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Genetically modified mouse models to study the role of metastasis-promoting S100A4(mts1) protein in metastatic mammary cancer

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Transgenic and knockout mouse models are extensively used to study the mechanisms of tumour formation. The availability of mouse models to study metastatic spread of tumours is although quite limited. S100A4(mts1), that belongs to the S100 family of Ca-binding proteins, has been shown to function as a metastasis-promoting protein. We generated strains of mice with modified expression of S100A4 in order to understand the mechanism by which S100A4 protein stimulates metastatic spread of the tumour cells. Transgenic mice over-expressing the *S100A4* gene in the mammary gland were crossed with GRS/A mice, characterized by a high incidence of spontaneous non-metastatic mammary tumours. The resulting hybrid mice developed metastatic tumours. Transgenic mice with ubiquitous expression of *S100A4* developed vascular tumours, hemangiomas and contained enhanced levels of the S100A4 protein in the blood. Based on these observations we demonstrated that extracellular S100A4 functions as an angiogenic factor. Study of tumour development in the S100A4 – deficient mouse model demonstrated key role of extracellular S100A4 in stimulation of tumour development and metastasis formation.

Keywords: Cancer, metastases, S100A4, transgenic mice, knockout mice.

Metastasis, the spread of tumour cells from the primary site followed by formation of secondary tumours in the other parts of the body, is the cause of death of most of the cancer patients. The process of tumour metastasis consists of a number or interrelated steps. This includes ability of cells to leave the primary tumour site, pass the basement membrane, enter the circulation, survive there, be arrested at a distant organ, survive in the novel microenvironment and proliferate in the foreign tissue. Metastatic process occurs as a result of cross-talk between tumour and host organism, that produces signals stimulating the spread of tumour cells (Fidler, 2002).

In the recent years a plethora of genetically modified mouse models was generated to study the mechanisms of tumour formation and progression. These models have an advantage that the tumours arise and progress in their normal context, closely imitate the native process of metastasis and enable study of the input of host organism to the stimulation of metastasis. Regrettably, the amount of reliable mouse models of metastasis is limited (Hirst & Balmain, 2004; Khanna & Hunter, 2004).

Metastasis-promoting S100A4(mts1) protein belongs to the S100 family of Ca^{++}-binding proteins that are implicated in a variety of activities such as cell proliferation and differentiation, cytoskeleton dynamics and apoptosis. Some members of S100 family including S100A4 can be released from the cells and exhibit various physiological activities, such as stimulation of neuronal differentiation, astrocyte proliferation and modulation of activity of inflammatory cells (Donato, 2003; Marenholz et al. 2004). Involvement of the S100 family members in cancer has been widely demonstrated (Weterman et al. 1993; Pedrocchi et al. 1994; Al-Haddad et al. 1999; Guerreiro et al. 2000; Feng et al. 2001). Much interest has been focused on S100A4 because of its association with stimulation of metastasis.

The *S100A4* gene was isolated as a gene differentially expressed in highly metastatic mouse mammary adenocarcinoma cells (Ebralidze et al. 1989). Introduction of the

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S100A4 gene into non-metastatic tumour cell lines and suppression of its activity in metastatic ones proved its involvement in metastasis formation (Mazzucchelli, 2002). Stimulation of tumour metastatic activity was demonstrated in two transgenic mouse models (Ambartsumian et al. 1996; Davies et al. 1996). In many human cancers the enhanced expression of S100A4 was associated with poor prognosis (Platt-Higgins et al. 2000; Yonemura et al. 2000; Davies et al. 2002; Rosty et al. 2002; Lee et al. 2004). All these data strongly relate the S100A4 gene expression with tumour progression and metastasis. The exact mechanism by which S100A4 stimulates metastasis formation although remains unclear.

