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Review

Metastasis Promoter S100A4 is a Potential Molecular Therapeutic Target

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Abstract. *S100A4, a member of the S100 family of proteins involved in calcium signalling, has come into the fore in recent years on account of its close relationship with tumour development and progression; therefore, S100A4 commends itself as a prime therapeutic target. The phenotypic effects of S100A4 are generated via diverse signalling pathways encompassing and incorporating the functions of cell cycle regulators, growth factor receptors, extracellular matrix components, and the inducers of angiogenesis and lymphangiogenesis. By virtue of this, S100A4 signalling can be specifically targeted to down-regulate the phenotypic activities that contribute to the growth, invasion and metastasis of cancer. Here, the discussion has focused on the signalling pathways that S100A4 uses, with a view to identifying the most effective targets to which drugs can be designed and specifically directed. Some approaches to the problem of inhibiting or deregulating the functions of S100A4, to control invasion and metastasis have been identified. S100A4 could provide a wide channel to control the growth, invasion and secondary spread of cancer and, thus, amply rationalise and validate it as an important therapeutic target.*

A major challenge in the search for new modes of cancer treatment is the identification of specific molecular targets to which anticancer drugs can be directed most effectively to control the growth and spread of cancer. Much effort has been focused on the identification of target macromolecules from profiles of molecular alterations and abnormalities of genetic expression, as well as from deregulation of signalling systems. The construction of these profiles of molecular alterations related to the biological behaviour of cancers has been amply described in recent years (1). From these, many novel targets are identifiable for the development of new drugs. There is general recognition that the management of patients, who have developed metastatic disease having failed first-line therapy, is a major problem. The identification of markers, whose expression is associated with metastatic disease, has become imperative so that therapy can be designed and directed at metastatic disease. Among metastasis markers known to date, S100A4 (also known as metastasin, 18A2/mts1, CAPL, PEL-98, 42A and p9Ka) commends itself as a prime therapeutic target. S100A4 is an important molecular marker for monitoring cancer progression. The expression levels of S100A4 have often been found to correlate with the size of the S-phase fraction or rate of cell proliferation. The changes in S100A4 expression correlate with the expression of growth factor receptors and might represent positive responses to growth factors. Thus, human tumour cells, which are high expressers of epidermal growth factor (EGF) receptors, tend to over express S100A4. Transfection and forced expression of S100A4 substantially increases cells proliferation and metastatic dissemination. Besides the ability to promote cell proliferation, S100A4 seems to inhibit apoptotic loss of cells. In breast cancer, S100A4 expression was found to correlate with nodal status and inversely with 5-year survival (see 1-3). Using artificial neural networks to analyse the significance of S100A4 in relation to the development of metastatic disease, it was shown that the relative expression of S100A4 and the metastasis suppressor nm23 genes is a most effective predictor of nodal status (4).

The phenotypic effects of S100A4 are generated *via* diverse signalling pathways encompassing and incorporating the functions of cell cycle regulators, growth factor receptors, extracellular matrix (ECM) components,

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and inducers of lymphangiogenesis and angiogenesis. By virtue of this, S100A4 signalling can be specifically targeted to down-regulate the phenotypic activities that contribute to the growth, invasion and metastasis of cancer. Significant contributions have been made towards the elucidation of the function of S100A4 in the pathological setting relating to cancer development and progression by several research groups, notably the Lukanidin group, the Rudland and Barraclough laboratory, the Zain group and the author's group (Table I). Hitherto, S100A4 has justifiably been seen as a marker of cancer progression, because its expression is deregulated and overexpression of S100A4 correlates significantly with disease progression.

Here, the discussion has focused on the signalling pathways that S100A4 uses, with a view to identifying the most effective targets to which drugs can be designed and specifically directed.

Table I. *Metastasis promoting properties of S100A4.*

Biological feature	References
Cell cycle regulation	7, 7a, 8-12
Influence on metastasis suppressors	19
Response to growth factors	18, 22, 19, 23
Invasion, motility, adhesion, ECM remodelling	37, 39a, 118, 51, 46, 52, 47
Modulation of cytoskeletal dynamics	37, 39-41
Angiogenesis	54, 10, 119, 54a

References are shown in chronological order

S100A4 and p53-mediated Regulation of the Cell Cycle

The progression of the cell division cycle is stringently regulated and monitored for DNA damage, in order that cells that carry abnormal DNA do not perpetually proliferate. Three checkpoints may be identified, *viz*. the G_1-S transition checkpoint, the S-phase checkpoint and the G_2-M checkpoint. The p53 suppressor protein regulates both G_1-S and G_2-M transitions. The retinoblastoma susceptibility Rb protein is another cell cycle regulator that functions at the G_1-S checkpoint and p53 functions in conjunction with Rb (see 5).

Several cellular proteins appear to co-operate with p53 in regulating the transition of cells past the checkpoints. These interact with and bind p53 and, in this way, abrogate the checkpoint control function of p53. Among these are the RAD proteins, SV40 large T-antigen and HPV E6 protein (see 2, 6). S100A4 was shown to sequester p53 and abrogate its checkpoint control function. This was demonstrated some time ago by the author's group (7) and confirmed later in other laboratories (8-11). S100A4 and S100β bind to the tetramerisation domain of p53 (residues 325-355); when binding occurs to p53 in lower oligomerised states, the tetramerisation of p53 is impaired, which conceivably could alter the subcellular localisation of p53 (12).

