EUROPEAN JOURNAL OF CANCER 47 (2011) 470–478

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

# Expression of a truncated *Hmga1b* gene induces gigantism, lipomatosis and B-cell lymphomas in mice

*Monica Fedele* [a,f], *Rosa Visone* [a,b,f], *Ivana De Martino* [a], *Dario Palmieri* [a], *Teresa Valentino* [a], *Francesco Esposito* [a], *Andres Klein-Szanto* [c], *Claudio Arra* [d], *Andrea Ciarmiello* [d], *Carlo M. Croce* [b], *Alfredo Fusco* [a,e,*]

[a] *Istituto di Endocrinologia ed Oncologia Sperimentale del CNR c/o Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università degli Studi di Napoli 'Federico II', 80131 Naples, Italy*

[b] *Division of Human Cancer Genetics, Comprehensive Cancer Center, Ohio State University, 43210 Columbus, OH, USA*

[c] *Experimental Histopathology, Fox-Chase Cancer Center, Philadelphia, PA, USA*

[d] *Istituto dei Tumori di Napoli Fondazione 'G. Pascale', 80131 Naples, Italy*

[e] *NOGEC (Naples Oncogenomic Center)-CEINGE, Biotecnologie Avanzate-Napoli, & SEMM, European School of Molecular Medicine, Naples Site, 80145 Naples, Italy*

ARTICLE INFO

*Article history:*
Received 3 June 2010
Received in revised form 23 September 2010
Accepted 30 September 2010
Available online 31 October 2010

*Keywords:*
Adipogenesis
Lymphoma
Experimental animal models
Oncogene
E2F1
High Mobility Group proteins

ABSTRACT

*HMGA1* gene rearrangements have been frequently described in human lipomas. *In vitro* studies suggest that HMGA1 proteins have a negative role in the control of adipocyte cell growth, and that *HMGA1* gene truncation acts in a dominant-negative fashion. Therefore, to define better the role of the *HMGA1* alterations in the generation of human lipomas, we generated mice carrying an *Hmga1b* truncated (*Hmga1b/T*) gene. These mice develop a giant phenotype together with a drastic expansion of the retroperitoneal and subcutaneous white adipose tissue. We show that the activation of the E2F pathway likely accounts, at least in part, for this phenotype. Interestingly, the Hmga1b/T mice also develop B-cell lymphomas similar to that occurring in Hmga1-knockout mice, supporting a dominant-negative role of the Hmga1b/T mutant also *in vivo*.



## 1. Introduction

*HMGA1* belongs to the *High Mobility Group A* (*HMGA*) gene family, which also includes the closely related *HMGA2* gene. The *HMGA1* gene codes, through alternative splicing, for two protein isoforms, HMGA1a and HMGA1b, which differ from each other for few aminoacids. The HMGA proteins are characterised by three DNA-binding domains containing short basic repeats, the so-called AT-hooks, capable of binding AT-rich sequences in the minor groove of the DNA, and an acidic carboxy-terminal tail that is believed to be important for protein–protein interactions.[1] They are non-histone chromatin-associated factors known as 'architectural transcription factors': by interacting with the transcription

* *Corresponding author:* Address: Istituto di Endocrinologia ed Oncologia Sperimentale (IEOS), Via Pansini 5, 80131 Napoli, Italy. Tel.: +39 081 67463602; fax: +39 081 2296674.
E-mail addresses: afusco@napoli.com, alfusco@unina.it (A. Fusco).

[f] Both authors contributed equally to the work.

0959-8049/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2010.09.045

machinery they alter chromatin structure and thereby regulate, negatively or positively, the transcriptional activity of several genes.[2] Both *HMGA* genes are widely expressed during embryogenesis, but expression becomes more restricted as foetal development progresses and is absent or low in adult tissues,[3,4] except for the stem cell compartment. In fact, it has been recently shown that HMGA2 is highly expressed in foetal and young-adult stem cells, being required for the self-renewal of foetal and young-adult stem cells relative to old-adult stem cells by negatively regulating $p16^{In4a}$ and $p19^{Arf}$ expression in foetal and young-adult, but not old-adult stages.[5]

This protein family is implicated, through different mechanisms, in both benign and malignant neoplasias.[6] In fact, unrearranged HMGA overexpression is a feature of malignant tumours and is also causally related to neoplastic cell transformation, as demonstrated by the ability of HMGA1 and HMGA2 overexpression to transform cells in culture and induce tumours *in vivo*.[7,8] Conversely, rearrangements of *HMGA* genes, mainly the *HMGA2* gene, are a feature of most benign human mesenchymal tumours.[9]

Indeed, they are frequent in human benign neoplasias of mesenchymal origin, including lipomas, uterine leiomyomas, pulmonary chondroid hamartomas and others[9]; breakpoints in *HMGA2* preferentially cluster in the third large intron of the gene, resulting in deregulation of its expression, truncation or, more frequently, generation of fusion genes encoding chimeric transcripts containing the first three exons of *HMGA2* and ectopic sequences from other genes. Truncation of *HMGA2* with the loss of its carboxy-terminus, including the 3′ untranslated region (3′ UTR), seems to account for *HMGA2* gene overexpression in these tumours.[7,10,11] Indeed, 3′ UTR of *HMGA2* contains multiple target sites for let-7 microRNA, and therefore, its loss would result in the lack of the let-7 negative regulation of HMGA2.[12,13]

Rearrangements of *HMGA1* in various benign mesenchymal tumours consist of breakpoints located either upstream or downstream of the gene sequence, and molecular analyses of *HMGA1* transcripts revealed common deletions of the carboxy-terminal region and/or parts of the 3′ UTR.[14,15] The generation of transgenic mice overexpressing *HMGA1* and *HMGA2* genes confirmed an oncogenic role for these genes. In fact, transgenic mice carrying wild-type forms of either *Hmga2* or *Hmga1* develop NK/T-cell lymphomas and pituitary adenomas.[16,17] Conversely, the generation of knockout mice for the *Hmga1* gene revealed an unsuspected tumour-suppressor potential since they develop B-cell lymphomas and myelo-proliferative disorders.[18]

