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REVIEW

Epigenetic regulation of S100 protein expression

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Abstract S100 proteins are small, calcium-binding proteins whose genes are localized in a cluster on human chromosome 1. Through their ability to interact with various protein partners in a calcium-dependent manner, the S100 proteins exert their influence on many vital cellular processes such as cell cycle, cytoskeleton activity and cell motility, differentiation, etc. The characteristic feature of S100 proteins is their cell-specific expression, which is frequently up- or downregulated in various pathological states, including cancer. Changes in S100 protein expression are usually characteristic for a given type of cancer and are therefore often considered as markers of a malignant state. Recent results indicate that changes in S100 protein expression may depend on the extent of DNA methylation in the S100 gene regulatory regions. The range of epigenetic changes occurring within the S100 gene cluster has not been defined. This article reviews published data on the involvement of epigenetic factors in the control of S100 protein expression in development and cancer.

Keywords S100 proteins · Epigenetics · DNA methylation

The S100 proteins

The S100 protein family consists of small (10–12 kDa), acidic calcium-binding proteins that form noncovalent homo- or heterodimers. Each S100 protein monomer contains two EF-hand structures, specialized in binding calcium ions, which are linked by a central hinge region of variable length. The affinities of S100 proteins for Ca^{2+} are within the micromolar range, implying that they may bind calcium ions under physiological conditions in activated cells. For most S100 proteins, the binding of calcium ions results in a pronounced conformational change exposing regions engaged in protein–protein interactions. Many of the S100 proteins also bind Cu^{2+} and Zn^{2+} ions with high affinity, but the binding sites are poorly defined (for review, see Donato 2001; Marenholz et al. 2004; Santamaria-Kisiel et al. 2006).

Devoid of any intrinsic enzymatic activity, the S100 proteins can nonetheless exert their influence on many intracellular processes through interactions with diverse partners. Binding of an S100 protein can affect the target protein conformation, activity, ability to interact with other proteins or can interfere with its posttranslational modifications, for example, phosphorylation. Since the list of S100 protein targets is rather impressive, calcium-induced interactions involving S100 proteins may entail a wide spectrum of physiological consequences, including changes in cytoskeleton dynamics, cell mobility and adhesion, cell cycle, differentiation, etc. (Santamaria-Kisiel et al. 2006). Extracellularly, these proteins act as trophic and chemotactic factors and RAGE receptor ligands (Perera et al. 2010; Leclerc et al. 2009). Multiple experimental data strongly suggest that, in spite of a high structural similarity, subtle differences in calcium-binding affinities and in the amino acid sequence, especially in the C-termini, together with differences in expression profiles, result in specific non-redundant functions of the S100 proteins.

Phylogenetically, these proteins appear to be a young group present only in vertebrates (Shang et al. 2008). Interestingly, most of the genes coding for S100 proteins are localized in a cluster on human chromosome 1q21, mouse chromosome 3f2 (Schafer et al. 1995; Ridinger et al.

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1998), and chromosome 2q34 in the rat (Ravasi et al. 2004). The clustered organization of genes gave rise to the systematization of S100 proteins and unification of their nomenclature; proteins coded by genes located within the cluster on chromosome 1 in man were assigned as S100A proteins with numbers, e.g., S100A1 and S100A2, reflecting the position of the gene in the cluster (Schafer et al. 1995; Marenholz et al. 2006; Fig. 1). In man, the remaining S100 genes are located on chromosomes 21q22 (S100B), Xp22 (S100G), 4p16 (S100P), and 5q14 (S100Z). With few exceptions, the S100 genes consist of three exons and two introns. The first exon is not translated, and the remaining two encode one EF-hand structure each.