Further, S100A4 could be regulating G_1-S as well as G_2-M transition checkpoints and, at the latter checkpoint, S100A4 and stathmin could be implicated together with p53 in the monitoring process. Stathmin expression closely parallels that of S100A4. It has been postulated that S100A4 might be involved in both G_1-S and G_2-M transitions by sequestering p53. This suggestion is based on the down-regulation of stathmin by wild-type p53, and the demonstration that stathmin expression runs in parallel with that of S100A4. This pathway thus postulates p53 functioning by modulating microtubule (MT) dynamics (13). This could possibly provide a dual approach to specifically targeting the process of cell cycle regulation using a S100A4/stathmin strategy which would focus not only on the deregulated checkpoint function of p53, but also upon MT dynamics. It is needless to emphasise that the basic considerations have to be defined in designing drugs that can achieve this. The topoisomerase inhibitor topotecan alters the expression of p53- and DNA damage-induced genes and also genes negatively-regulated by p53. Apparently topotecan alters the expression of S100A4 independently of p53 (14). So the design or use of agents which modulate the expression of a broad spectrum of genes, albeit associated with the p53 pathway, might not meet the requirement for target specificity.

The possibility of using the p53 pathway to inhibit angiogenesis has arisen from recent work. The anti-angiogenic agent TNP-470, a fumagillin analogue, seems to inhibit endothelial cell cycle arrest *via* the p53/$p21^{cip1}$/cdk pathway and Rb-mediated cell cycle regulation (15-17). With the interaction of S100A4 with p53 signalling, it might be possible to target endothelial cell proliferation.

Growth Factor Receptors and S100A4 Expression

We pointed out many years ago that cells that express the EGF receptor (EGFr) at high levels also express S100A4 highly. Such a correlation was found in four human breast cancer cell lines (18). In another study, the expression of S100A4 was found to be inversely-related to the expression of the oestrogen and progesterone receptors (ER/PgR) in breast cancer, suggesting that in the more aggressive breast cancers that are ER/PgR-negative, EGFr might be implicated in promoting rapid growth (19). Compatible with this are the findings of Boerner *et al.* (20) that oestrogen negatively-regulates EGF-mediated STAT-5 signalling in

breast cancer cell lines that overexpress EGFr. They demonstrated that extraneous ERα and exposure of cells to oestrogen reduced the phosphorylation of STAT5b by EGF, STAT-5 mediated transcription and the stimulation by EGF of DNA synthesis in the cells. In non-small cell lung carcinoma cells EGFr signalling is activated in the absence of oestrogen (21).

Transfection of the steroid-responsive MCF7 breast cancer cells with S100A4 conferred oestrogen-independent growth characteristics on these cells (22). This might suggest the possibility of growth promotion by other growth factors, such as EGF, in the transfected cells; this is not incompatible with the inverse relationship between the expressions of these two classes of receptors. In this context, it might be recalled that tyrosine kinase receptors of the EGFr family were reported to be up-regulated together with S100A4 in rat pancreatic carcinoma cell lines (23). Normal pancreatic tissue and specimens from chronic pancreatitis and pancreatic cancer have been compared for the expression of growth factor receptors and cancer genes; this revealed up-regulated expression of S100A4, EGFr and also of metallo-proteinases in pancreatic cancer, as compared with chronic pancreatitis (24). It is not clear yet if any causal link is present between these two events. ErbB2 signalling in medulloblastoma cells involves S100A4. ErbB2 up-regulates the expression of S100A4 and the expression levels of these two genes show a strong correlation in primary medulloblastomas. It is also apparent from this study that erbB2 expression is higher in aggressive disease as compared with localised tumour. It is also interesting that S100A4 might contain erbB2 response elements (25). This is more than halfway towards rationalising the link-up between the functions of the two entities.

Receptor tyrosine kinase inhibitors. That EGF induces cancer invasion was recognised many years ago. In recent years, efforts have been made to unravel the signalling pathway of this function of EGF. EGF seems to increase invasion, apparently *via* the induction of MMP and urokinase type plasminogen activator (uPA). These effects are accompanied by enhanced NF-κB activity (26). A significant finding of this study is that inhibitors of NF-κB inhibit EGF-induced invasion.

The inhibition of signalling by EGFr family receptors is now being recognised as a viable cancer treatment strategy. Inhibitors that can target these receptors are being developed and evaluated. Two modes of approach have been followed. The erbB receptors are activated by ligand binding and are phosphorylated by dimerisation. One approach has been to inhibit this activation process. Small membrane permeable receptor kinase inhibitors have been or are being tested in clinical trials; the inhibitors can be administered parenterally with good tolerability and safety, and can be combined with radiation and chemotherapies (27-30). The inhibitors lressa (TM) (gefitinib, Astrazeneca) and Tarceva (TM) (erlotinib, OSI/Genetech/Roche) inhibit EGFr (erbB1) as well.