Several observations support a critical role for HMGA proteins in the development, and particularly, in the process of adipogenesis.[19] In fact, $Hmga2^{-/-}$ mice express a *pygmy* phenotype together with a drastic reduction (87%) in adipose tissue,[3] whereas transgenic animals overexpressing either wild-type or truncated Hmga2 show an opposite phenotype with a significant increase in weight and size, characterised by lipomas or abdominal/pelvic lipomatosis.[11,16,20] Moreover, the lack of Hmga2 expression prevents both diet- and gene-induced obesity.[21] Conversely, HMGA1 proteins exert a negative role on adipocytic cell growth.[22,23] In fact, *Hmga1* gene expression is induced in mouse 3T3-L1 fibroblasts, which rapidly differentiate to adipocytes upon treatment with several agents,[24] soon after differentiation.[22] Further, suppression of *Hmga1* or expression of a truncated *Hmga1b* (*Hmga1b/T*) gene, deprived of both the acidic tail and the 3′ UTR, dramatically increased growth rate and impaired adipocytic differentiation,[22,23] also suggesting that the Hmga1b/T mutant acts in a dominant-negative fashion.[23] Data obtained from our laboratory on Hmga1b-transgenic animal models, which do not develop lipomas, have so far supported this model.[17] To validate *in vivo* the putative lipomagenic activity of the *HMGA1b/T* gene, we generated transgenic mice carrying this mutant gene under transcriptional control of the cytomegalovirus promoter (Hmga1b/T mice). These mice are overgrown and accumulate abundant ectopic fat depots. Increased E2F activity has been detected in the transgenic mice compared to wild-type littermates, likely accounting, at least in part, for the phenotype of the Hmga1b/T mice. We also show that Hmga1b/T mice develop B-cell lymphomas with morphological, histological and molecular features similar to those observed in $Hmga1^{-/-}$ mice, again supporting the hypothesis of a dominant-negative role of HMGA1/T on the HMGA1 function.

## 2. Materials and methods

### 2.1. Generation of transgenic mice

The construct carrying the cDNA encoding the truncated form of the murine *Hmga1b* gene (pRc/CMV-*Hmga1*/T) was obtained by sub-cloning a 243-bp cDNA fragment (from +196 bp to +438 bp downstream the transcriptional starting site (TSS) of the mouse *hmga1b* cDNA) into the *HindIII* and *XbaI* sites of the expression vector pRc/CMV (Invitrogen), as previously described.[23] It was electroporated into ES AB2.2 cells,[25] and G418-resistant clones were selected and analysed by Southern blot hybridisation with a CMV promoter probe on genomic DNA digested with SspI (Fig. 1A). Seven positive clones were selected on the basis of the unique Southern band of 2000 bp corresponding to the expected length of the SspI fragment including the CMV-*Hmgab1*/T insert (asterisks in Fig. 1B). These clones were expanded and the expression of *Hmga1b/T* was evaluated by a semiquantitative RT-PCR assay, using a construct-specific primer set described in the following paragraph. The highest two *Hmga1b/T* expressing ES cell clones (asterisks in Fig. 1C) were microinjected into C57BL6/J mouse blastocysts and then transferred to pseudopregnant foster mothers (Thomas Jefferson University, Philadelphia, PA). Chimeric mice were crossed to wild-type C57BL6/J mice (Taconic Farm), and germ-line transmission of the transgene was checked by Southern blot analysis of tail DNA from agouti coat-coloured F1 offspring.

### 2.2. RT-PCR analyses

Tissues from transgenic animals were rapidly dissected, frozen on dry ice, and stored at –80 °C. Total RNA was extracted using TRI-reagent solution (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol and treated with DNase I (Invitrogen). One micrograms of RNA was reverse transcribed using random exonucleotides as primers (100 mM) and MuLV reverse transcriptase (Applied

