**RESEARCH ARTICLE**

**Hmga1/Hmga2 double knock-out mice display a “superpygmy” phenotype**

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**ABSTRACT**

The HMG1 and HMG2 genes code for proteins belonging to the High Mobility Group A family. Several genes are negatively or positively regulated by both these proteins, but a number of genes are specifically regulated by only one of them. Indeed, knock-out of the Hmga1 and Hmga2 genes leads to different phenotypes: cardiac hypertrophy and type 2 diabetes in the former case, and a large reduction in body size and amount of fat tissue in the latter case. Therefore, to better elucidate the functions of the Hmga genes, we crossed Hmga1-null mice with mice null for Hmga2. The Hmga1-/-/Hmga2-/- mice showed reduced vitality and a very small size (75% smaller than the wild-type mice); they were even smaller than pygmy Hmga2-null mice. The drastic reduction in E2F1 activity, and consequently in the expression of the E2F-dependent genes involved in cell cycle regulation, likely accounts for some phenotypic features of the Hmga1-/-/Hmga2-/- mice.

**KEY WORDS:** HMG1, HMG2, Pygmy, E2F

**INTRODUCTION**

The High Mobility Group A (HMG) family comprises four proteins: HMG1a, HMG1b, HMG1c and HMG2. The HMG1 gene codes for the first three proteins by alternative splicings, whereas the HMG2 protein is encoded by a distinct gene named HMG2 (Johnson et al., 1989). These proteins bind the minor groove of AT-rich DNA sequences through three short basic repeats, called “AT-hooks”, located at the NH2-terminal region of the proteins. HMGAs act exclusively as architectural proteins. Indeed, they have no transcriptional activity per se, but they are able to alter the chromatin structure by interacting with the transcription machinery, and thus they can negatively or positively regulate the transcriptional activity of several genes (Johnsson et al., 1989; Reeves and Nissen, 1990; Thanos et al., 1993).

Both HMG4 genes are widely expressed during embryogenesis, whereas their expression is absent or very low in adult tissues (Zhou et al., 1995; Chiappetta et al., 1996). In particular, Hmga2 has not been detected in any of the adult mouse and human tissues tested, apart from a very low expression in CD34-positive hematopoietic stem cells, mouse proadipocyte proliferating cells and meiotic and post-meiotic cells (secondary spermatocytes and spermatids) (Chieffi et al., 2002). Conversely, Hmga1 is expressed, albeit at low levels, in adult murine and human tissues. However, the expression of both HMG genes becomes abundant in malignant cells in vitro and in vivo (Giancotti et al., 1989; Wood et al., 2000; Fedele et al., 2001; Fusco and Fedele, 2007). Indeed, HMG proteins are highly expressed in all malignant tissues analyzed, namely, pancreas, thyroid, colon, breast, lung, ovary, uterine cervix, prostate and gastric carcinomas, squamous carcinomas of the oral cavity, and head and neck tumors (Fusco and Fedele, 2007). Moreover, HMG1 protein expression is associated with a highly malignant phenotype as shown by the significant correlation detected between high levels of HMG1 protein expression and both the presence of lymph-node metastasis and advanced clinical stage (Balcerzak et al., 2003; Flohr et al., 2003; Donato et al., 2004).

Several studies indicate that HMG1 gene expression plays a causal role in carcinogenesis. Indeed, blockage of their expression prevents thyroid cell transformation (Berlingieri et al., 1995; Berlingieri et al., 2002) and leads to the death of malignant cells (Scala et al., 2000). Moreover, HMG1 or HMG2 overexpression is able to transform mouse and rat fibroblasts (Fedele et al., 1998; Fedele et al., 2001), and transgenic mice overexpressing either HMG1 or HMG2 develop haematopoietic malignancies and pituitary adenomas (Battista et al., 1999; Arlotta et al., 2000; Fedele et al., 2001), and transgenic mice overexpressing either HMG1 or HMG2 develop haematopoietic malignancies and pituitary adenomas (Battista et al., 1999; Arlotta et al., 2000; Fedele et al., 2002; Fedele et al., 2005; Zaidi et al., 2006).

