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Hmga1 null mice are less susceptible to chemically induced skin carcinogenesis

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ABSTRACT

The HMGA1 proteins have a critical role in the process of carcinogenesis. They are overexpressed in most human malignant neoplasias, and the inhibition of their expression has been shown to prevent cell transformation and results in malignant cell death. To determine whether HMGA1 proteins are also required for *in vivo* carcinogenesis, we compared the tumour susceptibility of mice wild-type or knockout for the *Hmga1*-null allele using a two-stage chemical skin carcinogenesis protocol. *Hmga1*$^{-/-}$ mice exhibited a decreased number and a delayed onset of skin papillomas in comparison with wild-type mice. Moreover, the progression of skin papillomas to carcinomas was observed in only 5% of *Hmga1*$^{-/-}$ compared to 18% of wild-type mice. These results suggest a lower susceptibility of *Hmga1*$^{-/-}$ mice to skin carcinogenesis induced by chemical agents.

1. Introduction

The HMGA family of mammalian high mobility group consists of three related members: HMGA1a, HMGA1b (encoded by the same gene and generated by alternative splicing), and HMGA2 (derived from a different gene).[1] They are small nuclear polypeptides that bind to the minor groove of DNA through highly cationic regions called 'AT-hooks'.[2] HMGA proteins function as architectural transcription factors: they do not independently regulate gene transcription, but modulate gene expression through the formation of stereospecific complexes on the promoter/enhancer regions of genes. These complexes are formed by direct interaction with other transcription factors and through DNA-protein interactions that bend, unwind or distort the structure of DNA.[3] HMGA proteins seem to play their major physiologic role during embryonic development. In fact, HMGA is abundantly expressed during embryogenesis, while their expression is negligible

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in normal adult tissue.[4] The generation of *Hmga1*-ko mice, recently reported by our group,[5] has revealed the critical function of this gene in development. Cardiac hypertrophy and type 2 diabetes were observed in *Hmga1*-ko mice,[5,6] even in mice heterozygous for the gene, suggesting that a quantitatively appropriate HMGA1 expression is required for normal development. Interestingly, the severe insulin resistance of *Hmga1*-null mice resembles that shown by three diabetic patients with impaired HMGA1 function.[6]

Alterations in the structure and/or the expression of the HMGA proteins play an important role in benign and malignant tumours. In fact, *HMGA* gene rearrangements, due to chromosomal translocation, are frequently detected in human benign tumours of mesenchymal origin.[7,8] Moreover, HMGA1 overexpression was first documented in transformed thyroid cells,[9] then in experimental carcinomas,[10] and subsequently in human thyroid,[11] colorectal,[12] pancreatic duct cell,[13] uterine,[14] breast,[15] ovarian cancers,[16] and in glioblastomas.[17] Thus, HMGA1 protein overexpression appears to be a constant feature of malignant human neoplasias. The oncogenic activity of *HMGA1* gene overexpression has been demonstrated *in vitro* and *in vivo*.[18,19] Moreover, blocking HMGA1 synthesis prevents malignant transformation of rat thyroid cells by murine transforming retroviruses,[20] and an adenovirus carrying the *HMGA1* gene in antisense orientation induces apoptotic cell death of anaplastic human thyroid carcinoma cell lines but not of normal thyroid cells.[21] These findings demonstrate that HMGA proteins have a causal role in neoplastic transformation. Furthermore, the development of haematological malignancies, including B cell lymphoma and myeloid granuloerythroblastic leukaemia in *Hmga1*-knockout mice also revealed an unsuspected tumour suppressor role for this gene.[5]

To verify whether *Hmga1* gene plays a pivotal role in *in vivo* transformation, we evaluated the susceptibility of *Hmga1*-ko mice to chemically induced skin neoplasias. For this purpose, we used *Hmga1*-ko mice in a two-stage skin carcinogenesis protocol based on initiation with 7,12-dimethylbenz[*a*]anthracene (DMBA) followed by promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). Overexpression of HMGA1 in murine skin carcinomas[22] and regulation of HMGA1-specific transcription start sites by TPA[23] led us to choose this model of carcinogenesis. Moreover, this animal model of carcinogenesis allowed us to determine whether the *Hmga1* gene had a role in the early or late steps of tumour development.