Various intracellular interacting partners, such as heavy chain of non-muscle myosin (Kriajevska et al. 1994; Ford & Zain, 1995), liprinβ-1 (Kriajevska et al. 2002), p53 (Grigorian et al. 2001) and methionine aminopeptidase (Endo et al. 2002) were identified for the S100A4 protein, suggesting its participation in cell motility, adhesion and proliferation. These findings suggest that S100A4 participates in metastasis formation by interfering with any of these processes. On the other hand, when released into extracellular space S100A4 stimulates neurite outgrowth (Novitskaya et al. 2000) and exhibits angiogenic activity (Ambartsumian et al. 2001) by promoting motility and invasion of endothelial cells (Schmidt-Hansen et al. 2004a). This implies that S100A4 could contribute to tumour progression by stimulating neovascularization.

Expression of the S100A4 in an adult organism is restricted mainly to the cells of the lymphoid system, presumably T-lymphocytes and macrophages (Lukanidin & Georgiev, 1996). In embryonic development S100A4 also demonstrates a very distinct pattern of expression in several mesenchymal tissues and in foetal macrophages (Klingelhofer et al. 1997). The important role of S100A4 in embryogenesis also could be postulated from its high level of expression in mouse trophoblast (Ford & Zain, 1995).

One of the approaches applied in our research was generation of mouse models of the *S100A4* gene. Over-expression of S100A4 as well as its germ-line inactivation could lead to changes in whole organism that might help to understand its normal function and the mechanism of action of S100A4 in stimulation of metastasis.

We generated transgenic mouse model where the *S100A4* gene was placed under the control of mouse mammary tumour virus promoter (MMTV LTR). This promoter directs expression of a gene of interest to the mammary gland. The *3-hydroxy-3-methylglutaryl CoA-reductase* gene (HMGCR) promoter was used to express the *S100A4* gene in all the mouse tissues. Finally, a mouse model with germ-line inactivation of the *S100A4* gene was generated. Here we summarise the results of analysis of these models. Examination of these models enabled us to formulate a hypothesis of how S100A4 stimulates dissemination of tumour cells to the secondary sites.

S100A4 stimulates metastatic disease in transgenic mice over-expressing S100A4.

To test the possibility that *S100A4* over-expression stimulates metastatic spread of cancer cells we generated strains of transgenic mice where the *S100A4* coding sequences was placed under control of the MMTV LTR promoter (Ambartsumian et al. 1996).

Transgenic animals did not exhibit any observable phenotypic abnormalities during the first 18 months of life. Transgenic females never revealed any signs of mammary gland dysplasia or neoplasia. This indicates that S100A4 does not have an oncogenic effect on the mammary epithelia.

To study the effect of the *S100A4* transgene on metastasis of mammary tumours we crossed the MMTV/mts1 transgenic mice with the GRS/A mouse strain. This strain carries a MMTV provirus and thereby exhibits high mammary tumour incidence (Michalides et al. 1981). Important feature of the mammary adenocarcinomas in the GRS/A mice is that they rarely metastasize (Van der Valk, 1981). The incidence of mammary tumours could be enhanced in GRS/A mice by force-breeding. Mammary tumorigenesis in these mice involves activation of the *wnt-1* and *int-2* oncogenes by proviral integration. It is proposed that their expression is lost upon progression of the tumours (Mester et al. 1987; Roelink et al. 1992).

Transgenic, GRS/A-MMTV/mts1, hybrid females and their non-transgenic littermates were subjected to force-breeding. Most of the females developed mammary tumours. The percentage of females developing mammary tumours, the time of first appearance, mean number of mammary tumours per animal, and the average size of the mammary tumours were comparable in the transgenic and nontransgenic animals. Importantly, in contrast to the non-transgenic siblings, lung metastases were frequent in the animals inheriting the MMTV/mts1 transgene (40%; Table 1).

Affected lung tissue revealed the presence of foci of metastatic mammary adenocarcinoma in hybrid mice, signifying the involvement of S100A4 in this process. Analysis of tumour sections revealed concentration of the S100A4 positive cells with elongated morphology in the stroma of tumours developed both in transgenic and non-transgenic animals. It has been shown that the S100A4 protein is expressed in fibroblasts (Strutz et al. 1995). Therefore, we considered that the S100A4 positive stromal cells were fibroblasts (Fig. 1A, B). The transgenic tumour sections as well as metastatic lesions in the lungs exhibited a significant expression of S100A4 in the tumour cells. These observations link the over-expression of S100A4 to the aggressiveness of tumours developed in the hybrid transgenic mice (Fig. 1A, B). Loss of expression of the *wnt-1* gene, that is known to correlate with the progression of the MMTV-induced mammary tumours, was also detected in the S100A4 transgenic tumours (25%) compared with the non-transgenic ones (62%).