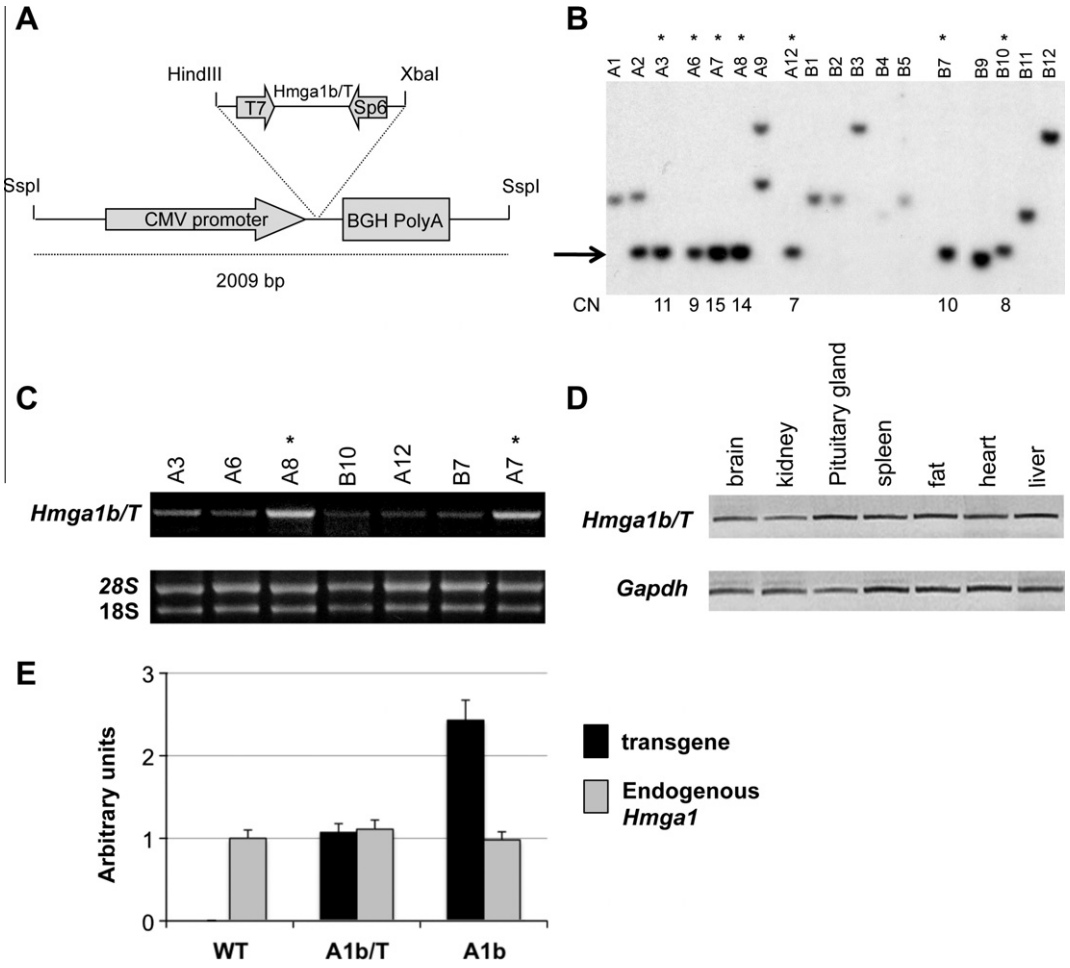


**Fig. 1 – Generation of Hmga1b/T transgenic mice. Schematic representation of the CMV-Hmga1b/T transgene vector construction (A). Analysis, by Southern blot, of the integration of the CMV-Hmga1b/T transgene in ES cell clones. Asterisks = selected clones for further analyses and CN = copy number (B). RT-PCR analysis of the expression of the CMV-Hmga1b/T transgene in the selected ES cell clones. Asterisks = selected clones for blastocysts microinjection (C). RT-PCR analysis of the expression of the CMV-Hmga1b/T transgene in a panel of adult tissues from transgenic mice (D). Quantitative RT-PCR for the relative expression of the *Hmga1b/T* and *Hmga1b* transgenes in Hmga1b/T (A1b/T) and Hmga1b (A1b) mice, respectively. The relative expression of the two transgenes in comparison to the endogenous *Hmga1b* gene is also shown in the two trangenic mouse models. The data represent the mean values ± SD of two spleens from each mouse line performed in duplicate (E).**

Biosystems). Five microlitres of cDNA were amplified as previously described.[11] Primers designed to amplify specifically the transcripts of the transgenic construct, as well as primers specific for the expressed enzyme glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) that was used as an internal control of the amount of cDNA tested, and primers to amplify *V-preB* and *rag2* genes were previously described.[18,23]

### 2.3. *Quantitative RT-PCR*

Each reaction was performed in duplicate in a final volume of 20 μl using 10 μl of 2× Power SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of each primer and 1 μl of each cDNA, using the following conditions: 10 min at 95 °C and then 45 cycles of 30 s at 95 °C and 1 min at 60 °C. At the end of the PCR run, a dissociation curve was constructed using a ramping temperature of 0.2 °C/s from 65 °C to 95 °C and a single melting point was obtained for each promoter. The $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels.[26] The following primer sequences were used: CMV-forward, 5′-tggcagtacatctacgtattagtcatc-3′; CMV-reverse, 5′-atccacgcccattgatgta-3′; hmga1-endog-forward, 5′-caagacccgggaaagtca-3′; hmga1-endog-reverse, 5′-cagaggactcctgggagatg-3′; cyclin E-forward, 5′-ctgagagatgagcactttctgc-3′; cyclin E-reverse, 5′-gagcttatagacttcgcacacct-3′; hprt-forward, 5-tcctcctcagaccgctttt-3′ and hprt-reverse, 5′-cctggttcatcatcgctaatc-3′.

### 2.4. *Protein extraction and Western blot*

Total protein lysates were obtained from transgenic (both normal and neoplastic) and wild-type spleens that had been homogenised in a solution containing 1% NP40, 1 mmol/l EDTA, 50 mmol/l Tris–HCl (pH 7.5) and 150 mmol/l NaCl, supplemented with complete protease inhibitors mixture

(Roche). Total proteins were separated by SDS–PAGE and transferred to nitrocellulose membrane. Membrane was blocked with 5% BSA in Tris Buffered Saline (TBS) and incubated with the specific primary antibodies (Ab) recognising the HMGA1 protein.[22] Bound Ab was detected by the appropriate secondary Ab, diluted in 5% nonfat milk in TBS solution containing 0.05% Tween 20 (TTBS), and revealed with an enhanced chemiluminescence system (Amersham Biosciences, Cologno Monzese, Italy).

### 2.5. MRI

Magnetic Resonance Imaging was performed at the 'Istituto dei Tumori di Napoli G. Pascale' on a 1.5-T magnet system using local receiver coils and an 8-cm field view. Coronal slices 3 mm thick were obtained with T1 weighting (TR/TE = 400/11 ms).

### 2.6. Plasmids, cell transfections and luciferase activity assay

The cyclin E-luciferase vector (cyclin E-luc) was generously provided by K. Helin (Biotech Research and Innovation Centre, University of Copenhagen, Denmark). Transfections for luciferase assays were carried out in 3T3-L1 mouse pre-adipocytes by using Lipofectamine 2000 method (Invitrogen), according to the manufacturer's instructions. A total of $2 \times 10^5$ cells were transiently transfected with 0.2 μg of cyclin E-luc and with 1 μg of each pCEFLHa-HMGA1,[22] pCEFLHa-HMGA1/T,[23] pCEFLHa-HMGA2 and pCEFLHa-HMGA2/T,[27] together with 0.5 μg of CMV-βgal and various amounts of the pCEFLHa plasmid (backbone vector) to keep the total DNA concentration constant. Cells were harvested 24 h post-transfection and lysates were analysed for luciferase activity. Transfection efficiency was normalised using the β-galactosidase activity. The assay was performed in duplicate and repeated in three independent experiments.

### 2.7. Electrophoretic mobility-shift assay

Protein/DNA-binding was determined by electrophoretic mobility-shift assay (EMSA) for E2F1 with protein extracts from wild-type and Hmgab1/T fat. Seven micrograms of each extract were pre-incubated in a buffer containing 20 mM Hepes pH 7.6, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.02% $NaN_3$, 40 mM KCl, 10% glycerol and 1 μg of sonicated salmon sperm for 10 min at RT. Then, 2 μl of the radiolabelled oligonucleotide was added and the mixture was incubated for 20 min. For supershift analysis, pre-incubation was run for 30 min in ice in the presence of the specific antibody. The DNA-protein complexes were resolved on 4% non-denaturing acrylamide gels and visualised by autoradiography. The double-strand oligonucleotide used was the E2F1-responsive element (sc-2508, Santa Cruz Biotech, Santa Cruz, CA). The antibodies used for the supershift analysis were: anti-E2F1 (sc-193, Santa Cruz) and anti-Rb (sc-50, Santa Cruz).

