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“Identification of the genes up- and down- regulated by the HMGA1 proteins: tissue specificity of the HMGA1-dependent gene regulation”

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Identification of the genes up- and down- regulated by the HMGA1 proteins: tissue specificity of the HMGA1-dependent gene regulation

LIST OF PUBLICATIONS RELATED TO THE THESIS

1. **Martinez Hoyos J**, Fedele M, Battista S, Pentimalli F, Kruhoffer M, Arra C, Orntoft TF, Croce CM, Fusco A. Identification of the genes up- and down-regulated by the high mobility group A1 (HMGA1) proteins: tissue specificity of the HMGA1-dependent gene regulation. Cancer Res 2004; 64 (16):5728–35.

2. Battista S, Fedele M, **Martinez Hoyos J**, Pentimalli F, Pierantoni GM, Visone R, De Martino I, Croce CM, Fusco A. High-mobility-group A1 (HMGA1) proteins down-regulate the expression of the recombination activating gene 2 (RAG2). Biochem J 2005; 389:91–7.

3. Donato G, **Martinez Hoyos J**, Amorosi A, Maltese L, Lavano A, Volpentesta G, Signorelli F, Pentimalli F, Pallante P, Ferraro G, Tucci L, Signorelli CD, Viglietto G, Fusco A. High mobility group A1 expression correlates with the histological grade of human glial tumors. Oncol Rep 2004; 6:1209–13.

Manuscript in preparation:

Josefina Martinez Hoyos, Angelo Ferraro, Silvana Sacchetti, Monica Fedele, and Alfredo Fusco.
Hand1 gene expression is negatively regulated by the High Mobility Group A1 (HMGA1) proteins and is drastically reduced in human thyroid carcinomas.

ABSTRACT

HMGA1 proteins play their major physiological role during embryonic development and have a critical role in neoplastic transformation.

To identify new pathways in which HMGA1 is involved we searched for genes regulated by HMGA1 proteins using microarray analysis in embryonic stem (ES) cells bearing one or two disrupted *hmga1* alleles. We identified 87 transcripts increased and 163 transcripts decreased of at least four fold in *hmga1*-/- ES cells. For some of them an HMGA1-dose dependency was observed, since an intermediate level was observed in the heterozygous ES cells. When the expression analysis of these genes was extended to embryonic fibroblasts and adult tissues, such as heart, spleen and liver, from *hmga1*-knockout mice, contrasting results were obtained. In fact, aside some genes showing the same HMGA1 regulation observed in ES cells, there were some genes that did not modify their expression, and some other showing a HMGA1-mediated regulation but in opposite direction. These results clearly indicate that HMGA1-mediated gene regulation depends on the cellular context.

Then, we focused on HMGA1-regulation of *Hand1*, a transcription factor crucial for differentiation of trophoblast giant cells and heart development. We were able to demonstrate that HMGA1 proteins bind directly to *Hand1* promoter in vitro and in vivo resulting in the inhibition of the *Hand1* promoter activity. Since the HMGA1 proteins are over-expressed in malignant neoplastic tissues, we have also investigated Hand1 expression in human thyroid carcinoma cell lines and tissues: an inverse correlation was found between HMGA1 and Hand1 expression. Since the down-regulation of the Hand1 gene expression was not associated neither to loss of heterozygosity nor to alteration of the methylation pattern, it is reasonable to hypothesize that HMGA1 over-expression may play a critical role in Hand1 silencing in thyroid carcinomas.

1 BACKGROUND

1.1 HMG superfamily

The high mobility group proteins (HMG) is a superfamily of relatively abundant and highly conserved nuclear proteins, consisting of 3 families, HMGA, HMGB and HMGN. HMG families have been grouped based on similarity in physical and chemical properties and because all act as architectural elements that affect multiple DNA-dependent processes in the context of chromatin. In the nucleus, all HMG proteins are highly mobile, bind transiently to chromatin and compete with histone H1 for nucleosome binding sites. However, each family is clearly unique, has a characteristic functional motif, induces specific changes in their binding sites, and performs unique cellular functions. It is now clear that HMGs impart structural and functional plasticity to the chromatin fiber; however, their biological function seems complex and their effects on the cellular phenotype are still not fully understood.

HMGA proteins interact with a wide range of nuclear components including transcription factors, components of the splicing machinery, proteins involved in replication and chromatin assembly factors. HMGA proteins are easily detectable in cells of early embryos and in undifferentiated or proliferating cells. In fully differentiated cells their expression is sharply down-regulated except in malignant and benign tumours (Fedele et al. 2001).

HMGB protein family includes HMGB1, HMGB2 and HMGB3 all of which are expressed in early embryos. HMGBs were shown to perform important functions both as intranuclear and as extra cellular regulatory proteins. In the nucleus, HMGBs are viewed as major architectural components of chromatin that affect numerous activities including transcription, recombination and DNA repair. As an extracellular component, HMGB1 affects cell migration and tumor invasiveness, and also acts as a cytokine that mediates the response to infection, injury and inflammation (Lotze et al. 2005).

HMGN proteins, which are expressed only in vertebrates, bind specifically the generic structure of the 147 base pair nucleosome core particle, the building block of the chromatin fiber. The interaction of HMGN with chromatin reduces the compaction of the chromatin fiber and affects the rate of transcription, replication and DNA repair in chromatin templates. By binding to nucleosomes, HMGNs affect the ability of regulatory molecules and nucleosome remodeling complexes to reach and modify their chromatin target. HMGN protein family includes HMGN1, HMGN2, HMGN3, HMGN4 and NSBP1 which are expressed in a tissue and developmental specific manner (Shirakawa et al. 2000)

The wide range of cellular activities affected by HMG proteins supports the general notion that these proteins act as structural components of chromatin but at the same time raises the question as to which of the various cellular functions are most affected by these proteins. Indeed, numerous studies suggest

that HMG proteins also act as specific cofactors in distinct cellular pathways. For example HMGA1 facilitates the formation of an enhanceosome on the promoter of the interferon–β gene (Merika and Thanos 2001), HMGB1 stabilizes the binding of the glucocorticoid receptor to chromatin (Bianchi and Agresti 2005), and HMGN1 is specifically recruited by Cockayne syndrome protein A to the polymerase stalled at UV-damaged DNA sites (Fousteri et al. 2006).

1.2 HMGA family

HMGA protein family consists of *HMGA1* and *HMGA2* genes that encode four proteins named HMGA1a, HMGA1b, HMGA1c and HMGA2, being the first three proteins spliced forms of the *HMGA1* gene. *HMGA1* is located at chromosomal locus 6p21 in humans and in the t-complex locus on chromosome 17 in mice whereas *HMGA2* is located at chromosomal locus 12q14-15 in humans and at the *pygmy* locus on chromosome 10 in mice.

The HMGA1a, HMGA1b and HMGA2 proteins are composed of 107, 96 and 108 amino acid residues, respectively. Each protein contains three basic domains, named AT-hooks and an acidic C-terminal region (Figure 1). The HMGA1a protein differs from HMGA1b in that it has an additional insertion of 11 amino acid residues between the first and the second AT-hook domains. The structure of HMGA2 protein is very similar to that of HMGA1b; however, the first 25 amino acid residues are totally different. Moreover, in HMGA2 there is a short peptide of 12 amino acid residues between the third AT-hook and the C-terminal acidic tail (Fedele et al. 2001).

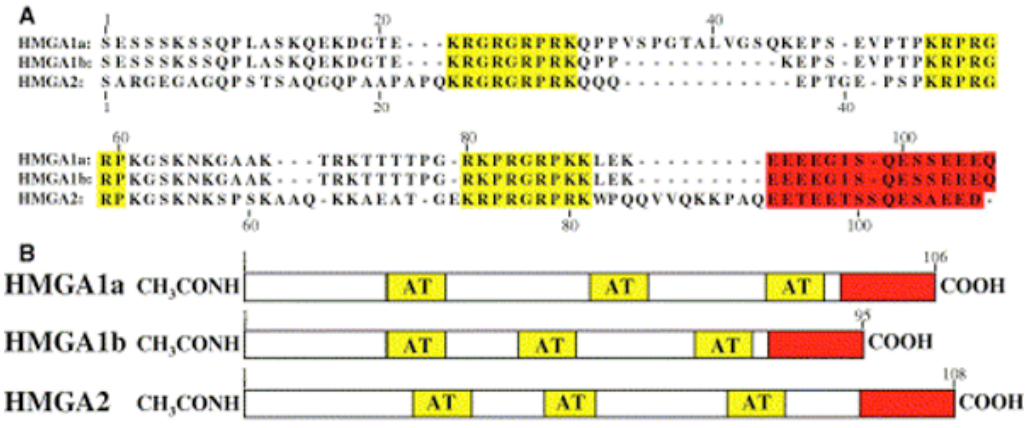


Figure 1 Scheme of the sequences of HMGA proteins

These proteins are very well conserved during evolution, and only a few differences can be detected between the human and the murine HMGA sequences. HMGA1c has a deletion of 67 nucleotides compared with the HMGA1a sequence. This deletion results in a frameshift so that the two proteins are identical in their first 65 amino acids and differ thereafter. Little is known about this form that, however, appears to be the only isoform present in normal human and mouse testis (Fedele et al. 2001).