Table 1. Characteristics of tumours induced in hybrid GRS/mts females

Strain	Total amount of animals	Animals with tumour	Tumour number per animal	First detection, pregnancy	Tumour size, mm	Metastasis
Tg463,	31	75·6% (23)	1·44±0·3	2–3	19·95±8·8	39·1% (9)
Tg507	9	55·5% (5)	1·8±0·44	2–3	17·1±8·5	40% (2)
control	31	67·7% (21)	1·42±0·83	2–3	19·9±8·8	4·7% (1)

Number of animals in each group is shown in the brackets.

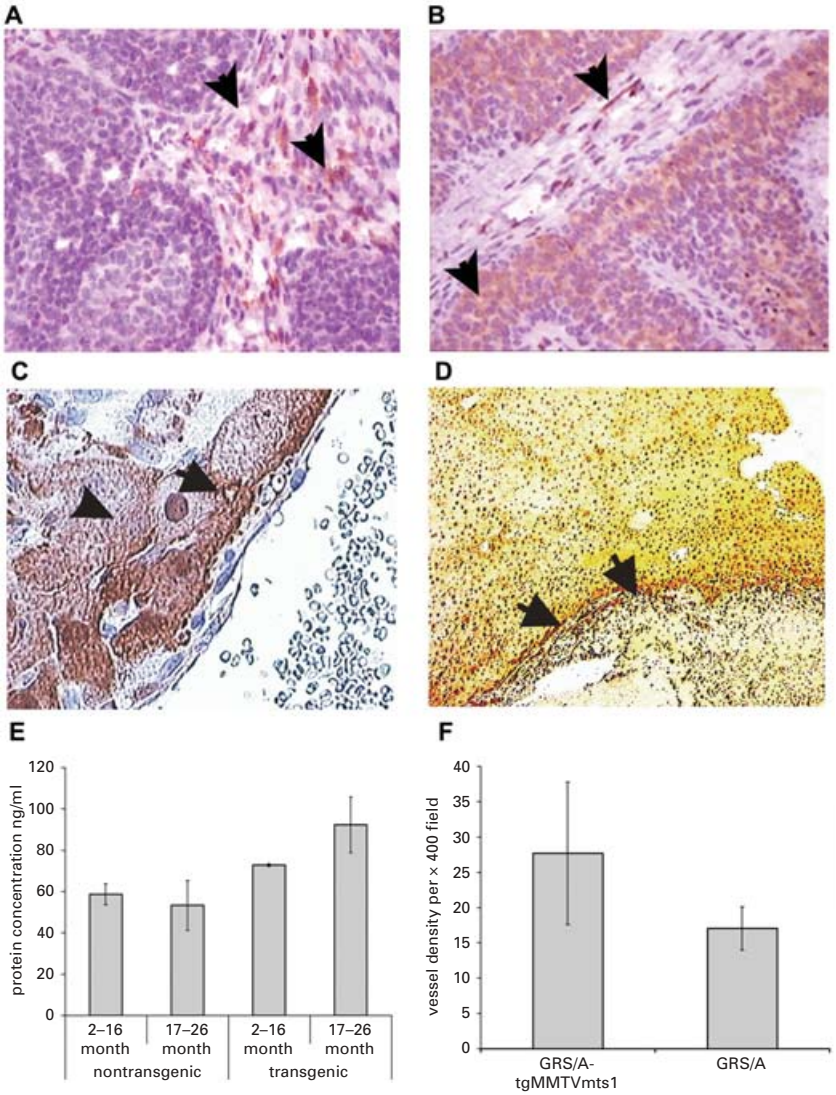

Fig. 1. Expression of S100A4 in the MMTV/mts1 and HMGCR/mts1 transgenic mice. A and B – expression of the S100A4 protein in the GRS/A-MMTV/mts1 hybrid transgenic animals. A – Immunohistochemical detection of the S100A4 in tumour-associated fibroblasts of the nontransgenic tumours. B – Immunohistochemical detection of the S100A4 both in the tumour cells and in tumour-associated fibroblasts. C and D – expression of S100A4 in the hepatocytes flanking the liver hemangioma developed in the HMGCR/mts1 mice. D – low magnification (×40) image demonstrating gradual increase of expression on the border of the lesion. C – high magnification demonstrates absence of expression in the endothelial cells forming hemangioma. E – Increased amount of the S100A4 protein in the serum of aged HMGCR/mts1 transgenic animals determined by sandwich ELISA. F – Average blood vessel density in the GRS/A-MMTV/mts1 hybrid transgenic tumours compared to the nontransgenic controls. Vessel density was determined by counting CD-31 positive capillaries in 10 random fields of 10 different tumours (P=0·024).

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The progressed status of tumours over-expressing the S100A4 was furthermore confirmed by transplantation of the tumours to the Balb/c nu/nu mice. Three of five transplanted metastatic transgenic tumours sustained their metastatic ability. Two non-metastatic transgenic tumours did cause lung metastases after transplantation. Two non-transgenic nonmetastatic tumours did not cause metastases, but the solely non-transgenic metastatic tumour continued to be metastatic.

Summarizing, the MMTV/mts1 transgenic mouse model allowed us to demonstrate on the level of whole organism that S100A4 overexpression is needed to confer aggressive properties to the mammary tumours.

Study of the function of S100A4 in mice ubiquitously expressing the S100A4 gene