### 2.8. Histological analysis and immunohistochemistry

For histological examination, dissected tissues were fixed by immersion in 10% formalin and embedded in paraffin. Mounted sections (5 μm thick) were stained with haematoxylin and eosin using routine procedures.

### 2.9. Analysis of splenocyte cell surface antigens

Spleens removed from mice were dissociated into single cells and RBCs were lysed by hypotonic shock using Tris–$NH_4Cl$ buffer ($NH_4Cl$ 0.14 M, Tris 0.017 M, pH 7.2). Cells were washed in PBS supplemented with 2% FCS, 0.2% sodium azide and labelled with FITC-, PE- and Cy5-conjugated appropriate antibodies for 30 min at 4 °C. Cells were then washed and analysed on FACS-Calibur flow cytometer (Becton Dickinson, Buccinasco, Italy). All the Abs used were obtained from Pharmingen/BD Biosciences (Buccinasco, Italy).

### 2.10. Statistics

The results are expressed as mean ± SD. For the comparison of statistical significance between two groups, Student's *t*-test was used. A $P$-value $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Generation of transgenic mice with Hmga1b/T

An ES cell-mediated strategy has been used to generate transgenic mice, as previously described.[18] A truncated *Hmga1b* cDNA (*Hmga1b/T*), deprived of the COOH-terminal tail, under the transcriptional control of the CMV promoter (Fig. 1A) was transfected into the ES cells AB2.2. G418-resistant clones were selected and analysed by Southern blot hybridization with a CMV promoter probe (Fig. 1B). Seven positive clones were selected on the basis of the expected 2009 bp fragment recognised by the Southern blot assay (asterisks in Fig. 1B). The expression of the *Hmga1b/T* transgene was evaluated by RT-PCR. As shown in Fig. 1C, all selected clones expressed the transgene. The two clones with the highest levels of *Hmga1b/T* mRNA (asterisks in Fig. 1C) were microinjected into C57BL6/J mouse blastocysts, which were then transferred to pseudopregnant foster mothers. Several chimeric mice, identified by the mixed black/agouti coat colour, were obtained and crossed with wild-type C57BL6/J mice. Two independent Hmga1b/T strains, identified by Southern blot hybridization, were generated and examined. Both mouse lines showed the same phenotype. The *Hmga1b/T* expression was detected by RT-PCR in all the analysed tissues of transgenic animals (Fig. 1D), without any significant difference among them. Western blot analyses confirmed the expression of the Hmga1b/T protein in transgenic mice (data not shown). Expression of the *Hmga1b/T* transgene was compared to that of the *Hmga1b* transgene from Hmga1b/T and Hmga1b mice, previously generated in our laboratory,[16] respectively. The results, shown in Fig. 1E, indicate a higher expression of the transgene in Hmga1b mice then in Hmga1b/T mice. Moreover, we also show that the expression of the endogenous *Hmga1b* gene is not changed with respect to wild-type littermates in both transgenic mice models (Fig. 1E).

### 3.2. *Hmga1b/T mice exhibit a giant phenotype associated with abdominal/pelvic lipomatosis*

Most Hmga1b/T mice (derived from both lines) exhibited a giant and obese phenotype. At 12 months of age, 70% of them, both males and females, showed an average 10% increase in body length (naso-anal), compared to sex-matched wild-type littermates (Fig. 2B). The remaining 30% of Hmga1b/T mice was indistinguishable from the wild-types. Moreover, starting from the third month of age, both male and female transgenic mice revealed a significant increase of the body-weight growth curves compared to the controls (Fig. 2C and D). MRI of the transgenic and wild-type mice was performed to evaluate the extent of fat deposition and the presence of other abnormalities (Fig. 2A). Transgenic mice showed a drastic expansion of the abdominal and subcutaneous white adipose tissue. To confirm MRI results regarding fat mass, 14-month-old male and female mice were sacrified and three