The high expression of HMG proteins during embryogenesis suggests that they exert an important role in development. Indeed, the phenotypic characterization of mice knocked out (KO) for each of the Hmga genes revealed that these proteins play crucial roles in various aspects of development (Fusco and Fedele, 2007). Cardiac hypertrophy and type 2 diabetes were observed in Hmga1-null (A1-KO) and heterozygous mice suggesting that an appropriate amount of HMG1 protein is required for cardiomyocytic cell growth and regulation of the insulin pathway (Foti et al., 2005; Fedele et al., 2006a). Conversely, Hmga2-null (A2-KO) and heterozygous mice showed a pygmy phenotype with a decrease in body size of 60% and 25%, respectively, compared with the wild-type (WT) mice, and a drastic reduction of fat tissue (Zhou et al., 1995; Anand and Chada, 2000), which suggests that HMG2 plays an important role in the control of body size and adipocyte proliferation and differentiation.

However, since many genes are regulated by both HMG proteins, it is obvious that several functions exerted by one member of the HMG family are, at least partially, compensated...
by the other family member. Therefore, the aim of our work was to generate mice carrying an impairment of both Hmga genes. These mice were generated by crossing the Hmgal-null mice (A1-KO) (Fedele et al., 2006a) with mice null for Hmga2 (A2-KO) (Zhou et al., 1995; Scala et al., 2001) and the resulting phenotype was analyzed. The Hmgal/Hmga2-null (A1/A2-KO) mice presented reduced vitality (most of them died in utero) and a very small size, even lower than that of the pygmy A2-KO mice. We demonstrate that E2F1 activity is impaired in A1/A2-KO mouse embryonic fibroblasts (MEFs) and tissues, an event that results in a drastic reduction of the expression of E2F1-dependent genes, which probably accounts for the “superpygmy” phenotype of the A1/A2-KO mice.

MATERIALS AND METHODS
Generation and genotyping of mutant mice
The A1/A2-KO mice were generated crossing A1-KO (Fedele et al., 2006a) and A2-KO (Zhou et al., 1995; Scala et al., 2001) null mice. The genotype of the A1-KO, A2-KO and A1/A2-KO mice was analyzed as previously described (Fedele et al., 2006a; Zhou et al., 1995).

A1-KO mice, originally generated in a mixed genetic background C57/Sv129, were backcrossed for more than 10 generations in C57Bl/6J. These mice can be estimated to derive more than 99% of their genes from the C57Bl/6J strain. A2-KO is a spontaneous mutant pygmy mouse obtained from the Jackson Laboratory (Bar Harbor, ME). The original strain was C3H/HeNcrWf. A1-KO and A2-KO were crossed to obtain double mutant mice. These mice were intercrossed for more than 20 generations. Thus, all the analyzed mice have the same genetic background.

All mice were maintained under standardized non-barrier conditions in the Laboratory Animal Facility of the Istituto dei Tumori di Napoli (Naples, Italy) and all studies were conducted in accordance with Italian regulations for experiments on animals.

Isolation of mRNA, Northern blot analysis and RT-PCR
Total RNA was extracted using TRI-reagent solution (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. cDNA was synthesized from total RNA using random hexamers (100 nM) and MuLV reverse transcriptase (Perkin Elmer, Santa Clara, CA). Semi-quantitative PCRs were run using the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA). Specific PCR conditions are available upon request. RNA samples that had not been reverse transcribed before PCR served as negative control. For semi-quantitative PCR, reactions were optimized for the number of cycles to ensure product intensity within the linear phase of amplification. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide. Gels were scanned with Chemidoc (Bio-Rad, Hercules, CA). The primer sequences used to amplify the indicated mouse genes are available upon request.