The *S100A4* gene is expressed in a restricted cell types and tissues both in the adult organism and during embryonic development. To study the consequences of *S100A4* over-expression in all the mouse tissues we generated transgenic mouse strains where *S100A4* expression was controlled by the ubiquitous promoter of the 3-hydroxy-3-methylglutaryl CoA-reductase gene (HMGCR/mts1). Strains of HMGCR/mts1 transgenic mice were characterized by a down-regulation of S100A4 expression at the post-translational level (Ambartsumian et al. 1998–1999). An increased level of the S100A4 RNA was documented in all the mouse tissues tested, whereas the corresponding level of the protein was detected only in the organs normally expressing S100A4, such as spleen and thymus. Therefore we were unable to achieve the goal to over-express S100A4 protein in all the tissues of the mouse. Excitingly, aging HMGCR/mts1 mice developed diverse pathological conditions. Among pathological manifestations the most striking ones were tumour-like formations located mainly in the liver. The histopathological analysis demonstrated that they represent vascular tumours – hemangiomas. Generally, hemangiomas are extremely rare in the populations of laboratory mice. The incidence of spontaneous hemangiomas in mice of different strains throughout their natural life ranges from 0·16% to 0·6% (Peters et al. 1972; Frith & Wiley, 1982; Booth & Sundberg, 1995), whereas in the aged HMGCR/mts1 mice the incidence of hemangiomas was 14%.

Hepatocytes both in the wild type and in the HMGCR/mts1 mice do not express S100A4. In contrast, immunohistostaining of liver hemangiomas with anti-S100A4 antibodies demonstrated that hepatocytes in the affected area, but not the endothelial lining cells, expressed S100A4. Moreover, we observed a gradual increase of the S100A4 expression in hepatocytes towards the lesion (Fig. 1C, D). This observation indicated that the repression of the S100A4 expression was deteriorated in the aged mice resulting in accumulation of the S100A4 protein in non-permissive tissues with a subsequent development of pathological changes. Since the endothelial cells in hemangioma unlike the surrounding hepatocytes, did not express S100A4, we proposed that it was secreted from the hepathocytes and exhibited stimulatory effect on endothelial cells. Indeed we were able to detect increased amount of the S100A4 protein in the serum of aged transgenic animals. The amount of S100A4 in the serum of 17–26 month old transgenic animals was 1·73 times higher than in the control (Fig. 1E). *In vitro* studies with the recombinant S100A4 protein indeed demonstrated that it stimulates motility and invasion of endothelial cells (Ambartsumian et al. 2001; Schmidt-Hansen et al. 2004a). Moreover treatment of endothelial cells with the S100A4 protein resulted in activation of the NF-κB transcription factor with consequent stimulation of transcription and secretion of matrix metalloproteinases, in particular collagenase 3 (MMP-13).