1.3 Physical and biochemical properties of the HMGA proteins

Originally the HMGA proteins were characterized by their small sizes (10,6-12 kDa), their solubility in dilute (5%) acids, their unusually high concentration of basic, acidic and proline aminoacid residues, their highly phosphorylated state *in vivo* and their rapid mobility during electrophoretic separations. Subsequently, HMGA proteins were shown to bind to the minor groove of short stretches of AT-rich DNA. Given these DNA-binding properties, it was somewhat surprising when biophysical techniques indicated that the HMGA proteins, as free molecules, have very little, if any, secondary structure. HMGA1a protein has relatively little α-helix or β-sheet content and exhibits greater that 70% random coil or other structural characteristics when free in solution. Nevertheless, when specifically bound to other molecules, such as DNA or protein substrates, the HMGA proteins assume induced structural features. For example, the DNA-binding regions of the HMGA proteins assume a planar, crescent-shaped configuration called the "AT-hook" when specifically bound to the minor groove of short stretches of AT-rich DNA. Each HMGA protein has three similar, but independent, AT-hook peptides with the consensus sequence of Pro-Arg-Gly-Arg-Pro (with R-G-R-P being variant) flanked on either side by a number of positively charged lysine/arginine residues. The core of the AT-hook motif is highly conserved in evolution from bacteria to humans and is found in one or more copies in a large number of other, non-HMGA proteins, many of which are transcription factors or components of chromatin remodeling complexes (Reeves 2001).

HMGA binding can induce structural changes in bound DNA substrates. Depending on the sequence, the organization, the topology or the length of the substrate itself, HMGA binding can bend, straighten, unwind and induce looping in linear DNA molecules. They are also able to introduce supercoils into relaxed plasmid DNAs *in vitro* and induce localized changes in the rotational setting of DNA on the surface of isolated nucleosome core particles. Interestingly, HMGA proteins also participate in protein-protein interactions and induce structural changes in the bound protein substrates. One of HMGA partners is PU.1, a member of the Ets transcription factor family and an important regulator of the immunoglobulin heavy chain mu (IgGμ) enhancer in

B-lymphocytes. There is evidence suggesting that the interaction of PU.1 with HMGA1a induces a structural change in the PU.1 protein that increases its mu enhancer binding affinity, resulting in an up-regulation of IgGμ gene transcription. Although the molecular mechanisms that mediate such induced structural changes in bound proteins are unknown, as with DNA substrates, they are likely to revolve around the intrinsic disorder and flexibility of the HMGA proteins (Reeves 2001).

In addition to their other distinguishing characteristics, the HMGA proteins are among the most extensively modified proteins found in the mammalian nucleus. A variety of techniques have been employed to demonstrate that the HMGA proteins are subject to *in vivo* post-synthetic phosphorylation, acetylation, methylation and poly-ADP-ribosylation reactions. These secondary biochemical modifications are dynamic, rapidly responding to both intra- and extracellular signaling events, and markedly influence both the substrate-binding properties of the HMGA proteins and their biological activities. HMGA proteins undergo phosphorylation by various protein kinases, including protein kinase C (PKC), cdc2 and casein kinase II. A link between apoptosis induced in leukaemic cells and the degree of phosphorylation of HMGA1a protein has been described (Diana et al. 2001). At the early stages of the apoptotic process, the HMGA1a protein is hyper-phosphorylated. Subsequently, when the apoptotic bodies are formed, the HMGA protein becomes almost completely de-phosphorylated.

HMGA proteins are also regulated by acetylation. The transcriptional coactivator CBP/p300 (CREB-binding protein) and P/CAF (CBP-associated cofactor) acetylate HMGA1a at distinct lysine residues, causing distinct effects on transcription. In the context of the human ß-interferon gene expression, acetylation of HMGA1a by both CBP and P/CAP is required for the enhanceosome activation, whereas only CBP acetyltransferase activity is required for enhanceosome destabilization and post-induction turn-off (Munshi et al. 1998).

HMGA proteins have the ability to physically interact with a large number of proteins, most of which are transcription factors: AP-1, ATF-2/c Jun heterodimer, IRF-1, c-Jun, NF-kB p50/p65 heterodimer, C/EBPß, Elf-1, NF-AT, NF-kB p50 homodimer, NF-kB p65, NF-Y, Oct-1, Oct-2A, PIAS3, PU.1 RNF4, SRF and Tst-1/Oct-6. Recently, other molecular partners were discovered using a proteomic approach (Table 1), suggesting an involvement of HMGA proteins in other chromatin functions, such as RNA-processing, DNA replication, chromatin structural organization and remodeling. (Sgarra et al. 2005).

Table 1 HMGA molecular partners identified by MS analysis

Protein name	Accesion number
CBF-beta	Q13951
SF3a120	Q15459
hnRNP K	Q07244
hnRNP H	P31943
hnRNP F	P52597
hnRNP	MP52272
Ku80	P13010
RBBP-4	Q09028
RBBP-7	Q16576
RuvB-like 2	Q9Y230
Tubulin alpha-1 chain	P05209.
Tubulin beta-1 chain	P07437
CapZ alpha-1	P52907
NPM	P06748
Heat shock cognate 71 kDa protein	P11142
GRP 78	P11021
GRWD	Q9BQ67
REC14	Q9GZS3

1.4 HMGA proteins and gene transcription

Until 2004 there were reports of over 50 different eukaryotic and viral genes whose transcriptional expression was regulated by HMGA proteins *in vivo* (Table 2). The vast majority (>35) of these are positively regulated and their inducible expression is controlled by a variety of biological and environmental stimuli. The promoter regions of many of the positively regulated genes contain multiple stretches of AT-rich sequence. Transcriptional activation of these types of promoter often involves the formation of an "enhanceosome", a stereo-specific, multi-protein complex that includes HMGA proteins and other transcription factor making specific protein-DNA and protein-protein contacts in intricate, but precise, ways. In the case where HMGA proteins act as negative regulators of gene transcription they often serve as inhibitors of enhanceosome formation, usually by sterically blocking the functional binding of other crucial transcription factors to their recognition sites in gene promoters. (Reeves 2001). Very recently, in our laboratory, we have described the down-regulation of the recombination activating gene (RAG2) by HMGA1 proteins (Battista et al. 2005).

Table 2 Genes Regulated by HMGA Proteins

Positive Regulation		Negative Regulation
Vascular Endothelial Tissue	CD44	β-Globin
	E-Selectin	IL-4
	IGFBP-1	IgE
	iNOS	GP 91-phox
	COX-2	TCRα
	SM22α	BRCA1
Immune System	MGSA/GROα	RAG2
	CXCL1	
	IFN-β	
	GM-CSF	
	TNF-β	
	IL-2	
	IL-2Rα	
	IL-15	
	HLA-II	
	c-fos	
	IgG Heavy Chain	
Viral Genes	HIV-1 LTR	
	HSV-1 IE-3	
	HSV-1 EBNA1	
	BV EBNA1	
	VHP 18	
	JV virus Early & Late Genes	
Other	Leptin	
	GATA-1	
	Hum Insulin Receptor	
	α-ENaC	
	Tyrosinase	
	Rhodopsin	
	Neurogranin IRC3	
	PKCγ	
	mRANTES	
Plant Genes	Plastocyanin	
	Nodulin N23 gene	
	Ferredoxin	
	Phytochrome A3	
	Glutamine Synthetase	
	Soybean hsp17.5E	

One of the best-studied mechanisms of gene regulation in which HMGA proteins are involved is that of the interferon-β gene (IFN- β). The activation

of the IFN- β expression is due to a multifactor complex that assembles in the nucleosome-free enhancer region of the gene, formed by the factors NF-kB, IRF, ATF2/cJun, and the HMGA1a protein (Figure 2). HMGA1a plays a double function in this context: (i) induces allosteric changes in the DNA, thus increasing the affinity of the transcription factors for their binding sites and (ii) establishes protein-protein interactions with the same factors. This new structure, called enhanceosome, is responsible for the modification and the remodeling of a nucleosome that masks the TATA-box; consequently, transcription can start. This remodeling is triggered by the recruitment from the "enhanceosome" of GCN5/PCAF that acetylates the nucleosome and also HMGA1a at K64, the latter modification resulting in the stabilization of the enhanceosome. Later, another acetyltransferase called CBP modifies HMGA1a at K70 destabilizing the enhanceosome and, consequently, repressing transcription (Munshi et al. 1998).

