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Joining S100 proteins and migration: for better or for worse, in sickness and in health

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Abstract

The vast diversity of S100 proteins has demonstrated a multitude of biological correlations with cell growth, cell differentiation and cell survival in numerous physiological and pathological conditions in all cells of the body. This review summarises some of the reported regulatory functions of S100 proteins (namely S100A1, S100A2, S100A4, S100A6, S100A7, S100A8/S100A9, S100A10, S100A11, S100A12, S100B and S100P) on cellular migration and invasion, established both in culture and in animal model systems and the possible mechanisms that have been proposed to be responsible. These mechanisms involve intracellular events and components of the cytoskeletal organisation (actin/myosin filaments, intermediate filaments and microtubules) as well as extracellular signalling at different cell surface receptors (RAGE and integrins). Finally we shall attempt to demonstrate how aberrant expression of the S100 proteins may lead to pathological events and human disorders and furthermore provide a rationale to explain possibly why the expression of some of the S100 proteins (mainly S100A4 and S100P) have led to conflicting results on motility, depending on the cells used.

1. Introduction

Since their initial discovery half a century ago [1] as a group of low molecular weight acidic polypeptides (10 to 12kDa), the identification of new members of the family of S100 proteins has been gathering momentum. To date, approximately 25 different proteins have been assigned to the family which consists of 16 S100A proteins (S100A1-S100A16) as well as others (such as S100B, S100G, S100P and S100Z). These proteins exist as monomers (only calbindin is stable in this configuration), homo-, heterodimers or multimeric forms within cells and their extracellular matrices [2]. Their sequence identity data overall ranges from 16 to 98% with S100A3 and S100A7 having the lowest conserved identity and similarity (16% and 28%, respectively), whereas S100A7 and S100A15 share 95% of identical or similar sequences (Table 1). This high degree of similarity between the protein paralogues, averaging around 50% when looking across all the different members, is thought to be due to several rounds of gene duplication events during evolution [3]. Consequently, the genes encoding the majority of the S100 proteins (S100A1-S100A16) are clustered at the chromosomal locus, 1q21, into two subgroups, with S100A10 and S100A11 tightly linked in one chromosomal location and the remaining chromosme 1-members (S100A1-9 and S100A12-16) in another [4]. The genes encoding the remaining known S100 proteins, S100B, S100G, S100P or S100Z, are found on chromosomes 21, X, 4 and 5, respectively.

A feature common to all of these proteins is the presence of a pair of calcium-binding helix-loop-helix domains referred to as EF-hand calcium-binding regions towards either end of the protein and separated by a hinge region [5]. The C-terminal EF-hand motif, composed of 12 amino acids is a canonical calcium-binding domain and possesses a calcium affinity which is 10-50 times higher (Kd between 10 - 50μM) than that of the N-terminal loop [6-8], a 14 amino-acid long domain considered to be more S100 specific in its composition (referred to as S100 specific or pseudo EF-hand). The two calcium-binding motifs demonstrate the highest levels of amino acid conservation throughout the S100 proteins (Fig. 1). When considering the canonical EF-hand motif, amino acids at positions 1, 3, 5, 10 and 12 are essential for the formation of the calcium-binding loop [9] forming the consensus sequence

$D_1XN_3XD_5XXXXF_{10}XE_{12}$. This arrangement is found conserved in all S100 proteins, except for S100A10, S100A14 and S100B, where the observed sequences are $D_1XC_3XD_5XXXXF_{10}XS_{12}$, $G_1XC_3XD_5XXXXF_{10}XS_{12}$ and $D_1XD_3XD_5XXXXF_{10}XE_{12}$, respectively. The mutations and/or deletions of key residues result in the inactivation of the EF-hand motifs and the loss of their ability to bind Ca^{2+}, at least for S100A10 [10] and S100A14 [11]. The sequence of the pseudo EF-hand motifs shows that they are also highly conserved amongst the various human S100 proteins (Fig. 1). However, there is less stringency and identity than for the canonical loop, since Ca^{2+} binding to this motif is mostly accomplished through main-chain carbonyl groups, resulting in a weaker affinity for Ca^{2+} and a Kd of around 200-500μM [6].

Binding of calcium to these motifs, whenever possible, results in a conformational change that exposes a hydrophobic region of the proteins [12]. This amphipathic patch is predicted in the hinge region (Fig. 1) and the C-terminal portion of the S100 proteins [13,14]. Not surprisingly, these two regions show the least amount of sequence homology, perhaps highlighting their importance and specificity in binding to target molecules. It is through these interactions that the S100 proteins modulate the activity of other cellular components both intracellularly and extracellularly, since they themselves contain no intrinsic enzymatic activity. Sequence analysis also demonstrates that all S100 proteins lack the typical leader sequences required for endoplasmic reticulum entry and are consequently externalised independently of the orthodox endoplasmic reticulum and Golgi complex secretory route. Amongst the S100 proteins, the role of S100A13 in the non-classical secretory pathway is the best described, forming well characterised stress-dependent multimeric complexes with specific cytokines such as interleukin 1a and fibroblast growth factor 1 (FGF) (for example see [15]). However, the exact mechanisms in place to facilitate the release of S100 proteins generally remain unclear, but seem to require proper microtubule and actin cytoskeletal organisation, at least for some of the factors [16,17].

Because of the diversity of S100 proteins, and because they can regulate protein activities, both intracellularly as well as in extracellular spaces, a plethora of binding partners, and as a consequence many biological pathways, have been suggested to be affected by these proteins. Whilst there is some evidence that the presence of S100 proteins are associated with cell growth, division and differentiation (elegantly outlined in previous reviews [18-24] and some of the more recent contributions demonstrating such effects are summarised in Table 2), the presence of S100 proteins has been frequently associated with altered cell migration.

Thus, this review will focus on the reported regulatory functions of S100 proteins on cellular migration and cellular invasion, established both through cell culture work and in animal model systems, on a case by case basis. We shall present how, sometimes, contradictory roles of specific S100 proteins on cellular migration have been reported, possibly underlined by their presence as both cytoplasmic and/or extracellular pools (Table 3). We shall also summarise some of the possible mechanisms that have been proposed as potential regulators of such processes, including the targeting of cytoskeletal elements and provide a reflective rationale that could begin to explain the conflicting roles reported, on occasion, in different cell systems. Finally, through this work, we shall demonstrate how they regulate physiological processes and how, through aberrant expressions, they can also lead to pathological events and human disorders.

2. S100 proteins and their effects on cell migration

S100A1

S100A1 was, along with S100B, the earliest discovered member of the S100 proteins [25,26]. It is expressed in numerous tissues in the human body, but is specifically found at high concentration (micrograms/mg of soluble protein) in cardiac/skeletal muscle and brain [27]. Aberrant expression of S100A1 in these organs has been correlated with, but not necessarily causally, to pathological onsets, providing a new focus of therapeutic research to treat potentially neurological, heart and vascular disorders as well as diabetes mellitus and some types of cancer; these interactions are mainly intracellular (recently reviewed in Wright et al.[28]).

Loss of S100A1 expression in knockout mice *in vivo* has indicated that the animals do not suffer severe pathologies, but suggest a possible involvement for S100A1 in heart contractibility [29]. The same group has recently also proposed a function for S100A1 as an angiogenesis agent. Indeed S100A1 genetically ablated mice were found to present insufficient perfusion recovery following femoral artery resections [30]. S100A1 knockout endothelial cells isolated from the same animals demonstrated an impaired migration during scratch wound assay, suggesting that intracellular S100A1 may possess some motility-promoting effects in these cells. Altering levels of S100A1 in breast epithelial tumor cells

has, however, been shown to result in no apparent changes in migratory/invasive properties [31].

Whilst, as described above, the direct evidence of S100A1 in cellular motility, particularly *in vivo*, has been rather scarce, the reports of its expression on cytoskeletal structural remodelling have been numerous *in vitro*. Loss of S100A1 can regulate positively the levels of tubulin in rat pheochromocytoma cells, leading to an increase in neurite formation [32], whilst in astrocytes, addition of recombinant S100A1 resulted in the calcium-dependent disassembly of Triton-insoluble microtubular structures *in vitro* [33]. Consistent with these findings, purified S100A1 protein has been reported to inhibit microtubule assembly in a Ca^{2+} and pH dependent manner [6,34], where the C-terminal part of the protein is essential for their interactions [35]. Other cytoskeletal components of the intermediate filaments can also interact with S100A1 (reviewed in Garbuglia et al.[36]). Direct interaction with desmin, for instance, has been highlighted, resulting in the inhibition of desmin intermediate filaments [37].

Finally, interactions of intracellular S100A1 with the microfilaments have also been demonstrated in different cell types in culture. For instance, in the more specialised filamentous actin (F-actin) structure of the sarcomere, the formation of the titin-F-actin complex can be inhibited by S100A1 [38,39]. S100A1 can bind directly to the spring motif PEVK ((**P**)Proline, (**E**) glutamic acid, (**V**) valine, and (**K**) lysine) of the cardiac specific N2B titin variant both *in vitro* and *in situ*. Such interaction competes for the binding of titin to F-actin, resulting in the alleviation of the PEVK-based inhibition of the F-actin sliding mechanism. This competitive interaction, if proven at the organ level, may result in a significant reduction of passive tension during stretching of mouse left ventricular myocardium, providing another possible molecular explanation for the involvement of S100A1 in both cardiomyopathy and hypertrophy (reviewed in Ritterhoff and Most [40]).

The association of intracellular S100A1 with F-actin has also been documented in other cell systems, since both proteins could be seen colocalised on stress fibers in cultured vascular smooth muscle cells *in vitro* and a direct interaction, using purified proteins, was further indicated by co-sedimentation analysis *in vitro* [41]. Equally important are the regulatory effects of S100A1 on F-actin polymerisation. S100A1 has been reported to interact with Synapsin I, preventing its dimerisation and resulting in the synapsin I-dependent F-actin assembly [42].

All in all, S100A1 interactions with the various cytoskeletal components have now been well characterised. However, the physiological and biological consequences of such binding, at least in non-muscle cells, remain elusive.

S100A2

Initial findings suggested, maybe too enthusiastically, that expression of S100A2 was typically down regulated in tumors relative to normal tissue and consequently it may act as a tumor suppressor gene. The first series of reports implicating S100A2 in cellular motility came from work on human squamous carcinoma cell lines [43,44], where reduction in the levels of S100A2 mRNA by antisense technology increased cellular motility, whilst addition of exogenous extracellular S100A2 to the medium in the nanomolar range, or intracellular ectopic expression resulted in reduced rates of migration, implicating that both intracellular and extracellular pools of the proteins may influence cell motility. The biological explanations have not been unequivocally established, but initial experiments in these reports provided possible mechanisms to explain such observation. Thus, effects on cellular migration may be due to changes in the polymerisation dynamics of the actin filaments as well as a possible involvement of the receptor for advanced glycation end product (RAGE), a trans-membrane protein belonging to the immunoglobulin family [43].