In the present study we report that mice homozygous for the *Hmga1*-null allele develop fewer papillomas compared to wild-type mice, and fewer of these lesions progress to carcinomas. Moreover, we document reduced expression of c-Fos protein in the $Hmga1^{-/-}$ mice, suggesting an explanation of the decreased sensitivity of these mice to chemically induced skin carcinogenesis.

2. Materials and methods

2.1. Experimental skin carcinogenesis

Mice carrying a disrupted *Hmga1* gene were generated by homologous recombination in an ES cell line, as described;[5] the disrupted *Hmga1* gene was subsequently transferred into an inbred C57Bl/6J genetic background. Mice 6–7 weeks old were used. Each mouse was identified with an ear tag and genotyped by Southern blot analysis. The dorsal skin was shaved and treated with a single application of DMBA (7,12-dimethylbenz[*a*]anthracene; Sigma, St Louis, MO) (25 μg in 200 μl acetone), followed by twice weekly application of TPA (12-O-tetradecanoylphorbol-13-acetate; Sigma, St Louis, MO) (200 μl of 10^{-4}M in acetone). TPA treatment was stopped after 26 weeks. The number and size of skin tumours on each mouse were recorded at regular intervals. Mice were sacrificed after 32 weeks, and the neoplastic lesions were excised, and frozen or fixed for histolologic, immunohistochemical and molecular analysis. All the mice had been housed in the Animal facility of the Comprehensive Cancer Center at the Ohio State University, and their care was in accordance with institution guidelines.

2.2. Histological and immunohistochemical procedures

For light microscopy, tissues were fixed by immersion for 24 h in Bouin's solution and embedded in paraffin using standard procedures. Sections (5 μm) were stained with haematoxylin and eosin or haematoxylin and periodic acid–Schiff (PAS) reagent. Frozen sections (4–8 μm) of normal and pathological tissues were cut in a cryostat and allowed to dry for 1 h at room temperature before fixation in acetone for 10 min. The slides were air dried for 2 h at room temperature and then placed in a buffer bath (phosphate buffered saline, PBS) for 5 min before immunohistochemistry.

For immunohistochemical studies of paraffin-embedded samples, 3–4 μm paraffin sections were deparaffinised, placed in a solution of absolute methanol and 0.3% hydrogen peroxide for 30 min and washed in PBS. The slides were incubated overnight at 4 °C in a humidified chamber with antibodies diluted 1:100 in PBS. The slides were subsequently incubated with biotinylated goat anti-rabbit IgG for 20 min (Vectostain ABC kits, Vector Laboratories) and then with premixed reagent ABC (Vector) for 20 min. The immunostaining was performed by incubating the slides in diaminobenzidine (DAB-DAKO) solution containing 0.06 mM DAB and 2 mM hydrogen peroxide in 0.05% PBS (pH 7.6) for 5 min. After chromogen development, the slides were washed, dehydrated with alcohol and xylene, and mounted with coverslips using a permanent-mounting medium (Permount). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system. The antibodies used in this study were raised against the synthetic peptide SSSKQQPLASKQ specific for the HMGA1 proteins.[11] For HMGA2 immunohistochemistry, antibodies raised against the recombinant HMGA2 protein were used.[24] The specificity of the reaction was validated by the absence of staining when carcinoma samples were stained with antibodies pre-incubated with the peptide against which the antibodies were raised (data not shown). Similarly, no positivity was observed when tumour samples were stained with a pre-immune serum (data not shown).

2.3. Protein extraction and Western blot analysis

Frozen sections 10–15 μm thick from papillomas and carcinomas were resuspended in NIH lysis buffer [1% Nonidet P-40;

1 mM EDTA; 50 mM Tris-HCl (pH 8.0); 150 mM NaCl; 2 mM phenylmethylsulfonyl fluoride (PMSF); 50 mM NaF; 10 mM Na_3V_4; 20 mM NaPP; 1.5 mM aprotinin]. Tissue lysates were clarified by centrifugation at 12,000 rpm for 20 min at 4 °C. The supernatant was then used for immunoblotting. Western blotting was performed by standard procedures using the following antibodies: anti-c-Fos (Santa Cruz, sc-52-G), anti-vinculin (Santa Cruz, sc-7649), monoclonal anti-Rab11 (PharMingen/DB Biosciences) and mouse TrueBlot secondary antibody (eBiosceince, San Diego, CA). Briefly, the protein extracts were separated by 4–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Biorad). Membranes were blocked with 5% non-fat milk in TTBS and incubated with antibodies diluted in the same solution. Bound antibodies were detected by the appropriate horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham).