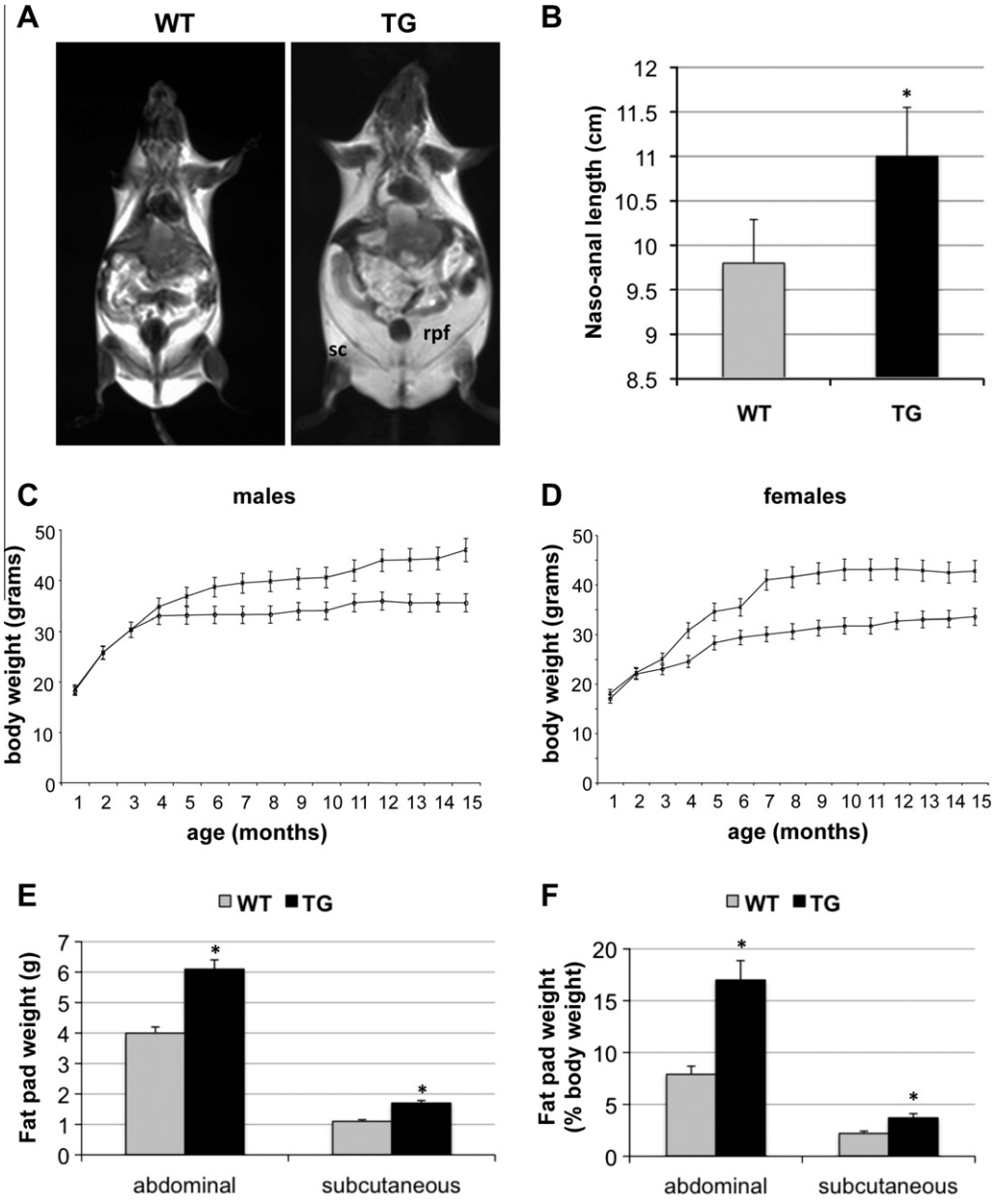


**Fig. 2 – Hmga1b/T mice develop a giant and obese phenotype. MRI of representative one-year-old wild-type and Hmga1/T transgenic mice. A frontal section of each is shown. rpf = retroperitoneal fat and sf = subcutaneous fat (A). Naso-anal length of a cohort of 20 mice, equally distributed between males and females, was measured at 12 months of age and the mean values ± SD were plotted as histograms. $^{*}P < 0.05$ (B). Increased weight of transgenic mice. Mean and 95% confidence intervals of weights of 10 wild-type (o) and 10 Hmga1b/T transgenic (×) male (C) and female (D) mice as a function of age. Weights of dissected fat pads in wild-type and Hmga1b/T mice. Abdominal fat includes gonadal, retroperitoneal and mesenteric fat pads; subcutaneous fat represents the inguinal fat pad. Values, expressed in grams (E) and % body weight (F) represent the means ± SD of 12-month-old male and female mice.**

intra-abdominal fat pads (gonadal, retroperitoneal, and mesenteric) and one subcutaneous fat pad (inguinal, in the groin) were dissected and weighed. As shown in Fig. 2D and E, the absolute and relative weights of the abdominal and subcutaneous fat depots were increased in transgenic mice compared with that in wild-type controls. Histological examination of the abundant abdominal fat mass observed in Hmga1b/T mice showed an overall hypertrophy of the adipose tissue in the abdominal/pelvic area of these mice, consistent with a diagnosis of abdominal/pelvic lipomatosis.

### 3.3. *Enhanced E2F activity in Hmga1b/T mice*

Retinoblastoma proteins (pRB) are known to be critical in controlling cell cycle in most cell types, including adipocyte cells. They prevent the entry of cells into S phase by binding, and thereby inactivating the E2F transcription factors.[28] We have recently reported that HMGA2 activates E2F1 by binding pRB and displacing HDACs from the inhibitory complex recruited by pRB onto E2F-responsive elements.[27] A similar mechanism has also been demonstrated in 3T3-L1 adipocytic cells by HMGA1.[29] We have also previously shown that the truncated *Hmga1b* gene dramatically increased the growth rate of the 3T3-L1 cells and is able to enhance the free E2F (i.e. not complexed with pRB) DNA-binding activity highly.[23] Herein, we demonstrate that cyclin E promoter, a common E2F target, was significantly induced upon transfection of a truncated *Hmga1b* gene, similarly to what occurs for both wild-type and truncated *Hmga2* genes (Fig. 3A). Interestingly, a wild-type *Hmga1b* construct is not able to enhance cyclin E promoter (Fig. 3A), and this result is consistent with the phenotype of the *Hmga1b*-transgenic mice, which are not overgrown and do not gain weight compared to their wild-type littermates.[17] Next, we analysed the E2F activity in *Hmga1b/T* mice by EMSA assays with white adipose tissue (WAT) lysates from transgenic and wild-type animals. The results show an increase of the faster migrating complex, corresponding to the free E2F1-DNA-binding, in transgenic *versus* wild-type mice (Fig. 3B, lanes 1 and 4), confirming *in vivo* the capacity of the *Hmga1b/T* mutant to enhance E2F1 activity. Supershift analyses with antibodies directed against pRB and E2F1 confirmed the specificity of the protein-DNA complexes (Fig. 3B, lanes 2, 3, 5 and 6). In accordance with these data, cyclin E was found overexpressed in adipose tissue of these transgenic animals (Fig. 3C).

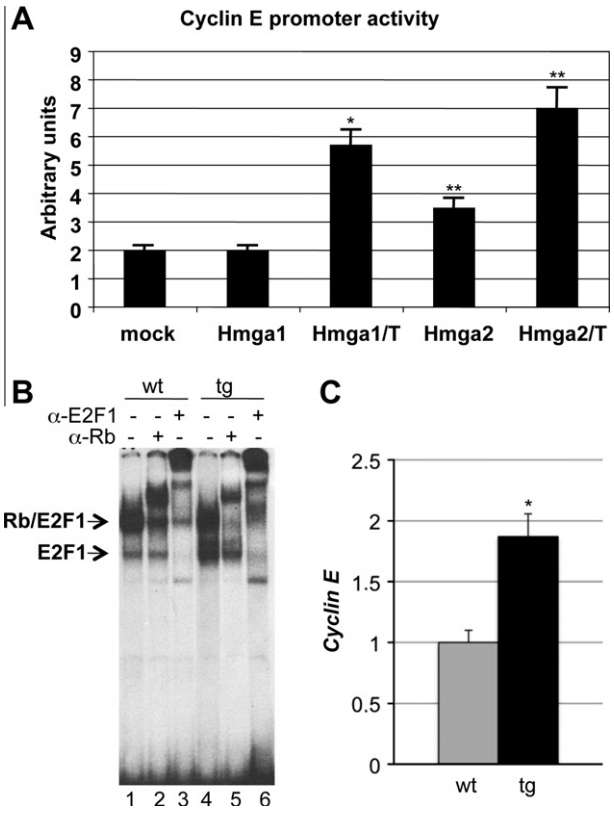


**Fig. 3 – Hmga1b/T mutant protein enhances E2F1 activity. Cyclin E transactivation, by luciferase assay, in 3T3-L1 cells transfected with the indicated gene expression vectors or the backbone vector (mock). The data represent the mean values ± SD of three independent experiments performed in duplicate. *P < 0.05 and **P < 0.001 (A). EMSA of white adipose tissue extracts from wild-type (lanes 1–3) and Hmga1b/T transgenic mice (lanes 4–6) using an E2F-responsive element sequence (E2FRE) as a probe. Samples in lanes 2, 3, 5 and 6 were pre-incubated with the indicated antibodies before the addition of the probe. Supershifted bands confirmed the presence of E2F1 and pRB in the protein/DNA complexes as indicated on the left (B). qRT-PCR of white adipose tissue samples as in B to detect expression of cyclin E (C).**