Growth curves of WT, A1-KO, A2-KO and A1/A2 KO MEFs
Primary MEFs were obtained from 12.5-day-old embryos. The MEFs were minced and used to establish single cell suspensions. They were grown in Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Carlsbad, CA) containing 10% fetal bovine serum (Sigma, St Louis, MO), 1% glutamine (GIBCO), 1% penicillin/streptomycin and 1% gentamicin (GIBCO). Three MEF preparations for each genotype were plated in triplicate in a series of 6-cm culture dishes (4×10^4/dish) and counted daily with a cell counter for 6 consecutive days to extrapolate growth curves. The values represent means ± SEM of the three MEF preparations for each genotype.

Body weight, body length and growth analysis
The mice were generally kept and bred with ad libitum access to water and pelleted standard food (Mucedola, Milano, Italy). The body weight was measured monthly, beginning at 4 weeks of age, for 12 months. Body length was measured by manual immobilization, extension and measurement of the naso–anal length by a ruler.

Senescence associated-β-galactosidase assay
4×10^4 MEFs, plated 24 h before the assay, were washed twice with PBS and immersed in fixation buffer [2% (w/v) formaldehyde, 0.2% (w/v) glutaraldehyde in PBS] for 7 min. After three additional PBS washes, the cells were allowed to stain overnight in staining solution (40 mM citric acid/sodium phosphate pH 6.0, 150 mM NaCl, 2.0 mM MgCl2, 1 mg/ml X-gal) at 37°C without CO2 to avoid changes in pH. We used three MEF preparations for each genotype, plated in triplicate. Pictures of each preparation of MEFs were done at least three times for each replicate. The values represent the means ± SEM of three MEF preparations for each genotype. We represent percentage of MEFs stained for β-galactosidase activity versus the total of MEFs. Pictures were taken on a bright field microscope (Zeiss, Oberkochen, Germany) at a magnification of 50×.

Protein extraction and Western blotting
Protein extraction and Western blotting procedures were carried out as reported elsewhere (Federico et al., 2009; Esposito et al., 2010). Briefly, for total cell extracts, cells were lysed in RIPA buffer (20 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P40, and a mix of protease inhibitors), and clarified by centrifugation at 13,000 rpm at 4°C for 30 min. Total proteins were directly resolved on 12.5% polyacrylamide gel under denaturing condition and transferred to nitrocellulose filters for western blot analysis. Membranes were blocked with 5% BSA in TTBS and incubated with the specific primary antibodies. The antibodies used were: anti-β-actin (sc-1616, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-γ-tubulin (sc-17787, Santa Cruz), anti-vinculin (sc-7649, Santa Cruz), anti-p21 (sc-397, Santa Cruz), anti-p16ink4a (ab-54210, Abcam, Cambridge, UK), anti-p-19 (Ab102848, Abcam), anti-p-27 (610241, BD Transduction Laboratories™), anti-cyclin E (sc-481, Santa Cruz), anti-cyclin A (sc-751, Santa Cruz). Antibodies versus the HMGAI and HMGAI2 proteins have been previously described (Pierantoni et al., 2003; Chiapetta et al., 2008). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:3000) for 60 min at room temperature and the reaction was detected with a western blotting detection system (ECL) (Promeaga, Fitchburg, WI).

Statistical analyses
All values were tested for normal distribution using the D’Agostino–Pearson test for all variables. Since all values passed the test (P > 0.05), parametric tests were used for the analysis. We used Chi squared to compare the Mendelian inheritance of A1/A2-KO mice. The association between body weight and body length in mice, MEF growth curves, senescence with the different genotypes were determined by one-way ANOVA followed by the Bonferroni post-test. The statistical analyses were performed using GraphPad Prism v.6.0 (La Jolla, CA, USA). A P value < 0.05 was considered statistically significant.