2.4. Statistical analysis

Data are expressed as mean ± SEM. Differences were analysed by Student's *t* test. Kaplan–Meyer survival curves were used to analyse the percentage of tumour-free mice in dependence of the weeks of treatment. Differences were analysed by Log-rank test. *P* values $\leqslant 0.05$ were considered significant for both statistical assays.

3. Results

3.1. The onset of papillomas is delayed in Hmga1-null mice

To address the role of HMGA1 in cancer development, we applied a two-stage chemical skin carcinogenesis protocol to *Hmga1*-null mice. Sixteen $Hmga1^{+/+}$, 22 $Hmga1^{+/-}$ and 19 $Hmga1^{-/-}$ mice were given a single initiating treatment with DMBA and tumour promotion was stimulated by TPA application twice weekly. After 8 weeks, the number of $Hmga1^{-/-}$ mice developing skin papillomas was 26%, which was significantly lower then the number of wild-type mice (68%) ($P < 0.01$) (Fig. 1a). The $Hmga1^{+/-}$ mice showed an intermediate phenotype, with a tumour incidence of 37%. A significant difference in the number of papillomas *per* mouse in $Hmga1^{-/-}$ and $Hmga1^{+/+}$ mice at specific time points was also observed (Fig. 1b). These differences were statistically significant at the 12th and 16th week, when the average number of papillomas *per* mouse was reduced by approximately 60% in $Hmga1^{-/-}$ mice compared to $Hmga1^{+/+}$ mice [average ± SE: 1.2 ± 0.3 and 2.8 ± 0.4 ($P < 0.05$) after 12 weeks from the beginning of the treatment; 2.1 ± 0.3 and 4.1 ± 0.4 ($P < 0.001$) after 16 weeks]. The $Hmga1^{+/-}$ mice again showed an intermediate phenotype [average ± SE: 2.1 ± 0.3 at the 12th week; 2.4 ± 0.5 at the 16th week].

3.2. HMGA1 deficiency resulted in decreased malignant transformation

In the two-stage mouse skin carcinogenesis, papilloma regression is a common event; however, a small percentage of benign lesions progress to form squamous cell carcinomas (SCC). At 32 weeks after DMBA treatment, the incidence of mice bearing SCC was 18% ± 5 for wild-type mice and 5% ± 3 for $Hmga1^{-/-}$ mice with a significant *P* value of 0.0419; thus, wild-type mice developed many more carcinomas than $Hmga1^{-/-}$ mice (Fig. 2). Therefore, these data suggest that HMGA1 proteins also have a role in the malignant progression of skin tumours.

At the histological analysis, papillomas appeared as discrete exophytic masses consisting of a central connective tissue core forming papillae covered by thickened epithelium. This epithelium showed orderly differentiation and was covered by a keratinised layer of variable thickness (Fig. 3a and b). Some mice also developed, with a reduced incidence, benign epidermal tumours resembling human keratoacanthomas. These tumours consisted of large cup-shaped, keratin-filled central regions surrounded by a rim of very well differentiated squamous epithelium, which formed a distinct lip (data not shown). Conversely, SCC were composed of cords and islands of well-differentiated keratinising neoplastic epithelium that invaded the dermis and subcutis and penetrated the panniculus carnosus (Fig. 3c and d). No microscopic or macroscopic differences in appearance were observed between tumours of the same type in the different animal groups.