The logical consequence of the notion that S100A4 is an angiogenic factor is that it might contribute to the tumour metastasis by enhancing the tumour vascularization. Indeed, evaluation of the vascularization indices of mammary tumours of the GRS/A nontransgenic and GRS/A-mts1 hybrid transgenic mice showed that transgenic tumours characterized by aggressive metastatic behaviour had 1·5 times higher vascularization index than the non-transgenic ones (Fig. 1F).

Therefore we propose that tumour progression and metastasis formation are stimulated by the extracellular S100A4 that acts as an angiogenic factor.

S100A4 knockout mice repress tumour growth and metastasis formation

In the developing tumour various cells are able to release S100A4. Tumour-associated fibroblasts express high levels of S100A4. Activated macrophages, that are regarded as cells producing angio-stimulatory and angio-inhibitory molecules (Polverini, 1996) up-regulate expression of S100A4 (Grigorian et al. 1994). Cultured tumour cells and rat fibroblasts secrete the S100A4 protein (Ambartsumian et al. 2001; Watanabe et al. 1992). Moreover, tumour cells produce factors that stimulate release of S100A4 from fibroblasts and macrophages (Schmidt-Hansen et al. 2004b and our unpublished data). One can presume that S100A4 is released from the macrophages and fibroblasts, which are recruited to the tumour to create tumour-associated stroma, and by that play an important role in tumour progression. A mouse model with germ-line inactivation of *S100A4* could help to determine the role of host-derived stroma cells expressing S100A4 in stimulation of tumour progression and metastasis.

We found that deletion of *S100A4* in the mouse does not affect embryonic development and postnatal life. However part of the S100A4-deficient mice were prone to spontaneous tumour development suggesting participation of S100A4 in tumour suppressor function (El-Naaman et al.

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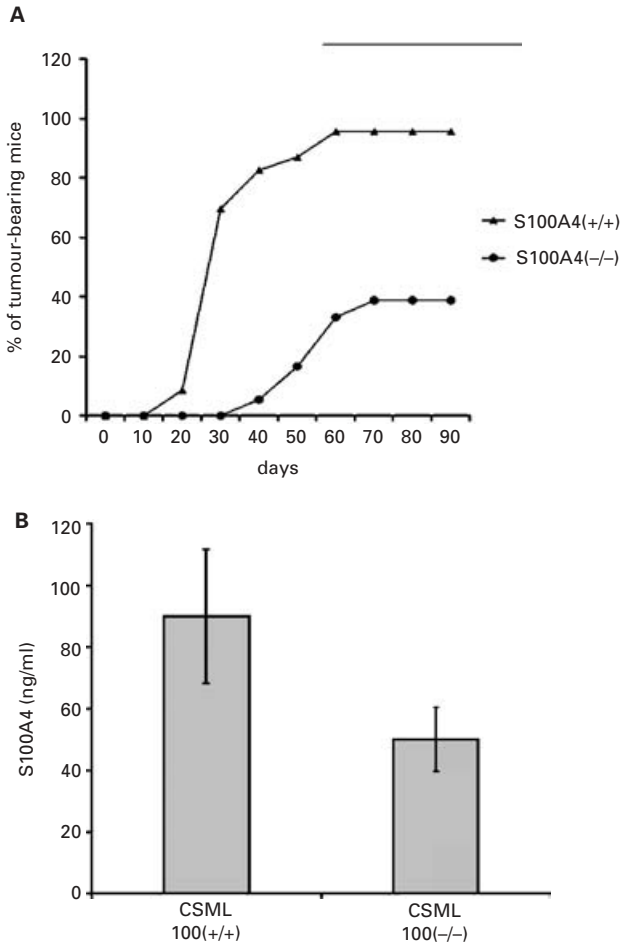

Fig. 2. Repression of tumour development in the S100A4-deficient mice. A. Percentage of tumour-bearing S100A4(+/+) and S100A4(−/−) mice grafted with CSML100 tumour cells. Both tumour incidence and uptake are repressed in the S100A4 deficient mice. B. Amount of the S100A4 released by CSML100 tumours developed in the knockout and wild type mice. Amount of S100A4 was determined in the tumour interstitial fluids by Sandwich ELISA.

