A truncated HMGA1 gene induces proliferation of the 3T3-L1 pre-adipocytic cells: a model of human lipomas

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The high mobility group A (HMGA) proteins are non-histone chromosomal proteins implicated in the organization of chromatin structure and in the assembly of protein complexes on the promoters of several inducible genes. Rearrangements of HMGA1 and HMGA2 genes, consequent to chromosomal translocation, have been frequently detected in human benign tumours of mesenchymal origin including lipomas. We have demonstrated previously that 3T3-L1 adipocytic differentiation is associated with increased HMGA1 protein levels, and that the block of HMGA1 synthesis dramatically increases the growth rate of 3T3-L1 cells and suppresses adipocytic differentiation. Here we have examined the role of a truncated HMGA1 gene in adipocytic cell growth. We have found that expression of the truncated Hmga1 gene (Hmga1/T) dramatically increases 3T3-L1 cell growth without blocking adipocytic differentiation. The Hmga1/T 3T3-L1 cells had higher E2F activity than the wild-type cells, and a deregulated cell cycle. In fact, the Hmga1/T cells had a reduced G0/G1 fraction, and a greater number of cells in S-phase. However, consistent with the benign nature of tumours associated with HMGA1 rearrangements, the Hmga1/T 3T3-L1 cells did not acquire the malignant phenotype. These results suggest a critical role played by HMGA1 rearrangements in the generation of human lipomas.

Introduction

The HMGA family consists of three members: HMGA1a and HMGA1b, which are products of alternative splicing of the same gene, named Hmga1 (1), and HMGA2, which is encoded by a separate gene (2). Each HMGA protein has three separate DNA binding domains referred to as ‘AT-DNA hook’ motifs. The C-terminal region of HMGA proteins contains a highly acidic tail that is believed to be important for protein–protein interactions. HMGA1 proteins have no transcriptional activity per se (3), but they participate in the assembly of protein complexes on the promoters of several inducible genes and can thus be defined ‘architectural factors’ (4–7).

HMGAs gene alterations play an important role in the generation of benign and malignant tumours. In fact, Hmga1 is abundantly expressed during embryonic development (8, 9), but negligible levels in normal adult tissues. Conversely, Hmga1 over-expression is a common feature of malignant tumours (10–21). A high level of HMGA expression is crucial for expression of the malignant phenotype. Indeed, block of HMGA synthesis prevents rat thyroid cell transformation by murine transforming retroviruses (22, 23) and an adenovirus carrying the HMGA1 gene in an antisense orientation induces programmed cell death in carcinoma cell lines derived from human thyroid, lung, colon and breast cancers (24).

Rearrangements of the HMGA2 and HMGA1 genes are frequent in human benign neoplasias of mesenchymal origin, including lipomas (25–29). These gene rearrangements are caused by chromosomal translocations involving regions 12q13-14 or 6p21, where the HMGA2 and HMGA1 genes are located, respectively. Consequent to HMGA2 translocations, breaks occur within the third intron of the gene thereby resulting in chimeric transcripts containing HMGA2 exons 1–3 (including the AT-hook domains) and ectopic sequences from other genes (25, 26). In most tumours with 6p21 rearrangements, the breakpoints are located at the 3’ end of HMGA1 gene, and molecular analyses of the HMGA1 transcripts revealed common deletions of the C-terminal region and/or parts of the 3’UTR (27–29).

We showed recently that these rearranged forms of Hmga2 can transform NIH3T3 cells, and that acquisition of ectopic sequences is not required for the transforming ability. This suggests that truncation of the HMGA2 gene, rather than its fusion to other genes, is the event responsible for cell transformation (30). Moreover, we and others have shown that transgenic mice carrying an Hmga2 truncated construct develop the giant phenotype associated with pelvic/abdominal lipomatosis (31, 32). Conversely, a Hmga1/T construct did not exert a transforming activity on NIH3T3 cells (M.Fedele, unpublished data).