3.3. Expression of Hmga1 and Hmga2 in the skin papillomas and carcinomas induced in Hmga1-null mice

The expression of both HMGA1 and HMGA2 proteins is induced during cell transformation *in vitro* and *in vivo*.[24] As shown in Fig. 4, we analysed the expression of the Hmga proteins in papilloma and carcinoma samples originating from wild-type and *Hmga1*-null mice. As expected, immunohistochemical analysis of papilloma and carcinoma samples did not show any expression of the Hmga1 protein in neoplasias of the *Hmga1*-null mice (Fig. 4b and d, respectively), while its induction was observed in all of the papillomas and carcinomas from the wild-type mice (Fig. 4a and c, respectively). Hmga2 protein expression was also detected in the carcinoma samples from both $Hmga1^{+/+}$ and $Hmga1^{-/-}$ mice (Fig. 4g and h, respectively), whereas it was absent in the papillomas from the same groups (Fig. 4c and d). These findings suggest that Hmga1 induction occurs earlier than Hmga2 induction during the process of skin tumour development.

3.4. The expression of Rab11a and c-Fos is decreased in normal skin and papillomas of Hmga1-null mice

Previous studies have demonstrated that HMGA1 enhances *c-fos* transcription in eukaryotic cells.[25] Moreover, *in vivo* carcinogenesis studies showed that c-Fos plays a crucial role in the transition from benign to malignant skin tumours by regulating expression of Rab11a.[26,27] Therefore, we analysed the expression of c-Fos and Rab11a proteins first in the untreated back skin of $Hmga1^{-/-}$, $Hmga1^{+/-}$ and $Hmga1^{+/+}$ mice (Fig. 5a and data not shown). The levels of c-Fos and Rab11a proteins were significantly lower in *Hmga1*-null skin compared to that of wild-type mice (Fig. 5b), and intermediate between $Hmga1^{-/-}$ and $Hmga1^{+/+}$ in heterozygous mice (data not shown). Then, we analysed c-Fos and Rab11a expression in SCC and

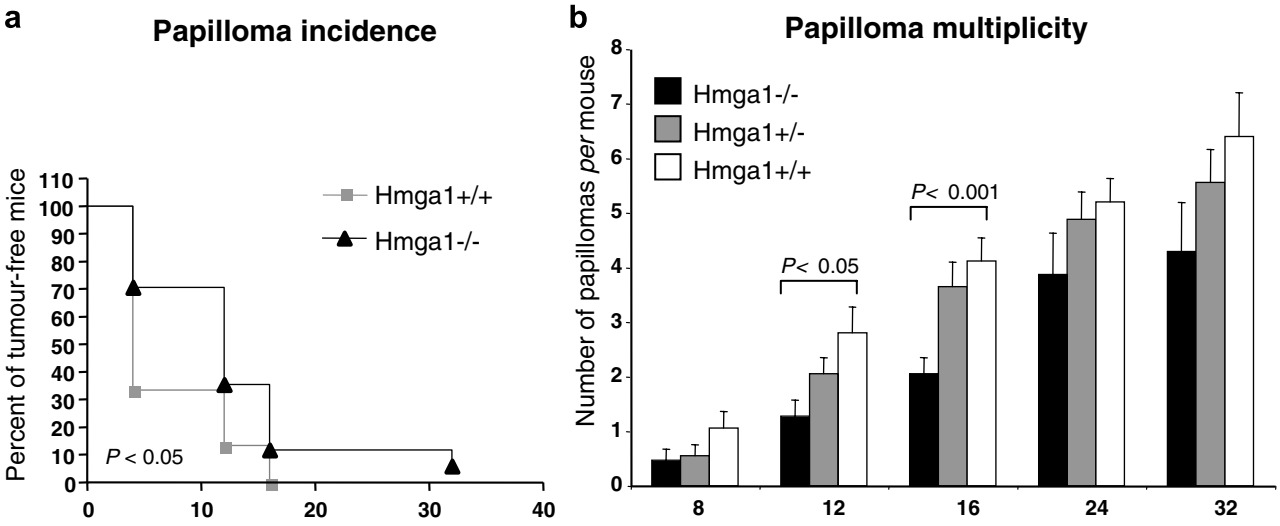

Fig. 1 – *Hmga1*$^{-/-}$ mice develop fewer skin tumours than wild-type mice. Mice of the indicated genotypes were subjected to a two-stage DMBA/TPA skin carcinogenesis protocol. (a) Papilloma incidence, i.e. percentage of mice with papillomas, was analysed using Kaplan–Meyer curves showing the tumour-free survival. A significant difference was observed between the two groups indicated ($P < 0.05$). (b) Papilloma multiplicity, i.e. the number of papillomas *per* mouse, calculated each week until the end of the treatment, is shown for each mouse group as indicated. The differences between *Hmga1*$^{-/-}$ mice and their respective wild-type controls were significant at 12 weeks ($P < 0.05$) and 16 weeks ($P < 0.001$) from the beginning of the carcinogenesis protocol.