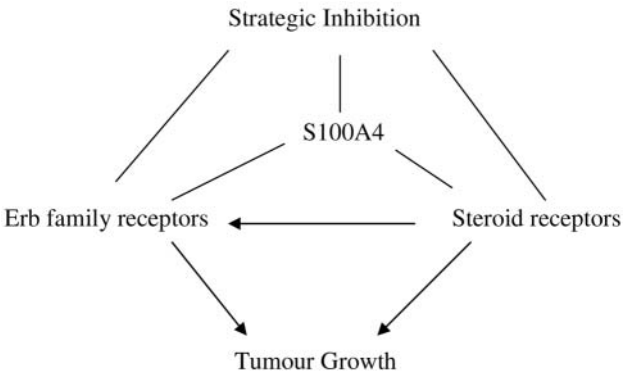

Figure 1. *Postulated scheme of how strategic inhibition of S100A4 and erb family of tyrosine kinase receptors or their combination might achieve tumour growth inhibition.*

Monoclonal antibodies against HER2 (erbB2/neu) receptors. The second approach is the development of anti-HER2 antibodies. The erb/EGFr family receptor HER2 is amplified or the protein is overexpressed in breast cancer. HER2 is overexpressed in 15-30% of invasive breast cancer. Herceptin has been approved as an anticancer agent for the treatment of advanced breast cancers that overexpress the receptor. Herceptin (Trastuzumab) is a recombinant humanised anti-HER2 monoclonal antibody that binds the extracellular domain of the receptor and blocks intracellular signalling. Several studies have demonstrated the effectiveness of Trastuzumab in patient management (31). A new class of HER2 inhibitor, *viz*. Pertuzumab, is also being evaluated, which inhibits the homo- as well as hetero-dimerisation of HER2. Having shown much promise in phase I studies, Pertuzumab is being further evaluated (32, 33). Some of these inhibitors clearly have potential as agents that can be tested in combination with S100A4 inhibition, an approach which might have the advantage of inhibiting erbB as well as ER-mediated signalling pathways, and which might generate synergistic responses (Figure 1). S100A4 has been regarded as a metastasis promoter rather than a causal factor in the metastatic process. S100A4 interacts with several other cellular proteins in order to influence tumour progression, *e.g.,* EGFr and ER/PgR (18, 19). More direct evidence has come in the interim from Davies *et al.* (34), who showed that the HER2 expression background is a factor in the promotion of tumour progression by S100A4.

S100A4-mediated Regulation of Intercellular Adhesion and Cell Motility

As discussed above, S100A4 significantly alters cytoskeletal dynamics and possesses the potential to affect cell division. Modulation of cytoskeletal dynamics can also profoundly influence invasive behaviour. That enhancement of S100A4 expression is accompanied by increased cell motility is now well established. The expression of several ECM transmembrane components are modulated by S100A4, *e.g.,* cadherins and CD44 are both intricately involved with cell motility. CD44 induces cell motility and changes in motility might be attributed to the redistribution and clustering of CD44v6 hyaluronidase receptors on the cell surface. This occurs as a consequence of the up-regulation of S100A4 expression (5).

Invasion and CCN3 interaction with S100A4

CCN3 is a member of the CCN (cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed) family that has generated much interest in recent years. The CCN proteins could be metalloproteins and have been implicated in cell growth, motility and cell spreading, and these processes are possibly regulated by the CCN proteins by regulating the mobilisation of intracellular Ca^{2+} and the influx of Ca^{2+} into the cell (35). Both CCN3 and CCN2 transiently increase the levels of intracellular Ca^{2+}[i], this alteration of Ca^{2+}[i] affecting the expression of S100A4. Inhibition of Ca^{2+} mobilisation and inhibition of its influx up-regulate S100A4 (36). From these findings, a functional link can be inferred between S100A4 and CCN family members.

CCN3 has salient effects on cell behaviour, which appear to be attributable to its ability to interact with a variety of intracellular and ECM molecular constituents. CCN3 can interact with S100A4; the latter itself appears to be able to promote cell motility by interfering with cytoskeletal dynamics and regulate cell proliferation in cancer cells (37, 38). That S100A4 might interfere with cytoskeletal assembly was subsequently confirmed in other systems (39-41). CCN3 has been found to enhance cell motility by reducing intercellular adhesion (42), prompting the suggestion that this effect is due to the interaction of CCN3 with S100A4. Statins were identified as potential inhibitors of CCN2 expression some time ago (43), but this was not pursued further. It might be useful to explore this on its own, or in combination with S100A4 down-regulation. Rho family GTPases are required for the functioning of CCN proteins (43). The activation of RhoA might play an important role in angiogenesis, since TNP-470 inhibits angiogenesis and also RhoA activation in endothelial cells (44). S100A4-mediated cytoskeletal depolymerisation might also involve Rho family GTPases (41). Fritz (45) has strongly advocated the use of statins as anticancer agents. Therefore, the inhibition of the Rho GTPases would be worth investigating, not only from the point of view of inactivating CCN proteins, but also from the viewpoint of possibly inhibiting angiogenesis.