However, HMGA1 proteins are critical for adipocytic cell growth and differentiation (33). In fact, we found that suppression of Hmga1 expression by antisense technology dramatically increased growth rate and impaired adipocytic differentiation of 3T3-L1 cells. We found that HMGA1 proteins bind C/EBPβ in vitro and in vivo, and strongly potentiate the capacity of C/EBPβ to transactivate the leptin gene promoter, an adipose-specific gene (33).

The aim of the present work was to investigate the oncogenic effect exerted by a truncated Hmga1 gene deprived of the acidic tail, on pre-adipocytic 3T3-L1 cells. We show that the truncated Hmga1 gene dramatically increased the growth rate of the 3T3-L1 cells without affecting adipocytic differentiation. However, these cells did not acquire the malignant phenotype. 3T3-L1 cells that expressed Hmga1/T had higher E2F activity than normal cells, and their cell cycle was deregulated.

Abbreviations: EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate-dehydrogenase; HMGA, high mobility group A.
Materials and methods

Cell culture, plasmids and transfections

The mouse NIH 3T3-L1 pre-adipocytic cells used in this study were generously supplied by Dr E.Santos (National Cancer Institute, NIH, Bethesda, MD). They were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% calf serum (Gibco BRL, Life Technologies, Gaithesburg, MD). Induction of adipocytic differentiation of 3T3-L1 cells was essentially performed as described elsewhere (34). Briefly, confluent 3T3-L1 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% calf serum until confluency. Two days later, they were grown in DMEM supplemented with 10% fetal calf serum and 0.5 mM 1-methyl-3-isobutylxanthine, 10 μM dexamethasone and 10 μg/ml insulin for 48 h. Cells were further cultured in the same culture medium devoid of dexamethasone and methylisobutylxanthine for 6 days. For the serum-stimulation experiment, cells werestarved of serum, maintained with 0.5% serum for 3 days, and then induced to re-enter the cycle with 10% calf serum.

A 243-bp cDNA fragment encoding the truncated Hmgal gene was subcloned into the HindIII and XbaI sites of the expression vector pRc/CMV (Invitrogen, Life Technologies, Milan) in the sense orientation (pCMV-Hmgal/T). A 1500-bp cDNA fragment corresponding to the entire coding sequence of the Hmgal gene was subcloned into the HindIII sites of the expression vector pRc/CMV in the sense orientation (pCMV-Hmgal). cDNA encoding the mutated Ret (Cys634 to Tyr), associated to MEN2A, was inserted in the HindIII sites of pRc/CMV vector (35).

The 3T3-L1 cells were transfected by the calcium phosphate technique as described previously (36), and subjected to G418 selection (400 μg/ml, Invitrogen Life Technologies).

Colonies

3T3-EA cells were seeded at a density of 2 × 10³ cells per 100-mm dish. The next day they were transfected with pRc/CMV, pCMV-Hmgal and pCMV-Hmgal/T as described previously. Forty-eight hours post-transfection, cells were split and selected in G418. About 15 days later, cells were stained with 0.1% crystal violet in 20% methanol, and counted. The results are the average of three independent experiments performed in duplicate.

Growth curves

For standard growth curves, 3T3-L1 cells were seeded at a density of 10⁴ in 60-mm plates (Falcon) and grown in DMEM supplemented with 10% calf serum. Medium was renewed every 2 days, and cells counted as indicated in Figure 3. The doubling time was measured when the cells were in the logarithmic phase of growth. Statistical analysis was performed by use of the Student’s t test using the GB-STAT program. A two-sided P value < 0.05 was considered to be statistically significant.

Electrophoretic mobility shift assay

Cells were lysed 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 Na3VO4; 20 mM NaPP; 1.5 mM aprotinin] as described previously (33).

DNA binding was determined by electrophoretic mobility shift assay (EMSA) for E2F1, as described elsewhere (37). Briefly, 8 μg of total extracts were incubated in 20 mM HEPES pH 7.5, 40 mM KCl, 5 mM glycerol in a volume of 20 μl containing 1 μg of poly(dI-dC) and 5 mM spermidine, for 10 min at room temperature.