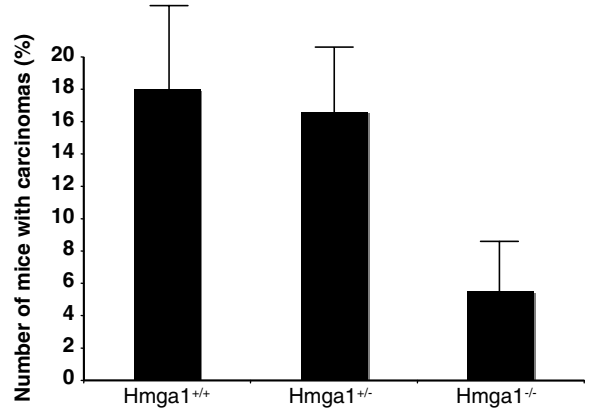

Fig. 2 – Malignant transformation of papillomas to carcinomas is less frequent in *Hmga1*-null mice than in heterozygous and wild-type mice. Percentage of carcinoma incidence *per* mouse was calculated for each mouse group at the end of the 32nd week from the beginning of the carcinogenesis protocol. The difference between *Hmga1*$^{-/-}$ mice and their respective wild-type controls were significant ($P < 0.05$).

papillomas from *Hmga1*-null, -heterozygous and wild-type mice. As shown in Fig. 5b, Western blot analysis revealed lower levels of c-Fos in both *Hmga1*$^{-/-}$ SCC and papillomas than in the corresponding tumours from wild-type mice. Intermediate levels were observed in the Hmga1$^{+/-}$ samples. Similarly, Rab11a expression levels were decreased in Hmga1$^{+/-}$ SCC and papillomas in comparison with wild-type samples, and further decreased in Hmga1$^{-/-}$ papilloma but unexpectedly increased in Hmga1$^{-/-}$ SCC compared to wild-type.

This finding suggests that the lack of Hmga1 expression in the keratinocytes of *Hmga1*$^{-/-}$ mice reduced malignant transition of papillomas to SCC by interfering with the c-Fos/Rab11a pathway. It is likely that the increase of Rab11a expression in SCC, in the absence of Hmga1 protein, must be considered an epiphenomenon associated to the neoplastic phenotype.

4. Discussion

It is widely accepted that the HMGA proteins play a crucial role in the development of malignant tumours. HMGA proteins have been found to be overexpressed in almost all of the malignant neoplasias so far analysed, and there is a correlation between HMGA expression levels and the grade of malignancy.[13,28] Moreover, a causal role in cell transformation has been demonstrated for these proteins. In fact, HMGA overexpression can transform cells *in vitro* and *in vivo*,[18,19] and blocking HMGA2 expression by antisense methodology prevents malignant transformation of rat thyroid cells by *v-mos* and *v-ras-Ki* oncogenes.[24] However, when mice carrying a disrupted *Hmga2* gene (pygmy mice) were either treated with radioactive iodine or crossed with transgenic mice carrying the E7 papilloma virus oncogene under the transcriptional control of thyroglobulin gene promoter, they developed thyroid carcinomas with the same frequency as wild-type mice, and the tumours they developed were indistinguishable from tumours in wild type mice. Therefore, these results indicated that *HMGA2* gene expression is not required for *in vivo* thyroid cell malignant transformation,[29] suggesting that HMGA1 proteins, rather than HMGA2, may be required for cell transformation. This hypothesis is also supported by the evidence