The cadherins, which are regarded as invasion suppressors, are another target for S100A4. The expressions of these are inversely-related and it would appear that S100A4 down-regulates E-cadherin expression (46). Moriyama-Kita *et al.* (47) have amply confirmed this recently. They transfected oral squamous cell carcinoma cell lines expressing E-cadherin with S100A4, and showed that the constitutive expression of the latter gene down-regulated the expression of E-cadherin. Gastric cancers with high S100A4 and low cadherin expression are highly invasive and show peritoneal dissemination. In contrast, low S100A4 and high E-cadherin expressions are found in well-differentiated adenocarcinomas (48). Human melanoma cells exhibiting high S100A4 and low E-cadherin expressions are highly metastatic when xenografted into murine recipients (49). Indeed, low/negative S100A4 and high E-cadherin expression status is indicative of good prognosis in melanoma patients (50).

Matrix metalloproteinases (MMP) have been closely identified with cell motility (2). MMPs also show an inverse relationship with S100A4 expression, as demonstrated in human glioma cell lines some years ago in our laboratory (51), and this relationship has been confirmed subsequently in osteosarcoma cells (52).

Targeting of S100A4-mediated Angiogenesis

Angiogenesis and lymphangiogenesis are essential elements of normal developmental processes, tissue repair, as well as of pathogenesis. Angiogenesis is essential for supporting tumour growth. Lymphatic/vascular channels are required for successful metastatic dissemination, which the tumour appears to be able to induce intratumorally, as well as in association with tumours (53). There is a considerable body of documented evidence that the expression of S100A4 shows significant correlation with the presence of tumour in the regional lymph nodes in breast cancer patients. This is compatible with the recently assimilated evidence that S100A4 markedly influenced the angiogenic process. Ambartsumian *et al.* (54) found a high incidence of benign haemangiomas in S100A4 transgenic mice, greater tumour-associated vascular density, and enhanced endothelial migration and induction of vascularisation *in vitro*. A significant presence of S100A4 in endothelial cells of vessels in the proximity of breast tumour, and also a significant association with vascular density, have been reported recently (54a). Therefore the

angiogenic pathway is viewed as a potential target to inhibit S100A4-mediated promotion of tumour spread.

Inhibition of angiogenesis. The targeting of angiogenic components that might be involved with the mediation of S100A4 function is one of the strategies that have recently been evolved. Among them is methionine aminopeptidase (MetAP2). The MetAPs function seems to promote the intracellular translocation of newly-synthesised proteins by removing the initiator N-terminal methionine. The inhibition of MetAPs has cytostatic effects.

The induction of angiogenesis is a complex phenomenon that involves a wide spectrum of biological activities. New vessels are formed from pre-existing microvessels with endothelia and pericytes. The pericytes need to be detached; there is a turnover of the ECM; there is proliferation and migration of endothelial cells and the formation of new endothelial tubes, pericyte attachment and vascular stabilisation. Aminopeptidases produce N-terminal modification of proteins and peptides involved in these biological processes (55). Together with other proteinases, these aminopeptidases have been implicated in angiogenesis and other physiological function. Thus, although MetAP2 inhibitors have often been described as specifically effective in inhibiting angiogenesis, it is rather difficult to reconcile this perceived specificity with the fact that the biological activities involved in vascular reconstruction also occur in other physiological and pathological situations. Nonetheless, the interest in the present discussion is the influence that S100A4 brings to bear on all or a majority of the biological features and signalling pathways associated with neovascularisation.

S100A4 seems to interact with the N-terminal half of MetAP2. MetAP2 possesses S100A4 binding sites in the region spanning amino acid residues 170-229. *In vivo*, the interaction was also demonstrated by their co-precipitation and co-localisation in cells (56). The interaction could be regulating the function of MetAP2 by altering its cellular location and, in this way, altering its activity.

Several inhibitors, *e.g.,* fumagillin and ovalicin, that specifically target MetAP2, have been identified. The drug called TNP-470, which is related to fumagillin, catalytically inactivates MetAP2 and inhibits cell cycle progression and inhibits angiogenesis (57). The cell cycle inhibition by TNP-470 has been shown to involve interference with the cell cycle regulatory function of p53. It induces p53 activation that results in the expression of $p21^{waf1/cip1}$, leading to growth arrest (15, 16). The fungus *Neosartorya* sp. produces an angiogenesis inhibitor called RK-805 (6-oxo-6-deoxyfumagillol) which specifically inhibits MetAP2 (58). A-357300 is a reversible inhibitor of MetAP2, which produces cell cycle arrest in the G1-phase specifically of endothelial cells and inhibits angiogenesis (59). Another new class of reversible inhibitors, the 4-aryl-1,2,3-triazoles, have been described recently; these seem to inhibit endothelial cell growth and angiogenesis (60). Although many of these inhibitors are reported to inhibit angiogenesis *in vivo*, the model systems used to test and make these claims need to be rigorously examined.

Angiogenic inhibition and inhibition of tumour growth is exerted by IDR-803, IDR-804, IDR-805 and CKD-732, which are powerful inhibitors of MetAP2 (61). In this case inhibition of angiogenesis is determined in xenografted murine tumours. Here again, inhibition of cell proliferation is induced by a p53-mediated mechanism, as indicated by the increased expression of $p21^{waf1/cip1}$. Certain benzimidazole derivatives have also been described to possess specific inhibitory effects on endothelial growth and angiogenesis (62). Other modes of action have been attributed to TNP-470. Nitric oxide synthase was implicated in the process (63).