RESULTS
Generation of A1/A2-KO mice
To generate A1/A2-KO mice, we crossed A1-KO mice with Hmga2<sup>+/-</sup> mice. The A2-KO mice in our animal house are infertile consequent to the block of spermatogenesis (Chieffi et al., 2002). We were able to produce mice with different combinations of Hmgal and Hmgal2 null alleles, including the A1/A2-KO mice. Mating A1<sup>+/+</sup>A2<sup>+/+</sup> × A1<sup>−/+</sup>A2<sup>−/+</sup> mice we generated 47 A1-KO/A2<sup>−/+</sup> mice (37%), 64 A1-KO/A2<sup>−/−</sup> mice (50%) and 17 A1/A2-KO mice (13%) of 128 total mice. Therefore, the generation of the double KO mice was much lower than expected according to Mendelian laws thereby indicating lethality of these embryos (P < 0.05).

To verify the absence of Hmgal and Hmgal2 gene expression in the A1/A2-KO mice we performed RT-PCR and Western blot analyses. As shown in Fig. 1A, there was no Hmgal expression in kidney and spleen from the A1/A2-KO mice. Similarly,
Fig. 1. Lack of Hmga1 and Hmga2 expression in A1/A2-KO mice. (A) RT-PCR expression analysis of the Hmga1 gene in WT, A1-KO, A2-KO and A1/A2-KO spleen and kidney tissue. (B) RT-PCR expression analysis of Hmga2 gene in WT, A1-KO, A2-KO and A1/A2-KO testis tissue. (C) RT-PCR expression analysis of Hmga1 and Hmga2 genes in WT, A1-KO, A2-KO and A1/A2-KO MEFs at passage 4. G6PD gene expression was used as internal control. (D) Western blot analysis of Hmga1 and Hmga2 proteins in WT, A1-KO, A2-KO and A1/A2-KO protein extracted from MEFs at passage 4. β-tubulin was used as loading control.

Fig. 2. Body weight and size analysis of A1/A2-KO mice. (A,B) Representation of body weight, from 1 to 12 months, and naso–anal length, measured at 12 months of age, of cohorts, males or females, of 10 WT (circle), A1-KO (square), A2-KO (triangle) and A1/A2-KO (diamond) mice. Values are means ± SEM. *P<0.05, **P<0.01 versus WT. (C) Gross appearance of a representative 6-month-old A1/A2-KO mouse and a sex-matched WT sibling.
Hmga2 expression was not detected in the testis (one of the few adult tissues expressing Hmga2) of A1/A2-KO mice, whereas it was present in the testis of WT and A1-KO animals (Fig. 1B). The same results were obtained from the analysis of the expression of Hmga1 and Hmga2 in MEFs from A1/A2-KO mice (Fig. 1C). Western blot analysis using antibodies raised against the Hmga1 and Hmga2 proteins did not reveal these proteins in the A1/A2-KO MEFs thereby confirming the RT-PCR data (Fig. 1D).

The A1/A2-KO mice display a striking reduction of body size

At birth, the A1/A2-KO mice were smaller than the WT, A1-KO and even the A2-KO littermates, which have been described as “pygmy mice” (Zhou et al., 1995). The differences in body size among these mice remained evident in adulthood (Fig. 2). Therefore, we monitored the weight and growth-length of the WT, A1-KO, A2-KO and A1/A2-KO mice. The A1/A2-KO mice weighed much less than each single KO mice, even one month after birth. At 12 months of age, body weight was reduced by 27%, 55% and 75% in the A1-KO, A2-KO and A1/A2-KO mice, respectively, compared with WT (*P<0.05, **P<0.01) (Fig. 2A). Also body length (nose-to-tail), at 12 months was markedly reduced: A1-KO mice were 25%, A2-KO 40% and A1/ A2-KO 60% shorter than WT mice (**P<0.01) (Fig. 2B). Interestingly, the size and the weight of livers and spleens of A1/A2-KO mice are about one forth of the WT organs (data not shown). These results suggest that the Hmga1 and Hmga2 genes play a critical role in controlling body growth. A representative A1/A2-KO mouse is shown in Fig. 2C. We decided to call the A1/A2-KO mice “superpygmy” to distinguish them from the A2-KO pigmy mice.

