane or Boyden chamber assay (t.a). Fibroblasts were also tested on the 3D collagen gel assay (3D gel). The filters used in the transmembrane assay were coated with the same native type 1 collagen used to make the 3D gels [Schor et al 1996, 2003]. The response of fibroblasts and endothelial cells followed a bell shape, whereas tumour cells reached a plateau. Using the same native collagen substratum, fibroblast response was more pronounced in the 3D collagen gel than in the transmembrane assay.
B. Effects of the substratum: Permissive and non-permissive matrices. The motogenic effect of MSF was tested on the same cell types using the transmembrane assay. In this case the filters were coated with native type I collagen, denatured collagen (gelatin) or a mixture of 90% native collagen and 10% thrombospondin 1 (collagen-TSP1). The full range of MSF concentrations was tested (as in A); results show the maximum effect observed (usually with 100pg/ml-1ng/ml). Collagen-TSP1 matrices were also non-permissive for fibroblast migration; they were not tested on tumour cells.

A, B. Results are shown for FSF44 fibroblasts, Endo 742 microvascular endothelial cells and HSG salivary gland tumour cell line. The same results were obtained with various lines of adult skin fibroblasts, microvessel and large vessel-derived endothelial cells (human and bovine) and tumour cells (MDA-MB231, A549, HaCaT-MAL, TYS, B88, BHY, HNt). Controls were incubated in serum-free media without MSF. To compare different experiments, the number of cells that migrated in the presence of MSF is expressed as fold-stimulation relative to controls (shaded area).

Fig 16. Motogenic determinants within MSF are different for fibroblasts, endothelial cells and tumour cells. Recombinant mutant MSF proteins were produced by PCR-mediated mutagenesis. The motogenic effect of wild type MSF+aa and the mutant proteins was assessed in the transmembrane assay using filters coated with native type 1 collagen. A range of concentrations were tested on adult skin fibroblasts, endothelial cells and tumour cells. Results show the maximum effect observed (usually with 100pg/ml-1ng/ml). The same results were obtained with human and bovine endothelial cells and tumour cell lines HSG, TYS, and HNt. Controls were incubated in serum-free media without any of the proteins (shaded area).
DGI: IGD sequence was reversed to DGI in the type I module indicated (3, 5, 7 or 9) [Schor et al, 2003].
FE: FEEGF. The two histidine residues of the HEEGH sequence, present in module I8, were mutated to phenylalanine [Houard et al 2005].

Fig 17. Inhibition of MSF motogenic activity by NGAL and IGFBP7. Distinct functionality of MSF+aa and MSF-aa.
The motogenic effects of rhMSF+aa and rhMSF-aa on adult skin fibroblasts were assessed on their own (control) and in the presence of Mab pepQ (MSF function-neutralising Ab), rhNGAL and rhIGFBP-7.
Irrespective of the migration assay (3D-gel or transmembrane), MSF+aa and MSF-aa stimulated cell migration on their own to the same extent (controls). This activity was neutralised by co-incubation with Mab pepQ. NGAL inhibited MSF+aa-stimulated migration to baseline levels, but had no effect on the activity of MSF-aa.

IGFBP-7 inhibited the migration stimulated by both MSF isoforms when tested in the 3D gel assay, but had no effect in the transmembrane assay.
Baseline migration (in the absence of any of the proteins tested) is indicated by the shaded area. NGAL and IGFBP-7 did not affect baseline migration when tested on their own.

Fig 18. The presence of MSF and an inhibitor of MSF in serum.

A. Detection of bioactive and immunoreactive MSF in serum from breast cancer patients and controls.

Serum samples were fractionated by gel filtration chromatography. Each fraction was examined for motogenic activity in the 3D collagen assay and immunoreactivity by MSF-specific ELISA (Fig 5).
The motogenic activity of each fraction is shown in the histogram. As previously reported [Picardo et al 1991], MSF bioactivity was detected in the serum of cancer patients (fractions 10 and 11), not in serum from age- and sex-matched controls. Circles indicate MSF immunoreactivity measured by ELISA. This was detected in fractions 10 and 11 in both control and patient sera. These fractions correspond to approximate molecular weight 70-100kDa.

B. Separation of MSF and an inhibitor of MSF (serum MSFI) by gel electrophoresis.

Serum fractions 10 and 11 from gel filtration chromatography (A) were run in SDS-PAGE gels under reducing or non-reducing conditions. The gels were then sliced and each slice was eluted and tested for motogenic activity. MSF bioactivity was found in serum of controls only under reducing conditions, and in serum of patients under both reducing and non-reducing conditions (indicated in red, approx 70kDa). MSFI bioactivity was found in both control and patient serum under reducing conditions only (indicated in blue, approx 25kDa). Serum-MSFI was identified as NGAL. Serum MSF was identified by affinity to heparin, affinity to Mab VSI and neutralisation of bioactivity by Mab pepQ.

Fig 19. Functionally distinct MSF isoforms present in serum and produced by tumour cell lines.

MSF isolated from serum (**A**) and MSF produced by tumour cell lines (**B**) were tested for motogenic activity in the 3D collagen assay in the absence (control) or in the presence of NGAL and the MSF-function neutralising Mab pepQ, as indicated.

A. MSF from serum of controls is, like MSF+aa, inhibited by NGAL to baseline levels. In contrast, MSF from serum of cancer patients is not inhibited by NGAL (like MSF-aa, see Fig 17).

B. MSF produced by tumour cell line A549 was fully inhibited by NGAL whereas MSF from HaCaT-MET was not inhibited. MSF from T47D, MDA-4355 and HaCaT-MAL was partly inhibited by NGAL.

MSF bioactivity was inhibited by Mab pepQ in all cases. Baseline migration (in the absence of any of the proteins tested) is indicated by the shaded area.

Fig 20. The induction of angiogenesis in the chick yolk sac assay by MSF and IGDS. Different concentrations of MSF and IGDS were incorporated into methyl cellulose gels (MCGs). These were then dried and applied to the chick embryo yolk sac membrane. The induction of a radial disposition of vessels 24hr later was scored as a positive angiogenic response. Results are expressed as the percentage of positive angiogenic responses elicited by various concentrations of MSF and IGDS. A significant response was induced over a broad range (0.5-500ng/MCG) for both agents. Maximal response was induced by MSF at doses from 1ng to 50ng and by IGDS from 0.5ng to 500ng (maximal concentration tested). Phorbol ester PMA (phorbol 12-myristate, 13-acetate) at 300ng/MCG was used as positive control. Negative controls included MCGs alone and the reverse peptide SDGI. MSF-function neutralising Mab pepQ abolished the angiogenic activity of MSF and IGDS. Upper and lower horizontal bars indicate values achieved by the positive and negative controls, respectively (mean and SD).

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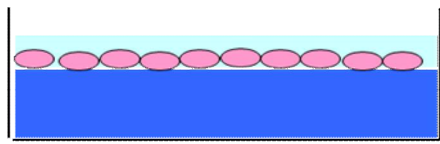
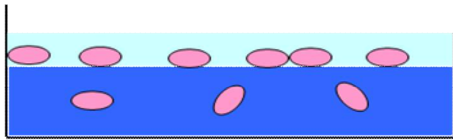
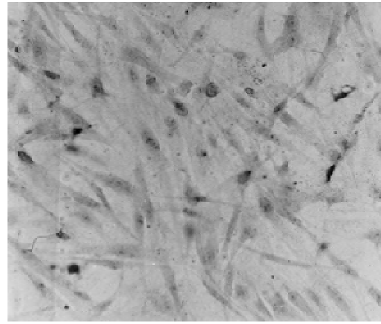
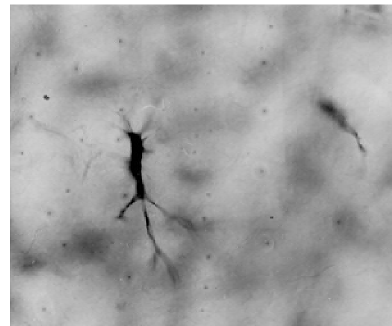
3D collagen gel assay