Forced overexpression of S100A2 in squamous cell carcinoma cells *in vitro* has been linked to differential expression of numerous genes, some of which are involved in cytoskeletal organisation and migration [44], for example reduced level of the inflammatory-associated, cyclooxygenase-2 (Cox-2). Re-expression of Cox-2 protein in S100A2-expressing cells partially reversed S100A2- dependent loss of invasion and growth in soft agar [44].

The concept of S100A2 as a tumor suppressor gene has, however, since been challenged by more recent reports which have also highlighted its aberrant overexpression as an essential step towards tumorigenesis and metastasis in experimental cell systems [45] (reviewed in Wolf et al. [46]). Studies aiming to determine the biological consequences of its intracellular expression in different human carcinomas highlight both its cytoplasmic and nuclear location [47] and its interaction with p53 and its p67 and p77 orthologs, at least *in vitro* [48,49], thereby providing a possible model to regulate the intracellular functions of the p53 family proteins in growth arrest and apoptosis.

S100A2 expression has also been linked to enhanced chemotaxis and cellular migration and invasiveness in both physiological and pathophysiological conditions. As

early as 1996, the presence of extracellular S100A2 in the medium of eosinophils was shown to promote chemotaxis over a wide range of doses between 10^{-10} to 10^{-5}M [50]. Forced overexpression of intracellular S100A2 in stably transfected, non-small cell lung cancer cell lines can also result in enhanced migratory and invasive properties using transwell and trans-endothelial assays [51,52]. More importantly, high expression of intracellular S100A2 in non-small cell lung cell lines promoted their metastasis *in vivo* [51]. Concomitant with a role in invasion, reducing the levels of intracellular S100A2 through the use of short hairpin RNA (shRNA) in these same cells was also sufficient to prevent any further spreading of the tumor cells from the initial lesion [51]. Transforming growth factor-β1 (TGF-β1)-induced motility and invasion of hepatocellular carcinoma cell lines were significantly reduced when intracellular levels of S100A2 were knocked down using specific shRNA and small interfering RNA (siRNA) technologies [53]. Further analysis of the data indicates that the impairment in migration and invasive abilities were also seen without treatment with TGF-β1 (Discussion with Kondaiah P. and Naz S.), demonstrating a direct role of intracellular S100A2 in motility at least *in vitro*.

Biological mechanisms to explain the conflicting effects of S100A2 on cell motility and invasion in different cell systems are still missing. Unfortunately, only limited direct links between S100A2 and components of the motility apparatus have so far been reported. Interactions of S100A2 with tropomyosin have been demonstrated *in vitro* and appear to be Ca^{2+} dependent. Colocalisation of intracellular S100A2 protein with the actin cytoskeleton has only been reported in the microvilli region of the kidney epithelial LLC PK1 cells grown to high density [54].

In contrast S100A2 has been shown recently to interact with the cell surface receptor, RAGE, with a Kd in the micromolar range using surface plasmon resonance experiments with recombinant GST-RAGE proteins [55]. However, a direct correlation between their interactions and any changes in cellular motility remain to be demonstrated, providing no direct route to explain any relationship between the level of extracellular S100A2 and cellular migration. Thus overall, the links between S100A2 levels and cell migration appear contradictory in various cell systems and lack a consistent molecular explanation.

S100A4

Originally named mts1, 18A2, CAPL, FSP1, Metastatin, p9Ka, PEL98, 42A, Calvasculin and Placental Calcium Binding Protein, S100A4 is one of the S100 proteins that has received constant attention in the field of carcinogenesis, due to its significant role in directly promoting the metastatic process, first established by us in 1993 [56](see review Mishra et al. [57]). Indeed, since our original results [58], S100A4 has now been confirmed to be a very potent marker for cancer prognosis, acting as a predictor for poor outcome [59] particularly in high risk patient groups [60]. Consequently, the biological functions of S100A4 have primarily been studied in cancer systems, whether cellular or animal. S100A4 expression can provoke increased motility and invasion in cancer cell lines originating from breast, colorectal, pancreatic, lung and esophageal squamous epithelia to list just a few recently published contributions [61-69].

In non-cancerous tissues, S100A4's presence intracellularly, is increased in human endometriosis, a pathological condition in which endometrial tissue migrates to ectopic sites [70]. Similarly, expression of intracellular S100A4 protein is also seen in cells of the stromal compartment of the normal mammary gland of adult humans and during active ductal development, possibly acting as one of the mediators of mammary gland development [71], where it was originally discovered as a marker of epithelial to mesenchymal differentiation towards a myoepithelial-like phenotype [72]. Experimentally S100A4 was shown to increase the invasion of epithelial cells into the fat pad during branching morphogenesis *in vivo* in a TGF-α mediated pathway, possibly through the regulation of levels of matrix metalloproteinase (MMP)-3 and E-cadherin [73].

In other non-disease states, high levels of the intracellular protein and/or mRNAs are primarily found in motile cells *in vivo*, such as those of the immune system (peritoneal macrophages, neutrophils and human lymphocytes [74-76] as well as mesenchymal fibroblastic cells [77,74]). The true biological consequences of S100A4's presence in normal physiological processes remain to be fully characterised, since mice overexpressing [78] or lacking the expression of S100A4 [79] do not exhibit overt abnormalities compared to wild-type animals. It was only when the increased level of S100A4 expression occurred in the presence of a coupled oncogene product that gross pathologies were observed [80]. With more scrutiny, however, some changes in cellular motility have now been reported, both *in vitro* and more importantly *in vivo*, when studying different cell types and tissues. When intracellular S100A4 is depleted, macrophages are significantly impaired in their ability to reach sites of inflammation in mice, whilst bone marrow macrophages isolated from the same animals possess a reduced chemotactic motility *in vitro* [81]. At the cellular level, loss of

intracellular S100A4 expression resulted in severe loss of lamellipodia stability and pronounced random migration, suggestive of defects in cell polarisation [81]. Such observations identify the S100A4 protein as an important intracellular agent capable of regulating cellular migration in physiological conditions. Intracellular S100A4's ability to regulate cell migration *in vitro* has been further supported by work on renal proximal tubular epithelial cells in culture, where altered levels of S100A4 forced either by stable transfection or epidermal growth factor (EGF) and TGF-β1 stimulation led to a more mesenchymal fibroblastic morphology [82,83]; repressing its intracellular expression using antisense technology following EGF and TGF-β1 treatments were sufficient to repress these phenotypes [83].

Again phenotypic similarities have been observed in cancer cells. Our original work [56], confirmed by others [84,83,85], showed that overexpression of intracellular S100A4 in tumor cells *in vitro* leads to severe changes in cell architectures to a more mesenchymal type signature. Changes in motility protrusions and overall organisation of actin were also observed, with a large number of lamellipodial extensions and forward protrusions at the cell front [86,61].

S100A4 is found primarily intracellularly, at a concentration as high as 10 μM [87], with no specific sub-localisation, being observed both in the cytoplasm and in the nucleus. Traces of the protein have also been detected in the extracellular space, both in culture [17,88], in tumor interstitial fluids [89] and in the serum of ageing mice [90]. The biological functions of externalised S100A4 are unknown at present, but initial experiments suggest that addition of recombinant S100A4 (in the micromolar range) in the extracellular environment is sufficient to promote cellular migration, at least *in vitro*. Enhanced motility was therefore seen in endothelial cells [90], in human pulmonary artery smooth muscle cells [91,92] and in T-lymphocytes and fibroblasts [17,93]. In such instances, S100A4 is thought to promote these activities through either the secretion and activation of MMP, such as MMP-13 [94], and/or possibly through regulation of the activities of specific cellular receptors such as annexin 2/plasmin [88], RAGE [91,92] or possibly fibronectin deposition [17].

It is important to note, however, that not all cell types appear to respond in a similar manner to S100A4. Elevated concentrations of intracellular S100A4 protein have been shown to be inhibitory for cellular migration in astrocytes and that lowering its intracellular level through siRNA is sufficient to promote their migration, where MMP-9 and MT1-MMP may be involved [95]. This work was further supported by the fact that down regulation of

S100A4, either through siRNA treatment in astrocyte cultures, or in S100A4 knockout transgenic mice, was sufficient to promote cellular migration in response to injury, resulting in a reduction in glial scar formation in animals [96]. This potential pathophysiologic role of S100A4 in the central nervous system has recently been challenged. Indeed high expression of S100A4 by astrocytes has now been demonstrated in response to traumatic brain injury (in both human and rodent systems) and brain excitotoxicity. Such trauma resulted in the subsequent release of S100A4 from these cells into the extracellular environment, inducing neuroprotective effects, possibly through the regulation of metallothionein I and II [97]. The exact mechanisms leading to such protective functions remain to be fully elucidated, but initial experiments indicated the presence of two neurotrophic motifs on S100A4 which resulted in the activation of the Janus kinase/STAT pathway to prevent neurodegeneration [97].

Overall the direct molecular pathways that are responsible for the regulation of cellular motility remain to be fully characterised, since numerous pathways have now been suggested to regulate such a property (above remarks and herein). S100A4 has been shown to interact with proteins involved in the cytoskeletal architecture which may be a possible link with motility. Indeed S100A4 has been reported to bind directly to tropomyosin [98] and to F-actin [99,100]. The direct biological consequences of such interactions still remain to be elucidated *in vivo*, since other studies have demonstrated much lower binding affinities using biometric analysis in the case of tropomyosin [101].

More recently intracellular S100A4 was shown to interact with the Rho binding and regulating protein, Rhotekin, through pull-down and immunoprecipitation experiments [102] in a complex where RhoA was also present. These proteins have been intimately linked with cell polarity and migration [103-107]. Reduced expression of intracellular S100A4 or Rhotekin by targeted knockdown led to diminished invasion and migration of MDAMB231 breast cancer cells through an increase in contractile F-actin stress fibers. This new finding suggests that intracellular S100A4 and Rhotekin possibly share a cooperative signalling event resulting in the regulation of the RhoA pathway, at least in cultured cells.