### 3.4. *Hmga1b/T mice develop B-cell lymphomas*

From 12 months of age, a high percentage (75%) of transgenic Hmga1b/T mice showed splenomegaly. This was histologically and cytologically diagnosed as B-type lymphoma (51%) (Fig. 4, Panels Aα and β, and C) or lymphoid/myeloid hyperplasia (24%) (Table 1). In a cohort of 37 transgenic mice, the B lymphoma subtypes, as defined by Morse et al.[30] were: 11 pre B-cell, 4 follicular B-cell, 3 diffuse large B-cell histiocyte-associated and 1 small B-cell lymphomas, as defined by immunohistochemical analysis with antibodies against specific B and T surface antigens and cytoplasmic proteins and further confirmed by flow cytometric analysis (data not shown). In most of the cases, lymphomas were also observed in other organs such as lymph nodes, liver (Fig. 4Aγ), biliary tract, prostate, pancreas, kidney, lung and thymus. We further characterised transgenic spleens by semiquantitative RT-PCR, looking at genes specifically altered in their expression. As shown in Fig. 4D, similarly to what we previously described for lymphomas developed by *Hmga1*-knockout mice,[18] Hmga1b/T splenocytes showed up-regulation of both *V-preB* and *Rag2* genes compared to wild-type controls. These results support a positive role of the *Hmga1b/T* mutant gene in B-cell expansion and strongly suggest its dominant-negative role, with respect to the wild-type Hmga1 protein, in the pathogenesis of this lymphoproliferative disease.

Finally, as summarised in Table 1, in our cohort of animals, analysed between the ages of 18 and 22 months, we also

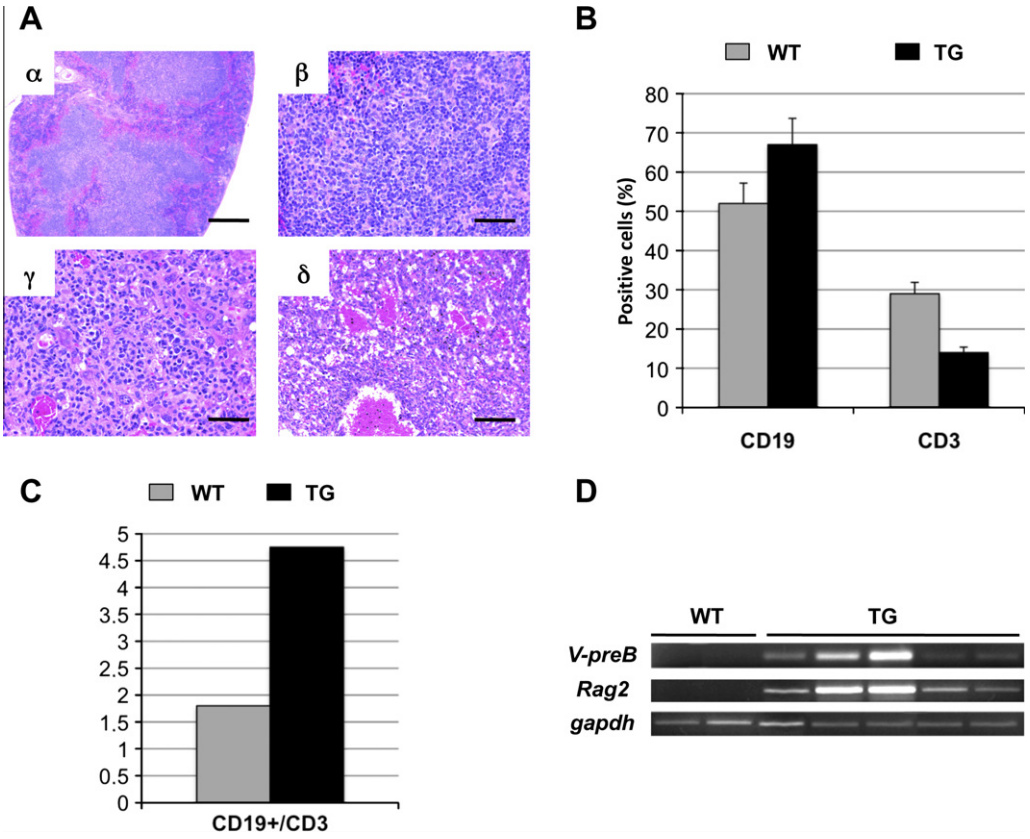


**Fig. 4 – B-cell lymphomas in Hmga1/T mice. Histologic analyses of representative neoplasms developed by Hmga1b/T mice (A): spleen early lymphoma (α); higher magnification of sample shown in α (β); liver lymphoma (γ); hemangiosarcoma (δ). Scale bars: 100 μm (α), 50 μm (β, γ, δ). Analysis of B- and T-cell surface antigens by flow cytometry. The relative percentages of CD19$^+$ (B-lymphocytes) and CD3$^+$ (T-lymphocytes) cells were plotted as histograms, showing their changes in transgenic (TG) versus wild-type (WT) samples. The results represent the mean ± SE of seven different spleens for each genotype ($P < 0.05$) (B). The ratio between B- and T-cells has been represented to show the increase of the B-cell population in TG versus WT animals (C). RT-PCR analysis showing the expression of *V-preB* and *Rag2* genes in TG and WT spleens. Gapdh expression was evaluated as an internal control of RNA used (D).**