VEGF signalling, S100A4 and angiogenesis. The vascular endothelial growth factors (VEGF) together with their receptors (VEGFR) constitute a major signalling pathway of angiogenesis. Genetic alterations and overexpression of genes coding for these growth factors and their receptors have significant implications for lymphangiogenesis. They seem to function *via* the downstream phosphoinositide 3-kinase (PI3K) family effectors. PI3K regulates many endothelial functions such as vascular permeability and stability. VEGF occurs as five isoforms, which bind to specific tyrosine kinase receptors, VEGF-R1 (flt-1) and VEGFR2 (flk-2), that occur in the plasma membrane of the endothelial cells. The activation of the receptors by the ligand results in the expression of serine/cysteine proteinases and MMPs. VEGF activation can also lead to the modulation of ECM composition, such as the expression of specific integrins. This ECM remodelling leads to the induction of cell proliferation and directed migration of endothelial cells. The VEGF/VEGFR signalling pathway and its components are regarded as important targets in cancer and in certain other pathological conditions, such as ulcerative colitis.

Ets family transcription factors in angiogenesis and lymphangiogenesis. The Ets family transcription factors appear to actively participate in many biological processes such as cell differentiation, proliferation and apoptosis, and are also involved in the pathological scenario of neoplastic transformation and dissemination. The Ets transcription factors are overexpressed in many cancers and the gene itself shows reorganisation in leukaemia and Ewing family sarcomas. The formation of chimeric EWS-Ets proteins results in alterations in the transcriptional regulation of several genes (64). Ets transcription factors

have been implicated in regulating the expression of several genes in the metastatic cascade.

Angiogenesis and lymphangiogenesis are essential components of tumour dissemination. The induction of angiogenesis/lymphangiogenesis is another arena of Ets involvement. It is overexpressed in a number of human neoplasms, *e.g.,* carcinomas of the prostate and the breast. Ets is not expressed in normal breast epithelium or in non-invasive cancers, but intense staining is seen in invasive cancers. The expression of the protein correlated with the Bloom grading (65). However, there was no correlation between Ets expression and clinical stage of the disease. According to Buggy *et al.* (66), Ets-1 mRNA levels were similar in fibroadenomas and primary breast carcinomas, but higher Ets protein expression was detected in the cancers compared to fibroadenoma. They detected four forms of the Ets-1 protein, *viz*. p33, p42, p51 and p52, which they regard as significant in terms of cancer dissemination, because the expression of these proteins correlated with the expression of urokinase-type plasminogen activator. Earlier, Span *et al.* (67) had reported that, in breast cancer, Ets-1 expression significantly correlated with VEGF and indeed Ets expression was a powerful predictor of prognosis. Benign prostatic hyperplasia and normal prostate tissue show no Ets expression, while both latent prostatic carcinoma and overt carcinomas do. Also, latent cancer differs markedly from clinically overt cancer with respect to expression (68). Ets-1 expression is markedly higher in peritoneal metastases; also the degree of expression in endothelial cells has been correlated with microvessel density (69). Microvessel density correlated with Ets-1 mRNA expression in both primary and metastatic ovarian cancers. Earlier, Fujimoto *et al.* (70) reported a negative correlation of Ets expression with prognosis for cervical cancer patients. In colorectal cancer, microvessel density and VEGF expression correlated with Ets-1 expression. The latter was greater in patients with nodal involvement. Ets expression inversely-correlated with survival rates (71).

Several modes of Ets function in angiogenesis can be identified. As already stated, an early step in the induction of angiogenesis is the destabilisation of vascular endothelia. Ang (angiopoietin)-2 actively participates in this process. The Ang-2 promoter contains elements that control the endothelium-related functions of Ang-2 gene.

The human Ang- 2 promoter contains several Ets-binding sites near the transcription start site, which act as positive regulators as well as negative regulators (72, 73).

The induction of angiogenesis by Ets-1 is apparently closely linked with VEGF function. Ets-1 seems to up-regulate the expression of VEGF and HGF (hepatocyte growth factor), both *in vivo* and *in vitro* conditions, by virtue of the presence of Ets-binding sites in the promoter regions of both the HGF and VEGF genes (74). The Ewing's sarcoma-specific EWS-Ets proteins activated both cyclin D1 and VEGF promoters (75). Significant differences have been found between metastatic and non-metastatic testicular germ cell tumours in terms of Ets-1 expression (76). Higher microvessel densities were recorded in more advanced tumours and in the context of enhanced Ets-1 expression. In oesophageal squamous cell carcinoma, the simultaneous expression of Ets-1 and VEGF was associated with high microvessel density and correlated with poor disease prognosis (77).

Besides aiding the transcription of genes involved in the induction of angiogenesis, Ets-1 seems to be able to promote cell migration and invasion (78), presumably attributable to the ECM remodelling that takes place as a consequence of the induced expression of MMPs and integrin receptors that regulate cell adhesion properties. Tsutsumi *et al.* (79) examined normal gastric epithelium and gastric cancers. Ets-1 was not expressed in the normal epithelium. In gastric tumours, Ets expression was not related to histological grade, but related quite markedly with the presence of tumour in the lymph nodes and distant metastases, and histologically with lymphatic and venous invasion. The promoter regions or enhancer elements of many proteinases have Ets-binding sites, and so Ets would be able to regulate the transcription of these genes.