The cytoskeletal complex with which intracellular S100A4 interacts that has received most interest is undoubtedly the non-muscle myosin (NM) heavy chains [108-110,101]. The NM heavy chains, stabilised by the essential light chains and controlled by the regulatory light chains, form fully functional myosin structures present in all non-muscle eukaryotic cells. They play essential roles in cellular processes where force generation and movement are required. Among others, the NM composed of the isoforms IIA, IIB and IIC (NMIIA,

NMIIB and NMIIC) are seen as crucial components of cell polarity and migration, participating in the remodelling of the actin cytoskeleton [111,112]. Whereas NMIIA force generation is responsible, at least in part, for the assembly of the actomyosin network in cellular protrusions and the dynamics of adhesion, NMIIB establishes front to back cellular polarity through the cross-linking of actin filaments in cultured cells [113,114]. Analysis at the biochemical level and using recombinant proteins indicates that S100A4 preferentially binds to and inhibits the assembly of NMIIA filaments, but has little effect on NMIIB organisation [108]. This specific intracellular interaction has been confirmed by fluorescence lifetime imaging microscopy in cultured cells [115]. Consistent with such observation, absence of intracellular S100A4 leads to an over-assembly of NMIIA complexes in cultured bone marrow macrophages, possibly leading to the instability of the different cellular protrusions formed [81]. Moreover overexpression of intracellular S100A4 in breast carcinoma cell lines results in large cellular lamellipodia formed at the leading edge, but a general loss of filopodial extensions and focal adhesion assembly and maturation [61]. These latter effects may indeed be due to the ability of intracellular S100A4 to interact with NMIIA, since the expression of a truncated form of the protein, which prevents its binding to NMIIA [116,117], leads to loss of filopodial extensions and assembled focal adhesions. The resulting mechanisms are still not entirely clear [87], but it is logical to suggest that, since intracellular S100A4 is thought to affect NMIIA disassembly by binding to the unstructured NMIIA tail [118,110,119], its absence may lead to an over-assembled network of NMIIA [81], whilst its intracellular overexpression and binding may prevent and even unzip the overall organisation of NMIIA filaments [118].

It is also important to note that the motility-promoting effects of S100A4 might not be exclusively due to direct regulation of the cytoskeleton architecture. Evidence of a more general regulatory function have come to light recently demonstrating the involvement of the AKT/slug pathways in S100A4 mediated cell migration [67], where specific down regulation of intracellular S100A4 in esophageal squamous cell carcinoma resulted in low activity of AKT, low expression of the transcription factor slug and in parallel an increase in E-cadherin levels. Loss of E-cadherin and activation of slug transcription factor are seen as hallmarks of epithelial mesenchymal transition [120]. The inverse association between S100A4 and E-cadherin expression is not novel and has been described in carcinoma cell lines [121], but the demonstration that such effects may be regulated through AKT activation is new. Other S100 proteins have been shown equally to regulate AKT activity, as part of a more complex signalling cascade [122-124].

The Wnt/β-catenin pathway is also associated with S100A4-mediated cell migration [68], where the presence of a T cell factor (TCF) binding site in the 5'-untranslated region of the S100A4 promoter has been identified. Furthermore, β-catenin has been shown to bind to this region and consequently increase the expression of the intracellular S100A4 protein, resulting in subsequent enhanced migration and invasion of colon carcinoma cells [125]. This regulatory mechanism has recently been utilised to isolate drugs that might ultimately lead to the reduction of S100A4 expression, namely calcimycin [68] and sundilac [126], possibly through regulation of the β-catenin pathway; these have been shown to have potential therapeutic effects on colon carcinogenesis.

S100A6

The role of S100A6 protein (calcyclin) has been linked to changes in cellular motility and cytoskeletal reorganisation, but obtaining a clear picture has been challenging to ascertain, since its expression seems to lead to cell-specific as well as different *in vivo*/*in vitro* phenotypes. The most consistent results reported that when NIH-3T3 fibroblastic cells were forced to express low levels of intracellular S100A6 by knockout technology, there was a vast reorganisation of the actin cytoskeleton with an extensive cortical network of actin filaments and tropomyosin structures [127,128]. In parallel, the number of focal adhesions was seen to be significantly increased at the cell periphery, as determined by immunofluorescent staining for vinculin. These factors, may, therefore, be responsible at least in part, for the large increase in lamellipodia and possibly for the enhancement in cellular motility seen when intracellular S100A6 levels are knocked down [127].
The involvement of S100A6 in the motility of cancer cells has also been reported, albeit with contradictory outcomes. Manipulating intracellular S100A6 levels in osteosarcoma cells, either by down-regulating or up-regulating its expression, led to increased or decreased migration, respectively, as measured by the wound healing assay, further suggesting a role for S100A6 as an inhibitor of cell motility in cultured cells [129,130].

However, S100A6 has also been shown to promote cellular motility in pancreatic cancer cells. Thus, reduction of its normally upregulated intracellular levels in this type of cultured tumorigenic cell leads to a reduction in their migration and lower invasive properties [131,132] by a mechanism that is dependent on the presence of annexin 2. Such data is supported by other correlative experiments performed on animals and tissues, where elevated

levels of intracellular S100A6 have been shown to be associated with tumorigenesis (reviewed in Lesniak et al. [133]) and the ability of colorectal adenocarcinoma cells [134] and Ras transformed NIH 3T3 cells [135] to metastasize to form secondary lesions.

The molecular mechanisms utilised by S100A6 to regulate cell motility have remained elusive. Direct interaction between S100A6 and the tropomyosin-actin complex has been shown *in vitro* following cross-linking experiments [136], but remains to be confirmed *in vivo*, since the only current evidence seems to suggest that S100A6 acts as a down-regulator of tropomyosin expression [132]. Given the current uncertainties as to the role of tropomyosin in cellular motility [137], a direct correlation between their interactions and migratory properties is no more than conjecture. Other components of the actin cytoskeletal architecture, in the form of the myosin ATPase inhibitors, caldesmon [138,139] and calponin [140] can also interact with S100A6 *in vitro*, but no mechanistic link to cell motility has been demonstrated.

S100A7

S100A7 (psoriasin) is regarded as an inflammation-associated protein and to have chemoattractant properties, promoting migration of granulocytes, monocytes, macrophages and lymphocytes *in vitro* in the Boyden chamber assay, when added extracellularly at nanomolar concentration or if present in conditioned media [141,142]. Its action on enhancing cellular motility *in vitro* has also been reported in other non-hematopoietic cells as well as cells from pathophysiological conditions such as osteosarcoma, oral squamous and breast carcinoma [143-148]. It is currently unclear where S100A7 is located in these cells and the molecular pathways required. In some cases, these results suggest a role for the extracellular pool of S100A7 promoting cell migration, at least *in vitro*. Indeed an increase in cellular motility could be reduced by addition of an antibody to S100A7 in the culture medium and was dependent on the RAGE receptor, since abrogating the receptor function using antibodies directed against it or by specific siRNA down-regulating the RAGE receptor resulted in suppressed migration and chemo-attraction [141,143,144].

In contrast, intracellular S100A7 can also interact with the multifunctional c-jun activation domain binding protein 1(Jab1) in human breast cancer cell lines [145]. Interestingly, the expression of a triple mutated form of the S100A7 protein in breast MDAMB231 cells that is unable to interact with Jab1, but has retained its ability to form dimers, demonstrated reduced ability to induce cellular migration, suggesting that the

intracellular S100A7-Jab1 interaction may play a role in such an event *in vitro* [147]. Another intracellular binding partner for S100A7 is the integrin subunit β6 identified through a proteomic approach using the β6 subunit cytoplasmic tail as bait. Immunostaining strengthens the case for a possible interaction between these two molecules, since they were seen to colocalise at the cell membrane and in intracellular vesicles in cultured cells [146]. Such interaction may also play some role in cellular migration and invasion. Thus, disruption of their binding, either by reducing the levels of intracellular S100A7 with siRNA or by the use of a membrane permeable TAT peptide conjugated to the C-terminal β6 residues containing the S100A7 binding sites, were both sufficient to inhibit αvβ6-dependent invasion *in vitro* [146].

Important evidence supporting a role for S100A7 in promoting migration/invasion *in vivo* has also recently come to light through the use of the mouse paralog of S100A7, mS100a7a15 inducibly expressed in a transgenic mouse model and aggressive MVT-1 cells (derived from mice doubly transgenic for MMTV-c-Myc and MMTV-VEGF)[142,149]. Induction of high levels of mS100a7a15 in the transgenic animal was shown to increase dramatically the metastatic abilities of MVT-1 cells, resulting in the formation of secondary lesions in the lung. The direct mechanisms are not fully understood, but changes in the expression patterns of molecules such as MMP-9 and vascular endothelial growth factor (VEGF) in MVT-1 cells and/or the recruitment of macrophages to the sites of the primary lesion have been put forward as possible explanations [142].

S100A7 has also been shown to act as a potential tumor suppressor and to inhibit cellular migration when expressed. For instance, overexpression of S100A7 was found to decrease significantly the chemotactic and migratory abilities of MCF7 and T47D breast cancer cell lines through a possible loss of lamellipodia [150]. Further analysis suggests that high expression of S100A7 down-regulates the β-catenin/TCF4 pathway through an enhanced interaction of β-catenin and E-cadherin. Supporting the role of S100A7 as an inhibitor of cellular migration in cancer cells, further work with MDAMB468 breast cancer cell line demonstrated that down-regulation of S100A7, using a specific short hairpin RNA, resulted in a reproducible and consistent increase in cell motility and invasion in Matrigel-lined chambers [151]. In this work, S100A7's ability to regulate the expression of MMP-13 and VEGF were advanced as potential mechanisms towards increasing motility *in vitro*.

S100A8 and S100A9:

High levels of S100A8 and S100A9 proteins have been correlated with increased cellular motility and migration through biomembranes using different cultured cell systems, including leukocytes (recently reviewed in Goyette and Geczy [152]). These properties have been linked to both intracellular and extracellular roles for such proteins, using methods to regulate their concentration in cells or through the use of purified proteins or specific antibodies to enhance or counteract their functions, respectively. For instance, the presence of either recombinant S100A8 or S100A9 proteins in the medium (10^{-12} to 10^{-9}M) was sufficient to activate neutrophils and induce a significantly raised chemotactic response in modified Boyden chambers, whilst the sole addition of antibodies raised against either one was also enough to prevent cellular invasion *in vitro* [153]. It is, however, important to relate these findings to a potential physiological role. Interestingly, concentrations of S100A8 and S100A9 in human serum are found to be in the nanomolar range [154], concentrations that are therefore much higher than the ones used in this study. It is further thought that higher levels of S100A8/A9 found at sites of inflammatory conditions [155-157] are, at least in part, responsible for the subsequent infiltration of neutrophils and activation of monocyte/macrophages, casting some doubt on the biological relevance of this *in vitro* data.

Other cell types are similarly affected by the presence of S100A8 and S100A9 in their extracellular environment. Thus HUVEC endothelial cells and PNT1A SV-40 immortalised normal human prostate cells in culture can penetrate through transwell membranes and/or migrate more efficiently on addition of purified S100A8 or S100A9 at concentrations in the micromolar range or obtained from conditioned media [158-160]. This extracellular motility enhancing property is also conserved in some tumor cells, since addition of S100A8 or S100A9 to the medium provoked dramatic increases in migration of the rectal cancer cell line SW837 [161]. Furthermore, genomic ablation of S100A9 resulted in mice that had significantly decreased tumor incidences and reduced rates of metastasis following spontaneous tumor formation using or after ectopic injection of MC38 colon tumor cells [162], importantly supporting a role for this protein in motility and invasion of cancer cells *in vivo*. Different cellular receptors have now been highlighted as potential mediators of the extracellular S100A8-S100A9-dependent migration. Indeed evidence indicates that the S100A8/A9 protein dimers interact or colocalise with RAGE at the surface of colon, prostate and melanoma tumor cell lines [162,160,163]. Their colocalisation appears to be important for the enhancement of cell motility, since addition of RAGE antibody to murine metastatic melanoma B16F10 cells in culture is sufficient to counteract the migration-promoting effects

of picomolar concentrations of either S100A8 or S100A9 [163]. In other cells from non-pathophysiological conditions, S100A9 at concentrations around 10^{-7} M is thought to promote human neutrophil chemotaxis through activation of the β_2 integrin, Mac-1 receptor [164]. S100A9 (but not S100A8) can also interact specifically with the cell surface glycoprotein EMMPRIN (BASIGIN) and that high expression of this receptor is required to induce the migration of melanoma cells, possibly through increases in MMP1 expression [165].