Binding reactions were incubated for 15 min after the addition of 2.5 fmol of a 5′P-end-labelled oligonucleotide (sp. act., 8000–20 000 c.p.m./fmol). The oligonucleotide used was the E2F1-responsive element (sc-2508, Santa Cruz Biotech, Santa Cruz, CA), and a 100-fold molar excess of specific unlabelled competitor oligonucleotide was added. EMSA was performed also by pre-incubating extracts with 0.5 μg of anti-E2F1 rabbit polyclonal antibodies (Santa Cruz Biotech) at 4°C for a minimum of 3 h. After addition of the labelled oligonucleotide and a 15-min incubation at room temperature, the products were resolved on 6% PAGE, and autoradiographed. E2F1 DNA binding activity was measured and quantified by Phosphorimag densitometric scanning. The relative level of E2F1 activity was assessed by comparison with the corresponding E2F1 value in parental and Hmgal transfected cells.

RT–PCR analysis

Total RNA was extracted by RNAzol (Tel-Test, Friendswood, TX). One microgram of total RNA, digested with free-Rnase DNase, was reverse transcribed using random hexanucleotides as primers (100 mM) and 12 U AMV reverse transcriptase (Gibco). Subsequent PCR amplification was as follows: 200 ng of cDNA were amplified in a 25 μl reaction mixture containing Taq DNA polymerase buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.4 mM of each primer, 1 U Taq DNA polymerase (Perkin-Elmer-Cetus, Co., Branchburg, NJ). The PCR amplification was performed for 30 cycles (94°C for 30 s, 55°C for 1 min and 72°C for 1 min). To detect exogenous Hmgal, primers were designed to specifically amplify the transcripts of the transfected constructs. The forward primer 5′-AGGAGAGTTAGGAGAGTCG-3′ overlapped the 5′ of the cloned gene; the downstream primer 5′-AGTGGAGGCTGTAGGGCGAG-3′ overlapped the vector pRc/CMV. In addition, a set of primers specific for the expressed enzyme glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was added to each reaction after 20 cycles of PCR as an internal control of the amount of cDNA tested. The GAPDH specific primers were: 5′-ACATGGTTCATTAGTACTCC-3′ (forward), corresponding to nucleotides 194–214, and 5′-TGGACTCCACGCTACTCAG-3′ (reverse), corresponding to nucleotides 356–336. The primers used for adipo- cytic lipin binding protein gene (pLp) gene expression were 5′-GATGC-CTTTTTGGAACCTCGG-3′ and 5′-AAGTAGCTTTAAGGCGGTC-3′, corresponding to nucleotides 109–138 and nucleotides 427–408, respectively (38). For the expression of the obese gene (ob), which encodes the leptin protein, the primers used were 5′-CCTGCTCCAGCTGCTCAAG-3′ and 5′-CGTGTGTAATGCTGATGATGCC-3′ (39). The primers used for amplification of lipoprotein lipase gene were 5′-GACCTCTGATTTCTCCCT-3′ and 5′-GGTGGATAATCTGCGGCA-3′, corresponding to nucleotides 1262–1278 and nucleotides 211–194, respectively (40). For the detection of the phosphoenolpyruvate carboxykinase gene (PEPCK), the primers were 5′-ACAGTTGGAATGATCCG-3′ and 5′-CTGATGGGATGGACAGATCA-3′, corresponding to nucleotides 1491–1508 and nucleotides 191–171, respectively (41). For the peroxisome proliferator-activated receptor gamma gene (PPARγ), the primers were 5′-CCCGTGAAGGACGAAAGGTT-3′ and 5′-CTCCTGACGCTCTTCAGGAT-3′, corresponding to nucleotides 541–562 and 1045–1024, respectively (42). The reaction products were analysed on a 2% agarose gel, and then transferred by blotting to GeneScreen plus nylon membranes (Dupont, Boston, MA). RNA that was not reversed transcribed before PCR amplification was also amplified (data not shown). Membranes were hybridized with Hmgal, ob, p2, PEPCK, LPL and PPARγ DNA CDNA probes. The relative levels of expression were assessed by comparison with the level of GAPDH in the same sample. The hybridization signal was quantified using a Molecular Dynamics Phosphorimager. The images recorded by the Phosphorimag er were analysed by volume integration with the ImageQuant software.