The enhanced expression of Ets proteins might contribute to metastasis by promoting the invasive properties of cancers. MMPs and their TIMP (tissue inhibitors of metalloproteinases) have long been known to be overexpressed in and associated with cancer invasion (2). TIMPs inhibit angiogenesis (80, 81). In cervical carcinoma cell lines, MMP expression correlates significantly with VEGF expression, this correlation occurring in the invasive phenotype, but not in normal cervical tissue (82). Similar correlations of MMP and some isoforms of VEGF have been reported in the more aggressive alveolar rhabdomyosarcoma (83). Behrens *et al.* (84) presented evidence that MMPs (1, 9) are up-regulated together with Ets-1 in the fibroblastic stroma during the progression of sporadic colorectal adenomas to the invasive carcinoma stage. However, in comparison, HNPCC (human non-polyposis colon cancer), which is comparatively less invasive, showed significantly reduced expression of Ets-1 and the MMPs. On the other hand, MMP-3, -7, -12 and also -9 reportedly can induce the expression of angiostatin and, in this way, limit angiogenesis. HGF is known to induce angiogenesis and a close association was noted between the expression of the HGF receptor tyrosine kinase encoded by the c-met gene and tumour progression (2). The induction of angiogenesis and of c-met expression also correlate with the induction of MMPs (85). The MMPs, as well as Ets-1,

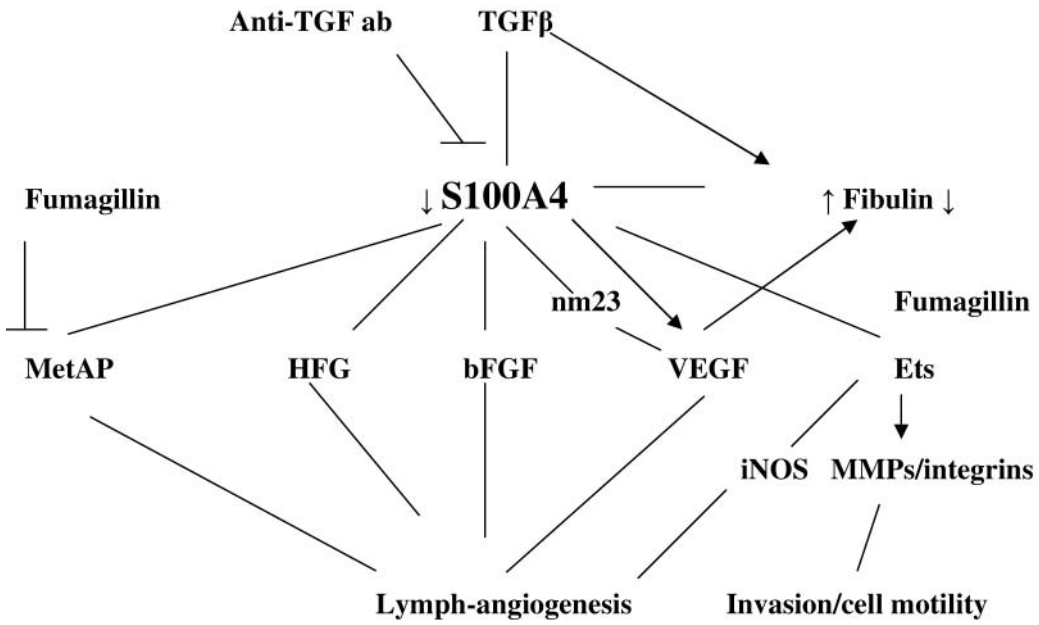

Figure 2. *Potential pathways of S100A4 signalling in angiogenesis, emphasising the possibility that S100A4 can influence virtually all the significant pathways of angiogenesis.*

are differentially-expressed in lymphatic and vascular systems, which might be one of the mechanisms by which angiogenesis and lymphangiogenesis might be independently- and differentially-regulated in tumour progression. This was demonstrated in a murine transgenic model (86).

Another pathway which appears to be mediated by Ets-1 is the stimulation of angiogenesis by nitric oxide (87). In colon cancer and colon cancer cell lines, the Ets family transcription factor E1AF expression correlated not only with MMPs, but also with COX (cyclooxygenase)-2 and iNOS (inducible nitric oxide synthase). COX-2 expression related closely with tumour size and pathology (88).

Hypoxia also induces the expression of several angiogenic effectors; among them are genes that are relevant in the context of this review. TGF-β, VEGF-R1 and FGF-2 are induced by chronic hypoxia (89). Some genes, such as S100A4, possess putative hypoxia responsive elements and so are differentially-expressed. Although this might be coincidental, one cannot ignore the possibility of some link-up between the induction of angiogenic factors and S100A4.

Several putative pathways of information flow from S100A4 that could lead to the induction of angiogenesis (Figure 2). The Ets-1 mediation of angiogenesis is possibly initiated by aberrant expression of S100A4. VEGF is a downstream target of S100A4 (10). Ets-1 expression induced by VEGF in HUVEC (human umbilical vein endothelial cells) cells is inhibited by fumagillin. Fumagillin is an inhibitor of MetAP which, as discussed elsewhere in this review, has S100A4-binding sites. However indirect it might seem, this observation does link Ets function in angiogenesis with S100A4.