A1/A2-KO MEFs have a lower growth rate than WT, A1-KO and A2-KO MEFs

We analyzed the growth rate of the MEFs derived from WT, A1-KO, A2-KO and A1/A2-KO mice, established from embryos at 12.5 dpc, by performing a growth curve. As shown in Fig. 3, growth rate was much lower in A1/A2-KO MEFs than in WT MEFs (*P<0.05). The analysis of the genes coding for proteins involved in cell cycle control revealed higher p27 protein levels in the A1/A2-KO MEFs than in WT MEFs (Fig. 4A). Since we found no differences at mRNA level (Fig. 4A), it is likely that post-transcriptional mechanisms are involved in the control of this protein in A1/A2-KO MEFs. Conversely, cyclin A and cyclin E expression was decreased in all Hmga-null MEFs particularly in those deriving from the A1/A2-KO mice (Fig. 4B).

Next we examined the susceptibility to senescence of the Hmga-null MEFs at different culture passages by measuring senescence-associated β-galactosidase (SA-β-gal) activity. As shown in Fig. 5A at passage 4, the number of cells showing SA-β-gal activity was significantly higher in A1/A2-KO MEFs than in WT (**P<0.01 versus WT). A representative picture of the senescence assay is shown in Fig. 5B.

Senescent MEFs express elevated levels of p16, p19 and p21 consequent to replication and culture stress (Narita et al., 2006). These genes may cooperate to inhibit Rb phosphorylation and maintain growth arrest in an irreversible state. Therefore, we evaluated p16, p19 and p21 mRNA and protein levels in WT, A1-KO, A2-KO and A1/A2-KO MEFs by quantitative RT-PCR and Western blot analysis. As shown in Fig. 6, the mRNA and protein expression levels of p16, p19 and p21 at passage 7 were significantly higher in A1/A2-KO than in WT MEFs.
Reduced E2F1 activity may account for the “superpygmy” phenotype

We previously demonstrated that HMGA1 and HMGA2 overexpression enhances E2F1 activity by binding to RB and displacing HDAC from the RB/E2F complex (Seville et al., 2005; Fedele et al., 2006b). Therefore, we envisaged that the “superpygmy” phenotype of A1/A2-KO mice could result from decreased E2F1 activity that would, in turn, lead to reduced cell growth and, maybe, embryonic stem cell self-renewal.

Since E2F1 acetylation reflects its activation augmenting DNA binding and stabilizing the protein (Martínez-Balbás et al., 2000) thereby enhancing its transcriptional activity, we evaluated the amount of acetylated E2F1 in WT, A1-KO, A2-KO and A1/A2-KO MEFs by western blot analysis using antibodies specific for the acetylated form of E2F1. A representative experiment is shown in Fig. 7A. Densitometric analysis deriving from two Western blot experiments showed that the levels of acetylated E2F1 were about 60% lower in the A1-KO and A2-KO MEFs than in the WT MEFs (Fig. 7B). A further decrease was observed in the A1/A2-KO MEFs, where it is almost undetectable. Consistently, the expression of cyclin A (Fig. 4B) and cyclin E (Fig. 4B), which are specific targets of E2F1, paralleled the levels of acetylated E2F (Fig. 7).

DISCUSSION

Because proteins HMGA1 and HMGA2 proteins share the ability to regulate a number of common genes, some functions might be compensated by the expression of the other member of the HMGA family. Therefore, to identify all the activities of the HMGA proteins we generated mice KO for both the genes by crossing the A1-KO mice with A2-KO mice. Only a few A1/A2-KO animals were generated, which is indicative of embryonic lethality. Moreover, most of them died before one year after the birth. Therefore, we postulate that the HMGA proteins play a critical role in controlling some functions that are essential for embryonic and/or fetal development. It is noteworthy that the presence of the other family member was sufficient to prevent embryo lethality in the A1-KO and A2-KO mice. Preliminary results from our laboratory indicate that most of the deaths were due to haemorrhagic events that may result from increased levels of angiogenic factors (A. Federico and F.E., unpublished data).