In vitro and in vivo analysis of the transformed phenotype

The colony-forming assay was performed as described previously (43). In vivo tumourigenicity was measured by inoculating mock-, pCMV-Hmgal and pHmgal/T transfected cells (2 × 10⁶ cells/mouse) subcutaneously into four athymic nude mice. CMV/RET-MEN2A was used as a positive control in the same experiment (44). The reaction products were analysed on a 2% agarose gel, and then transferred to GeneScreen plus nylon membranes (Dupont, Boston, MA). RNA that was not reversed transcribed before PCR amplification was also amplified (data not shown). Membranes were hybridized with Hmgal, ob, p2, PEPCK, LPL and PPARγ DNA CDNA probes. The relative levels of expression were assessed by comparison with the same sample. The hybridization signal was quantified using a Molecular Dynamics Phosphorimager. The images recorded by the Phosphorimag er were analysed by volume integration with the ImageQuant software.

Immunoblotting

Protein extracts were prepared from undifferentiated or terminally differentiated fibroblasts as described previously (44). The protein extracts separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were transferred to Immobilon-P Transfer membranes (Millipore). Membranes were blocked with 5% non-fat milk proteins and incubated with antibodies at the appropriate dilutions. The antibodies against E2F1, cyclin D3, p27Kip1, cyclin A and CDK4 proteins were from Santa Cruz Biotech, CA. The rabbit polyclonal antibodies directed against the Hmgal proteins have already been described (9,15). Equal amounts of protein lysates were loaded, as demonstrated by staining the membranes with Red Ponceau. To confirm equal loading, the same western blots were incubated with antibodies versus γ-tubulin (Sigma-Aldrich Corporation, St Louis, MO). Bound antibodies were detected by the appropriate horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, UK).

Flow-cytometric analysis

Wild-type and pHmgal/T transfected 3T3-L1 cells were analysed for DNA content as described previously (45). Cells were collected and washed in PBS. DNA was stained with propidium iodide (50 mg/ml) and analysed with a FACSscan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (Palo Alto, CA). The CELL-FIT program (Becton Dickinson) was used to analyse cell cycle data.

Results

Colonies

3T3-L1 cells transfected with a truncated Hmgal construct

To investigate the role of the truncated form of the Hmgal gene in adipocytic cell transformation, we first transfected...
pre-adipocytic 3T3-L1 cells with constructs carrying either the truncated \textit{Hmga1} cDNA (Hmga1/T ) or the wild-type \textit{Hmga1} cDNAs (used as a control). The truncated \textit{Hmga1} gene contains the three DNA binding domains of the protein and lacks the acidic domain (46). The wild-type and the truncated \textit{Hmga1} cDNAs were inserted into the expression vector pRc/CMV that carries the gene for resistance to neomycin (Figure 1A). The empty vector served as a control. After transfection, cells were subjected to G418-selection. Several resistant clones were isolated and further characterized. First, we demonstrated that the \textit{Hmga1} and Hmga1/T cDNA sequences were expressed in the transfected cells by an RT–PCR assay (Figure 1B) using a primer on the \textit{Hmga1} gene and another on the backbone vector. The expression of the constructs was verified also by western blotting (Figure 1C).