**Table 1 – Neoplastic lesions in Hmga1b/T transgenic mice.**

| Pathology | TG | | | WT | | |
|---|---|---|---|---|---|---|
| | Females | Males | Total | Females | Males | Total |
| B-type lymphoma | 13/19 | 6/18 | 19/37 | 2/16 | 1/16 | 3/32 |
| Spleen lymphoid/myeloid hyperplasia | 1/19 | 8/18 | 9/37 | 1/16 | 1/16 | 2/32 |
| Heart hypertrophy | 3/19 | 1/18 | 4/37 | 0/16 | 0/16 | 0/32 |
| Spleen angiosarcoma | 1/19 | 2/18 | 3/37 | 0/16 | 0/16 | 0/32 |
| Lung adenocarcinoma | 2/19 | 0/18 | 2/37 | 0/16 | 0/16 | 0/32 |
| Lung adenoma | 1/19 | 0/18 | 1/37 | 0/16 | 0/16 | 0/32 |
| Uterine leiomyosarcoma | 1/19 | NA | 1/19 | 0/16 | NA | 0/16 |
| Endometrial hyperplasia | 1/19 | NA | 1/19 | 0/16 | NA | 0/16 |
| Mammary adenocarcinoma | 1/19 | 0/18 | 1/37 | 0/16 | 0/16 | 0/32 |
| Hepatocellular carcinoma | 1/19 | 0/18 | 1/37 | 1/16 | 0/16 | 1/32 |
| T-type lymphoma | 0/19 | 1/18 | 1/37 | 0/16 | 0/16 | 0/32 |
| Brain choroid papilloma | 1/19 | 0/18 | 1/37 | 0/16 | 0/16 | 0/32 |

NA = not applicable.

found a certain number of other neoplastic lesions, including lung adenocarcinomas and spleen angiosarcomas (Fig. 4Aδ), further supporting the oncogenic potential of this mutant form of the *Hmga1* gene.

## 4. Discussion

Rearrangements of *HMGA1* by chromosomal aberrations of 6p21 have been frequently described in human benign

tumours of mesenchymal origin, including lipomas, uterine leiomyomas and pulmonary chondroid hamartomas.[6] The chromosomal breakpoints were located either upstream or downstream of the gene sequence, and sometimes within an 80 kb region surrounding *HMGA1*, but no intragenic rearrangements have ever been reported.[15] Therefore, it has been hypothesised that truncation of HMGA1 is not a pre-requisite to gain oncogenic potential. In contrast with this, we previously demonstrated the capacity of a construct expressing a truncated *Hmga1b* gene, devoid of the C-terminal tail (*Hmga1b/T*), to enhance proliferation of 3T3-L1 mouse fibroblasts.[23] A similar finding was obtained by blocking expression of *Hmga1*.[18] These data prompted us to hypothesise that the truncated Hmga1b mutant might have an oncogenic potential probably acting with a dominant-negative effect on the wild-type protein function. To validate this hypothesis we generated transgenic mice for the *Hmga1b/T* mutant gene. Characterisation of their phenotype confirmed the key role of the truncation of HMGA1 in the development of adipose tissue and in the acquisition of a neoplastic phenotype. Indeed, these mice are giant, obese and develop lymphomas of the same subtype developed by Hmga1-knockout mice,[18] once again suggesting a dominant-negative effect of this truncated form on wild-type HMGA1 proteins.

These mice represent one more wedge in the studies that independent groups have previously reported about Hmga1- and Hmga2-transgenic mice. Indeed, it has been reported that: (i) transgenic mice overexpressing either a wild-type or a truncated *Hmga2* cDNA, under two strong and essentially ubiquitous promoters, developed abdominal/pelvic lipomatosis and/or an abnormally high incidence of lipomas, NK-T/NK cell lymphomas and mixed growth hormone/prolactin cell pituitary adenomas[10,11,16,20]; (ii) transgenic mice with *Hmga1b* under control of the CMV promoter developed the same phenotype apart from the absence of lipomatosis[17]; (iii) transgenic mice that misexpressed full-length or truncated human HMGA2 transcript under control of the differentiated mesenchymal cell (adipocyte)-specific promoter of the adipocyte P2 (Fabp4) gene, produced neoplastic phenotype, including fibroadenomas of the breast and salivary gland adenomas[31]; and (iv) transgenic mice overexpressing *Hmga1a* flanked by the H-2K promoter and immunoglobulin intronic enhancer develop T-cell lymphoid malignancy and uterine sarcomas.[32,33]

Consistent with the behaviour of 3T3-L1 cells overexpressing the Hmga1b/T mutant,[23] mice expressing the *Hmga1b/T* transgene show enhanced E2F activity and accumulation of fat tissue, likely due to the enhanced adipocyte proliferation. This phenotype, not present in mice transgenic for the wild-type *Hmga1b* gene,[17] is quite similar to that observed in mice transgenic for either *Hmga2* or *Hmga2/T*.[11,16] Accordingly, in 3T3-L1 cells, overexpression of Hmga1b/T, Hmga2 and Hmga2/T but not Hmga1b is able to enhance the activity of an E2F-responsive element, such as cyclin E promoter. Moreover, differently from Hmga2-, Hmga2/T- and Hmga1b-transgenic mice, all the sharing the development of pituitary adenomas and T/NK lymphomas,[10,16,17] Hmga1b/T mice develop neither pituitary adenomas nor T- but B-type lymphomas, like Hmga1-knockout mice.[18]

Therefore, it appears that HMGA1b/T mutant has different biological effects in comparison to wild-type HMGA1b protein, depending on the cellular context. In adipocyte cells, even though both Hmga1b and Hmga1b/T are able to interact with pRB and displace HDAC1 from E2F1-responsive promoters,[27] only Hmga1b/T is able to enhance E2F1 activity, resulting in accumulation of fat tissue *in vivo*. Conversely, it appears that in lymphocytes Hmga1b/T has a dominant-negative effect on the endogenous Hmga1 protein. In fact, as for Hmga1$^{-/-}$ mice,[18] Hmga1b/T animals show higher levels of B-cell-specific genes and up-regulation of the *Rag2* gene expression compared with wild-type compounds, suggesting that B-cell lymphomas in Hmga1b/T mice may develop as a result of deficient T-cell function, as already hypothesised in Hmga1$^{-/-}$ mice.[18]

In conclusion, we validated *in vivo* the oncogenic role of the truncated *HMGA1* gene and its key role in the control of adipocyte proliferation. Moreover, our data indicate that HMGA1b/T might behave as a dominant-negative mutant in lymphocytic cells.