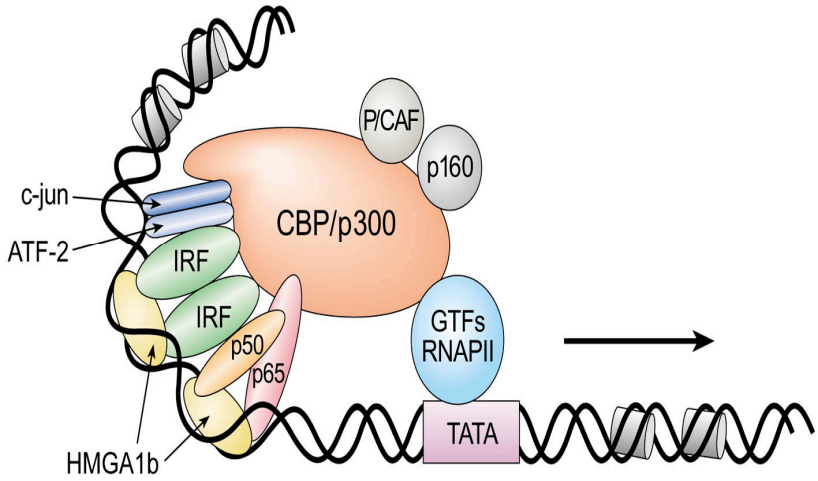


Figure 2 HMGA proteins facilitate the assembly of specific multiprotein complexes required for gene promoter activation

1.5 HMGA expression in normal and neoplastic tissues

The *HMGA2* gene is not expressed in any of the several adult mouse and human tissues tested. A very low expression has been observed in CD34 positive hematopoietic stem cells, and in mouse pre-adipocytic proliferating cells. The *HMGA1* gene is expressed at low levels in adult murine and human tissues: a higher expression was observed in testis, skeletal muscle ant thymus. Conversely, both the genes are widely expressed during embryogenesis.

Hmga1 and Hmga2 over-expression was first described in rat thyroid transformed cells and in experimental thyroid tumours. Over-expression of the HMGA proteins was then found to be a common feature of experimental and human malignant neoplasias, including thyroid, prostate, uterus, breast, colorectum, ovary and pancreas carcinomas (Fedele et al. 2001). Recently, in our laboratory, we have correlated HMGA1 expression with the histological grade of human glial tumors (Donato et al. 2004). Moreover, the expression level of the HMGA proteins is significantly correlated with parameters of poor prognosis in patients with colorectal cancer. In all of these epithelial/endothelial cell-derived malignant tumors, the over-expressed proteins are full-length non-mutants forms of the HMGA proteins. In contrast to the situation in carcinomas, benign tumors of mesenchymal origin (lipomas, leiomyomas, fibroadenomas, aggressive myxomas, pulmonary hamartomas and endometrial polyps) often contain chromosomal rearrangements that result in the creation of new hybrid genes that code for chimeric proteins in which the AT-hooks of the HMGA proteins are fused to ectopic peptidic sequences (Hess 1998).

Over-expression of the HMGA proteins is a necessary event in *in vivo* cell transformation. This was demonstrated by experiments in which Hmga expression was blocked by transfecting rat thyroid cells with antisense Hmga constructs. When these cells were infected by the myeloproliferative sarcoma virus and the Kirsten murine sarcoma virus carrying the v-mos and v-ras-Ki oncogenes, respectively, they did not acquire the typical markers of neoplastic transformation (ability to grow in soft agar and induce tumors after injection into athymic mice). Conversely, these markers were shown by the untransfected rat thyroid cells infected with the same murine retroviruses (Berlingieri et al. 1995). Over-expression of HMGA1 proteins is also essential in the development of cancer in humans. In fact, 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 (Scala et al. 2000). Moreover, it has been reported that the over-expression of HMGA1a or HMGA2 leads to neoplastic transformation of both Rat-1a fibroblasts and CB33 cells, whereas the decrease of HMGA1a/b expression abrogates transformation in Burkitt's lymphoma cells (Wood et al. 2000).

1.6 HMGA proteins in embryogenesis and differentiation

In normal cells the expression of HMGA proteins is restricted to embryogenesis, it decreases with organogenesis and in normal adult cells is very low or almost absent. In particular, both genes are expressed at high levels in the entire embryo until 8.5 dpc (Chiappetta et al. 1996, Zhou et al. 1995). At later stages, the expression pattern becomes more restricted; in particular, HMGA1 expression is confined to specific body organs of ectodermal,

mesodermal and endodermal origin, while HMGA2 expression is restricted to mesenchymal tissues. A role for both factors in development has been demonstrated: studies carried out by Chada's group demonstrated that mice showing a pygmy phenotype carry a disrupted *Hmga2* gene and are characterized by a large reduction of fat tissue and the same group demonstrated that HMGA1 is required for normal sperm development (Zhou et al. 1995, Liu et al. 2003). Fusco's group demonstrated that suppression of HMGA1 expression impairs differentiation of pre-adipocytic cells and loss of *Hmga1* gene function affects lymphohematopoietic differentiation. We reported that *hmga1*-/- ES cells generate less T-cell precursors than do wild type ES cells upon *in vitro* specific differentiation. Indeed, they preferentially differentiate to B-cells, probably consequent to decreased IL-2 expression and increased IL-6 expression, that are both directly regulated by the HMGA1 proteins. Moreover, a lack of HMGA1 expression results in altered hemopoietic differentiation, i.e., there is a reduction in the monocyte/macrophage population, and an increase in megakaryocyte precursors, erythropoiesis and globin gene expression. Re-expression of the *hmga1* gene in *hmga1*-/- ES cells restores the wild type phenotype (Battista et al. 2003). These results indicate that drastic changes occur in the transcriptional activity of the *hmga1*-/- cells, and presumably they depend on the modification of the expression of HMGA1-regulated genes.

1.7 Factors that regulate HMGA gene expression

The structure of the human *HMGA1* gene is complex and consists of eight transcribed exons that produce multiple forms of transcripts as a result of a complicated pattern of alternative mRNA splicing. Additionally, the gene has four different promoter/enhancer regions that are capable of independently initiating transcription depending on the cell type and the nature of the stimulatory signal. Among these agents that induce *HMGA1* gene transcription are: serum, transforming growth factor α (TGF-α), epidermal growth factor (EFG), platelet-derived growth factor (PFDG), fibroblast growth factor (FGF), phorbol esters and calcium ionophores, interferon-β1 (IFN- β1) and endotoxin, retinoic acid, morphine and hypoxia, as well as the transcription factors AP-1, c-Myc and the human papillomavirus E6 proteins (Reeves and Beckerbauer 2001). From this partial list of inducing agents it is obvious that many different signal transduction pathways, and a variety of transcription factors, participate in the *in vivo* control of expression of HMGA genes thus paving the way for a plethora of possible mechanisms that might led to their aberrant regulation and pathological expression.

2 AIMS OF THE STUDY

The first aim of the study was to identify new pathways in which HMGA1 proteins are involved. For this reason, using oligonucleotide microarray hybridization technique, we analyzed the expression profile of embryonic stem (ES) cells in which one or both *hmga1* alleles were disrupted. In this way we could identify the genes that are regulated, positively or negatively, by HMGA1 proteins.