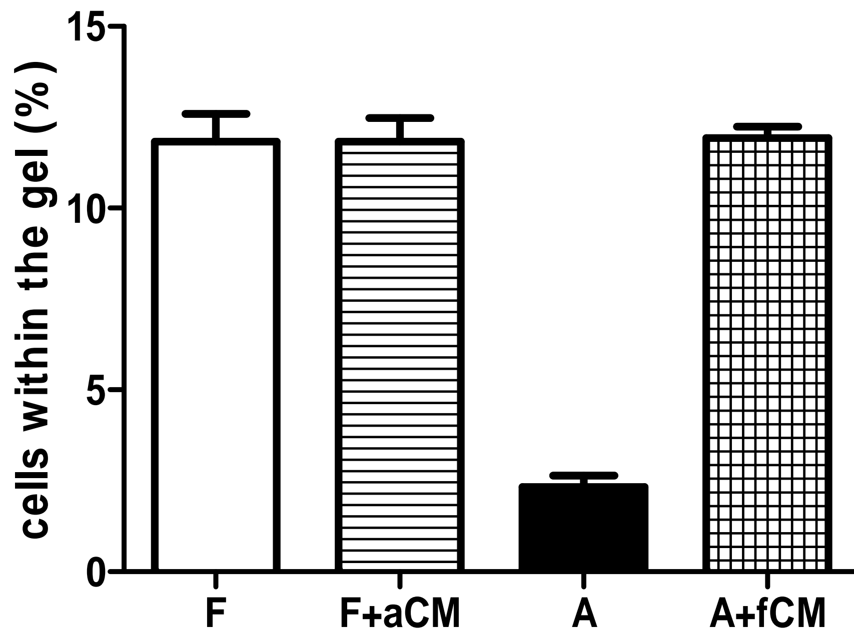


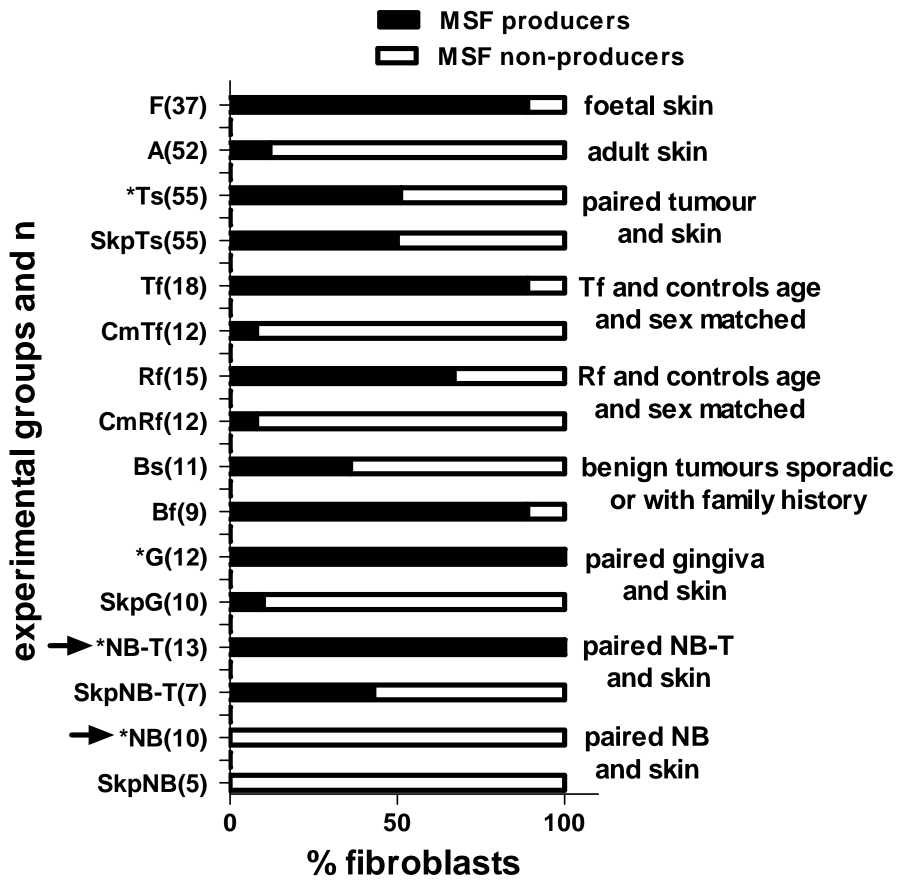
Surface of gel

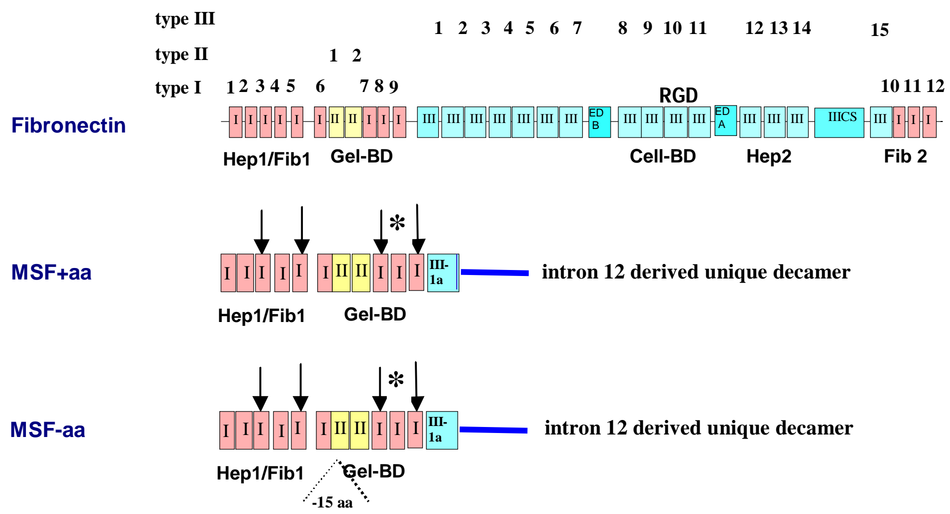


Within 3D gel

Migration of foetal and adult fibroblasts into 3D gels




MSF producers
MSF non-producers
experimental groups and n
F(37)
A(52)
*Ts(55)
SkpTs(55)
Tf(18)
CmTf(12)
Rf(15)
CmRf(12)
Bs(11)
Bf(9)
*G(12)
SkpG(10)
*NB-T(13)
SkpNB-T(7)
*NB(10)
SkpNB(5)
foetal skin
adult skin
paired tumour
and skin
Tf and controls age
and sex matched
Rf and controls age
and sex matched
benign tumours sporadic
or with family history
paired gingiva
and skin
paired NB-T
and skin
paired NB
and skin
0
50
100
% fibroblasts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type II
1 2
type I
1 2 3 4 5 6 7 8 9 10 11 12
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Fibronectin
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IIICS
Hep1/Fib1
Gel-BD
Cell-BD
Hep2
Fib 2
MSF+aa
III-1a
intron 12 derived unique decamer
Hep1/Fib1
Gel-BD
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III-1a
intron 12 derived unique decamer
Hep1/Fib1
Gel-BD
-15 aa

MSF+aa

https://www.ncbi.nlm.nih.gov/nuccore/AJ535086.1

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MSF-aa

(15 amino acid deletion in module II-1)