Although the genes of S100 proteins are located in a cluster, there is no evidence that their expression is by any means synchronized either in a cell-specific or developmental manner. Quite the opposite—there are many reports showing that in a given cell type, a certain S100 protein may be abundant while the one encoded by a neighboring gene is expressed at a low level or absent. Therefore, studies which compared the expression of a panel of S100 proteins in a given cell type or tissue, or in a set of normal versus cancerous tissues, led to the conclusion that, despite structural similarities and clustered genes, each S100 protein has a very specific expression pattern (Pedrocchi et al. 1994; Elder and Zhao 2002; Cross et al. 2005). Another interesting feature of S100 proteins is that expression of an individual protein may be completely different between cell lines, even those derived from related sources. Attempts aimed at identifying cell-specific transcription factors that would underlie this phenomenon have failed because exogenously introduced promoter constructs appeared to be equally active in cells differing in endogenous expression of a given S100 protein (Tulchinsky et al. 1992; Wicki et al. 1997; Lesniak et al. 2000). These observations turned the attention to epigenetic factors that could be involved in the control of S100 protein expression.

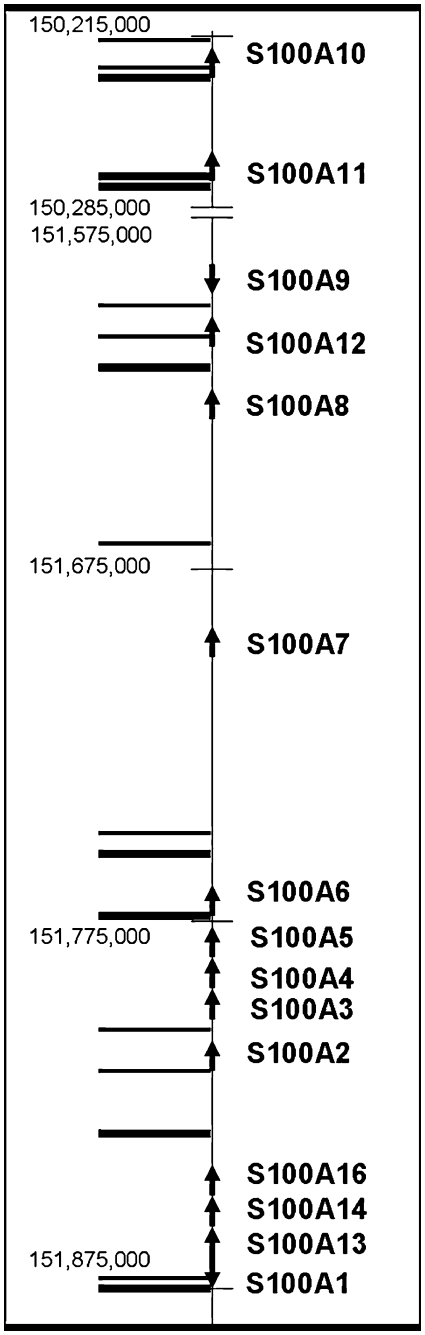

Fig. 1 Localization of CpG islands (*bars*) within the S100 gene cluster on human chromosome 1q21. The CpG Island Searcher and CpGplot programs were used. Only islands fulfilling the following criteria: CG content >55%, expected CpG/observed CpG >0.65, length >200 bp and identified by both programs are shown. Islands longer than 500 bp are indicated by *thick bars*. Positions of the examined regions are given according to the USCS Genome Browser

Epigenetic features of the S100 gene cluster

First indications that epigenetic mechanisms may be important in the regulation of S100 protein expression came mainly from the observations that in non-expressing cells, the synthesis of a given S100 protein could be reactivated in response to DNA methyltransferase inhibitors such as 5-aza-cytidine. For example, reexpression of S100A4 was described for lymphoma cells (Tulchinsky et al. 1995) following an earlier observation of differential sensitivity of the S100A4 gene 5′ flanking region to HpaII digestion in S100A4 expressing and non-expressing mouse adenosarcoma cell lines (Tulchinsky et al. 1992). Likewise, S100A2 reexpression upon 5-aza-cytidine treatment was observed in tumor-derived mammary epithelial cells (Lee et al. 1992) and that of S100A6 in non-expressing HepG-2 cells (Leśniak et al. 2000). These proteins as well as S100A3, S100A10, S10011, and S100P could be detected in various medulloblastoma cell lines after DNA demethylation (Lindsey et al. 2007).