The most impressive feature of the A1/A2-KO mice is their size: they are about 75% smaller than the WT littermates. We called these mice “superpygmy” since they are even much smaller than the pygmy A2-KO mice. This result is not surprising since the Hmga2 gene is involved in defining the body size in mice and, even in humans. Indeed, mice overexpressing Hmga2 showed a giant phenotype that was a mirror image of the pygmy phenotype (Battista et al., 1998; Battista et al., 1999; Baldassarre et al., 2001). Moreover, HMGA2 is an important genetic determinant for human adult height (Weedon et al., 2008; Buyssse et al., 2009; Hodge et al., 2009; Yang et al., 2010; Hendriks et al., 2011; Carty et al., 2012; Makvandi-Nejad et al., 2012). Consistently, an 8-year-old boy carrying a truncated HMGA2 gene displayed extreme somatic overgrowth with features strikingly similar to those observed in the Hmga2 transgenic mice (Ligon et al., 2005). However, our results implicate also Hmga1 in body size determination. In fact, a slight
reduction in size occurred also in the A1-KO mice, while impairment of both Hmga genes leads to a drastic reduction in body size. Moreover, we found a reduced body size already at birth; in fact, A1/A2-KO MEFs grew slower than both the WT MEFs and those carrying the impairment of a single Hmga gene.

We also found that E2F1 activity was impaired in the absence of HMGA expression, as shown by its very low acetylation status. Impaired E2F1 activity may account for the “superygmy” phenotype of the A1/A2-KO mice and the slower growth of the corresponding MEFs. Indeed, E2F1 activity was very low in A1/A2-KO MEFs. This result is consistent with our previous finding that HMGA2 displaces histone deacetylase 1 from the pRB/E2F1 complex thereby resulting in enhanced acetylation of both E2F1 and DNA-associated histones, and thus promoting E2F1 activation. Accordingly, the level of the acetylated form of E2F1 was drastically lower in all the Hmga KO mice, particularly in the A1/A2-KO mice, than in the WT. This is consistent with the reduced expression of the E2F-dependent genes, namely, cyclin A and cyclin E, in the A1/A2-KO MEFs. However, it cannot be excluded that other genes regulated by the HMGA genes might contribute to the phenotype of the A1/A2-KO mice.

In contrast with Narita et al., who reported that HMGAI reduces cell lifespan (Narita et al., 2006), A1-KO and A1/A2-KO MEFs became senescent earlier than WT MEFs. This result suggests that the cellular context plays a critical role in determining the effect of the HMGA proteins on cell growth. In the case of A1-KO MEFs, the context may be considered more physiological than that in which HMGA1 was ectopically overexpressed in IMR90 (Narita et al., 2006). It is likely that HMGA overexpression causes drastic changes in the chromatin architecture that causes non-cancerous cells to become senescent. Moreover, the different experimental approach, one in vivo and the previous one in vitro, may account for these contradictory results. Indeed, significant discrepancies were found between in vitro and transfection approaches in a study of the p53 pathway (Toledo and Wahl, 2006). Noteworthy, the behaviour of the Hmga-null MEFs in our study is in agreement with the notion that HMGA overexpression plays an oncogenic role, which is a feature of malignant neoplasias.

In conclusion, the generation of mice carrying the impairment of both the Hmga genes is an excellent model with which to shed light on the functions of these genes.

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Competing interests
The authors have no competing interests to declare.

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Fig. 7. E2F1 acetylation in A1/A2-KO MEFs. (A) Western blot analysis of acetylated and total form of E2F1 in representative WT, A1-KO, A2-KO and A1/A2-KO MEFs. Vinculin was used as loading control. (B) Densitometric analysis of the levels of acetylated E2F1 versus E2F1 total in WT, A1-KO, A2-KO and A1/A2-KO MEFs. Level of acetylated E2F1 versus E2F1 total in WT was assumed as 100 (arbitrary units). These values represent the means ± SEM of two experiments. *P<0.05 versus WT.
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