https://www.ncbi.nlm.nih.gov/nuccore/AJ276395

https://www.ncbi.nlm.nih.gov/protein/12053817

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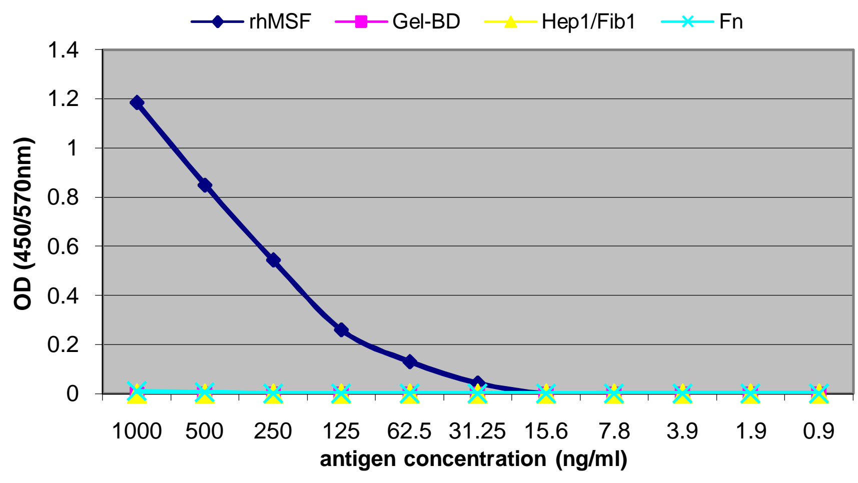
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0.8
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0.4
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OD (450/570nm)
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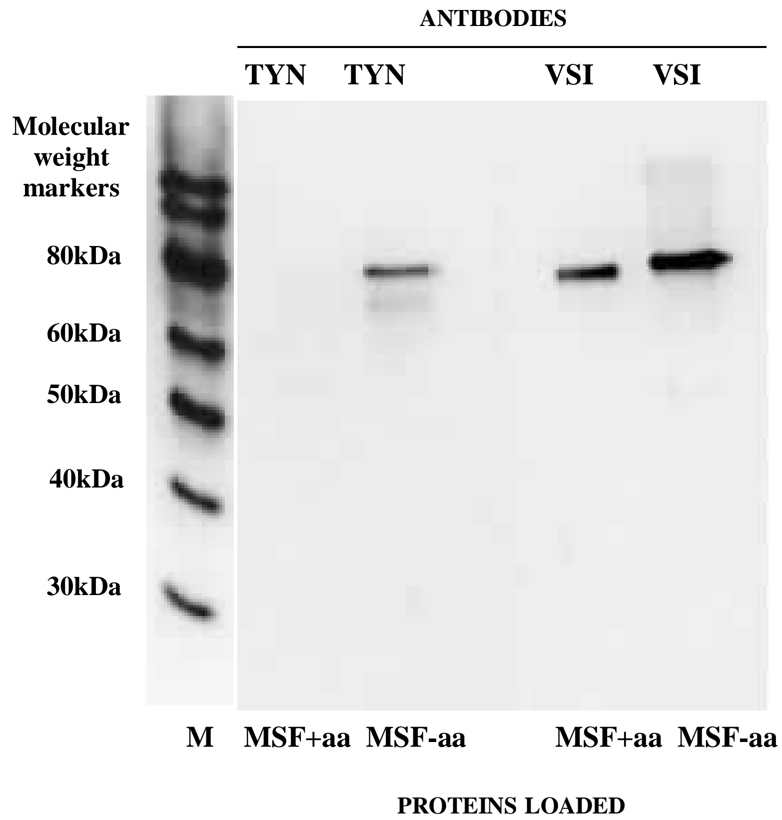
7.8
3.9
1.9
0.9
antigen concentration (ng/ml)


ANTIBODIES
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VSI
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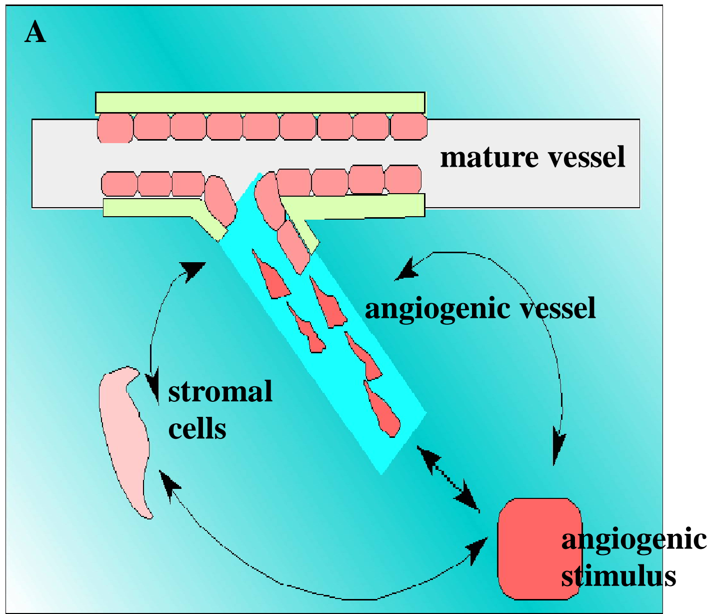
PROTEINS LOADED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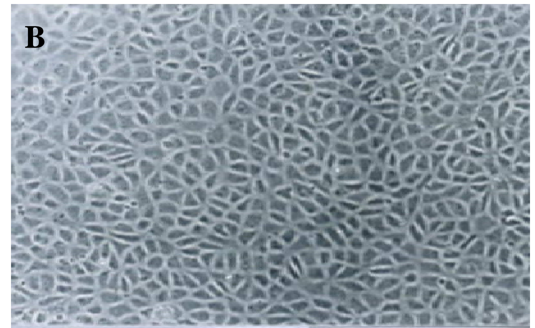
angiogenic vessel
stromal
cells
angiogenic
stimulus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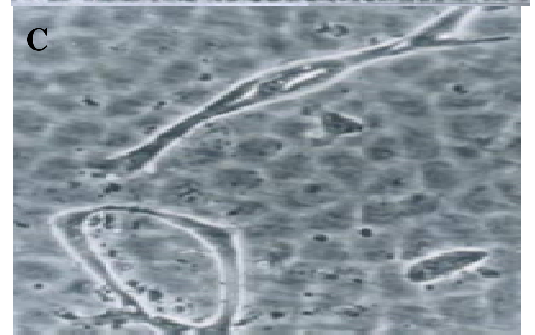
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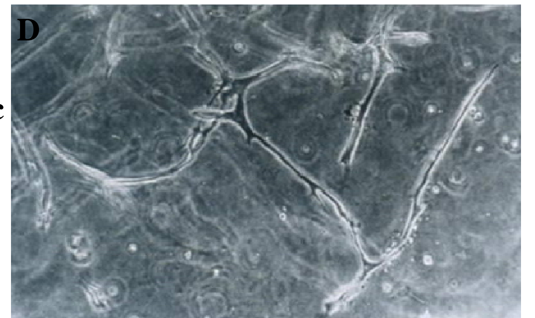
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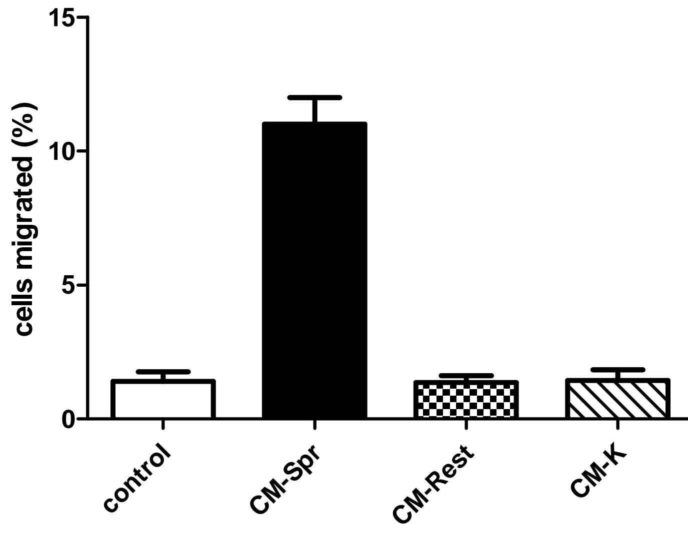
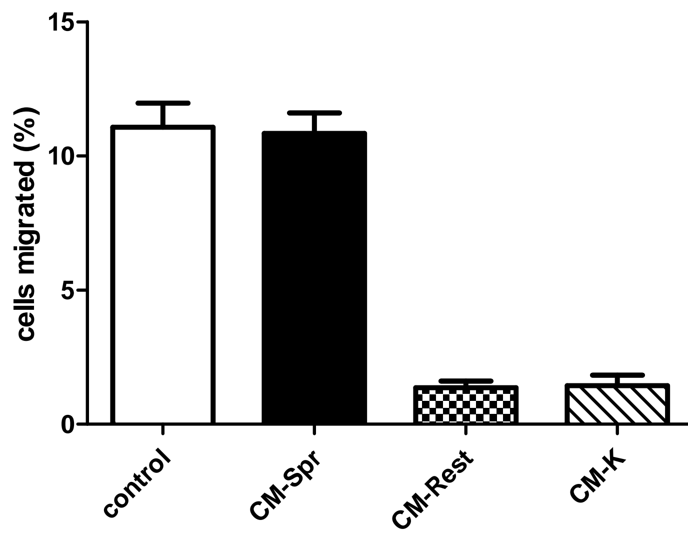
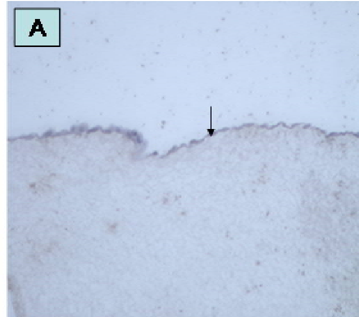
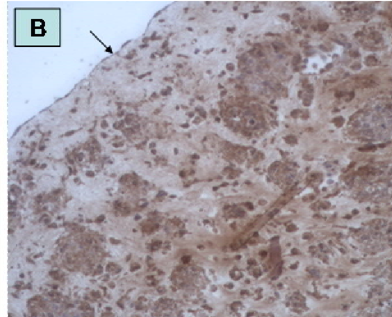
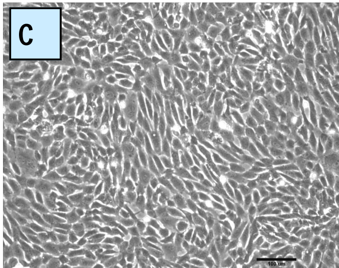
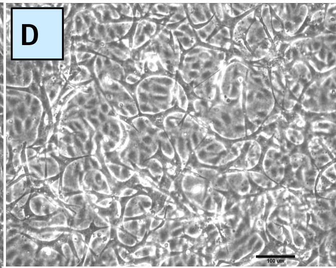