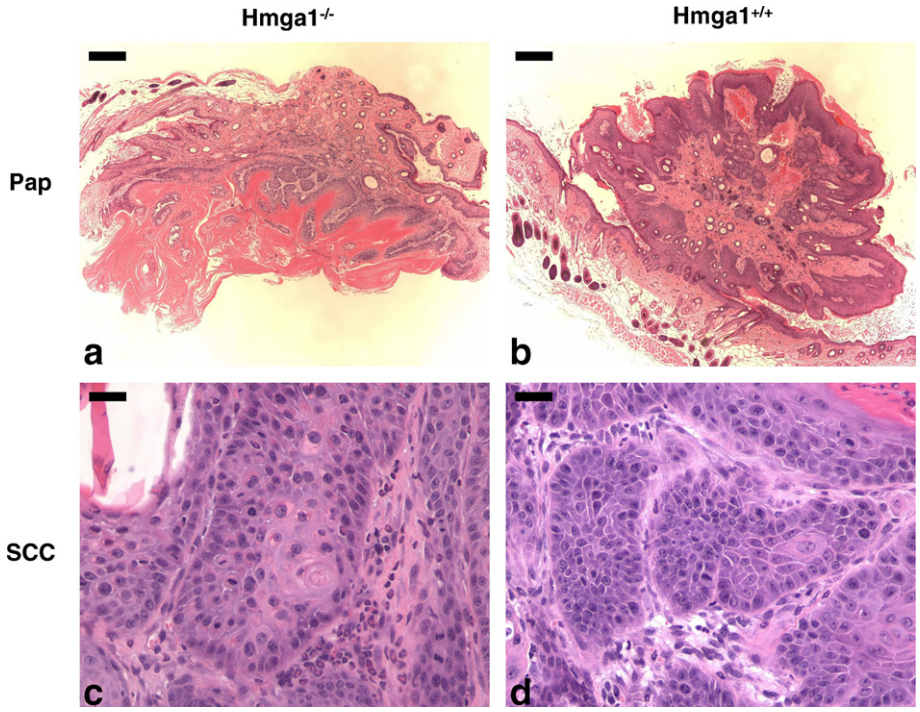

Fig. 3 – Histological examination of papillomas and carcinomas in *Hmga1*$^{-/-}$ and *Hmga1*$^{+/+}$mice. (a,b) Papillomas (bar scale = 100 μm). (c,d) Squamous cell carcinomas (bar scale = 50 μm). Pap = papillomas; SCC = squamous cell carcinomas.

that blocking HMGA1 protein synthesis using an adenovirus carrying HMGA1 antisense sequences prevents transformed thyroid cell growth;[21] in addition, rat thyroid cell transformation by the Kirsten murine sarcoma virus does not take place in the absence of the HMGA1 protein induction.[20] Alternatively, the expression of only one of the *HMGA* genes may be required to transform thyroid cells to the malignant phenotype. The absence of *HMGA1* gene induction in HMGA2 antisense virally infected thyroid cells[24] is consistent with this hypothesis.

The recent generation of *Hmga1*-knockout mice[5] offers an excellent opportunity to define the role of individual *HMGA* genes in the process of carcinogenesis *in vivo*. In the present study, mice homozygous and heterozygous for the *Hmga1*-null allele and wild-type mice were treated with DMBA and TPA in a classical two-stage skin carcinogenesis protocol. After 8 weeks of treatment *Hmga1*$^{-/-}$ mice had significantly fewer skin tumours than wild-type mice. Moreover, at the 12th and 16th weeks of chemical treatment, the total number of papillomas *per* mouse was significantly higher in wild-type than *Hmga1*$^{-/-}$ mice. Taken together, these findings indicate that HMGA1 plays a critical role in the development of chemically induced skin tumours. Furthermore, malignant progression of papillomas to carcinomas was observed in only 5% of *Hmga1*$^{-/-}$ mice compared to 18% of wild-type mice, suggesting a further role for HMGA1 in tumour malignant progression. This result is consistent with the literature data that show a frequent association of HMGA1 expression and the presence of a highly malignant phenotype.[11–17]

Although we show evidence that HMGA1 plays a critical role in development of the malignant phenotype, our results also demonstrate that malignant tumours can still arise in the absence of the HMGA1 proteins. Thus, it is likely that HMGA2, which we showed to be induced in skin carcinomas, may substitute for HMGA1. Of course, we cannot exclude that other genes, as yet unidentified, may duplicate some functions of the HMGA1 proteins.