Overall, a powerful case can be made for developing strategies for the inhibition of Ets-1 transcribed genes; however, the wide spectrum of genes that Ets family factors activate makes it unlikely to achieve specific targeting of tumour-associated processes alone. Nonetheless, since Ets is associated with the activation of genes determining angiogenic ability, cell motility and the invasive behaviour of cancer cells, the identification of components of Ets as targets is a worthwhile pursuit, especially in endometrial cancer where more than one angiogenic factor might be involved. Ets-1 is said to regulate VEGF in well-differentiated endometrial cancers, but in poorly-differentiated tumours bFGF (basic fibroblast growth factor) was linked with Ets-1 transcription function (90). Thus, in advanced cancers, the potential of an Ets suppression strategy might be an important option, with the possibility that there might be an angiogenic switch in the progression of well-differentiated tumours to the poorly-differentiated, more aggressive, state.

Cytoskeletal dynamics, S100A4 and angiogenesis. Another potential downstream target of S100A4 is cytoskeletal dynamics, which is demonstrably altered and regulated by

S100A4. TNP-470 is an anti-angiogenic agent derived from fumagillin. It inhibits vascular permeability and also inhibits VEGF-mediated phosphorylation of VEGFR-2 activation, Ca^{2+} influx and RhoA activation in endothelial cells (44). Therefore, it is interesting to note in this context that TNP-470 can alter the cytoskeletal dynamics, which seems to involve cofilin and HSP27 (91). It might be recalled that S100A4 can itself regulate the function of cofilin and HSP27. Cofilin possesses actin-depolymerising and severing properties, its function being inactivated or regulated by phosphorylation. The LIM-motif-containing protein kinase (LIMK1) regulates actin cytoskeletal dynamics in progression of the cell cycle (3, 92). S100A4 promotes cell proliferation, and up-regulation of cofilin expression may often be found in parallel with enhanced expression of S100A4, *e.g.,* in the C6 glioma (93), although no direct relationship between these two features has been determined to date. It should also be noted that cofilin expression was down-regulated and S100A4 up-regulated in a hepatocellular carcinoma cell line with high metastatic potential (94). On the other hand, stathmin appears to be involved in the function of S100A4 in relation to the modulation of cytoskeletal dynamics. Therefore, it might be not profitable to look at correlations of this kind in isolation and without demonstrable biological effects.

Nm23, S100A4 and angiogenesis. The expression of the metastasis suppressor gene nm23 is closely related to and probably regulated in close correspondence with that of S100A4. In experimental systems, as well as in breast cancer specimens, nm23 appears to function as a counterpoint in S100A4 expression, and the net functional effect of the activity of these two genes was correlated with metastatic ability. Interestingly, nm23 features among the genes regulated by VEGF. VEGF was found to transiently up-regulate the expression of two isoforms of nm23/NDP kinase in endothelial cells in ovarian angiogenesis, and *in vitro* in human umbilical vein endothelial cells (95). This is, however, not compatible with the promotion of progression since up-regulation of nm23 causes down-regulation of S100A4. The earlier findings of Ohta *et al.* (96) indicated, on the contrary, that enhanced VEGF expression was not only associated with enhanced nodal dissemination of non-small cell lung cancer, but also with the down-regulation of nm23. It may be that these findings (96) are more realistic than those of Zippo *et al.* (95), since the latter related to *in vitro* endothelial cell culture models. Furthermore, it was reported some years ago that, in breast cancer, nm23 inversely correlated with inducible nitric oxide synthase (iNOS) and iNOS correlated directly with nodal dissemination (97).

Fibulins and S100A4. The fibulins are secreted glycoproteins. They possess EGF-like repeats and a uniquely structured C-terminus. The fibulin family consists of six genes, which generate nine proteins by alternative splicing. The fibulins modulate cell adhesion and migration and also influence cell proliferation by virtue of being components of the ECM. Fibulin-5 binds to elastic fibres and participates in vascular remodelling and in the formation of neointima. It might be involved in angiogenesis (98). TGFβ up-regulated its expression in endothelial cells and this effect was negated by VEGF, which in fact was able to down-regulate fibulin expression. In some experimental systems, the inhibition of TGFβ results in a reduction of S100A4 expression accompanied by an inhibition of proliferation (99).

Fibulin expression is deregulated, indeed down-regulated, in many forms of cancer. Its antagonistic action against vascularisation and endothelial functions requires that it is down-regulated to promote tumour progression. However, the deregulation of fibulin expression in cancer might be more complex than it would seem. Fibulins might be differentially processed in tumour tissues (100), and extreme caution needs to be exercised before pronouncing on the expression status of fibulins in cancer. Nonetheless, a tenuous thread might link the fibulins with the tumour promoter S100A4. One of the genes that is differentially up-regulated in S100A4 transgenic mice encodes fibulin-5 (101). Therefore an auto-regulatory loop might exist in the involvement of S100A4, VEGF and fibulin (Figure 2).

S100A4 a Potential Therapeutic Target

Can inhibition of S100A4 be designed as a preventive and/or treatment modality? With the clear demonstration of the participation of S100A4 in manifold biological activities in cancers, the question arises as whether this might be a viable option. Essentially the imperative is to investigate whether inhibition of S100A4 influences the expression and function of genes associated with cell proliferation, apoptosis, angiogenesis and lymph-angiogenesis, invasion and secondary spread with which S100A4 seems to be associated. Does S100A4 inhibition down-regulate or up-regulate the relevant genes as required for promotion of tumour progression? Has S100A4 graduated from being a mere marker of progression and prognosis to an important therapeutic target that might be employed for designing treatment modalities?