Without MSF
cells migrated (%)
15
10
5
0
control
CM-Spr
CM-Rest
CM-K


With MSF
cells migrated (%)
15
10
5
0
control
CM-Spr
CM-Rest
CM-K


A

B

C

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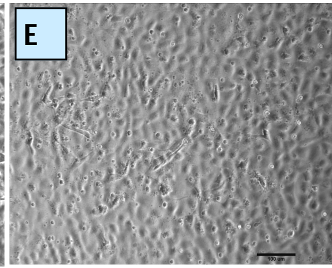
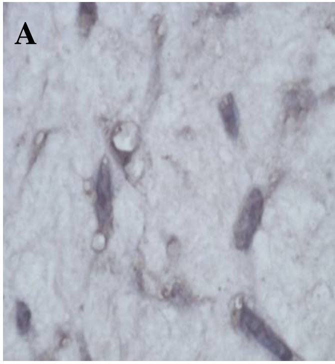
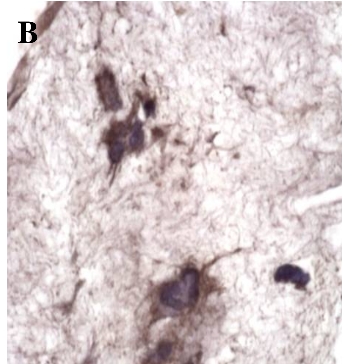