It has been demonstrated that benign skin tumours do not progress to malignant skin tumours in mice lacking c-Fos protein and that this failure is due to Rab11a inactivation.[26] Moreover, it has been shown that HMGA1 enhances SRF-dependent activation of the *c-fos* promoter and that TPA mediates the activation of Rab11a in mouse skin in a c-Fos-dependent manner.[25,26] We analysed Rab11a and c-Fos expression in both *Hmga1*$^{-/-}$ and *Hmga1*$^{+/+}$ mice. Notably, decreased expression of Rab11a and c-Fos was found in keratinocytes and papillomas of the *Hmga1*$^{-/-}$ mice, in comparison with the wild-type mice, suggesting that the reduced expression of c-Fos in *Hmga1*-null mice might contribute to the decreased susceptibility to the development of malignant skin tumours.

The correlation among HMGA1, c-Fos and Rab11a expression suggests a mechanism by which HMGA1 could be involved in malignant progression. Nevertheless, other biological roles of the HMGA proteins, such as their reported capacity to inhibit DNA repair,[30,31] may be taken into consideration. In fact, the results shown here could also be explained by considering that HMGA1 overexpression would increase the incidence of mutations that facilitate the process of carcinogenesis.

In conclusion, the data reported here indicate that the expression of the *HMGA1* gene plays a critical role in the process of chemically induced skin cancer development.