Intracellular molecules can also be specifically regulated in the presence of high micromolar levels of extracellular S100A8/S100A9 in cultured cells. As such, treatment of cells with recombinant S100A8 resulted in dramatic changes in the actin polymerisation of human polymorphonuclear neutrophils (PMN) and WEHI 265 monocytoid cells, where F-actin accumulation within pseudopodia was profoundly affected, possibly explaining the changes observed in cell shape and cell size [166]. Lewis Lung carcinoma cells treated with recombinant S100A8 or S100A9 proteins ranging from 10^{-13} to 10^{-8}M also demonstrated significant morphological rearrangements with the formation of large cellular protrusions, possibly pseudopodia and invadopodia, which were dependent upon activation of mitogen-activated protein kinase p38 [167]. Supporting this observation, the phosphorylation of the complex S100A8/S100A9 by p38 was shown to regulate its association with F-actin *in vitro*. This colocalisation could also be seen in cultured human neutrophils in the actin-rich regions of lamellipodia following stimulation with the strong chemoattractant fMLP [168]. Others, however, have suggested that S100A9-deficient PMN cells demonstrated abnormal polarised cell shape with strong accumulation of F-actin in pseudopods [169], therefore blurring somewhat the true roles of these proteins in actin remodelling.

Besides these changes induced by extracellular levels of the S100A8/S100A9 discussed so far, other evidence suggests that the intracellular localised S100A8/S100A9 pools can equally affect these proteins' functions. Thus, interactions of the S100A8/S100A9 complex with the microtubule network and intermediate filaments have been reported in cultured hematopoietic cells [170,171]. The direct interaction between tubulin and S100A8/S100A9 takes place in a calcium-dependent manner, resulting in an increase in the number and stability of tubulin filaments [170] with follow-on studies demonstrating that the formation of a $(S100A8/S100A9)_2$ tetramer is essential for the promoting effects of these proteins on microtubule formation [172]. Cell work has further demonstrated that S100A9-deficient phagocytes contain lower levels of polymerised microtubule filaments, an observation that may explain differences in migratory properties seen in such cell backgrounds [170]. Neutrophils isolated from S100A9-deficient mice also showed migration

rates that were lower than those from wild-type mice, particularly when they were stimulated with Interleukin IL-8; their ability to cover greater distances than unstimulated neutrophils was also reduced [169]. In contrast another study demonstrated that treatment of the same S100A9 null cells with other chemokines (FMLP, KC and MIP-2) did not result in any significant change in chemotaxis [173].

S100A9 has been shown to be important for transendothelial migration of granulocytes following activation by arsenite, since S100A9 -/- cells showed no acceleration of their migratory properties when compared to their wild type counterparts [170]. Importantly such inhibition of neutrophil/granulocyte motility could also be observed *in vivo*. Thus when LPS was injected into the murine air pouch, it resulted in a rapid accumulation of neutrophils. However this recruitment of neutrophils could be efficiently prevented with an antibody to S100A8, indicating the importance of extracellular S100A8 in neutrophil accumulation [174]. S100A9 is also a vital regulator of granulocyte migration in a wound healing model, since these cells from the knock-out mice demonstrated severe reduction in their ability to infiltrate neighbouring tissues and resulted ultimately in a decelerated closure of skin wounds when compared to control animals [170].

S100A10

S100A10 has recently been shown to regulate macrophage invasion both *in vivo* and *in vitro*. Thus when macrophages from S100A10-/- transgenic mice were isolated, they exhibited a dramatic reduction in invasion through the Matrigel barrier in a Boyden chamber, but no changes in overall migration [175]. Similar observations were made when studying the recruitment of leukocytes into intraperitoneal cavities, with a much lower number of the S100A10-/- cells able to reach such cavities, highlighting an important regulatory role for S100A10 in such infiltration *in vivo*.

Changes in expression of S100A10 by these macrophages have also highlighted a crucial role for S100A10 in carcinogenesis *in vivo*. Thus, tumor growth from T241 fibrosarcomas or murine Lewis lung carcinomas was significantly impaired in another study using S100A10-/- null mice due to a loss of macrophage recruitment at the tumor site [176].

However, it is thought that some of the cancer-promoting abilities of S100A10 may, in fact, be due to another mechanism. Thus, upregulation of intracellular S100A10 expression has been demonstrated in high grade and basal-type breast cancers compared to low grade and non-basal types, suggesting a possible role for this protein in the migratory and/or invasion steps required for dissemination of the tumor cells [177]. Such a suggestion has been

further strengthened by reports showing that S100A10 can play a role during invasion and migration *in vitro*, although there is some uncertainty in the latter. Thus when the level of intracellular S100A10 was knocked down, invasion of colorectal cancer cells and human HT1080 fibrosarcoma cells through Matrigel membranes in the presence of plasminogen was reduced, but surprisingly cellular migration was unaffected [178,179]. Two other independent reports have since presented the ideas that S100A10 plays an essential role during cell motility at least *in vitro,* since down regulation of its intracellular expression in a human epithelial squamous carcinoma cell line and in aggressive lung cancer cells led to a significant reduction in cellular migration using the scratch wound assay [180,181].

The ability of S100A10 to remodel the actin cytoskeleton is not novel. Initially S100A10 was shown associated in a heterotetrameric complex with annexin 2 at the plasma membrane [182,183]. Subsequent experiments highlighted the ability of this complex to bundle actin filaments in a calcium-dependent manner [184,185]. S100A10 can play a major role in overall actin remodelling and motility in a human epithelial squamous carcinoma cell line, since down-regulation of its expression using specific siRNA led to a disorganisation of actin filaments and impaired cellular migration when using the *in vitro* scratch wound assay [180]. The Rho GTPase-activating protein DLC1 protein interacts with S100A10. This interaction recruits S100A10 away from annexin 2 and targets it to ubiquitin-dependent degradation, therefore reducing its steady state level, leading to lower cell migration and invasion of the aggressive lung cancer cell lines *in vitro* [181].

S100A11

The S100A11 protein has been linked to changes in cellular motility and cytoskeletal reorganisation, as well as involvement in tumorigenesis, but a clear picture has not emerged (recently reviewed in [24]). Its overexpression is observed in a large variety of carcinomas, suggesting that S100A11 plays an important regulatory role in carcinogenesis and cell proliferation [124,186], whilst others suggest it possesses tumor suppressing abilities [187]. It is thought that its presence, whether intracellular or as a stimulus from the extracellular environment, as well as its actual subcellular location, may be responsible, at least in part, for the observed antagonist effects of the protein [188].

S100A11 has been demonstrated recently to promote cellular migration in response to cell treatment with hypoxia-induced mitogenic factor. Thus depleting levels of intracellular S100A11 using siRNA technology was sufficient to compromise significantly the migration

rates of smooth muscle cells following treatment with hypoxia-induced mitogenic factor, a protein that promotes cellular motility. This change in cellular motility also coincided with the translocation of S100A11 from the cytosol to the plasma membrane and the nucleus [189]. Changes in S100A11 subcellular location have also been reported following the formation of cell-cell contacts and have been linked to the phosphorylation status of the protein [187]. High expression of S100A11 has been demonstrated equally to lead to an increase in cell protrusions and pseudopodia, possibly through the control of actin organisation [187]. Supportive of these findings is the fact that S100A11 can interact with actin both in cultured cells and in the test tube and that their association is regulated by phosphorylation in response to cell-cell contacts, since phosphorylated S100A11 was found to occur in the nucleus [187]. Other analyses have demonstrated the interactions of S100A11 with annexin 1 [190] and the annexin 2 receptor at the cell membrane. [189]. The consequences of these interactions on cellular motility remain to be elucidated.

S100A12

S100A12 is present in the myeloid cell lineage, since it is found in abundance in granulocytes [191] as well as monocytes [192,193] and lymphocytes [194] in human but is not expressed in mouse counterparts. Some of the biological functions related to S100A12 are mediated by its association with the RAGE receptor, at least in cultured cells [195], but other receptors such as those of the G-protein-coupled family may also be important [196]. Extracellular S100A12 can induce directional migration and chemotactic responsiveness of monocytes and neutrophils *in vitro* [193], however, it is not known whether these effects relate to physiological extracellular concentrations of S100A12. Furthermore, injection of S100A12 intraperitoneally into mice led to increased recruitment of leukocytes at the site of administration, highlighting its potential role in regulating both migration and chemotaxis *in vivo* from the outside of a cell. However, since these cells may have expressed S100A8/9, which can also affect migration, these results may have be confounded by the presence of other active S100 proteins.

The mechanisms whereby S100A12 promotes chemotaxis have not been clearly established. One of the key steps towards chemotaxis and migration from the blood to the inflammatory site is the adhesion of cells of the leukocyte lineage. S100A12 has been shown to promote monocyte, neutrophil and lymphocyte adhesion *in vitro* [195,193,197]. Such properties were, at least in part, due to activation and increased expression of the Mac-1

integrin [197], a molecule that can interact with fibrinogen and has equally been shown to be regulated by S100A9 [164].

Other transmenbrane proteins that are activated by S100A12 are ICAM-1 and VCAM-1, as well as the RAGE analogue [195], and all of these may be important for leukocyte recruitment, at least *in vitro*. At the intracellular level, S100A12 can increase actin polymerisation, also associated with calcium flux in monocytoid cells [193].

S100B

The S100B protein is highly abundant in the brain, where it localises to astrocytes, and can be found both intracellularly and extracellularly, where it is believed to exert different biological roles. For example, it can induce severe changes in cellular proliferation, apoptosis and cell differentiation, through different pathways; these pathways have recently been reviewed [18] and will only be briefly discussed here with regard to their effects on cellular migration (effects on proliferation of myoblasts/lung adenocarcinoma cells and on differentiation of chondrocytes/myeloblasts are summarised in Table 2).