2004). We associated this phenomenon with the modulation of activity of the tumour suppressor p53 protein which is a target for S100A4 (Grigorian et al. 2001).

To study tumour development and metastasis formation in the S100A4-null mice we used CSML100 mammary adenocarcinoma cell line that form highly metastatic expressing S100A4 tumours in the mice (Ebralidze et al. 1989). CSML100 tumour cells were injected subcutaneous to the wild type and S100A4-deficient mice. All of the wild type mice rapidly succumb due to the tumours and multiple lung metastasis, while tumour development in the S100A4 −/− mice was severely impaired. Approximately 60% of the S100A4-deficient animals never grew tumours. Not only the percentage of tumour-bearing animals was less among the knockout mice, but the time of tumour uptake was significantly delayed (Fig. 2A). Most important, tumour-bearing S100A4 −/− mice did not develop lung metastases. Immunohistochemical analysis of tumour sections showed that tumours developed in the S100A4-deficient mice contained aberrant stroma. The blood vessel density was decreased approximately two times and macrophages and lymphocytes were mostly concentrated in the outer edge of the tumour.

Insufficiency of stroma development in the knockout tumour-bearing animals was associated with the level of the S100A4 in tumour microenvironment. The amount of S100A4 released by tumour developed in the wild type mice is approximately 2 times higher that the one produced by tumour developed in the S100A4-deficient mice (Fig. 2B). Tumour stroma composing cells do not produce S100A4 in the S100A4(−/−) mice CSML100 cells themselves fail to secrete enough amounts of the S100A4 protein, therefore sufficient local concentration of S100A4 could not be achieved and subsequently tumour development and its spread to the secondary sites cannot be supported.

Mouse models with genetically modified *S100A4* gene demonstrated the effectiveness of *in vivo* approach to study the mechanisms of stimulation of the metastatic spread of tumour cells directed by single gene.

We were able to demonstrate using an *in vivo* approach that S100A4 expression indeed stimulates metastatic spread of tumour cells. GRS/A-mts1 hybrid transgenic females with the mammary gland-specific overexpression of S100A4, in contrast to the controls, developed metastatic mammary tumours.

Transgenic mice with ubiquitous expression of S100A4 in all the tissues (HMGCR/mts1) revealed a complex pattern of regulation of S100A4 expression. In particular, we have shown a tissue-specific post-translational down-regulation of the S100A4 expression. Together with the observation that the *S100A4* gene has very definite species-specific expression pattern (Davies et al. 1995) this observation points to the existence of strict repression mechanisms that limit the expression of S100A4 to certain cells and tissues.

Analysis of the pathological changes observed in the aged HMGCR/mts1 transgenic mice proved the importance of angiogenic function of extracellular S100A4 in stimulation of metastatic disease. To answer a question which cells in the developing tumour are responsible for S100A4 release and consecutive stimulation of tumour dissemination we generated a S100A4 – deficient mouse model. Excitingly, tumour development and metastatic spread of S100A4 expressing metastatic mammary carcinoma cells grafted to the S100A4(−/−) mice was substantially repressed. Therefore this mouse model demonstrated the vital importance of S100A4 expression in the host-derived stroma cells for tumour fate.

It has been shown that tumour cells are able to stimulate the release of S100A4 from the cells of host-derived stroma (Schmidt-Hansen et al. 2004b). Accumulation of S100A4 in the tumour microenvironment triggers a

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sequence of events that finally leads to tumour dissemination. By interaction with a putative receptor S100A4 stimulates signal transduction pathway leading to activation of transcription factors, such as NF-κB, which in turn will stimulate transcription of a wide variety of genes. In particular, it has been shown that S100A4 stimulates production of matrix degrading proteases (MMPs) as well as invasion and motility of endothelial cells (Schmidt-Hansen et al. 2004a, b). This leads to remodelling of extracellular matrix, development of new blood vessels and penetration of stroma cells into the tumour. Thus a favourable microenvironment for tumour dissemination is generated.

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