Six 3T3-L1-Hmga1/T clones were selected for further analysis. We selected four clones transfected with the empty vector and the wild-type \textit{Hmga1} plasmid. All transfected 3T3-L1 clones responded in the same manner. Therefore, the data reported here refer to one representative clone for each construct. The other clones were examined, and gave consistent results. In fact, the transfected cells were stained with crystal violet, and, as shown in Table I, the expression of a truncated \textit{Hmga1} gene determined a remarkable increase in the number and growth of colonies. In fact, an almost confluent

![Fig. 1.](https://academic.oup.com/carcin/article-abstract/24/12/1861/2390348)
monolayer of G418-resistant cells was observed after transfection with pHMga1/T construct.

Morphology of the 3T3-L1 cells transfected with HMga1/T

The 3T3-L1 cells expressing the HMga1 truncated construct (HMga1/T 3T3-L1) showed only slight morphological changes. As shown in Figure 2, these cells are more refractile, and form star-like structures. They resemble transformation foci that are induced in NIH3T3 cells by oncogenes, such as ras and others. No morphological changes were observed in the empty vector or in the wild-type HMga1-transfected 3T3-L1 cells (Figure 2).

Analysis of growth and neoplastic phenotype of 3T3-L1 transfectants

We investigated whether HMga1/T affected the growth profile of the selected clones. Figure 3 shows the growth curve of two representative clones transfected with pHMga1/T compared with the growth of pCMV and wild-type pHMga1-transfected cells. The HMga1/T 3T3-L1 cells had a sharp increase in growth rate compared with control cells (Figure 3). The decrease in the cell number of the parental and wild-type HMga1 transfected 3T3-L1 cells after 6–8 days might be due to the fact that the cells are becoming confluent. Conversely, the HMga1/T transfected cells, smaller in comparison with the parental and HMga1 transfected cells, become confluent at a higher cell density.

As shown in Table II, all the HMga1/T 3T3-L1 cell clones had a much shorter doubling time (18 h) and an increased growth rate compared with the parental cells (doubling time, 23 h) and with cells transfected with the backbone vector (doubling time, 23 h). Conversely, the HMga1 cell clones

<table>
<thead>
<tr>
<th>Cell clones</th>
<th>Number of colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty vector</td>
<td>31 ± 1.5</td>
</tr>
<tr>
<td>CMV-HMga1</td>
<td>45 ± 3.5</td>
</tr>
<tr>
<td>CMV-HMga1/T</td>
<td>255 ± 22.9</td>
</tr>
</tbody>
</table>

Each value is the mean ± SE of at least three independent experiments performed in duplicate.

Table II. Cell-doubling time of 3T3-L1 transfectants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (h)</th>
<th>Median</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3-L1</td>
<td>23.1 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV CI 1</td>
<td>23.8 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV CI 2</td>
<td>23.0 ± 0.25</td>
<td>22.9 ± 0.17</td>
<td>0.8</td>
</tr>
<tr>
<td>3T3-L1 CMV CI 3</td>
<td>23.2 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV CI 4</td>
<td>22.9 ± 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1 CI 1</td>
<td>28.4 ± 0.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1 CI 2</td>
<td>27.7 ± 0.11</td>
<td>28.2 ± 0.35</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1 CI 3</td>
<td>28.2 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1 CI 4</td>
<td>28.5 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1/T CI 1</td>
<td>18.5 ± 0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1/T CI 2</td>
<td>17.7 ± 0.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1/T CI 3</td>
<td>17.3 ± 0.18</td>
<td>17.9 ± 0.51</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1/T CI 4</td>
<td>18.1 ± 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1/T CI 5</td>
<td>18.3 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3T3-L1 CMV-HMGA1/T CI 6</td>
<td>17.9 ± 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Each value is the mean ± SE of at least three independent experiments performed in duplicate. Statistical analysis was performed by use of the Student test. A two-sided P value <0.05 was considered to be statistically significant.
showed an opposite phenotype, with an increased doubling time (28 h) and a reduced growth rate (Figure 3 and Table II). The growth properties of Hmga1 and Hmga1/T clones were significantly different from the parental and the backbone vector transfectants (P < 0.0001).