Overall and to the best of our knowledge, S100B has been proven to be an important inducer of cell motility in most, if not all, cell systems used *in vitro*. A direct correlation between its expression and cellular migration has, however, remained elusive *in vivo*, except in disease states. Indeed mice where S100B expression has been ablated via gene targeting have demonstrated very little problematic physiological consequences and no clear changes in tissue structures of the brain [198]. High level expressions of S100B have been linked to carcinogenesis *in vitro*, particularly melanoma, as well as brain-derived astrocytomas and glioblastomas, where S100B is thought to induce cell proliferation through interaction with p53 [199].

It is now well accepted that one of the more direct regulatory effects of extracellular S100B, at micromolar concentrations, on cellular migration is promoted through its interaction with RAGE, both in cultured cells and in cell-free systems using purified proteins (see references herein and [200]). The cascades of signalling pathways activated by the coupling of these two proteins have, however, been shown to be different, depending on the type of cultured cells studied. For instance, in neurons, the S100B-RAGE complex has been linked to extension of neurite outgrowth in a Cdc42-Rac1 dependent manner [201], whilst in murine microglia and vascular smooth muscle cells, this effect is promoted by the activation

of a myriad of effectors, including Src kinase [202,203]. The downstream effectors following on were, however, not identical, some activating the Ras pathway, whilst the MAPKs (p38MAPK and ERK1/2) and transcription factor NF-kB were activated in others. The use of inhibitors directed towards either Src or p38/MEK kinase have clearly established their importance in both vascular smooth muscle cell [203] and Schwann cell migration *in vitro* [204].

Besides the extracellular role of S100B through the RAGE receptor, recent reports have highlighted other possible pathways where S100B may encourage cellular motility *in vitro*. A reduction in the levels of S100B in astrocytoma cell lines, obtained by siRNA technology, resulted in reduced migration, possibly through the rapid collapse of F-actin at the plasma membrane. Such changes may to be due to the loss in intracellular levels of the S100B protein, since addition of extracellular recombinant S100B, in the nanomolar range, was not sufficient to reverse these phenotypic changes [123]. Similar observations regarding S100B expression and motility were also observed in cancer cell lines. When the expression of S100B in non-small cell lung cancer PC14 cells was altered following either transfection with episomal plasmids or with siRNA, it affected cellular migration in transwell assays as well as invasion using Boyden chambers. Thus, increased levels of S100B could promote motility, whereas reducing its levels correlated with a significant reduction in cell movement *in vitro* [205,206]. In these two cases, it is unclear whether the phenotypic changes were preferentially due to intracellular or extracellular pools of the S100B proteins.

Interactions of S100B with numerous components of the cytoskeleton have also been reported. Using purified proteins, S100B has been shown to interact directly with components of the actin cytoskeleton such as CapZ [207] and caldesmon [208], the microtubule protein tubulin [209] and tau [210]. Recent studies have reported the colocalisation of S100B with different cytoskeletal architectures [211,212], but proof of their interactions in living cells has been more difficult. The direct biological implications of such subcellular locations have only been linked to motility through coincidental observations so far. For instance, the RhoA/ROCK pathway has been put forward as a possible mediator of cellular migration activated by S100B [123], whilst similarly, the formin protein, diaphanous-1, is also recruited and is essential for any observed migratory enhancement produced by S100B [202].

S100P

As with some of the other S100 proteins discussed above, the role of S100P in neoplastic progression has generated much interest over the last decade [213,214] and was recently reviewed in Gibadulinova et al. [215]. S100P expression is, however, not restricted to carcinogenesis, since it can be seen readily in most human tissues, particularly in the placenta and oesophagus [216]. The direct physiological implication of its expression is currently unclear, although a recent investigation has proposed a role for S100P in endometrial implantation [217] and the regulation of its expression in the endometrium has been demonstrated further to vary according to the ovarian cycle [70]. Similarly, an emerging consensus has now clearly linked S100P expression with promoting cellular motility and invasion in numerous disease states, such as cancer (discussed below) and endometriosis, but the direct demonstration that the protein retains similar properties in healthy cells has so far, and to the best of our knowledge, not been reported. It is therefore through studies of different carcinomas, in animals, tissues and at the cellular level, that most information on this protein has been acquired, sometimes through coincidental observations of the aberrant levels of S100P and the carcinogenic and metastatic nature of the tumors studied [218]. Recent work in culture has indeed demonstrated that direct ectopic overexpression of intracellular S100P is sufficient to promote cellular motility of rat mammary and human HeLa cells [219], a human lung squamous carcinoma cell line HTB-58 [220], human pancreatic carcinoma cell lines [221] and human breast carcinoma cell lines [222]. The reverse experiments also appear to hold true and specifically reducing the aberrantly high levels of intracellular S100P in cancer cell lines, obtained from the colon [223,224] and the pancreas [225,221], reduce both their migratory and invasive properties *in vitro*. Importantly direct evidence has also been presented in animal models, where inducing high S100P expression is sufficient to promote carcinogenesis and metastasis [225,213,221], whilst down regulating its level is enough to impede normally highly malignant cells from forming secondary lesions [223].

The molecular mechanisms for the S100P-dependent effects on cellular migration and invasion have been the focus of different investigations, generating different outcomes, depending on the cultured cell types used. Cellular targets that could contribute to such phenotypic changes include intracellular components of the actin cytoskeleton, and over the years, S100P has been reported to affect directly the properties of a number of proteins

involved in remodelling of the actomyosin network. The direct interaction between ezrin and S100P was first demonstrated *ex vitro* through affinity chromatography. This binding resulted in the cosedimentation of the complex along with F-actin. Further *in vitro* studies on the human lung squamous carcinoma cell line HTB-58 suggested a correlative link between S100P-ezrin interaction and transendothelial migration, in that ectopic expression of a S100P mutant, incapable of binding to ezrin, was similarly unable to promote cellular invasion which was observed when expressing the wild type counterpart [220]. Another actin regulator IQGAP1, which is thought to promote actin reorganisation through the Cdc42 and Rac1 pathways can also interact with high affinity with S100P in pull-down, co-immunoprecipitation and surface plasmon resonance experiments (Kd=0.2μM) [226]. The biological consequences of their binding on migration is not clear, since expressing S100P appeared not to induce significant changes in the overall actin organisation of HeLa cells (although no actual staining was provided) and no data was given relating to their migratory properties.

In contrast, upregulating intracellular S100P expression in other cancer cell lines, such as pancreatic, Panc-1 and colon, LS174T cells has resulted in significant changes in cellular morphology and cytoskeletal organisation along with enhanced cellular migration [227,224], suggesting that S100P expression may induce different cell specific phenotypes. Indeed, ectopic expression of S100P in Panc-1 cells was found to correlate with the down regulation of several cytokeratins, but a robust phosphorylation level of cofilin along with an increase in S100A6 and cathepsin D proteins. The latter was further shown to be, at least in part, responsible for the invasive abilities of the S100P-expressing cells. Reducing S100P levels in colon LS174T cells by shRNA technology, resulted in severe abrogation of cellular protrusions (referred to by the authors as invadopodia structures) and reduced cell motility *in vitro* [224].

In our hands, and using an inducible system, intracellular S100P expression was found to affect dramatically F-actin organisation in cultured rat mammary and HeLa cells, resulting in a severe disruption of the stress fibers stretching through the cytoplasm [219]. This loss in actin filaments was also shown to lead to a dramatic reduction in focal adhesion formation and stability. Such effects were demonstrated to be caused, at least in part, by direct interaction of S100P with the non-muscle myosin IIA isoform *ex vitro* and *in vitro*, suggesting that, as with S100A4, S100P expression could disassemble the myosin IIA network, resulting in possible loss of stress fiber contractility and reduced maturation and

formation of focal adhesions. Such changes would, in turn, result in increased cellular motility, a mechanism that was supported by experiments in which either down-regulation of myosin IIA or vinculin using siRNA technology, resulted in a similar non S100P-dependent increase in motility *in vitro*.

However extracellular targets for S100P have also been identified as important inducers of some of its migratory activities *in vitro*, suggesting that it may also have physiological roles outside the cell. In support of this argument, S100P has been shown to be secreted from pancreatic Panc-1 cell lines where it activates RAGE, resulting in increased cell proliferation [221]. The wild type Panc-1 cells also acquired migratory and invasive abilities through the addition of recombinant S100P proteins in the nanomolar range, although no quantification of the motility was provided and a direct connection between S100P-RAGE was not presented [221]. Independently, migration of SW480 colon cancer cells through the Transwell motility assay was found to be significantly improved following treatment with nanomolar concentration of recombinant S100P protein [228], whilst the addition of an antagonist of the RAGE receptors blocked this effect, suggesting a possible role for S100P-RAGE inn cellular motility, possibly through the ERK1/2 and NF-kB pathways, at least *in vitro*.

2. S100 proteins, cellular migration and diseases

Through decades of research, the family of S100 proteins has been linked to numerous pathologic conditions which have been comprehensively reviewed [229,84,230-232] and other reviews herein, to cite just a few). A few points related to specific S100 proteins, migratory properties and diseases will be succinctly summarised here.

Although the large majority of S100 proteins have been reported to be associated with cellular motility and to be involved, at least coincidentally, in a plethora of diseases, direct evidence has, to our knowledge, been reported unambiguously only in carcinogenesis/ metastasis and other "physiological" invasions, such as fibrosis, where they are usually considered to be relevant markers of disease progression [28,46,57,133,215].

Intracellular expression of S100A4 (reviewed in Schneider et al. [84]) and to a lesser extent S100P [233,234], have now illustrated the possible transition of epithelial tumor cells to a more mesenchymal morphology. These, along with the expression of specific MMPs such as MMP-3, -9 and -13 can start to account for the increased motility and invasive properties respectively, seen during the steps of metastasis. Other concepts also support the

role of S100A4 and other S100 proteins, through a change in the tumor micro-environment, providing cues and stimuli that encourage outgrowth of overt metastases, in a series of events usually referred to as the metastatic niche [235,20]. Indeed, S100A4 expressing fibroblasts may be needed at tumor sites to facilitate carcinogenesis, possibly through release of extracellular S100A4 in the tumor environment, inducing local inflammation [236]. Through a reciprocal influence of tumor and stroma cells, this extracellular S100A4 may trigger pro-metastatic cascades, involving the p53 protein and the down regulation of the pro-apoptotic bax, along with the angiogenesis inhibitor thrombospondin-1 and MMP-13, in tumor cells [237].

The establishment of the metastatic niche, in the context of S100A4, may also be encouraged by the recruitment of T cells and macrophages into the tumor microenvironment [238,239]. How extracellular S100A4 may contribute to such accumulation of myeloid cells is not clear, but both the chemotactic properties of the protein and its ability to promote cellular migration [81] may be considered as a prime driver of such a phenotype. In this context, other S100 proteins, such as S100A8 and S100A9 have also been implicated in tumor progression, regulating various processes during chronic inflammation [240]. Through their expression in many epithelial tumors and infiltrating myeloid cells [241], they may promote infiltration of immune cells within the tumor stroma, in a process that appears to be critical in tumor progression. However the direct molecular events taking place remain unclear, but could possibly be due to the S100A8/S100A9 dependent enhancement in leukocyte adhesion and migration discussed in earlier sections [169,153].