E

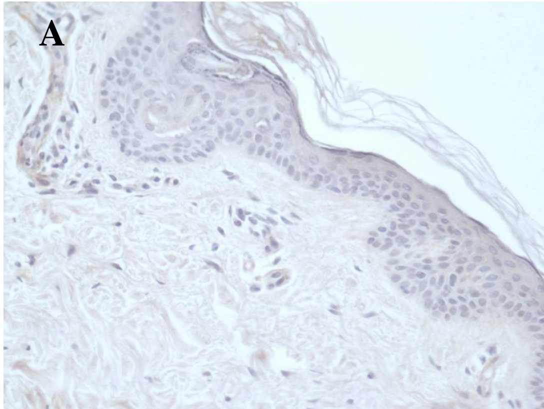

A

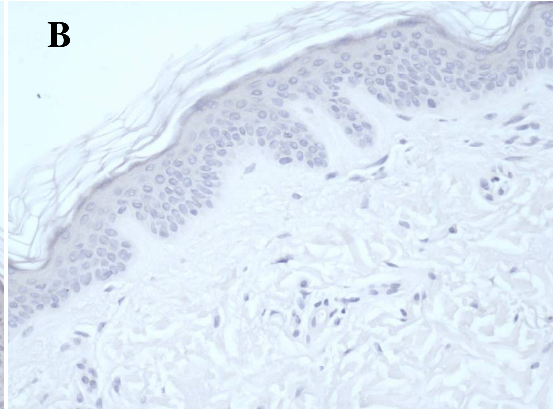
Treatment 1

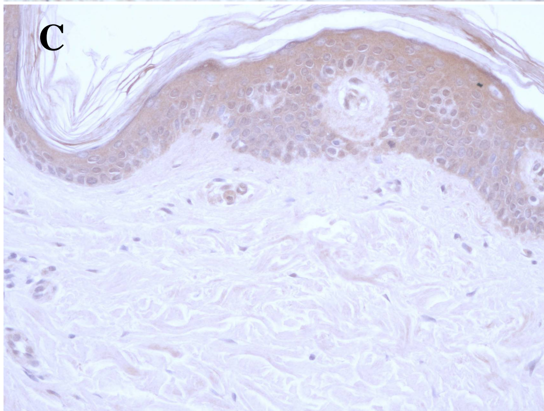

B

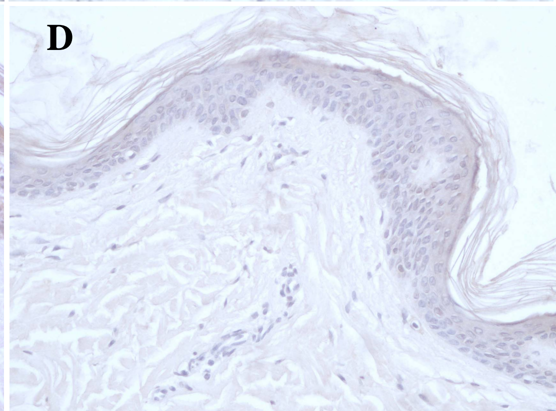
Treatment 2

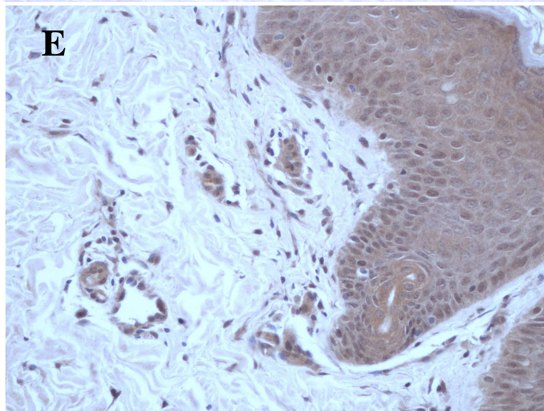
Total MSF **MSF-aa**

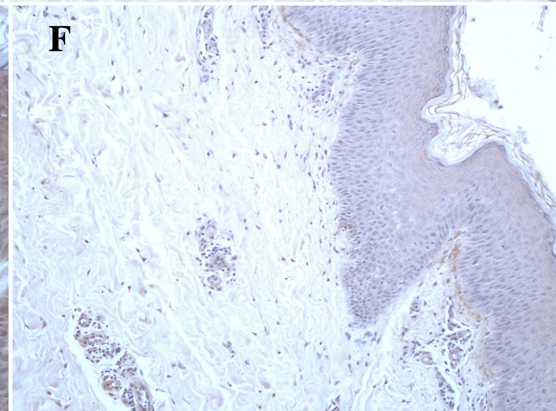




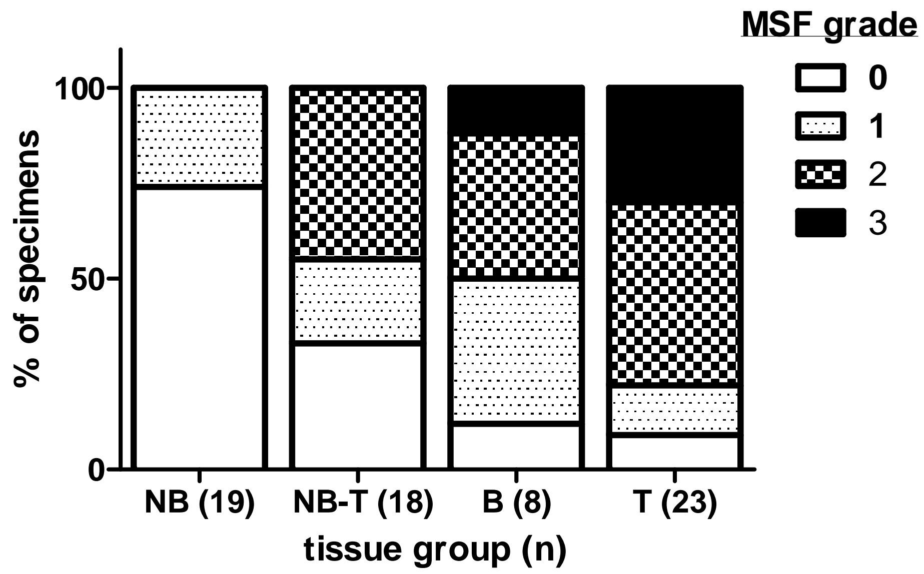




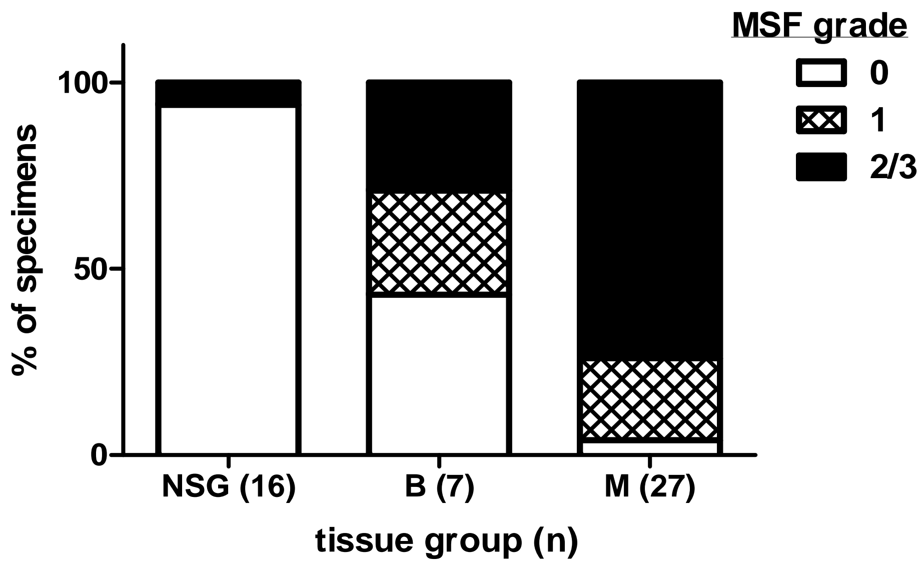


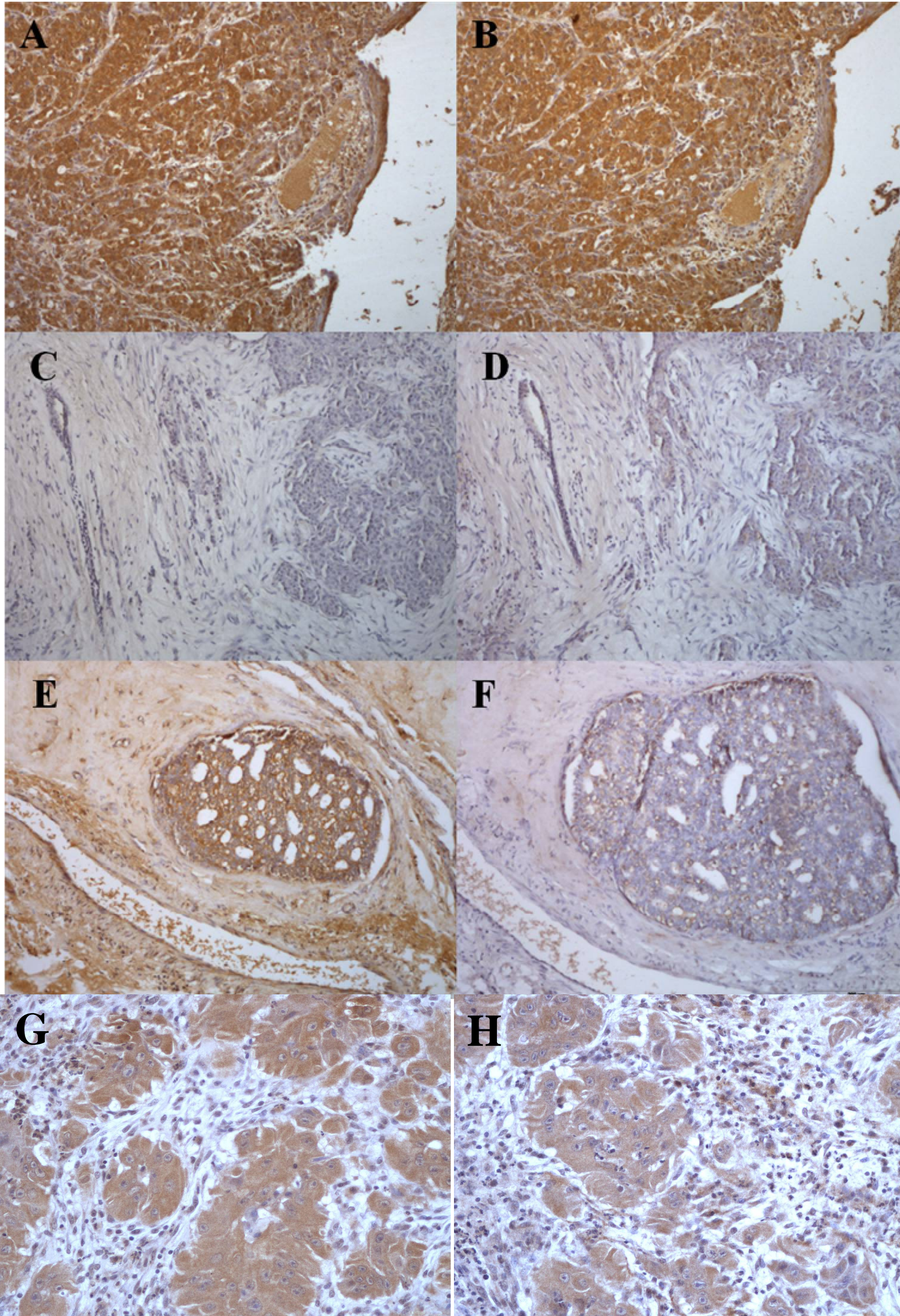


A

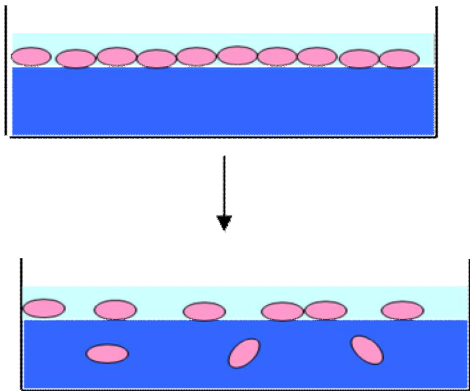


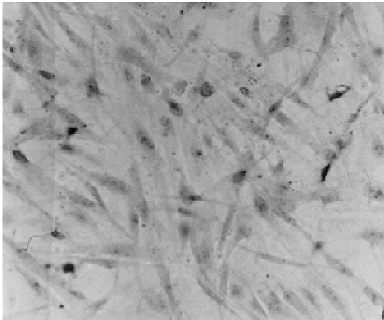
B



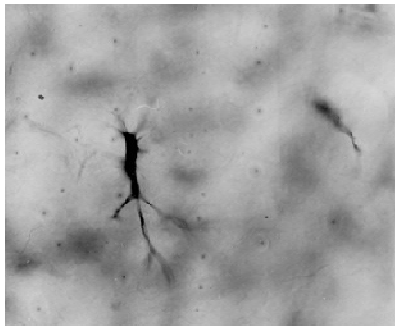

Total MSF