To characterize the phenotype of the Hmga1/T 3T3-L1 stable clones, we analyzed their ability to grow in soft agar and to induce tumors in athymic mice. Despite the morphological changes and the increased growth rate, the Hmga1/T-transfected cells did not acquire any of the typical markers of neoplastic transformation (Table III). Ret-MEN2A transdifferentiated NIH3T3 cells were used as positive control.

**Cell cycle profile of Hmga1/T expressing 3T3-L1 cells**

To verify whether the truncated Hmga1 gene expression caused a deregulated cell cycle in 3T3-L1 cells, the cell cycle distribution in parental and Hmga1/T-expressing 3T3-L1 cells was analyzed by flow cytometry at 0, 12, 18 and 24 h after treatment. When both cells are starved of serum for 2 days, they become quiescent (G0). The cells were then stimulated to reenter the cycle with serum. The percentage of Hmga1/T-expressing 3T3-L1 cells in the S-phase compartment increased 12 h after treatment, reaching a peak at 18 h (Figure 4A). Conversely, the 3T3-L1 cells were still in G0/G1 phase 12 h after treatment, and the fraction of parental cells in S-phase at 12 h of treatment was about half with respect to the Hmga1/T-transfected cells. Moreover, the serum stimulation induced a major percentage of Hmga1/T cells in G2/M phase of the cell cycle with respect to the 3T3-L1 cells (Figure 4A).

Progression through the cell cycle requires the coordinated sequential activation of cyclin/CDK complexes (47) and the binding of CDK inhibitors CDKI (48). To investigate the effects exerted by the truncated HMGAI protein on 3T3-L1 cells, we determined the expression of cyclins, CDKs and CDKI in wild-type and Hmga1/T 3T3-L1 cells (Figure 4B). Cells were serum starved, stimulated to re-enter the cycle after 3 days, and then lysed at time 0, 6, 12, 18, 24 and 36 h. The expression of cell cycle proteins was detected by western blotting. Cyclin D3 was expressed at a low level in starved cells, we determined the expression of cyclins, CDKs and CDKI in wild-type and Hmga1/T 3T3-L1 cells (Figure 4B). Cells were serum starved, stimulated to re-enter the cycle after 3 days, and then lysed at time 0, 6, 12, 18, 24 and 36 h. The expression of cell cycle proteins was detected by western blotting. Cyclin D3 was expressed at a low level in starved cells, whereas it was much higher in transfected cells (Figure 4B). Expression of other cyclins and CDKs was not altered in quiescent or proliferating cells (Figure 4B for CDK4 expression and data not shown). p27kip1 expression was greatly reduced in Hmga1/T 3T3-L1 cells after 12 h of stimulation, 6 h earlier than in 3T3-L1 cells (Figure 4B). No differences in the expression levels of other CDK inhibitors were detected (data not shown). These findings are in agreement with the observation that an increased fraction of Hmga1/T 3T3-L1 cells are in S- and G2/M-compartments with respect to wild-type cells.

**Increased E2F activity in Hmga1/T 3T3-L1 cells**

Retinoblastoma proteins are known to be critical in controlling the 3T3-L1 cell cycle. Rb is able to induce cell cycle arrest by negatively regulating the E2F family of transcription factors (49,50). Therefore, we first analyzed the E2F activity in parental, in Hmga1 and in Hmga1/T 3T3-L1 cell clones. We prepared extracts from exponentially growing normal and transfected 3T3-L1 cells, and performed an EMSA with an oligonucleotide containing the E2F1 consensus binding site. As shown in Figure 5A, E2F1 DNA binding activity was dramatically increased in all three Hmga1/T-expressing 3T3-L1 cell clones (lanes 4–6) compared with parental 3T3-L1 cells (lane 2) and with Hmga1 expressing cells (lane 3). We determined the specificity of the band by adding a 100× molar excess of unlabelled probe (lane 7). The addition of anti-E2F1 antibodies to Hmga1/T 3T3-L1 cells significantly reduced the