There are numerous studies relating S100A4 expression or the lack of it to tumour growth, invasion and metastasis. However, there are few studies on the expression status of S100A4 where tumour growth and progression have been inhibited; this line of exploration is an important pre-requisite if S100A4 is to be regarded as a potential therapeutic target. Also required are studies aimed at

investigating the effects of S100A4 inhibition on tumour development and progression. One can identify a few significant avenues of approach to the problem of inhibiting or deregulating the functions of S100A4 with a view to deregulating specific invasion and metastasis- related tumour cell faculties. Of these, negative regulation of S100A4 expression by genes that function in specific pathways of signalling towards a given phenotypic property can cover a wide spectrum of the biological activities of the cancer cell. The inhibition of S100A4 by antisense strategy or by using anti-S100A4 ribozyme is fairly obvious, but with the involvement of this protein in both normal physiology as a transducer of calcium signalling and in pathogenesis, achieving target specificity might prove to be difficult. Nonetheless, a vast array of opportunity is afforded by S100A4 to control the growth, invasion and secondary spread of cancer.

Among these are S100A4 target genes such as CCN3 and the cadherins. As stated earlier, these seem to be able to regulate cell motility. The MMPs are another example of inducers of cell motility that are related to S100A4.

The association of MetAP with S100A4 suggests that the inhibition of MetAPs might be a valuable approach to achieve cytostatic and anti-angiogenic effects by combining MetAP inhibitors with S100A4 down-regulation. As noted earlier, fumagillin family inhibitors, which markedly inhibit the proliferation of HUVEC cells, might be regarded as potentially useful in this approach.

Direct approaches to the down-regulation of S100A4 have been attempted. BKLF (basic Kruppel-like factor/Kruppel-like factor 3 /KLF3), a member of the Kruppel-like factor family of zinc finger proteins, is a potent transcriptional repressor that recruits a CtBP (C-terminal-binding protein) co-repressor. It is expressed in a variety of human tissues. BKLF targets many transcriptional regulators, recognizing CACCC boxes in the DNA (102, 103). BKLF binds to the S100A4 promoter and the binding of CtBP2 to BKLF can then inhibit S100A4 expression (104). The occurrence of BKLF in two states has been envisaged, of which one requires high levels of CtBP2 and inhibits transcription, while the second requires low CtBP2 and activates transcription. However, the wide-spread expression of BKLF/CtBt in human tissues might make it impractical to achieve S100A4 inhibition with any degree of specificity. S100A4 is synthesised and secreted by periodontal ligament cells and is believed to inhibit mineralization. In this system, S100A4 expression can be inhibited by a short interfering RNA (siRNA) (105). Ribozyme-mediated degradation of S100A4 mRNA is another possibility that has been explored (52, 106). Antisense S100A4 mRNA was employed successfully many years ago (107). It does effectively inhibit expression upon transfection into cells with concomitant reduction in the expression of the metalloproteinases and inhibition of invasion (108). These methodologies have been used in the experimental setting. It is appreciated that these approaches would be subject to technical caveats, possibly not resolvable at present, for testing their effectiveness for down-regulating S100A4 in the clinical context.

Drug-mediated suppression of S100A4 expression might be a viable option deserving more attention. Interferon (IFN)-γ is able to do this (109). However, S100A4-expressing cells are more sensitive to IFN-γ-induced apoptosis in some cell systems (110), but not in others, and indeed protect cells from apoptosis (111). So the possibility arises of pursuing different signalling pathways of IFN effect in the presence or absence of S100A4 expression. Admittedly, there is much scope for elucidation of the apoptotic signalling and the involvement of S100A4 in this, which might strengthen the case of S100A4 as a therapeutic target.

Epigenetic silencing of S100A4 is also worth examining in this context. S100A4 is hypomethylated in many tumours. Hypomethylation of this gene occurs frequently in invasive pancreatic carcinomas and less frequently in high-grade pancreatic intraepithelial neoplasia, but not in low-grade neoplasms or normal pancreatic tissue. Three CpG sites in the first intron of the S100A4 gene were highly methylated in cells of the normal pancreatic duct but, in contrast, these CpG islands were hypomethylated in 11/12 pancreatic cancer cell lines (112). Hypomethylated S100A4 can be inactivated by 5-aza-2'-deoxycytidine or with trichostatin A, individually or in combination (113). In clinical trials with myelodysplastic syndrome patients, azacytidine significantly improved survival and, upon prolonged, treatment restored normal haematopoiesis (114). The possible significance of other epigenetic modifications has not received much attention with regard to the expression and function of S100A4.

A final aspect is the function of S100A4 and its molecular conformation. S100A4 and other members of the S100 family of proteins possess the property of homo- and heterodimerisation (115, 116). How important dimerisation is for the functionality of these proteins in terms of their interaction with other cellular proteins is as yet uncertain. The C-terminal EF hand appears to be essential for the interaction of S100A4 with its target proteins, mutations of which totally negate its biological function (117). Interference in the binding of the protein with its targets is an obvious suggestion that arises from the above discussion.

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