MSF-aa
A
B
C
D
E
F
G
H

3D collagen gel assay



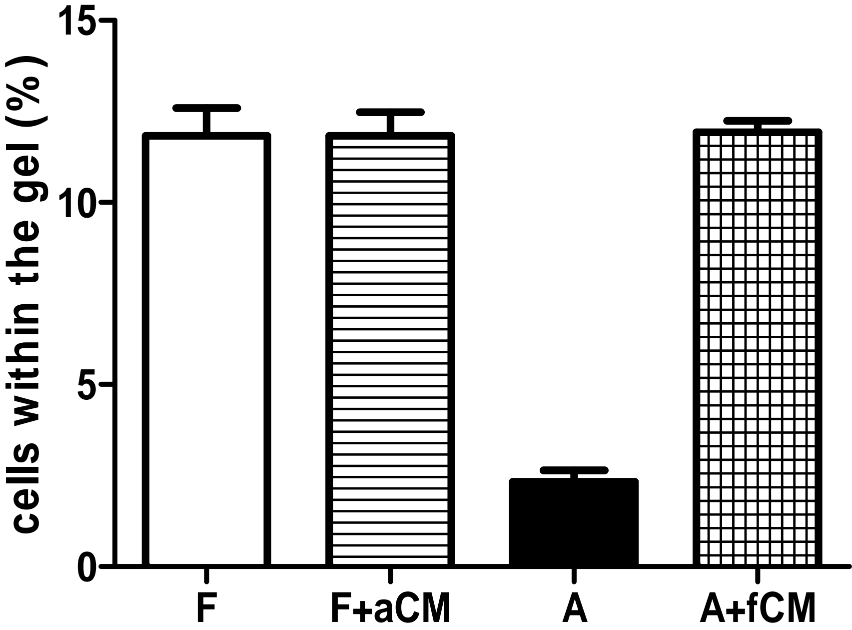


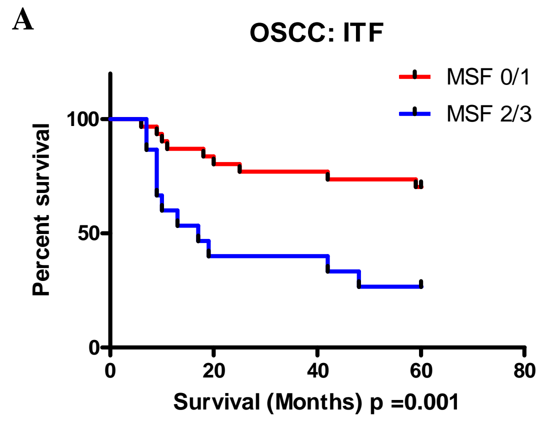
Surface of gel

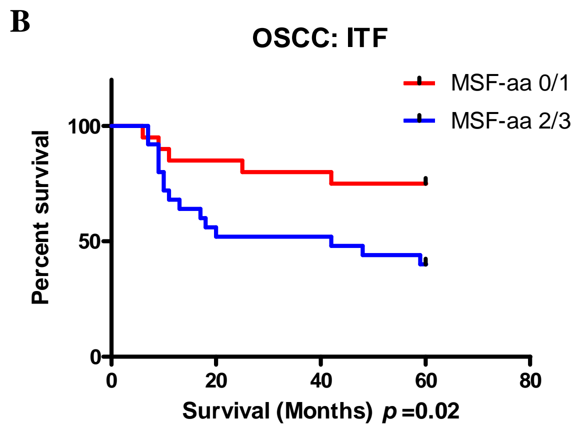


Within 3D gel

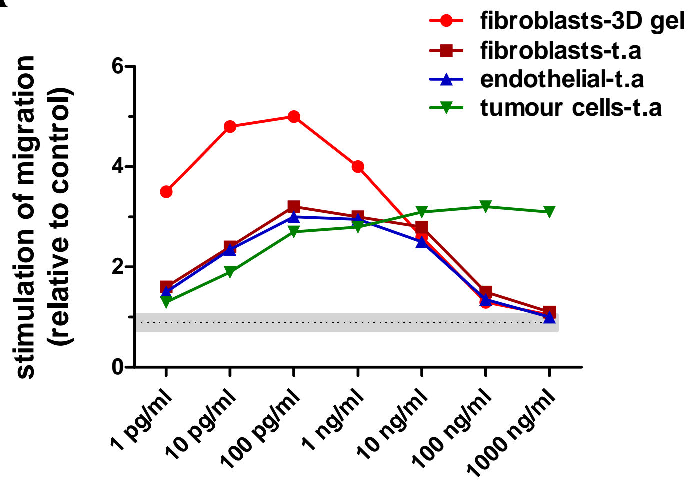
Migration of foetal and adult fibroblasts into 3D gels



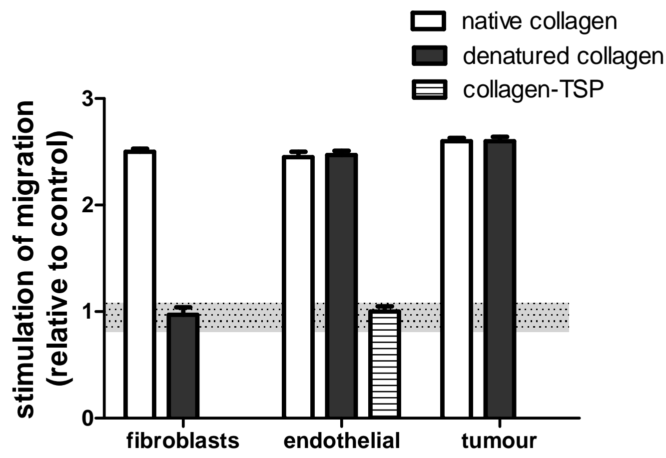

A
OSCC: ITF
MSF 0/1
MSF 2/3
Percent survival
100
50
0
0
20
40
60
80
Survival (Months) p =0.001

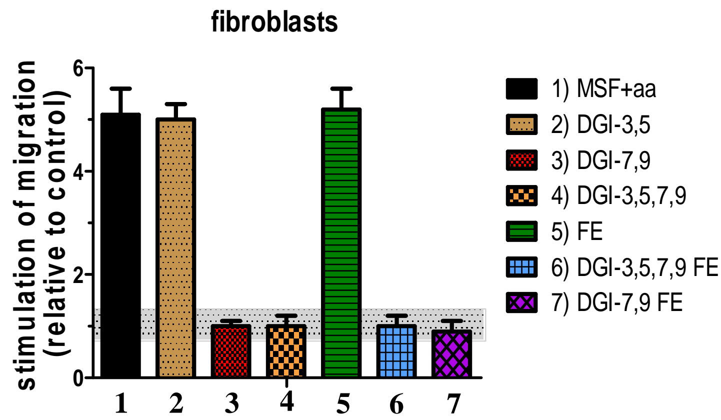

B
OSCC: ITF
MSF-aa 0/1
MSF-aa 2/3
Percent survival
100
50
0
0
20
40
60
80
Survival (Months) p =0.02