The second aim of the study was focused on one of the genes identified, Hand1, a transcription factor crucial for differentiation of trophoblast giant cells and heart development. We investigated if *Hand1*-regulation by HMGA1 proteins, could have significance in transformation. For this purpose we analyzed Hand1 expression in thyroid rat transformed cells, in human thyroid cell lines and in human thyroid carcinomas.

3 MATERIALS AND METHODS

3.1 Microarray analysis

Microarray analysis was performed as described in detail at http://www.cancergenetics.med.ohio-state.edu/microarray. Briefly, cRNA was prepared from 8 μg of total RNA, hybridized to MG-U74 Affymetrix oligonucleotide arrays (containing 13,059 murine transcripts), scanned, and analyzed according to Affymetrix (Santa Clara, CA) protocols. Scanned image files were visually inspected for artifacts and normalized by using GENECHIP 3.3 software (Affymetrix). Comparisons were made for each mutated sample versus wild type sample, taking the wild type sample as baseline by using GENECHIP 3.3. The fold-change values, indicating the relative change in the expression levels between mutated samples and wild type sample were used to identify genes differentially expressed between these conditions.

3.2 Plasmids

To study *Hand1* promoter, the region -2424 – -2728 of the mouse *Hand1* gene was amplified using as primers gggatacacgaaggtcagtttt (forward) and ctgagatcccagatcactca (reversed), cloned in TA Cloning Vector (Invitrogen) and subcloned in pGL3 (Promega) KpnI-XhoI cloning site. The point mutations in the HMGA binding site of the *Hand1* promoter (*Hand1MUT*prom-luc) were generated using the QuikChange Mutagenesis Kit (Stratagene) in accordance with the manufacture's protocols. The primers used were tattttaactaattaGGtaataacagagtctcctcctgcc forward) and ggcaggaggagactctgttattaCCtaattagttaaaata (reversed). Point mutations are shown in uppercase type. *Hand1*, *HMGA1* and *HMGA2* expression plasmids were constructed by cloning the murine full-length cDNAs of *Hand1* or *hmga1b* or *hmga2* into the mammalian expression vector pcDNA3.1.

3.3 Cell culture and transfections

The generation and the culture of *hmga1*+/- and *hmga1*-/- Embryonic Stem cells are described elsewhere (Battista et al. 2003). FRTL-5, FRTL-5-KiMSV and FRTL-5–HMGA1as-KiMSV cells, and their culture conditions are reported elsewhere (Berlingieri et al. 1995). Five x 10^5 FRTL-5 cells were plated in 6- well plates and transfected after 48 hours with 1 μg of reporter plasmid (either *Hand1*prom-luc, *Hand1MUT*prom-luc or pGL3), by Lipofectamine 2000 (Invitrogen). Cells were harvested 24 hours post-transfection and lysates were analyzed for luciferase activity. Transfection efficiency was normalized using the β-galactosidase activity and fold of

activation were calculated by dividing for pGL3 luciferase activity. All the assays were performed in triplicate and repeated in three independent experiments.

Human thyroid primary culture and human thyroid carcinoma cell lines (TPC-1, WRO, NPA, ARO, FRO, NIM 1, B-CPAP, FB-1, FB-2, Kat-4 and Kat-18) are described elsewhere (Pallante et al. 2005).

3.4 Tissue samples

Neoplastic human thyroid tissues and normal adjacent tissue or the controlateral normal thyroid lobe were obtained from surgical specimens and immediately frozen in liquid nitrogen. Thyroid tumours were collected at the Service d'Anatomo-Pathologie, Centre Hospitalier Lyon Sud, Pierre Benite, France. The tumour samples were stored frozen until RNA extractions were performed. Tissues from *hmga1* +/- and *hmga1* -/- mice have been described elsewhere (Martinez Hoyos et al. 2004).

3.5 RNA extraction from tissues and cells

Tissues were snap-frozen in liquid nitrogen and stored at –80°C until use. Total RNAs were extracted from tissues and cell culture using TRI REAGENT® (Molecular Research Center INC) solution, according to the manufacture's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis (virtual presence of sharp 28S and 18S bands) and spectrophotometry.

3.6 Semiquantitative and quantitative RT-PCR

RNAs were treated with DnaseI (Invitrogen) and reverse-transcribed using random exonucleotides as primers and MuLV reverse transcriptase (Perkin Elmer). To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing the PCR on samples that were not reversed-transcribed but otherwise identically processed. For semiquantitative 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 and scanned using a Typhoon 9200 scanner. Digitized data were analyzed using Imagequant (Molecular Dynamics). Quantitative PCR was performed with SYBR® Green PCR Master Mix (Applied Biosystems) as follows: 95 °C 10 min and 40 cycles (95 °C 15 s and 60 °C 1 min). Each reaction was performed in duplicate. To calculate the relative expression levels we used the 2-DDCT method (Livak et al. 2001). The primers sequences are: murine F

gatgccttctcgagttaaaa and R aagtgtagcgacaagaagga, rat F gttcaggacccaaaaagg and R gcagagtcttgatcttggag, human F ctggctctttctctcttgtc and R cgtctggttctctttctcag.

3.7 Protein Extraction, Western Blotting and Antibodies

Tissues and cell culture were lysed in buffer 1% NP40, 1mM EDTA, 50mM Tris-HCl pH 7.5, 150 mM NaCl, supplemented with CompleteTM protease inhibitors cocktail (Roche Diagnostic Corp.). Total proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Biorad). Membranes were blocked with 5% nonfat milk and incubated with antibodies against Hand1 and Tubulin. Hand1 antibody was a generous gift from Dr. Peter Cserjesi (New Orleans, USA). Bound antibody was detected by anti-guinea pig secondary antibody and revealed with an enhanced chemiluminescence system (Amersham-Pharmacia Biotech).

3.8 Electrophoretic Mobility Shift Assay

The production of recombinant proteins was previously described (Baldassarre et al. 2003). Five or 20 ng of recombinant protein were incubated with radiolabeled double-strand oligonucleotides, corresponding to the region spanning bases -2658 to -2688 of the 5' untranslated region of the murine *Hand1* promoter (5'- atttattttattttaactaattaattaataa-3'). A 200-fold excess of specific unlabeled competitor oligonucleotide was added. The same oligonucleotides were also used in binding assays with total extract from wild type and *hmga1*-knockout murine ES cells. 8 µg of extracts were incubated in 20 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 0.5 mM MgCl2, 0.5 mM DTT, 0.1 mM PMSF in a volume of 20 µl containing 1 µg of poly(dC-dG), 2 µg BSA and 10% glycerol, for 10 min at room temperature. Binding reactions were incubated for 10 min after addition of 2.5 fmol of ^{32}P-end labeled oligonucleotides (specific activity, 8,000 to 20,000 cpm/fmol). The DNA-protein complexes were resolved on 6% non-denaturing acrylamide gels and visualized by exposure to autoradiographic films.

3.9 Chromatin immunoprecipitation

Approximately 3x10^7 wild type, *hmga1* +/- and -/- ES cells were grown on 75 cm^2 dishes. Chromatin immunoprecipitation was performed as previously described (Martinez Hoyos et al. 2004). Input and immunoprecipitated DNA were analyzed by PCR for the presence of *Hand1* promoter sequence spanning –2424 to -2927. The primers used were 5'- cttggtgacaagcacctt-3' (forward) and 5'- ctgagatcccagatcactca-3' (reverse).