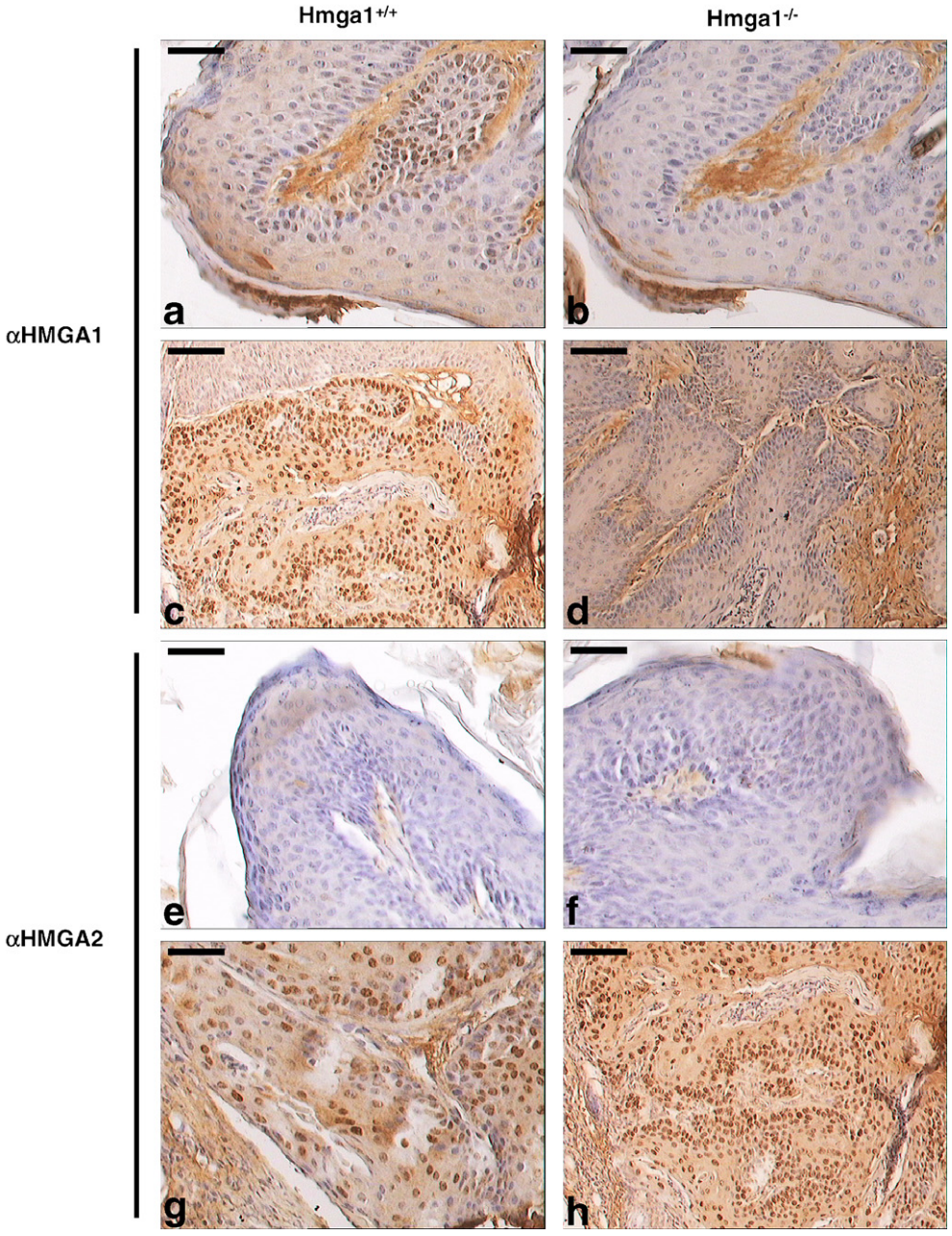

Fig. 4 – Immunohistochemical detection of Hmga1 and Hmga2 proteins in mouse skin tumours. Paraffin sections from benign and malignant mouse skin tumours arising in mice subjected to the carcinogenesis protocol were analysed by immunohistochemistry using antibodies raised against specific Hmga1 and Hmga2 peptides. (a) Immunostaining for HMGA1 of a representative skin papilloma developed by a *Hmga1*$^{+/+}$ mouse (bar scale = 50 μm). A positive nuclear staining was observed. (b) Immunostaining for HMGA1 of a representative skin papilloma developed by a *Hmga1*$^{-/-}$ mouse (bar scale = 50 μm). No immunoreactivity was observed. (c) Immunostaining for HMGA1 of a representative squamous cell carcinoma developed by a *Hmga1*$^{+/+}$ mouse (bar scale = 100 μm). A positive nuclear staining was observed. (d) Immunostaining for HMGA1 of a representative squamous cell carcinoma developed by a *Hmga1*$^{-/-}$ mouse (bar scale = 100 μm). No staining was observed. (e) Immunostaining for HMGA2 of a representative skin papilloma developed by a *Hmga1*$^{+/+}$ mouse (bar scale = 50 μm). No immunoreactivity was observed. (f) Immunostaining for HMGA2 of a representative skin papilloma developed by a *Hmga1*$^{-/-}$ mouse (bar scale = 50 μm). No immunoreactivity was observed. (g) Immunostaining for HMGA2 of a representative squamous cell carcinoma deriving from a *Hmga1*$^{+/+}$ mouse. A positive nuclear staining was observed (bar scale = 50 μm). (h) Immunostaining for HMGA2 of a representative squamous cell carcinoma deriving from a *Hmga1*$^{-/-}$ mouse. A positive nuclear staining was observed (bar scale = 100 μm).

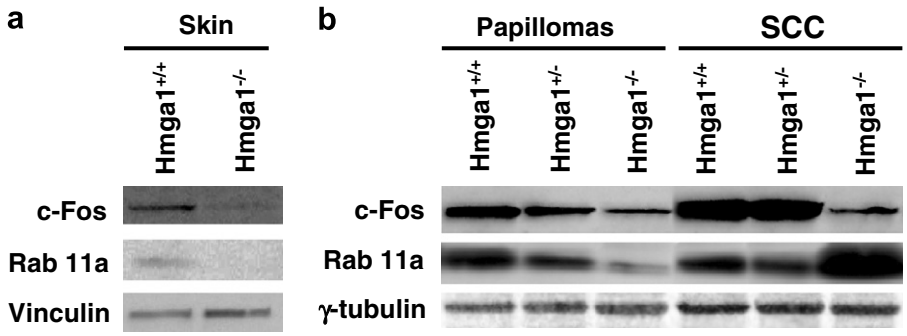

Fig. 5 – c-Fos and Rab11a expression is reduced in tumours and skin from *Hmga1*$^{-/-}$ mice. Western blot analysis showing the expression of c-Fos and Rab11a proteins in untreated skin (a) and papillomas (b) from *Hmga1*$^{-/-}$, *Hmga1*$^{+/-}$ and *Hmga1*$^{+/+}$ mice. Anti vinculin and γ-tubulin antibodies were used as control for equal protein loading, respectively.

Conflict of interest statement

None declared.

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