Reactivation of gene expression by DNA methyltransferase inhibitors implies that the gene has been silenced by methylation of cytosine residues within CpG pairs located, most presumably, in its regulatory regions (Curradi et al. 2002). Mammalian genomes are depleted in CpG pairs, except for short DNA stretches with higher than average CpG density, which are called CpG islands (Gardiner-Garden and Frommer 1987). CpG islands often coincide with gene promoters, and their methylation is associated with repressed chromatin state and transcription inhibition. Results of the analysis of the S100 gene cluster for the presence of CpG islands are presented in Fig. 1. CpG islands were identified in 5′ regulatory regions of S100A2, S100A6, S100A10, and S100A11 genes (Fig. 1, bars). They cover the proximal promoter, the first non-translated exon and part of the first intron (S100A6, S100A10, and S100A11), or the first exon and part of the first intron (S100A2). In addition to that, the S100A10 and S100A11 genes have another CpG island in their respective first introns, which makes them the most CpG-rich S100 genes. A CpG island is also found in the first intron of the S100A12 gene. CpG islands in the promoters of the S100A6, S100A10, S100A11 genes and in the first intron of the S100A11 gene fulfill the more stringent criteria described by Takai and Jones (2002), i.e., they are longer than 500 bp (Fig. 1, thick bars). Several CpG islands were found in the intragenic DNA regions within the S100 gene cluster and at the 3′ flanking regions of S100A1 and S100A10 genes (Fig. 1). Examination of the 5-kb vicinity of other S100 genes did not detect any promoter CpG islands. The S100P and S100Z genes proved to be CpG island-free, while S100B and S100G genes have CpG islands in 3′ flanking regions close to their third exons.

DNA methylation and S100 protein expression in cancer

Many S100 proteins have been reported to change their expression during cancer progression (for review, see Sedaghat and Notopoulos 2008; Salama et al. 2008) which gave rise to speculations about their causative role in various malignances. The most illustrative example of this presumption is the fact that S100A4, the level of which is often increased in metastatic cancers, was named metastasin (Grigorian et al. 1993) while the S100A2 protein, which is downregulated in many cancer tissues, is often referred to as a tumor suppressor protein (Wicki et al. 1997). Furthermore, S100B, S100A6, and some other S100 proteins were proposed as clinical markers of various malignances. Evidence has accumulated in recent years showing that changes in S100 protein expression in cancer are, in many cases, due to epigenetic mechanisms.

It is now well established that the genome of cancer cells is largely hypomethylated but that, parallely, some genes, including these coding for tumor-suppresor proteins, undergo hypermethylation (Łuczak and Jagodziński 2006). As described below and summarized in Table 1, the S100 proteins seem to be frequent subjects of this aberrant methylation status.

The S100A2 gene expression was found to be down-regulated in breast cancer cells (Lee et al. 1992, Wicki et al. 1997). Accordingly, methylation of several among the 14 CpGs present within the −1227/−201 fragment of the S100A2 gene promoter was shown, by bisulfite sequencing, to be increased in breast cancer cell lines and breast cancer biopsies when compared to normal epithelium (Wicki et al. 1997). There were, however, no changes in the extent of methylation in the further upstream promoter region (~−2,000 bp) and in the first intron (Wicki et al. 1997). On the other hand, the loss of S100A2 expression in prostate cancer tissues and cell lines could not be correlated with DNA methylation in the promoter fragment studied by Wicki et al. (1997) since the extent of methylation was similar in S100A2 expressing and non-expressing cells and tissues (Rehman et al. 2005). Downregulation of S100A2 expression was also observed by immunohistochemistry in lung cancer tissues (Feng et al. 2001). When studied in non-small cell lung cancer cell lines, diminished S100A2 expression was shown to correlate with methylation of CpGs within a 198-bp-long fragment of the first intron. Likewise, lower level of S100A2 in cell lines derived from lymph node metastases of head and neck cancer than in the parental non-metastatic cell line has been correlated with increased methylation within the intronic region (Zhang et al. 2007).