3.10 Methylation analysis using bisulphite genomic sequencing

The promoter region of human HAND1 gene was analyzed for CG content; a CpG island was determined based on a 200-bp lenght of DNA with a CG content of >50% and a CpG/GpC ratio of >0.5, using CpGplot program, available at http://www.ebi.ac.uk/emboss/cpgplot/. Bisulphite genomic sequencing was used to analyze the methylation patterns of individual DNA molecules. Sodium bisulphite conversion of genomic DNA (about 200 ng for each conversion) was obtained using EZ DNA Methylation Kit™ (ZYMO Research) following the manufacturer's instructions. The CpG islands identified as described previously were then PCR amplified using the following primers: *Pre-Nested PCR* 3FHand (-461 to -430) gtagagtagttggagttygggattgggaattg, 3RHand (+180 to +211) ctccatacrccccaaaaactaccraaaaccac. *Nested PCR* 3FnHand (- 275 to -249) ggaggggtggtagtaatagtttaggg, 3RnHand (+170 to +201) ccccaaaaactaccraaaaccacctataaactc. PCR reactions were carried out using FastStart Taq DNA polymerase (Roche) under the following conditions: 1) Pre-nested PCRs were normally carried out on 10 ng of bisulphite treated DNA in a final reaction volume of 50 ml, using standard conditions with 1,5 min at 95°C, followed by 5 cycles of 30 sec at 95°C, 30 sec at 59°C, and 40 sec at 72°C, then 25 cycles of 30 sec at 95°C, 30 sec at 57°C, and 40 sec at 72°C, then a final elongation of 6 min at 72°C before holding at 4°C. 2) Nested PCRs were performed in the same conditions, using 5 ml of the corresponding pre-nested PCRs in a final reaction volume of 50 ml. PCR final products (477 bp) were then cloned into the pGEM-Teasy vector provided by Promega pGEM®-T Easy Vector System II, following the supplier's procedures. The positive screened colonies contained the unique sequence of one individual DNA molecule. The plasmidic DNA from the selected positive colonies containing vectors with the insert was purified using the Qiagen plasmid Mini Kit. The purified plasmids were sequenced in both directions using T7 and Sp6 primers. 20 independent clones for each genomic preparation and fragment of interest were sequenced to determine the methylation pattern of individual molecules. Sequencing was performed at the CEINGE Sequencing Core Facility.

3.11 SNP-based Loss of Heterozygosity analysis

We performed LOH analysis using Single Nucleotide Polymorphisms. To identify the SNPs scattered in the genetic locus of *HAND1,* we input *HAND1* human gene name in the SNP database of NCBI and we found eight SNPs. The primers, SNP reference and alleles are: F- cgaaataggcaaacaggctc and R-aaagctcatccagggacga for rs924581 (A/G); F- gaagacccgatctgttttacct and R-cttcaaggctgaactcaagaa for rs4370323 (A/G), rs1846966 (C/G), rs11748765 (A/T); F- cgctgttaatgctctcagt and R- gtaaaacctgggatagcca for rs6880185 (A/G), rs13171812 (C/T), rs993098 (A/C), rs3822714 (A/G). The primers used for

PCR was also used for sequencing assays. PCR was performed using HotMaster Taq DNA Polimerase (Eppendorf AG, Germany) in a final volume of 25 μl. For amplification reaction we used 50 ng of genomic DNA, 0,5 unit of Hotmaster Taq DNA polymerase, a final concentration of each primer of 0.2 μM and 0.2mM of dNTPs and 2,5 μl of 10x HotMaster Taq DNA Polimerase Buffer with Mg2+. The conditions used for PCR was a initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 20 sec, 58°C for 10 sec, 70°C for 40 sec and a final extension of 5 min at 70°C. After amplification the size and quality of amplicons was checked loading 5 μl of reactions on agarose gel. 20 μl of each PCR was sequenced with specific forward and reverse primers used for amplification reaction.

4 RESULTS AND DISCUSSION

4.1 Gene expression profile analysis

RNAs extracted from wild type, *hmga1*+/- and *hmga1*-/- ES cells were hybridized to MG-U74 Affymetrix oligonucleotide arrays containing 13,059 transcripts. The expression profile of the heterozygous and homozygous ES cells was compared to that of the wild type ES cells that was used as a common reference. The number of transcripts increased or decreased in the heterozygous and the homozygous mutant versus wild type sample is shown in the Figure 3. Of the 13,059 transcripts represented on the array, 1,300 had a fold-change between two and three, 313 between three and four, 227 between four and ten, and 23 greater than ten. We examined the 250 transcripts (1.9%) that had a fold-change of four or more in the homozygous mutant versus the wild type sample. Among these 250 transcripts, 87 were increased and 163 were decreased, including 103 known genes (37 increased and 66 decreased), 118 ESTs (40 increased and 78 decreased), and 29 unknown genes (10 increased and 19 decreased). As a control of microarray analysis, we verified that the HMGA1 was not expressed in *hmga1*-/- ES cells. The genes with fold-change of four or greater in *hmga1*-/- ES cells were grouped according to their function: a) signal-transduction pathways, b) transcription factors, c) cell proliferation, d) extracellular-matrix and cellular-structure proteins, e) metabolic pathways, transport and secretion, f) growth factors and related proteins, g) genes with immune functions and h) other genes. The relative fold-changes in these genes, grouped as described above, are shown in Table 3. It is noteworthy that among the HMGA1-regulated genes we found Id3, lefty and Wnt-6 that are important in embryonic development and some oncogenes such as c-myc, junB, pim-2 and c-fos.

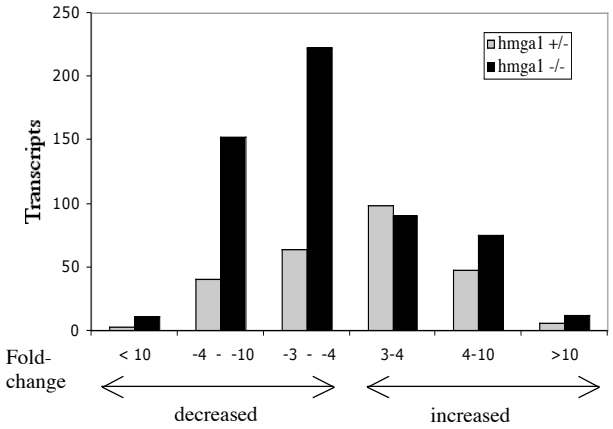


Figure 3 Gene expression profile in ES cells carrying one or two *hmga1*-disrupted alleles

4.2 Validation of microarray analysis

To validate the results obtained by microarray analysis we evaluated the expression of 50 transcripts by semiquantitative and quantitative RT-PCR in the wild type, *hmga1*+/- and *hmga1*-/- ES cells. For all of them, the differential expression associated with the expression of the HMGA1 proteins was confirmed. Some representative semiquantitative RT-PCR analysis are shown in Figure 4. The expression of some genes, i.e., TFEB, LKLF, and Id3 was Hmga1 dose-dependent. In fact, the changes in *hmga1*+/- ES cells were intermediate between those found in wild type and *hmga1*-/- ES cells. Conversely, the expression of other genes, i.e., cubilin, p96, D-9K, legumain and collagen was not modified in *hmga1*+/- ES cells in comparison to the wild type ES cells.

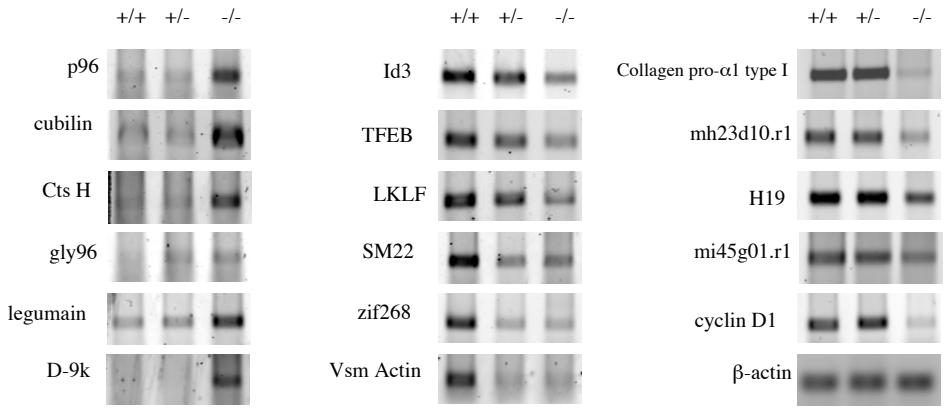


Figure 4 Validation of microarray data by semiquantitative RT-PCR

Thus, for some genes the level of *hmga1* expression may be critical for appropriate gene expression. In this case both alleles seem to be necessary to regulate the expression of these genes. For some other genes, the dependency on the *hmga1* expression levels was even more pronounced since the gene expression level in heterozygous ES cells was very close to that observed in homozygous cells. This type of regulation by *hmga1* expression levels may explain the appearance of pathologies, such as cardiac hypertrophy and B cell lymphomas, in mice heterozygous for *hmga1* gene disruption (Fedele et al, 2006). Several other genes showed the same expression level in wild type and heterozygous ES cells. In this case one *hmga1* allele is sufficient to regulate gene expression.