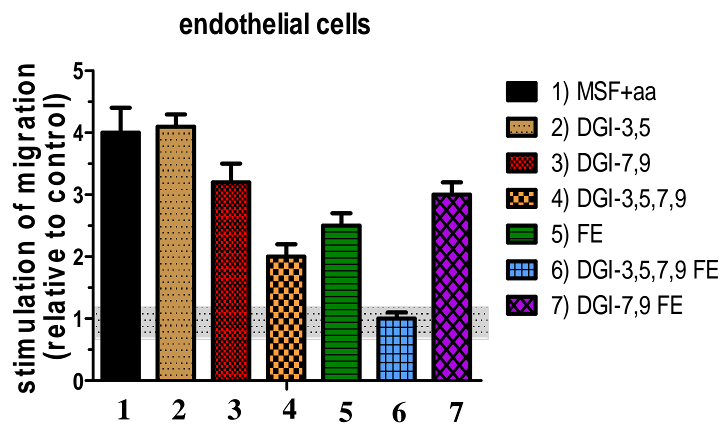
A

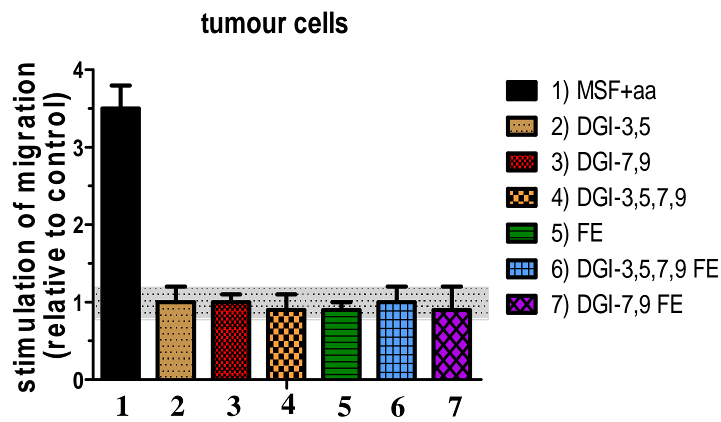


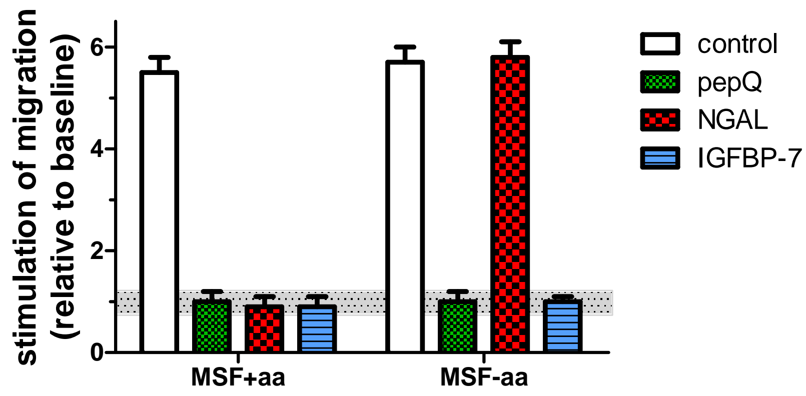
B

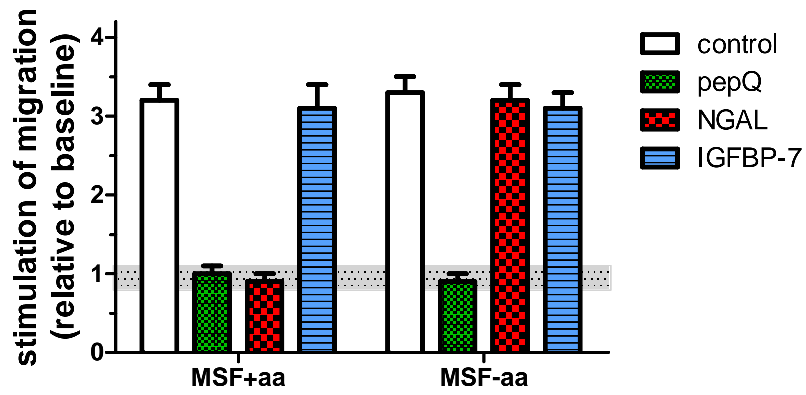



fibroblasts
stimulation of migration (relative to control)
0
2
4
6
1
2
3
4
5
6
7
1) MSF+aa
2) DGI-3,5
3) DGI-7,9
4) DGI-3,5,7,9
5) FE
6) DGI-3,5,7,9 FE
7) DGI-7,9 FE

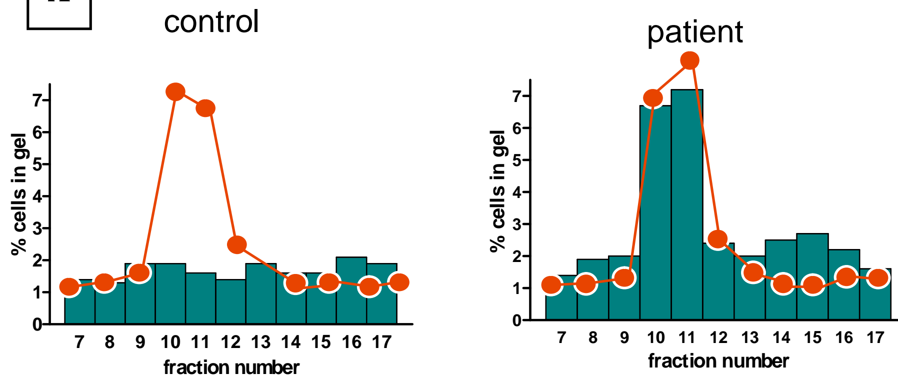

endothelial cells
stimulation of migration (relative to control)
0
1
2
3
4
5
1
2
3
4
5
6
7
1) MSF+aa
2) DGI-3,5
3) DGI-7,9
4) DGI-3,5,7,9
5) FE
6) DGI-3,5,7,9 FE
7) DGI-7,9 FE


tumour cells
stimulation of migration (relative to control)
0
1
2
3
4
1
2
3
4
5
6
7
1) MSF+aa
2) DGI-3,5
3) DGI-7,9
4) DGI-3,5,7,9
5) FE
6) DGI-3,5,7,9 FE
7) DGI-7,9 FE

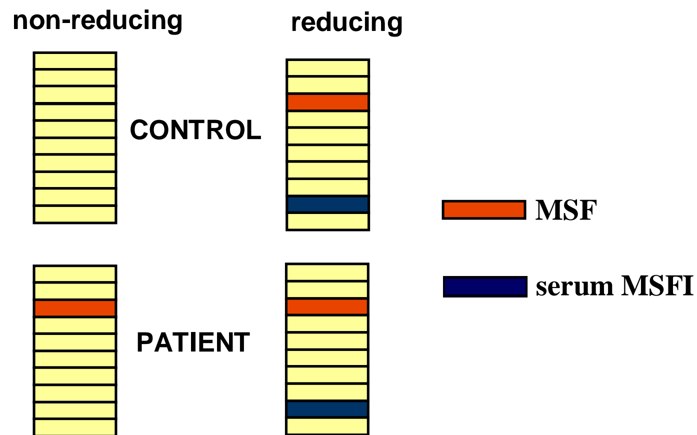

3D-gel assay
stimulation of migration
(relative to baseline)
0
2
4
6
MSF+aa
MSF-aa
control
pepQ
NGAL
IGFBP-7


transmembrane assay
stimulation of migration
(relative to baseline)
0
1
2
3
4
MSF+aa
MSF-aa
control
pepQ
NGAL
IGFBP-7