S100A8/S100A9, along with S100A4. are also associated with other cellular invasive processes leading to fibrosis, mainly of the kidney and liver [242,243](see review by Schneider et al. [84]), where the mesenchymal cellular organisation and therefore cell motility appear to be essential. This trait is mirrored by S100A6, and high levels of the protein are also observed in liver cirrhosis, biliaris and chronic renal disease [244,245]. Aberrant levels of S100A4 have also been linked to pulmonary disease, and transgenic mice expressing high levels of the protein develop severe pulmonary vascular obstructive disease and arterial hypertension [246,247]. Equally important is the involvement of extracellular S100A4 in the injured heart. In hypertrophic conditions, high expression of S100A4 by fibroblasts and invading macrophages and leucocytes is seen at the site of injury, possibly encouraging cardiac growth in the injured myocardium [248]. Aberrant levels of other S100 proteins during heart disease are also seen. Indeed altered expression of S100A1 has been linked to heart failure and hypertension, and is associated with cardiac performance, blood

pressure regulation [249] and during perfusion recovery following femoral artery resections [30], as for arthritis and other diseases affecting the human articulate cartilage [250,251] along with other S100 family members (S100A1, S100A2, S100A4, S1008, S100A9, S100A11, and S100B). Whether these cardio-changing associations of S100 proteins are directly linked to migration events or to other cascading signalling pathways associated with the S100 proteins is unclear. Even more important to consider is whether their expression is seen as causal mechanisms for such progression or limited to correlative observations mainly linked by association.

A final thought should be given to the important contributions of the S100 proteins to the regulatory mechanisms of inflammation, some of which have been discussed earlier, but now revisited here. S100A2 is a functional component in the immune response during periodontitis and may serve as a potential biomarker for periodontitis [252]. S100A7, initially identified as a protein up-regulated in inflamed hyperplastic psoriatic skin [253], has been linked to inflammation and hyperproliferation through differential expression profiling [254,23], where it is thought to promote anti-microbial activity [255,141]. Equally S100A8/S100A9 are released at the site of inflammation by phagocytes, monocytes, epithelial cells and endothelial cells [256], potentially acting as potent chemo-attractants in inflammatory processes and eliciting antimicrobial properties to various microbial pathogens [257]. Finally S100A4 appears also to be linked with inflammation resulting from microbial presence [251], but in this case, it does not possess direct bactericidal effects, but rather contributes to a reduction in bacterial accumulation at sites of infection, since the phagocytic capacity of ablated S100A4 leukocytes was impaired in the clearance of large amounts of *Staphyloccocus aureus.*

3. Rationalisation of role of S100A4/S100P in one single system

This review has shown that different S100 proteins and even the same S100 protein in different, largely *in vitro* cell systems, can cause either increases or decreases in one apparent cellular activity, that of cell migration/invasion, using a multitude of mechanisms to do so (Table 3). So is it possible to rationalise these effects and mechanisms from our own experience of S100A4/S100P in just one complete *in vitro* and *in vivo* system, that of the mammary gland?

In the mammary gland itself, we have shown that S100A4 expression occurs not in the epithelial cells themselves, but in the epithelial stem cells at the leading edge of growing budded structures, which penetrate and invade the surrounding fatty stroma [71,258]. S100A4 is also seen in myoepithelial cells, the smooth muscle-like cells which surround the epithelium and, in addition to stromal cells (e.g. endothelial cells, fibroblasts and lymphocytes), it also occurs extracellularly in insoluble structures resembling collagen/elastic fibres [74]. These results obtained *in vivo* were substantiated in our rat and human mammary cell lines *in vitro,* where S100A4 marked one of the first changes along the epithelial stem cell to myoepithelial-like cell lineage. Intermediate cells in this lineage isolated from benign tumors could also produce skeletal muscle, cartilage and bone precursors when reintroduced into syngeneic rats *in vivo* [258-260]. Moreover, overexpression of the transgene for S100A4 in and scrape-loaded addition of recombinant S100A4 to cultured rat mammary epithelial cells dramatically increased the production of elongated mesenchymal myoepithelial-like cells, the latter within 48 hours and there was no such effect upon addition of recombinant S100A4 without scrape loading [71]. These results establish a direct intracellular role for S100A4 in this process. In addition, the reduction in levels of the miRNAs commonly associated with epithelial to mesenchymal change is also observed in our cell lines isolated from a carcinogen-induced malignant metastasizing tumor TMT-081 [71] compared to their benign counterparts [261]; the former but not the latter also overexpress S100A4. These suppressor miRNAs include all 5 members of the miRNA-200 family and miR-205 and these miRNAs are often downregulated in highly invasive/metastatic breast and other cancers [262]. Our results suggest that one possible target for these suppressor miRNAs, either directly or indirectly, may be S100A4. Thus the normal production of S100A4 in the mammary gland could possibly trigger a natural development process of epithelial to mesenchymal cell conversion. This ability of S100A4 may help to explain the frequency of S100A4's expression in malignant cells of aggressive breast cancers [60] that are also normally predisposed to invade surrounding tissues [73].

The main cellular activity of Sl00A4/S100P in our hands is in stimulating cellular migration and not other cellular functions like cell proliferation [69]. Thus, direct overexpression of S100A4/S100P from transfected vectors caused rat mammary epithelial cells to migrate and invade through transwell membranes to invade local mammary tissues *in vivo* [69] and then to disseminate from the primary tumor to distant organs, particularly the lungs in intact syngeneic rats [56,213]. These results *in vitro* and *in vivo* were fully corroborated by mice transgenic for both MMTV promoter-controlled *neu* and normally

expressing rat S100A4 [80]. Although the overall process occurs in several steps, S100A4 or S100P, seem capable of inducing all of them even *in vivo*. The first step, that of cell migration, seems to occur via the intracellular pool in our S100P-inducible mammary cells. Thus addition of recombinant S100P to uninduced cells, even at high concentrations up to 1μM failed to stimulate this change; upon induction little or no S100P was secreted (<2nM), well below the 100 nM reported to be required in other cell systems [221,228]; and addition of the RAGE neutralising antibody or blocking peptide did not inhibit cell migration upon induction of S100P [219]. Thus rapidly produced intracellular S100P is sufficient to stimulate cell migration in our inducible rat mammary cell systems. However, it has recently been reported that addition of 100 nM extracellular S100A4 to the same rat mammary cells also stimulates cell migration, but this enhancement requires cross-linking of S100A4 via transamidation to produce higher-molecular-weight aggregates that work at the cell surface to enhance cell migration [263]. Whether sufficiently high external concentrations of S100 proteins are found *in vivo* is debatable, but since S100A4 [74] and S100P [213] are associated with insoluble extracellular structures *in vivo*, it is possible that insoluble aggregates could bind to cell surface receptors of whatever type and elicit a response. Thus two different routes for stimulation of migration can be identified under appropriate conditions, one intracellular and one at the cell surface and therefore may arise through different mechanisms in the same cells.

In the case of cellular migration produced by intracellular S100 proteins, most investigations reviewed herein implicate molecules in the cytoskeleton as key targets for the S100 proteins. In our hands S100A4/S100P can bind preferentially to NMIIA directly [101] and in cultured cells *in vivo* [115,219] with Kd's in the nanomolar to submicromolar range, then unzipping the NMIIA/actin filaments [118], and thereby dissolving and reorganising focal adhesion sites [219] to permit changes in cellular filopodial projections [61] in order to provide the necessary motive force. However, this is not the whole story, since S100 proteins including S100A4/S100P have been reported herein to interact with other intracellular molecules connected with the cytoskeleton and cell migration, some more weakly than others [101]. Although we have not followed up the S100P-ezrin interaction [220], in our hands S100P can also interact with α, β tubulins with affinities comparable to those with NMIIA, inhibit the rate of tubulin polymerisation and also stimulate migration [264]. These results suggest that at least one S100 family member can interact with more than one cytoskeletal target inside a cell to stimulate directly cell migration, and that it is their relatively unique dimeric structure [265] of the interacting domains [118] that permits such target promiscuity.

In the case of cellular migration produced by extracellular aggregates of S100 proteins, the jury is still out, although glycosaminoglycan (GAG) and integrin co-signalling pathways linked to activation of protein kinase C have been proposed to be responsible in our rat mammary cells [263].

One of the main problems with the whole field of S100 proteins and cell migration is the fact that certain S100 proteins can stimulate, while others can inhibit this cellular function, and some S100 members can even do both, depending on the cellular context (reviewed herein). Our published results suggest a possible explanation in that some S100 proteins, e.g. S100A1 and S100A2 could bind to S100A4 or S100P in cell-free and in cultured cells to form heterodimeric structures with higher affinity than that for self-association of either S100A4 or Sl00P alone. The formation of such heterodimers was also observed to compete away the homodimer interactions with cytoskeletal NMIIA and to inhibit S100A4 or S100P's stimulatory effects on cell migration in Boyden chamber assays and most importantly on invasion and metastasis *in vivo* in our syngeneic rat mammary model system [31,266,267]. Thus it is possible that the relative concentrations of different S100 proteins govern how an exogenously-expressed Sl00 protein may function with respect to its target molecules inside the cell, and whether it stimulates or inhibits cell migration.

As well as the inducible intracellular expression of S100P being capable of stimulating cell invasion through Matrigel, 100 nM of externally-added recombinant S100P stimulated cell invasion but not migration through the same gel [219]. Thus the mechanism of invasion in our cell systems, by contrast, would appear to be stimulatable by both intracellular and extracellular pools of S100A4/S100P. Since the amount of S100A4/S100P secreted in a transfected cell would be insufficient, at least in our cellular systems, to stimulate cell invasion from outside the cell (e.g. via RAGE receptors), the most likely molecules responsible are proteases, either of the cathepsin or metalloproteinase type [225,237]. We have evidence that primitive MMPs are produced in Ras and S100A4 overexpressing and invading optic nerve cells in transgenic fly larvae [268].

However, as stated earlier, S100A4 probably also exists *in vivo* in multimeric forms outside the cell anchored to extracellular molecules such as the GAG syndecan-4 [263] . The local concentrations may then be sufficiently high to enable such extracellular material released from host cells such as reactive myofibrobasts [58] and/or T lymphocytes [239] to stimulate cancer cell invasion. In this respect both S100A4 [259] and FGF2 [269] are secreted by the same cells intermediate between epithelial and myoepithelial-like cells and by the myoepithelial cells themselves in our rat mammary stem cell system *in vitro* by non-

classical secretory pathways. That the latter molecule is bound to extracellular GAGs [270,271] may suggest that S100A4 is bound to similar extracellular structures *in vivo*.