Table 3 The genes differentially expressed with a fold-change of 4 or higher in homozygous (-/-) *hmga1*-knockout ES cells grouped in families. Acc.No. = accession number; FC = fold change

DESCRIPTION	Acc. No.	FC +/-	FC -/-
A. Signal transduction			
M. musculus parathyroid hormone/parathyroid hormone related-peptide receptor	L34611	1.1	18.4
M.musculus mRNA for ryudocan core protein	D89571	3.9	10.6
M.musculus mitogen-responsive 96 kDa phosphoprotein p96	U18869	1.7	9.3
M. musculus mRNA for MAP kinase-activated protein kinase 2	BC063064	10	4.7
Mouse Wnt-6	M89800	1.1	4.1
M.musculus JIP-1	AF003115	-1.7	-4
Murine macrophage gene, encoding bmk (B cell/myeloid kinase)	J03023	-1.4	-4.1
M.musculus TGF-beta-inducible protein (TSC-36)	M91380	1.3	-4.2
M.musculus mRNA for retinoic acid receptor-alpha	X57528	1.1	-4.3
Mouse growth factor-inducible protein (pip92)	M59821	-2.5	-4.4
Mouse oxytocin-neurophysin I	M88355	1.7	-4.5
M.musculus patched	U46155	-1.2	-4.6
Mouse G protein alpha subunit (GNA-15)	M80632	-9.4	-4.7
Mouse mRNA for NBL4	D28818	-2.1	-5.1
M.musculus receptor protein tyrosine phosphatase-lamda (ptp-lambda)	U55057	1.3	-5.4
M.musculus protein-serine/threonine kinase (pim-2)	L41495	-1.7	-5.6
Mouse (clone M1) GTPase (Ran)	L32751	2.6	-5.7
M.musculus ShcC	U46854	1.3	-6.3
M.musculus syk mRNA for protein-tyrosine kinase	Z49877	-1.3	-6.3
M.musculus c-Src kinase (Csk)	U05247	-1.6	-8.6
B. Transcription factors			
M.musculus Thing1	U21226	3.8	14
M. musculus adult testis mRNA for truncated form of Sox17	D49473	1.3	13.7
M.musculus melanocyte-specific gene 1 (msg1)	U65091	1.4	9.7
M.musculus mRNA for fos-related antigen-2	X83971	3.1	4
M.musculus Sox4 (Sox4)	ET62444	-1.4	-4.1
Mouse c-fos oncogene	V00727	-12	-4.2
Mouse c-myc	L00039	-4.3	-4.3
M.musculus AP-2.2	X94694	-1.6	-4.4
Mouse mRNA for Zfp-57	D21850	-2.5	-4.6
M.musculus Kruppel-like factor LKLF	U25096	-1.4	-4.8
M.musculus transcription factor TFEB	U36393	-2.2	-4.9
M.musculus transcription factor NF-YC subunit	U62297	-2.6	-5.2
M.musculus DNA binding protein NFI-X (NfiX)	U57636	-1.9	-6.2
M. musculus mRNA for TIF1 beta protein	X99644	-5.8	-6.2
M.musculus transcription factor junB	U20735	-3.5	-6.4
M. musculus Oct-6 mRNA for octamer binding protein	X57482	-8.1	-6.5
Mouse helix-loop-helix protein (Id related)	M60523	-5.3	-7.4
Mouse growth factor-induced protein (zif/268)	M22326	-8.2	-24.9
C. Cell proliferation			
M. musculus chop-10	X67083	15.1	16.9
Mouse D-type cyclin (CYL2)	M83749	-2.5	9
M. musculus gly96	X67644	4.4	8
M.musculus mRNA for zyxin	Y07711	1.3	5.3
D. Extracellular-matrix and cellular-structure			
M.musculus laminin A chain	J04064	1.2	14
M.musculus alpha-2 type IV collagen	J04695	1.4	9.8
M.musculus alpha-1 type IV collagen (Col4a-1)	J04694	1.4	9.1
Mouse alpha-B2-crystallin	M73741	1.4	7.7
Mouse laminin B1	M15525	1.3	6.3
Mouse mRNA for cysteine-rich glycoprotein SPARC	NM_009242	1.2	5.5
Mouse gelsolin	J04953	1.5	4.5
M.musculus mRNA for hair keratin, mHb6	X99143	3.5	4.1

Mouse alpha-B crystallin	M63170	1	4
Mouse COL1A2 mRNA for pro-alpha-2(I) collagen	X58251	-3.8	-4.1
M.musculus rhoB	X99963	-7.4	-4.2
M.musculus neurofibromatosis 2 (Nf-2)	L27090	1.3	-4.5
M.musculus SM22 alpha	L41154	-1.5	-4.5
Mouse COL1A2 mRNA for pro-alpha-2 (I) collagen	NM_007743	-3.9	-4.7
M.musculus alpha 1 type I collagen	U50767	-1.9	-5.6
M.musculus mRNA for myosin I	X97650	-4.6	-6.3
Mouse tau microtubule binding protein	M18776	-1.3	-6.6
M.musculus FVB/N collagen pro-alpha-1 type I chain	U08020	-2.1	-7.5
Mouse mRNA for vascular smooth muscle alpha-actin	X13297	-2.1	-8.6
E. Metabolism, transport and secretion			
Mouse mRNA for mastocytoma proteoglycan core protein, serglycin.	X16133	2.6	40.9
M.musculus cathepsin H prepropeptide (ctsH)	U06119	1.5	17.3
M.musculus calcium binding protein D-9k	AF028071	2.3	9.8
Mouse serine protease inhibitor homologue (J6)	J05609	1.7	8.7
M. musculus heparan sulfate D-glucosaminyl 3-O-sulfotransferase-1 precursor	AF019385	-1.4	6.5
Mouse mRNA for preproinsulin-like growth factor IA	X04480	-1.5	6.2
M.musculus preprodipeptidyl peptidase I	U89269	1.2	5.9
M.musculus mRNA for cytoplasmic dynein heavy chain	ET63396	1.6	5.5
M.musculus steroid cytochrome p450 7-alpha hydroxylase	L06463	-1.1	-4
M.musculus very-long-chain acyl-CoA synthetase (VLCS)	AF033031	-2	-4
M. musculus (N-acetylglucosaminyltransferase I	L07037	2.5	-4.1
Mouse mRNA for inward rectifier K+ channel	D50581	1	-4.1
M.musculus preprocortistatin (Cort)	AF013253	2.7	-4.3
M.musculus extracellular superoxide dismutase (SOD3)	U38261	1.2	-4.6
Mouse mRNA for a preprothyrotropin-releasing hormone	X59387	-1.1	-4.7
M.musculus steroid sulfatase (Sts)	U37545	-2.3	-4.7
M.musculus mRNA for dihydropyrimidinase related protein 4	AB006715	5.7	-4.9
M. musculus putative chloride channel protein CLC6 (Clc6)	AF030106	-1	-4.9
Mouse placental alkaline phospatase	J02980	-1.1	-4.9
M.musculus Balb/c cytochrome c oxidase subunit VIaH	U08439	3.1	-5
Mouse metallothionein-III	M93310	1.4	-5.3
M.musculus carboxypeptidase E (Cpe)	U23184	-3.5	-5.8
M.musculus hormone-sensitive lipase	U08188	-1.7	-8
M.musculus ADP-ribosylation factor-like protein 4	U76546	-2.4	-8.7
F. Growth factors and related proteins			
M.musculus lefty	AJ000083	-1.1	-4.3
M.musculus mRNA for insulin-like growth factor binding protein-3	X81581	-3.2	-6.4
M.musculus mRNA for insulin-like growth factor binding protein-4	X81582	-1.4	-7.3
M.musculus acid labile subunit (ALS)	U66900	2.4	-7.4
Mouse Cyr61	M32490	-4.2	-8.4
M.musculus follistatin-like protein (mac25)	L75822	-2.6	-11
G. Immune functions			
Mouse gene for 47-kDa heat shock protein (HSP47)	D12907	1.1	5.7
M.musculus MHC class I B(2)-microglobulin	M84366	1.2	5
Mouse lymphocyte differentiation antigen (Ly-6.2)	M18184	1.7	4.9
M. musculus anti-digoxin immunoglobulin heavy chain variable region precursor	ET62206	2	4.7
Mouse CD19	M62553	3.3	-4.1
M.musculus (clone B6) myeloid secondary granule protein	L37297	-2	-4.3
M.musculus putative TNF-resistance related protein	U90926	-1.5	-6.5
Mouse mRNA for poliovirus receptor homolog protein soluble form	D26107	-3.7	-12.2
H. Other functions			
Mouse surfeit locus surfeit 3 protein	M14689	3.3	4.5
Mouse ERA-1-993	M22115	3.3	4.1
M.musculus imprinted in placenta and liver (Ipl)	AF002708	-1.7	-4.1
M.musculus H19	X58196	2.6	-5.9