## Conflict of interest statement

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

## Acknowledgements

This work was supported by grants from the Associazione Italiana Ricerca sul Cancro (AIRC) and the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MIUR).

We thank Ida Pellegrino and Michela Vitiello for their helpful contribution in some critical experiments, and the Associazione Partenopea per le Ricerche Oncologiche (APRO) for its support.

## REFERENCES

1. Reeves R, Nissen MS. The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. *J Biol Chem* 1990;**265**:8573–82.
2. Thanos D, Maniatis T. The high mobility group protein HMG I(Y) is required for NF-kappa B-dependent virus induction of the human IFN-beta gene. *Cell* 1992;**71**:777–89.
3. Zhou X, Benson KF, Ashar HR, Chada K. Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C. *Nature* 1995;**376**:771–4.
4. Chiappetta G, Avantaggiato V, Visconti R, et al. High level expression of the HMGI (Y) gene during embryonic development. *Oncogene* 1996;**13**:2439–46.
5. Nishino J, Kim I, Chada K, Morrison SJ. Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16$^{Ink4a}$ and p19$^{Arf}$ expression. *Cell* 2008;**135**:227–39.
6. Fusco A, Fedele M. Roles of HMGA proteins in cancer. *Nat Rev Cancer* 2007;**7**:899–910.

7. Fedele M, Berlingieri MT, Scala S, et al. Truncated and chimeric HMGI-C genes induce neoplastic transformation of NIH3T3 murine fibroblasts. *Oncogene* 1998;**17**:413–8.
8. Wood LJ, Maher JF, Bunton TE, Resar LM. The oncogenic properties of the HMG-I gene family. *Cancer Res* 2000;**60**:4256–61.
9. Hess JL. Chromosomal translocations in benign tumors: the HMGI proteins. *Am J Clin Pathol* 1998;**109**:251–61.
10. Baldassarre G, Fedele M, Battista S, et al. Onset of natural killer cell lymphomas in transgenic mice carrying a truncated HMGI-C gene by the chronic stimulation of the IL-2 and IL-15 pathway. *PNAS* 2001;**98**:7970–5.
11. Battista S, Fidanza V, Fedele M, et al. The expression of a truncated HMGI-C gene induces gigantism associated with lipomatosis. *Cancer Res* 1999;**59**:4793–7.
12. Lee YS, Dutta A. The tumor suppressor microRNA let-7 represses the HMGA2 oncogene. *Genes Dev* 2007;**21**:1025–30.
13. Mayr C, Hemann MT, Bartel DP. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science* 2007;**315**:1576–9.
14. Xiao S, Lux ML, Reeves R, Hudson TJ, Fletcher JA. HMGI(Y) activation by chromosome 6p21 rearrangements in multilineage mesenchymal cells from pulmonary hamartoma. *Am J Pathol* 1997;**150**:901–10.
15. Kazmierczak B, Dal Cin P, Wanschura S, et al. HMGIY is the target of 6p21.3 rearrangements in various benign mesenchymal tumors. *Genes Chrom Cancer* 1998;**23**:279–85.
16. Fedele M, Battista S, Kenyon L, et al. Overexpression of the HMGA2 gene in transgenic mice leads to the onset of pituitary adenomas. *Oncogene* 2002;**21**:3190–8.
17. Fedele M, Pentimalli F, Baldassarre G, et al. Transgenic mice overexpressing the wild-type form of the HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas. *Oncogene* 2005;**24**:3427–35.
18. Fedele M, Fidanza V, Battista S, et al. Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice. *Cancer Res* 2006;**66**:2536–43.
19. Hock R, Furusawa T, Ueda T, Bustin M. HMG chromosomal proteins in development and disease. *Trends Cell Biol* 2007;**17**:72–9.
20. Arlotta P, Tai AK, Manfioletti G, et al. Transgenic mice expressing a truncated form of the high mobility group I-C protein develop adiposity and an abnormally high prevalence of lipomas. *J Biol Chem* 2000;**275**:14394–400.
21. Anand A, Chada K. In vivo modulation of Hmgic reduces obesity. *Nat Genet* 2000;**24**:377–80.
22. Melillo RM, Pierantoni GM, Scala S, et al. Critical role of the HMGI(Y) proteins in adipocytic cell growth and differentiation. *Mol Cell Biol* 2001;**21**:2485–95.
23. Pierantoni GM, Battista S, Pentimalli F, et al. A truncated HMGA1 gene induces proliferation of the 3T3-L1 pre-adipocytic cells: a model of human lipomas. *Carcinogenesis* 2003;**24**:1861–9.
24. Student AK, Hsu RY, Lane MD. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J Biol Chem* 1980;**255**:4745–50.
25. Soriano P, Montgomery C, Geske R, Bradley A. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. *Cell* 1991;**64**:693–702.
26. Livak KJ, Schmittgen T. Analysis of relative gene expression data using real-time quantitative PCR and the 2(–ΔΔC(T)) method. *Methods* 2001;**25**:402–8.
27. Fedele M, Visone R, De Martino I, et al. HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity. *Cancer Cell* 2006;**9**:459–71.
28. Helin K, Harlow E, Fattaey A. Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein. *Mol Cell Biol* 1993;**13**:6501–8.
29. Esposito F, Pierantoni GM, Battista S, et al. Interaction between HMGA1 and retinoblastoma protein is required for adipocyte differentiation. *J Biol Chem* 2009;**284**:25993–6004.
30. Morse 3rd HC, Anver MR, Fredrickson TN, et al. Bethesda proposals for classification of lymphoid neoplasms in mice. *Blood* 2002;**100**:246–58.
31. Zaidi MR, Okada Y, Chada KK. Misexpression of full-length HMGA2 induces benign mesenchymal tumors in mice. *Cancer Res* 2006;**66**:7453–9.
32. Xu Y, Sumter TF, Bhattacharya R, et al. The HMG-I oncogene causes highly penetrant, aggressive lymphoid malignancy in transgenic mice and is overexpressed in human leukemia. *Cancer Res* 2004;**64**:3371–5.
33. Tesfaye A, Di Cello F, Hillion J, et al. HMGA1a up-regulates Cox-2 in uterine tumorigenesis. *Cancer Res* 2007;**67**:3998–4004.