As mentioned above, the S100A4 protein is often overexpressed in cancer, and its higher level is thought to contribute to increased cancer metastasis (Garret et al. 2006). Although the S100A4 gene appears to contain less than an average number of CpG sites, earlier observations indicated that its expression could be increased by 5-aza-cytidine (Chen et al. 1999). Examination of bisulfite sensitivity of cytosine residues in rat mammary cancer cell lines exhibiting different levels of S100A4 showed that the TATA box (−113/+36) and intronic (+135/+312) regions were differentially methylated while the upstream promoter region (−1404/−1227) revealed comparable sensitivity to bisulfite treatment (Chen et al. 1999). Similar results were obtained for human colon adenocarcinoma cell lines. The upstream promoter region (~−800) and the downstream region (+1124/+1439) were methylated regardless of the actual S100A4 expression level while three CpG sites (+35, +386, +777) within the intron were mostly unmethylated in S100A4 expressing cell lines and methylated in non-expressing ones (Nakamura et al. 1998). Likewise, a lower methylation level of cytosines at positions +315, +331, and +386 correlated with high S100A4 expression in pancreatic cancer cell lines (Rosty

Table 1 Changes in S100 gene methylation and expression in cancer

S100 protein	Cell/tissue	S100 protein expression	Gene region examined	Methylation (cancer vs. control)	Reference
S100A2	Breast cancer cell lines and biopsies	↓	Proximal promoter Upstream promoter 1st intron	↑ No change No change	Wicki et al. 1997;
	Prostate cancer cell lines and tissues	↓	Proximal promoter	No change	Rehman et al. 2005;
	Non-small lung cancer cell lines	↓	1st intron	↑	Feng et al. 2001;
	Head and neck cancer lymph metastases	↓	1st intron	↑	Zhang et al. 2007;
S100A4	Rat mammary cancer cell lines	↑	1st intron, TATA box region Upstream promoter	↓ No change	Chen et al. 1999;
	Colon adenocarcinoma cell lines	↑	1st intron Upstream promoter Downstream region	↓ No change No change	Nakamura et al. 1998;
	Pancreatic cancer cell lines	↑	1st intron	↓	Rosty et al. 2002;
	Endometrium grade III tumors and cell lines	↑	1st intron	↓	Xie et al. 2007;
	Medulloblastoma tissue and cell lines	↑	1st intron	↓	Lindsey et al. 2007;
	Epidermal cancer cell lines and squamous cell carcinoma	↓	1st intron	↑	Li et al. 2009
S100A6	Prostate cancer tissue and cell lines	↓	Promoter/1st exon	↑	Rehman et al. 2005;
	Medulloblastoma cell lines	↓	Promoter/1st exon	↑	Lindsey et al. 2007;
	Medulloblastomas	↓	Promoter/1st exon	No change	Anderton et al. 2008
	Gastric cancer tissue	↑	1st intron/2nd exon	↓	Wang et al. 2010;
S100A10	Primary human pituitary tumors	↓	Proximal promoter	↑	Dudley et al. 2008;
	Medulloblastoma tissue and cell lines	↓	Proximal promoter	↑	Lindsey et al. 2007; Anderton et al. 2008
S100P	Primary pancreatic adenocarcinomas	↑	Promoter/1st exon	↓	Sato et al. 2004;
	Prostate cancer cell lines	↑	Promoter/1st exon	↓	Wang et al. 2007

↑ increase, ↓ decrease

et al. 2002). Changes in the extent of CpG methylation within the intronic region were also observed in endometrial cancer cell lines and tissues. Methylation was detected in benign endometrium and grade I tumors expressing low levels of S100A4 but not in grade III tumors with high S100A4 expression (Xie et al. 2007). Expression-related hypomethylation of the intron in the S100A4 gene was also observed in 17% of medulloblastoma cases, versus normal cerebellum samples, and in 33% of medulloblastoma cell lines studied (Lindsey et al. 2007). An opposite situation, i.e., downregulation of S100A4 expression, was reported for various human epidermal cancers (Li et al. 2009). In that case, as exemplified by squamous cell carcinoma sample analysis, four CpG pairs within the gene intronic region became methylated in the cancerous tissues when compared with normal epidermis.