4.3 Some of the genes differentially expressed in *hmga1*–knockout ES cells depend on Hmga1 expression also in other cells and tissues

We next verified whether the genes differentially expressed in *hmga1*-knockout ES cells showed a differential expression also in embryonic fibroblasts isolated from *hmga1*-knockout mice. A semiquantitative RT-PCR analysis showed that the differential expression of some genes, i.e., D-9k, gly 96 and LKLF in *hmga1*-/- fibroblasts matched that found in *hmga1*-knockout ES cells, whereas the expression of other genes did not (Figure 5). We next evaluated the expression of the HMGA1-regulated genes in adult heart, liver and spleen tissue from *hmga1* +/- and *hmga1* -/- knockout mice.

Some genes, such as Id3 and p96 showed the same expression trend as in ES cells, being down-regulated and up-regulated by HMGA1, respectively, also in heart and spleen. Some other genes only changed in one type of tissue, for example, gly96 and LKLF expression was different only in spleen, TFEB and Laminin α1 only in heart. Interestingly, the regulation of some genes, such as TFEB and Laminin α1, in adult tissues was opposite to that found in ES cells. In fact, they were decreased in ES cells but increased in heart. These results suggest that HMGA1 function depends on the cellular context.

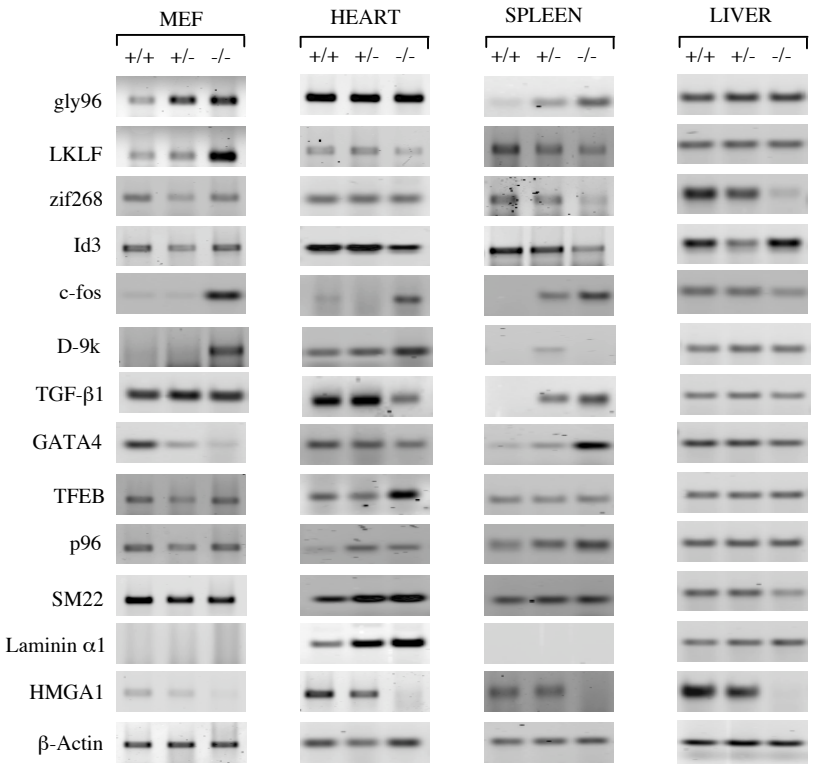


Figure 5 Gene expression in *hmga1*-knockout cells and tissues

It is known that by interacting with partner proteins, the HMGA1 proteins are able to enhance or suppress the effect of more "traditional" transcriptional activators and repressors. The fact that partner proteins are critical for HMGA1 activity, may account for the cell- and tissue- specific regulation exerted by the HMGA1 proteins.

4.4 Analysis of the HMGA1-dependent genes in transformed cells

We previously demonstrated that HMGA1 over-expression is a necessary event in cell transformation. In fact, when HMGA1 expression was blocked by transfecting rat thyroid cells (FRTL-5) with an antisense *hmga1* cDNA construct and infected with the Kirsten murine sarcoma virus (KiMSV) carrying the v-*ras*-*Ki* oncogene, they (FRTL-5-HMGA1as-KiMSV) did not acquire the typical markers of neoplastic transformation (ability to grow in soft agar and induce tumors after injection into athymic mice), even though the differentiation markers (i.e., TSH-dependency, ability to trap iodide, thyroglobulin synthesis and secretion) were lost. Conversely, the neoplastic markers were shown by the untransfected rat thyroid cells infected with the same murine retrovirus (FRTL-5-KiMSV). Therefore, as shown in Figure 6, we analyzed, by RT-PCR, the expression of some *hmga1*-dependent genes in FRTL-5 (lane 1), FRTL-5-KiMSV (lane 2) and FRTL-5-HMGA1as-KiMSV (lane 3) cells. The experiments revealed two sets of genes. Some genes showed the same regulation observed in the ES cells i.e., Carboxipeptidase E (Cpe) that decreased in *hmga1*-knockout ES cells and increased in the neoplastic cells or cathepsin H (ctsH) that increased in *hmga1*-knockout ES cells and decreased in the neoplastic cells compared to the wild type controls. Other genes were regulated in an opposite direction compared to ES cells, i.e., p96 and mac25 which demonstrated an increased and a decreased level, respectively, in both *hmga1*-knockout ES and FRTL5 KiMSV cells, compared to the respective controls, despite the fact that the HMGA1 proteins were expressed only in the latter cells.

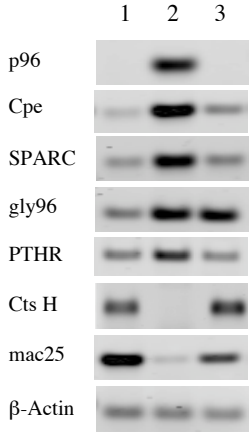


Figure 6 Gene expression in transformed cells over-expressing HMGA1

4.5 HMGA1 proteins bind to id3 and p96 promoters

The differential gene expression depending on the HMGA1 presence could depend on an indirect effect of the HMGA1 proteins in the sense that HMGA1 might induce some proteins which may interfere with the expression of some genes. To exclude this possibility and demonstrate a direct effect of HMGA1 on the regulation of some genes differentially expressed in *hmga1*-knockout cells, we performed EMSA and chromatin immunoprecipitation experiments.

We examined the Id3 and p96 genes because they were modified at RNA level in different cells and tissues and because their promoter regions contain AT-rich sequences that are a preferential binding site for the HMGA proteins.

To investigate whether the HMGA1 proteins were able to bind the AT-rich promoter regions of both Id3 and p96, we performed an electrophoretic mobility shift assay (EMSA) using oligonucleotides spanning nucleotides -632 to -615 of the murine Id3 promoter region and -901 to -872 of the 5' untranslated region of the murine p96 gene. As shown in Figure 7, a recombinant HMGA1 protein was able to bind directly to these regions. Binding specificity was demonstrated by competition experiments showing loss of binding with the addition of 200-fold molar excess of a specific, unlabeled oligonucleotide. Subsequently, we performed binding assays with total extract from wild type and *hmga1*-knockout murine spleens. Two specific complexes with a mobility corresponding to the HMGA1 proteins (isoforms A1a and A1b) were present in extracts from wild type and heterozygous (data not shown) spleens, while they were absent in extracts from homozygous *hmga1*-knockout mice. These complexes were specifically displaced by the incubation with an antibody directed against the HMGA1 proteins demonstrating that these complexes do consist of the HMGA1 proteins. A control gel shift for Sp1 was performed to normalize the spleen extracts used (Panel C).