In addition to stimulating cancer cell migration/invasion, some S100 proteins have also been reported to stimulate migration/invasion of endothelial cells and neovascularisation of cancer cells *in vivo* (reviewed herein) at relatively high 100nM to micromolar concentrations. The stimulation of invasion of the malignant cells and their neovascularisation may be attributable, in part, to S100A4/S100P produced by host cells in their vicinity. Thus, the S100 proteins may not only support the local invasive growth of cells from the primary tumor, but also their expansive growth in distant metastases. In this respect Sl00A4-transfected rat mammary cells can not only stimulate invasive growth in the primary tumor, but also dramatically enhance the number and size of lung colonies in syngeneic rats *in vivo* when introduced directly into the circulatory system via tail vein injections [272]. Both effects *in vivo* are abrogated by transfection of the cells with mutants of S100A4 that are incapable of binding to NMIIA and of stimulating cell migration *in vitro* [116,273]. Thus the S100 family of proteins may be relatively unique in being able to bind to several molecular targets associated with the cytoskeleton to stimulate cell migration at least in cultured cells. These proteins can also work from outside the cell to stimulate cancer cell invasion and endothelial cell migration *in vitro* and for these purposes may be produced from reactive host cells, although the evidence for this is less secure *in vivo*. Thus the intracellularly and extracellularly produced S100 proteins may work in concert but through different pathways, both to initiate the process of metastasis as well as to sustain migration/invasion of the metastatic lesions themselves.

4. **Concluding remarks**

The vast diversity of S100 proteins and their protein activities, both intracellularly as well as in the extracellular spaces, has led scientists to discover a multitude of biological pathways where these proteins may play vital functions, including cell motility, cell growth, and cell survival. Although the ablation of S100A8 gene highlights its essential function *in vivo*, targeted deletions of many of these S100 proteins in mice have been shown not to demonstrate any overt anomalies or adverse effects on the life of animals (S100A4, S100A9,

S100B), possibly because other S100 proteins can compensate for the loss of one family member. However, all of these S100 proteins have been shown to be capable of regulating cellular migration and sometimes cell invasion, at least *in vitro*. Of course, the limitations of such techniques do not necessarily reflect biological relevance *in vivo*, as demonstrated for S100A8 and S100A9, which can induce significant changes in cell behaviour at low concentration *in vitro* without necessarily leading to similar changes in physiological conditions when present at much higher levels [155-157]. Most cell migration assays presented here rely on planar cellular migration, a process that is readily accepted by the scientifical community, but only rarely seen in a true physiological environment. It is now well accepted that cellular migration *in vivo* will result from the arrangement of different cellular organisations where both mesenchymal and amoeboid migrations will play a part, along with other three dimensional cellular protrusions such as invadopodia [137]. Similarly, studying penetration of the basal lamina, an important aspect of cellular invasion, is also one of the more challenging to recapitulate *in vitro* as it requires dynamic interaction between the invading cells, especially when considering collective migration, and host cells from neighbouring and distant tissues, as well as the basal lamina and extracellular matrix itself. Consequently, and although most S100 proteins have been shown to be capable of regulating cellular migration and sometimes cell invasion, at least *in vitro*, the direct consequences of their expression, or lack of, to explain such biological relevance have remained elusive.
The direct correlation of some of the S100 factors and specific pathologic conditions have, however, highlighted their importance as markers, providing the scientific community with new molecules to use as potential drug targets or possible effectors of certain molecular pathways. Throughout this review, we have aimed to present the cellular consequences of the regulated expression of the S100 proteins and the cytological changes observed. It is apparent that some consensus can be drawn from such observations, at least in cultured cells. First, it seems clear that all S100 proteins induce some changes in the actin cytoskeleton organisation, however, this observation is only sometimes corroborated with direct interactions with actin or actin binding proteins (Table 3). As such, only S100A1 and S100A4 have been reported to bind to purified actin filaments, whilst S100A6, S100B and S100P have been demonstrated to interact directly with actin binding proteins, in the form of tropomyosin, CapZ, caldesmon and myosin IIA/ezrin, respectively. The regulation of microtubule organisation or the cell surface activation of RAGE is also a property that can be seen in multiple S100 proteins and may also play a significant regulatory role in cell migration. Analysis of the predicted amphipathic patch in the hinge region (Fig. 1) and the C-terminal portion of the S100 proteins

[13,14] are thought to be the direct regulator for the specificity in binding to other target molecules, but no obvious homology or similarity could be drawn from their sequences, in view of the different cellular targets highlighted here.

Strikingly, expression of some of the S100 proteins is shown to induce conflicting results on motility depending on the cells used, even *in vivo*. For instance S100A4 promotes migration of numerous cell systems, except astrocytes where its presence appears to be detrimental for such phenotypes. Similarly S100A6 is seen to reduce motility in most somatic/physiological balanced cells but accelerates movement in cancer cell lines, whilst S100A7 studies produce contradictory phenotypes in different breast cancer cell lines. It is experimentally unclear at the current time why such contradictory observations have been reported, but speculative arguments have been provided in this work, in regards to S100A4 and S100P expression to try to answer this conundrum. S100 proteins have also been shown to induce cellular response through different mechanisms and routes, i.e. extracellular/intracellular cascade of signalling that could also affect the pathways involved, depending on the concentration required to elicit such biological responses. Although in some cases the concentration of recombinant S100 proteins added to the different cell systems are in line with the levels expected in the extracellular space, but potentially not the amount of proteins released in the medium, there are still unanswered questions related to the true functions and biological consequences of such factors *in vivo*. Similarly, numerous S100 proteins have been shown to change cellular motility through activation of the RAGE pathway (Table 3). Yet homozygous deletion of RAGE in mice present no overt abnormalities in animal's viability and fertility [274], highlighting again whether any of these observations are of physiological developmental relevance *in vivo*.

Knowing the cellular effectors of the S100 proteins remains an area of intense research and consequently some of these proteins, or the antagonists that counteract their cellular activities are slowly making their way into plans of therapeutic avenues. For instance, because of its role as a key regulator of cardiac performance, cardiomyopathies and heart failure, S100A1 based gene therapy is being developed for clinical trials [275]. For S100A4, the S100 protein most closely linked to cancer progression, inhibitors of its activities have been used to identify new ways to combat its invasion-inducing capability. The results of such early work has identified the anti-helminth drug, niclosamide and a specific S100A4 antibody, as two molecules that have been demonstrated to inhibit S100A4 induced metastasis and stromal cell invasion, respectively [276,93], paving the way for the development of further anti-metastatic drugs with S100A4 as primary target.

Figure 1: S100 protein amino acid sequence alignment

Amino acids sequences of S100 proteins were aligned with the EF-hands and central regions indicated (number 1-12 in the canonical EF-hand motif refers to the position of essential amino acids for the formation of the calcium-binding loop). All sequences are human and the accession numbers are S100A1, AAH14392.1;
S100A2, EAW53305.1; S100A3, EAW53306.1; S100A4, CAG29341.1; S100A5, EAW53317.1; S100A6, EAW53326.1; S100A7, EAW53327.1; S100A8, EAW53330.1; S100A9, EAW53334.1; S100A10, NP002957.1; S100A11, NP005611.1; S100A12, EAW53332.1; S100A13, CAA68188.1; S100A14, AAM19206.1; S100A15, AAO40033.1; S100A16, EAW53304.1; S100B, NP006263.1 ; S100G, EAW98916.1; S100P, EAW82384.1 and S100Z, EAW95784.1. Sequences were aligned using the multalin sequence comparison program (http://multalin.toulouse.inra.fr/multalin/) and the resulting data shaded and presented using the boxshade integrated program (http://www.ch.embnet.org/software/BOX_form.html).

Table 1: Sequence identity between the different S100 proteins

Amino acid sequences of each S100 protein (figure 1) was analysed for homology (identity and similarity in brackets)) compared to all other members of the family. Highlighted in bold are the highest (S100A7 and S100A15) and lowest (S100A3 and S100A7) conservation seen between the different members.

Table 2: Potential roles of S100 proteins in cellular proliferation and/or differentiation

Summary of possible roles for S100 proteins in cellular proliferation and differentiation.

Table 3: S100 expression and examples in cellular migration/invasion *in vitro*

Summary of how the aberrantly regulated levels of S100 proteins affect cellular migration/invasion and the possible mechanisms involved.

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List of abbreviations

EGF	Epidermal growth factor
F-actin	Filamentous actin
FGF	Fibroblast growth factor
G-actin	Globular actin
GAG	Glucosaminoglycan
IL	Interleukin
MMP	Matrix metalloproteinases
NM	Non muscle myosin
PMN	Polymorphonuclear neutrophil
RAGE	Receptor for advanced glycation end product
siRNA	Small interfering RNA
shRNA	Short hairpin RNA
TGF	Transforming growth factor
VEFG	Vascular endothelial growth factor

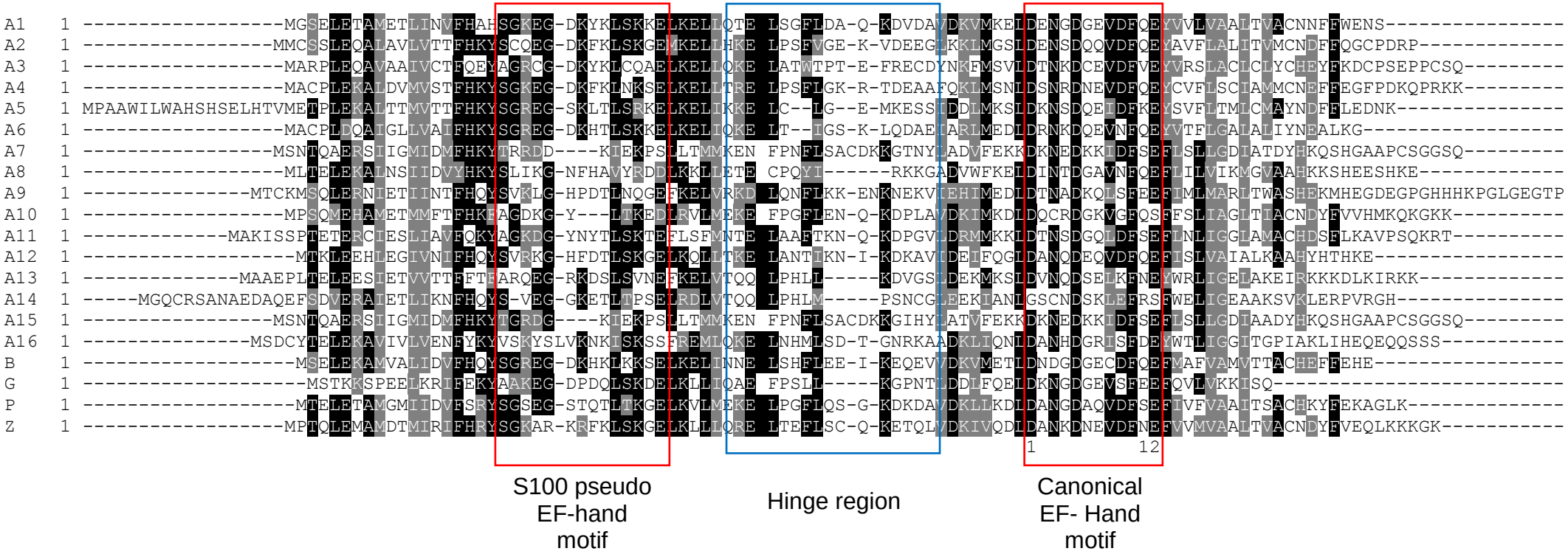
S100 pseudo EF-hand motif
Hinge region
Canonical EF- Hand motif