Evidence of both epigenetic silencing and induction in cancer has been obtained for the S100A6 gene. Rehman et al. (2004) observed S100A6 expression in all of the 66 studied cases of benign epithelium adjacent to prostatic adenocarcinoma and a complete loss of staining in adenocarcinomas. Loss of S100A6 expression was also observed in several prostate cancer cell lines. Subsequent examination of S100A6 gene promoter methylation in expressing and non-expressing cells and benign versus cancerous prostate tissues revealed increased methylation of cytosine residues within a 267-bp gene fragment covering a part of the promoter and of the first non-translated exon in non-expressing cell lines (Rehman et al. 2004) and in 52% of prostate cancer tissues examined (Rehman et al. 2005). Methylation of the same gene region was studied in medulloblastoma cell lines and primary medulloblastomas following the observation that S100A6 expression was increased after 5-aza-cytidine treatment (Lindsey et al. 2007). While in cell lines the extent of cytosine methylation was strictly correlated with the lack of S100A6 expression, the level of methylation was very low in primary medulloblastomas. Only five out of 40 studied tumors showed evidence of increased methylation of the S100A6 gene promoter/first exon when compared to methylation-free

normal cerebella. A subsequent study (Anderton et al. 2008) confirmed that the gene was methylated in medulloblastoma cell lines but not in 16 primary tumors studied, suggesting that methylation may concern cultured cells and not to occur to a great extent in primary tumors. DNA hypomethylation as the cause of S100A6 overexpression in gastric cancer has been reported by Wang et al. (2010). A slightly lower average methylation of four, among five, CpG sites present in the first intron/second exon region of the S100A6 gene was detected in 53 gastric cancer tissues examined when compared to adjacent non-neoplastic mucosa. This lower methylation rate corresponded to a higher level of acetylated histone H3 associated with the S100A6 gene promoter region.

S100A10 expression was found to be diminished due to methylation in non-expressing primary human pituitary tumors relative to normal pituitary. Five cytosine residues in a 146-bp-long fragment of the S100A10 gene promoter examined (−745/−600) were more often methylated than the corresponding cytosines in normal tissue (Dudley et al. 2008). Thirteen CpGs in a 252-bp-long promoter fragment (−652/−400) were also found to be more often methylated in medulloblastomas (Lindsey et al. 2007; Anderton et al. 2008).

S100P was found to be expressed, and the gene proximal promoter/first exon region to be hypomethylated, in seven dissected primary pancreatic adenocarcinomas when compared to normal pancreatic ductal epithelium in which the S100P gene was methylated (Sato et al. 2004). Accordingly, hypomethylation was detected in 30 of 34 xenografts and in pancreatic cancer cell lines. Lower methylation of the S100P gene proximal promoter/first exon region was also detected in prostate cancer cell lines (Wang et al. 2007).

DNA methylation and regulation of developmental and cell-specific expression of S100 proteins

In addition to data concerning aberrant S100 gene methylation in cancer, there is also increasing evidence that epigenetic events may accompany the developmental or stimulus-induced induction/upregulation of S100 protein expression. Malup et al. (2007) studied methylation of the −455/+131 gene promoter fragment in relation to cell and tissue-specific expression of S100B in brain astrocytes. Examination of four CpG sites (−207,−65,+5, +103) revealed a predominant absence of CpG methylation in the studied region in DNA clones isolated from total brain when compared to those isolated from other organs. Another study investigated the relation between S100B gene promoter methylation and the developmental stage-dependent expression of S100B in fetal mouse brain (Namihira et al. 2004). S100B expression was first observed in the subventricular layer of the telencephalic cortex of E14.5 mouse brains. Four cytosine residues (−818, −318, −207, −64), within a 860-bp-long promoter region studied, were highly methylated in E11.5 neuroepithelial cells which do not express S100B. Interestingly, methylation frequency at the −318 CpG site, but not at the remaining three cytosine residues, was significantly reduced in E14.5 neuroepithelial cells coinciding with the onset of S100B expression. Demethylation of cytosine at the −318 position corresponded with a reduced binding of MeCP2 to the promoter. The authors speculated that the binding of MeCp2 to the methylated −318 cytosine residue inactivated the gene while its demethylation relieved the inhibition and could be coupled to fetal brain cell differentiation into the astrocyte cell lineage (Namihira et al. 2004). Further work showed that demethylation of cytosine at position −318 in 11.5E neuronal precursor cells occurred in response to Notch signaling (NICD overexpression) and prompted the binding of the NFI transcription factor to a nearby binding site on the S100B promoter, leading to activation of S100B gene transcription (Namihira et al. 2009).