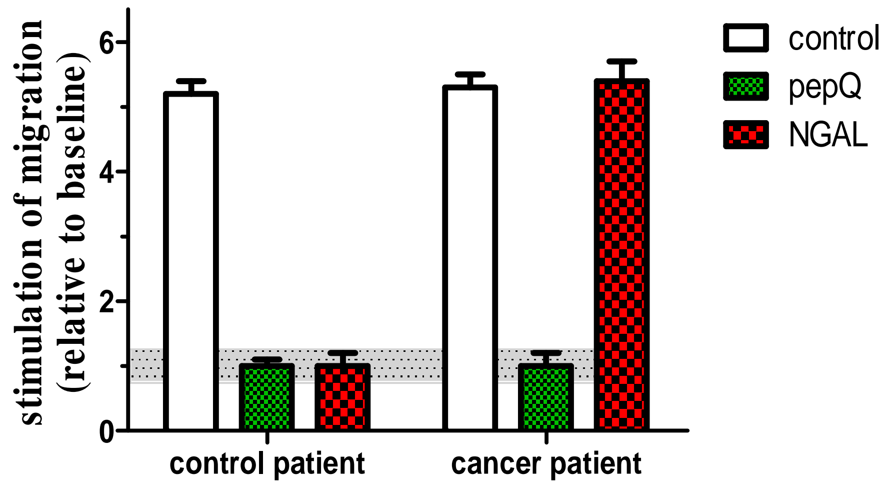
A



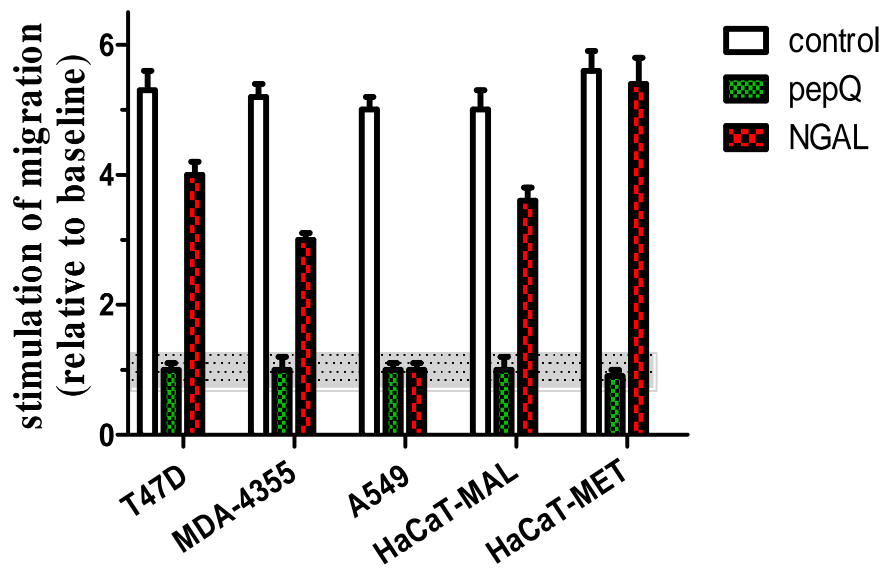
B

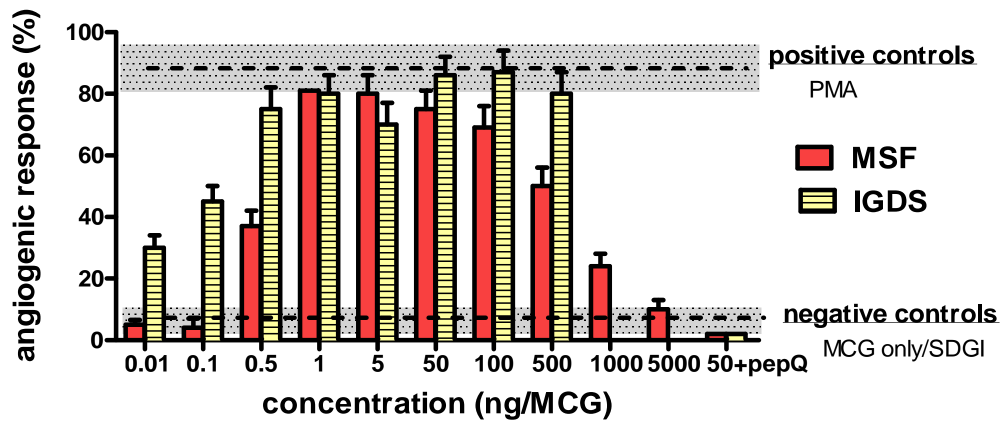


A



B




angiogenic response (%)
100
80
60
40
20
0
0.01
0.1
0.5
1
5
50
100
500
1000
5000
50+pepQ
concentration (ng/MCG)
positive controls
PMA
MSF
IGDS
negative controls
MCG only/SDGI

TABLES

TABLE 1

Peptide used as antigen	Ab code	Reactivity of Abs
VSIPPRNLGY 10 mer, MSF-unique C-terminus	VSI	Mab and Pab recognise MSF+aa and MSF-aa. Do not recognise Fn, Gel-BD or Hep 1/Fib-1 domains.
TNEGVMYRIGDQWDKQHDMGH 21-mer, IGD-containing peptide in module I-7	pepQ	Mab recognise MSF+aa, MSF-aa and Gel-BD. Do not recognise Fn.
TYNDRTDSTTSNY 13 mer, present in MSF-aa, II-1. In MSF+aa these amino acids are adjacent to the sequence deleted in MSF-aa (6 before and 7 after)	TYN	Mab and Pab recognise MSF-aa. Do not recognise MSF+aa, Fn or Gel-BD

Table 1. Overview of the antibodies raised and results obtained.

The peptides indicated were used as antigens to raise monoclonal (Mab) and polyclonal (Pab) antibodies; these were then tested for reactivity with MSF+aa, MSF-aa, full-length fibronectin (Fn) and the Fn domains Gel-BD and Hep 1/Fib-1, which are also present in MSF (Fig 3).

TABLE 2

Cell line - tumour of origin	Presence of MSF		Reference
	mRNA	protein	
MDA-MB435-breast	yes	yes	1
MDA-MB231-breast	yes	yes	1, 2
BT549-breast	yes	yes	2
Hs578T-breast	yes	yes	2
A549-lung	yes	yes	1, 3
HaCaT-MAL-skin	yes	yes	1
HaCaT-MET-skin	yes	yes	1
HSG-salivary gland	nd	yes	1
TYS-salivary gland	nd	yes	1
HSY-salivary duct	nd	yes	1
BHY-oral SCC	nd	yes	1
HNt-oral SCC	nd	yes	1
B88-oral SCC	nd	yes	1
SAS-HI-oral SCC	nd	yes	1
T47D-breast	NO	NO	2
T47D-breast	yes (low)	yes	1
MCF7-breast	NO	NO	1, 2
MCF7-p53	yes	yes	1
HT49-colon	NO	NO	1
PANC1-pancreas	nd	NO	1

Table 2. MSF expression by tumour cell lines in culture.

The presence of MSF mRNA was determined by RT-PCR or in-situ hybridisation. MSF protein was detected by IHC and/or by the expression of bioactive MSF [Houard et al 2005; Solinas et al 2010; Deng et al 2013;Schor et al, 2003 2012, 2020, unpublished]. In our laboratory, the presence of bioactive MSF was confirmed by neutralisation with Mab pepQ (MSF-function-neutralising antibodies) and by removal of the motogenic activity by affinity chromatography to MSF-identification antibodies (VSI) [Jones et al 2007; Schor et al, 2003 2012, unpublished]. An agreement between mRNA and bioactive protein expression was

found in all tumour lines examined. Results from different laboratories agreed regarding MDA-MB231, A549 and MCF7 cell lines, but not T47D.

(1) Results from our laboratory [Aljorani et al 2011, unpublished] (2) Houard et al 2005

(3) Deng et al 2013 SCC: squamous cell carcinoma; nd: not done

TABLE 3

Staining with Ab		**Comparison of MSF grades (VSI v TYN)**	**Pattern (no.) and specimens showing the pattern (n/79)**
VSI	**TYN**		
+ve	**+ve**	The same grades with both	no.1 (53)
+ve	**+ve**	Lower grades with TYN	no.2 (3)
+ve	**-ve**	No staining (grade 0) with TYN	no.3 (12)
-ve	**-ve**	No staining (grade 0) with both	no.4 (11)

Table 3. Expression of MSF isoforms in breast tumours.
Serial sections of archival breast carcinomas (n=79) were stained with Mab VSI and Mab TYN. Duplicate sections for each specimen and antibody were assessed by 2-3 independent observers and the final results were obtained by consensus. MSF staining was graded by comparison to calibration slides as negative (grade 0), weak (grade 1), moderate (grade 2) or strong (grade 3). The results are summarised in the Table as positive (**+ve;** grades 1, 2 and 3) or negative (**-ve;** grade 0). Mab VSI recognises total MSF (MSF+aa and MSF-aa isoforms) whereas Mab TYN only recognises MSF-aa, as indicated in Fig 6 and Table 1. Examples of the different staining patterns are shown in Fig 13. Our interpretation of the results obtained is as follows:

Pattern no.1: MSF-aa is likely to be the only isoform present in 67% of the tumours.
Pattern no.2: both MSF+aa and MSF-aa are present in 4% of the tumours.
Pattern no.3: MSF+aa is the only isoform present in 15% of the tumours.
Pattern no.4: Neither MSF+aa or MSF-aa are expressed in 14% of the tumours.

TABLE 4

		Comparison of survival curves (p value)	
MSF index / division for survival analysis	**survival analysed**	**MSF in the tumour stroma**	**MSF in the carcinoma cells**
% area stained / quartiles	Overall	0.01**	0.09*
% area stained / median	Overall	0.02**	0.13
final score (% area x intensity / median	Overall	0.06*	0.25
final score (% area x intensity / median	Disease-free	0.005**	0.81

Table 4. Analysis of of breast cancer patient survival according to MSF expression. Differential prognostic value of MSF-aa expression in the tumour stromal cells. Archival breast carcinoma specimens were obtained from a cohort of 71 patients with a follow- up of 10 years. Histological sections were stained with MSF-aa-identification Ab Mab TYN. The level of staining was quantified by image analysis in the carcinoma cells and in the tumour stroma by three MSF indices: (i) % area stained, (ii) intensity of the staining and (iii) final score, a combination of area x intensity. The values obtained were divided either in quartiles or by the median to analyse patient survival. Kaplan-Meier survival curves were compared by Log-rank and Gehan-Breslow-Wilcoxon tests (p values shown).
In the tumour stroma, high MSF-aa expression was associated with poor survival. The results were statistically significant for the percentage of area stained, but not for the intensity (not shown). MSF in the carcinoma cells followed the same trend but it did not reach statistical significance.
** statistical significant at 95% level of confidence. *near significant values (90% level).