Figure 1

Table 1: Sequence homology between the different S100 proteins

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	B	G	P	Z
A1		51 (62)	40 (51)	49 (58)	45 (57)	41 (57)	20 (38)	36 (52)	36 (52)	52 (63)	40 (58)	36 (48)	35 (46)	23 (41)	22 (41)	33 (47)	56 (65)	35 (47)	52 (65)	56 (73)
A2			42 (54)	59 (69)	49 (62)	44 (57)	24 (42)	26 (44)	34 (51)	34 (51)	30 (45)	33 (42)	30 (42)	26 (40)	22 (38)	29 (41)	41 (55)	32 (37)	38 (56)	39 (57)
A3				45 (58)	37 (46)	41 (50)	**16 (28)**	22 (35)	30 (45)	26 (40)	30 (45)	30 (36)	27 (35)	20 (35)	19 (36)	23 (36)	34 (45)	21 (29)	30 (43)	33 (48)
A4					49 (59)	46 (59)	22 (35)	28 (44)	32 (48)	34 (49)	28 (46)	33 (42)	32 (43)	23 (39)	24 (37)	31 (41)	42 (52)	29 (34)	39 (50)	42 (58)
A5						42 (52)	22 (34)	25 (42)	28 (43)	24 (37)	29 (43)	31 (43)	31 (39)	18 (38)	23 (34)	25 (36)	35 (48)	24 (36)	31 (44)	36 (54)
A6							25 (43)	25 (49)	33 (50)	26 (50)	31 (49)	39 (50)	30 (41)	24 (41)	27 (46)	28 (49)	41 (59)	31 (40)	37 (57)	38 (60)
A7								24 (34)	25 (42)	21 (34)	31 (42)	24 (42)	20 (35)	18 (32)	**95 (95)**	28 (40)	23 (41)	23 (33)	25 (40)	25 (42)
A8									27 (52)	27 (50)	27 (44)	43 (57)	23 (45)	18 (39)	27 (40)	31 (46)	32 (44)	27 (39)	41 (58)	30 (49)
A9										25 (42)	29 (42)	38 (47)	26 (38)	27 (36)	22 (38)	21 (43)	31 (43)	21 (34)	30 (43)	30 (47)
A10											37 (58)	30 (44)	22 (38)	26 (45)	25 (39)	24 (45)	35 (50)	22 (37)	41 (56)	46 (69)
A11												31 (44)	32 (43)	22 (33)	32 (43)	24 (36)	30 (47)	26 (35)	35 (51)	30 (52)
A12													30 (49)	26 (47)	26 (47)	25 (40)	38 (53)	30 (38)	45 (53)	39 (54)
A13														36 (58)	21 (38)	30 (43)	23 (42)	27 (36)	25 (42)	25 (44)
A14															18 (32)	21 (41)	24 (37)	17 (28)	21 (37)	22 (38)
A15																28 (42)	23 (40)	21 (31)	27 (41)	24 (40)
A16																	25 (42)	23 (33)	25 (44)	28 (47)
B																		30 (39)	50 (63)	43 (60)
G																			35 (49)	39 (54)
P																				49 (61)
Z																				

Table 2: Potential roles of S100 proteins in cellular proliferation and/or differentiation

Protein	Cell types	Possible cellular functions	References
S100A1	Neurons	Cell proliferation	[32]
	Chondrocytes	Differentiation	[212]
S100A2	Squamous carcinoma	Cell Proliferation	[44]
	Keratinocytes	Differentiation	[277]
S100A4	Thyroid/colorectal carcinoma Cardiac fibroblasts/ myocytes	Cell proliferation	[278,62] [279,248]
	Cardiac myocytes Neurons	Differentiation	[280] [281,282]
S100A6	Fibroblasts Osteoblasts	Cell proliferation	[128] [283]
S100A7	Endothelial cells Squamous carcinoma	Cell proliferation	[284] [285]
	Mammary epithelial cells	Differentiation	[286]
S100A8/ S100A9	Endothelial cells Keratinocytes	Cell proliferation	[159] [287]
	Keratinocytes Thyroid carcinoma	Differentiation	[287] [288]
S100A11	Lung adenocarcinoma Keratinocytes	Cell proliferation	[289] [124,186]
	Keratinocytes	Differentiation	[290]
S10A12	Hippocampal neurons	Differentiation	[291]
S100B	Myoblasts Lung adenocarcinoma	Cell proliferation	[292-294] [206,294]
	Chondrocytes myoblasts	Differentiation	[212] [293]
S100P	Fibroblasts Prostate carcinoma Pancreatic carcinoma	Cell Proliferation	[295] [296] [221]

Table 3: S100 expression and examples in cellular migration/invasion *in vitro*

Protein	Level in regulation	Cell type and changes in motility/invasion	Possible cellular mechanisms	References
S100A1	**Ablation** in knockout mice	**Reduced** in endothelial cells	None provided	[30]
	Up by overexpression	**No changes** in breast adenoma cells **Reduced** in breast carcinoma cells	None provided Antagonise S100A4 dimer formation	[31] [31]
S100A2	**Down** by antisense technology	**Increased** in head and neck squamous carcinoma cells	F-actin polymerisation dynamics / RAGE activation	[43]
	Down by shRNA/siRNA	**Reduced** in non-small cell lung cancer cells **Reduced** in hepatocarcinoma cells		[51] [53]
	Up by exogenous addition	**Reduced** in head and neck squamous carcinoma cells **Increased** in eosinophils	cyclooxygenase-2 (Cox-2) None provided	[44] [50]
	Up by overexpression	**Reduced** in oral squamous carcinoma cell **Increased** in non-small cell lung carcinoma cell	None provided	[44] [52,51]
S100A4	**Ablation** in knockout mice	**Reduced** in macrophages **Increased** in astrocytes	Myosin IIA/actin overassembly None provided	[81] [96]
	Down by shRNA/siRNA	**Increased** in astrocytes	MMP-9 and MT1-MMP regulation	[95,96]
	Up by exogenous addition	**Increased** in endothelial cells **Increased** in pulmonary artery smooth muscle cells **Increased** in fibroblasts **Increased** in T lymphocytes	RAGE activation RAGE activation Fibronectin deposition None provided	[90] [91,92] [17] [93]
	Up by overexpression	**Increased** in non-small cell lung carcinoma cells	Myosin IIA/actin overassembly	[66]

		Increased in esophageal squamous carcinoma cells **Increased** in colon carcinoma cells. **Increased** in breast carcinoma cells	AKT/Slug signal pathway Wnt/β-catenin pathway inhibitor MyosinIIA/actin MMP13 regulation Rhotekin/Rho	[67] [68] [56,86,116,61] [65] [102]
S100A6	**Down** by shRNA/siRNA	**Increased** in fibroblasts cells **Increased** in osteosarcoma cells **Decreased** in pancreatic carcinoma cells	Actin/tropomyosin remodelling None provided Annexin II None provided	[127] [129] [131] [132]
	Up by overexpression	**Reduced** in osteosarcoma cells	None provided	[130]
S100A7	**Down** by shRNA/siRNA	**Reduced** in oral carcinoma cells **Reduced** in breast carcinoma cells **Increased** in breast carcinoma cells	Integrin β6 subunit Jab1 interaction None provided MMP13/VEGF	[146] [147] [148] [151]
	Up by exogenous addition	**Increased** in macrophages **Increased** in leukocytes **Increased** in osteosarcoma cells	RAGE activation RAGE activation RAGE activation	[142] [141] [144]
	Up by overexpression	**Increased** in squamous carcinoma cells **Reduced** in breast carcinoma cells	RAGE activation β-catenin/TCF4 pathway	[143] [150]
S100A8/ S100A9	**Ablation** in knockout mice	**Reduced** in neutrophils (IL8 treatment) **Reduced** in phagocytes **Reduced** in granulocytes (arsenite treatment)	None provided Microtubule organisation None provided	[169] [170] [170]

	Up by exogenous addition	**Increased** in neutrophils **Increased** in macrophages **Increased** in human umbilical vein endothelial cells **Increased** in melanoma cells (S100A9 only) **Increased** in lung carcinoma cells	Integrin β2 subunit Mac1 activation None provided None provided EMMPRIN RAGE P38 dependant pseudopodia	[153] [167] [159,158] [165] [163] [167]
	Up by overexpression	**Increased** in prostate carcinoma cells	MAP kinase/NF-κB/RAGE	[160]
S100A10	**Ablation** in knockout mice	**Reduced** in macrophages (migration unaffected)	Plasmin	[175]
	Down by shRNA/siRNA	**Reduced** in colorectal carcinoma cells (migration unaffected) **Reduced** in fibrosarcoma cells (migration unaffected) **Reduced** in squamous carcinoma cells **Reduced** in lung carcinoma cells	Plasmin Plasmin microfilament organisation Annexin II/DLCI interaction	[178] [179] [180] [181]
S100A11	**Down** by shRNA/siRNA	**Reduced** in smooth muscle cells	Annexin II	[189]
S10A12	**Up** by exogenous addition	**Increased** in neutrophils **Increased** in monocytes	None provided None provided	[193] [193]
S100B	**Down** by shRNA/siRNA	**Reduced** in astrocytoma cells **Reduced** in lung adenocarcinoma cells	RhoA/ROCK/Microfilament None provided	[123] [206]
	Up by exogenous addition	**Increased** in microglia cells **Increased** in smooth muscle cells **Increased** in Schwann cells	RAGE/Src/Diaphanous-1 RAGE/Src RAGE/p38	[202] [203] [204]
	Up by overexpression	**Increased** in non-small cell lung carcinoma cells	None provided	[205]
S100P	**Down** by shRNA/siRNA	**Reduced** in pancreatic carcinoma cells **Reduced** in pancreatic carcinoma cells	None provided None provided	[221] [225]

		Reduced in colon carcinoma cells **Reduced** in colon carcinoma cells	Invadopodia None provided	[224] [223]
	Up by exogenous addition	**Increased** in colon carcinoma cells	ERK1/2 /NF-κB/RAGE	[228]
	Up by overexpression	**Increased** in breast and cervical cancer cells **Increased** in lung squamous carcinoma cells **Increased** in pancreatic carcinoma cells **Increased** in pancreatic carcinoma cells **Increased** in breast carcinoma cells	Myosin IIA Erzin interaction Cathepsin D RAGE None provided	[219] [220] [227] [221] [222]