Expression of S100A4 due to DNA demethylation induced as a result of integrin α6β4 signaling has also been reported (Chen et al. 2009). Five among seven CpGs within the +208/+662 intronic region studied were found to be largely methylation-free in MDA-MB-435 cells that stably expressed integrin α6β4 when compared to control cells. This demethylation coincided with the binding of the NFAT5 transcription factor to two sites located within the studied fragment.

Concluding remarks

All the above data seem to support the thesis that epigenetic factors play a role in regulating S100 protein expression both in development, as exemplified by data on S100B, and during malignant transformation. The latter is certainly true for cancer cell lines where the differences in DNA methylation between expressing and non-expressing cells are most manifested and can be unanimously correlated with protein expression. A criticism is often raised that DNA methylation pattern in established cell lines can be changed in response to culture conditions and does not reflect the in vivo situation. This criticism does not seem to hold for the S100 protein genes since with the exception of the S100A6 gene in medulloblastomas, examination of cancer tissue specimen confirmed that downregulation of S100 protein expression was accompanied by DNA methylation of the respective gene. Less pronounced differences in the methylation status, when compared to cell lines, can be attributed to higher heterogeneity of tissue samples which may contain both expressing and non-expressing cell types.

Interestingly, changes in the methylation status (and expression) concern the S100 genes independently of the CpG density in their regulatory regions. It becomes evident from the literature cited above that both the genes containing CpG islands (S100A6, S100A10, etc.) as well as relatively CpG-poor genes (S100A4) can be silenced by DNA methylation. The concept of DNA methylation envisages two possible ways by which methylated cytosine residues may interfere with DNA transcription: (1) they may abrogate the binding of a transcription factor and/or of the basal transcriptional machinery and (2) they may attract methyl-binding proteins, for example MeCP2, that, together with histone deacetylases and other co-repressors, rebuild chromatin to a tightly packed, transcriptionally inactive form (Bird and Wolfe 1999). The former mode implicates that even a single methylated cytosine residue can seriously disturb the transcription rate while the other concept would require that a larger fragment of DNA be methylated to achieve a stable inactive chromatin conformation. Concerning the first possibility, it was shown, for example, that binding of the upstream stimulatory factor, USF, to the E-box sequence in the S100A6 promoter was inhibited in cells in which the promoter DNA was methylated (Lesniak et al. 2007). Also, as noted above, loss of methylation facilitated the binding of NFI to the S100B gene promoter (Namihira et al. 2009) and of NFAT5 to the S100A4 gene promoter (Chen et al. 2009). Accordingly, changes in the methylation status of only several CpGs, as in, for example, the S100B (Namihira et al. 2004) and S100A4 genes (Nakamura et al. 1998), were reported to bring about a fundamental change in expression. On the other hand, it was shown that a complete lack of S100A6 gene expression in HEK293 cells was accompanied by extensive methylation covering the body of the gene and its proximal 5′ and 3′ regions (Lesniak et al. 2007). It is thus probable that not only the differentially methylated cytosine residues identified in a given study but also additional ones, located in other non-examined gene regions, may contribute to the observed differences in S100 protein expression.

An intriguing question that arises with regard to the clustered organization of the S100 genes is whether methylation/demethylation events regulating their expression are local or global. Although the available data on DNA methylation concern only some S100 genes and only limited DNA regions have been analyzed, it seems that, no matter how many CpGs should be methylated to cause effective gene silencing, the region involved does not surpass "the borders" of an individual S100 gene. Studies comparing methylation of several S100 proteins in a given cell line or tissue clearly show that genes lying only several kilobases apart differ in methylation and expression level (Tulchinsky et al. 1992; Wicki et al. 1997; Elder and Zhao, 2002). Thus, the conclusion, formulated based on S100A2 and S100A6 methylation/expression in fibroblasts and keratinocytes, that gene-specific rather than long-range effects on chromatin structure is decisive in the regulation of S100 gene expression (Elder and Zhao 2002), seems to apply to the S100 gene cluster in other tissues as well.

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