**Table III. Analysis of the transformed phenotype of the transfectants**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Colony-forming efficiency (%)a</th>
<th>Tumour incidenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty vector</td>
<td>0.5</td>
<td>0/4</td>
</tr>
<tr>
<td>CMV-Hmga1/3T3-L1</td>
<td>0.3</td>
<td>0/4</td>
</tr>
<tr>
<td>CMV-Hmga1/T/3T3-L1</td>
<td>0.6</td>
<td>0/4</td>
</tr>
<tr>
<td>CMV/RET-MEN2A/NIH3T3</td>
<td>80.0</td>
<td>4/4</td>
</tr>
</tbody>
</table>

aThe results were expressed as the ratio (number of colonies formed/number of plated cells) × 100.
bAssayed by injecting 2 × 10⁶ cells into athymic mice (4–6 weeks old). The animals were monitored for 8 weeks for the appearance of tumours at the inoculation site.
Transcriptional regulation of adipocyte differentiation requires the concerted activity of several transcription factors, which control growth arrest and the coordinated expression of adipocyte-specific genes. To understand the mechanisms underlying the increase in cell proliferation, we investigated E2F1 activity in normal and transected cells. Our results showed that E2F1 DNA binding activity was higher in Hmgal/T 3T3-L1 cells than in 3T3-L1 cells. Moreover, flow-cytometric analysis showed an accumulation of Hmgal/T 3T3-L1 cells in S- and G2/M phases of the cell cycle. Analysis of the cell cycle-specific genes in transected 3T3-L1 cells showed increased cyclin D3 expression, and a marked reduction of p27kip1 expression, with respect to untransfected 3T3-L1 cells, suggesting that these differences may account for the growth rate modifications of the Hmgal/T transfected cells. Moreover, the proliferative effect induced by the truncated Hmgal gene on adipocytic cells are consistent with the in vivo data obtained by the generation of transgenic mice carrying a truncated Hmgal gene. Indeed, these transgenic mice show a drastic expansion of the retroperitoneal and subcutaneous white adipose tissue (M.Fedele et al., 2004).
It is also interesting to observe that the phenotype of the Hmga1/T transfected cells, characterized by the maintenance of differentiation and by low invasiveness phenotype, recalls the phenotype of most human lipomas, which are normally well capsulated, differentiated and with a very low frequency of malignant progression and invasivity.

However, some questions might be raised. In fact we have demonstrated previously that the suppression of the Hmga1 gene led to an increased growth rate associated to loss of the adipocytic differentiation. These results might suggest a dominant negative effect of the truncated Hmga1 gene on the wild-type protein function, thereby preventing the negative effect of the wild-type protein on cell growth. Following this hypothesis, this putative dominant negative role would be limited to cell growth without affecting adipocytic differentiation.

However, other hypotheses might account for the increased growth rate of the 3T3-L1 cells. Since HMGA proteins function as architectural transcription factors by bending DNA and by directly interacting with other transcription factors, such as NF-κB, ATF-2, c-Jun (51), an excess of a truncated protein might allow the interactions with other transcription factors or alternatively deregulate the function of some transcriptional complexes. We recently found that HMGA1 proteins interact with RB proteins in vitro and in vivo (G.M.Pierantoni et al., in preparation). Therefore, HMGA1 may affect E2F-RB binding so causing an increased release of E2F. However, we have no data that could explain why the wild-type and the truncated form of Hmga1 operate in opposite ways. On the other hand this effect on the adipocytic cell growth is also confirmed by in vivo studies. In fact, transgenic mice over-expressing the HMGA1 wild-type protein showed a reduction of the fat tissue in contrast with the obese phenotype of the HMGA1/T mice (M.Fedele et al., in preparation).

Therefore, even though further experiments are required to elucidate the mechanisms underlying the increased adipocytic cell proliferation induced by the HMGA1 truncated form, the data shown here reinforce the concept that HMGA1 rearrangements are critical in the generation of some, likely a minority, human lipomas.

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