To verify that HMGA1 proteins bind to Id3 and p96 promoters *in vivo* we performed experiments of chromatin immunoprecipitation in MEF from wild type and *hmga1*-knockout mice. Chromatin prepared as described under "Materials and Methods" was immunoprecipitated with anti-HMGA1 or normal rabbit IgG antibodies. The results shown in Figure 7D demonstrate that HMGA1 proteins bind to these promoters. In fact, the Id3 and p96 promoter regions were amplified from the DNA recovered with anti-HMGA1 antibody in wild type and *hmga1*+/- but not in *hmga1*-/- MEFs. Moreover, no amplification was observed in samples immunoprecipitated with normal rabbit IgG.

We retain of particular interest the finding that p96 and Id3 are regulated by the HMGA1 proteins since they are believed to have a critical role in the process of carcinogenesis. In fact, even though no putative alterations on Id genes have identified in primary human tumours to date to certify Ids as true cellular proto-oncogenes, Id proteins, that are basic helix-loop-helix transcription factors, have been implicated in regulating a variety of cellular

processes, such as cellular growth, senescence, differentiation, apoptosis, angiogenesis, that regulate tumorigenesis (Sikder et al. 2003). In particular Id3 has been frequently found increased in human neoplasias. Equally, p96, a mitogen-responsive phosphoprotein cloned from a mouse macrophage cell expression library, is consistently down-regulated in mouse mammary carcinogenesis and in human ovarian carcinomas as compared to normal surface epithelium (Schwahn and Medina 1998, Mok et al. 1998). It is likely that Id3 up-regulation and p96 down-regulation in human neoplasias depends also on the HMGA1 over-expression, a feature of most of the human malignant neoplasias (Fedele et al. 2001).

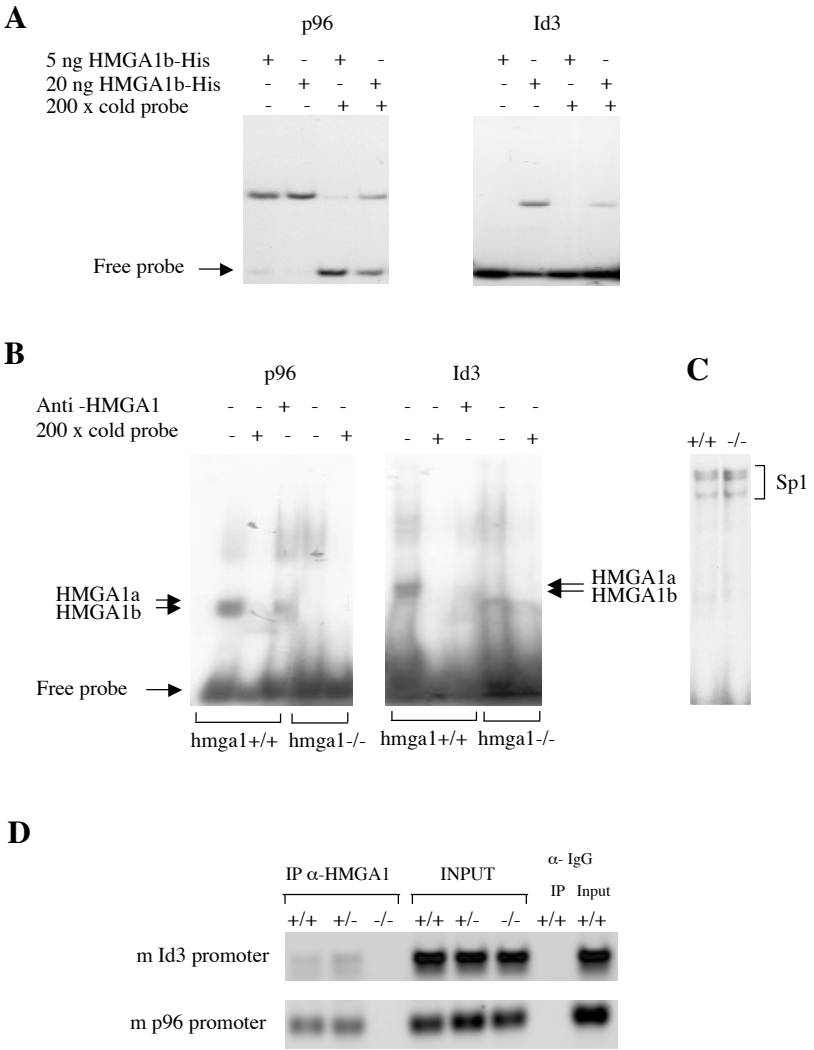


Figure 7 HMGA1 proteins bind Id3 and p96 promoters *in vitro* and *in vivo*.

4.6 Loss of HMGA1 correlates with an increased *Hand1* expression in murine ES cells, heart and thyroid tissues

Microarray analysis of the expression profile of embryonic stem (ES) cells bearing one or two disrupted *hmga1* alleles revealed the *Hand1* gene as a gene likely negatively regulated by HMGA1. In fact, the chip showed a 3,8 fold-change for heterozygous cells and a 14 fold-change for homozygous cells (Martinez Hoyos et al. 2004). Therefore, our first aim was to validate the results obtained by microarray analysis by semiquantitative and quantitative RT-PCR (Figure 8A and 8B). These analyses confirmed the differential expression between wild type and *hmga1*-knockout ES cells. They clearly showed that regulation of *Hand1* expression was HMGA1-dose dependent since an intermediate level of *Hand1* expression was observed in the heterozygous ES cells.

The analysis of the *Hand1* expression in heart and thyroid tissues derived from *hmga1*-knockout mice revealed the same kind of regulation by HMGA1 (Figure 8C). Conversely, no changes in *Hand1* expression were observed depending on the *Hmga1* expression when embryonic fibroblasts, brain, spleen, liver, kidney, pancreas and thymus from *hmga1* minus mice were analyzed (data not shown). This confirmed that HMGA1-mediated gene regulation depends on the cellular context. Interestingly, when we analyzed same tissues from the *Hmga2* minus mice, no changes in *Hand1* expression were observed indicating that *Hand1* regulation was HMGA1 specific.

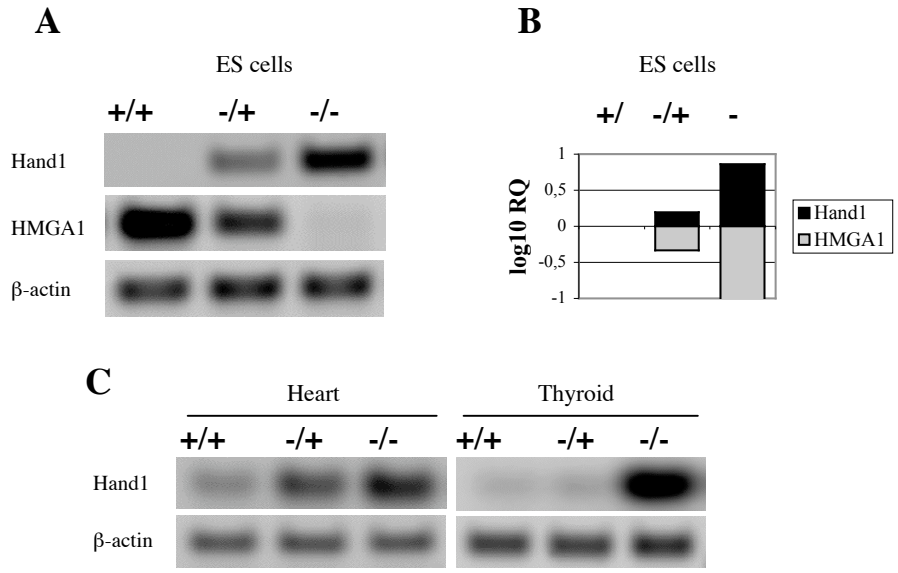


Figure 8 Hand1 expression in *hmga1* knockout cells and tissues

4.7 HMGA1 proteins bind to murine Hand1 promoter

To evaluate whether the differential gene expression was a direct effect of HMGA1 we performed an electrophoretic mobility shift assay (EMSA). In particular, we analyzed a region spanning nucleotides -2658 to -2688 of the 5' untranslated region of the murine *Hand1* gene containing AT-rich putative HMGA1 binding sites. As shown in Figure 9A, a recombinant HMGA1 protein was able to bind directly to this region. Binding specificity was demonstrated by competition experiments showing loss of binding with the addition of 200-fold molar excess of specific, unlabeled oligonucleotides. Subsequently, we performed binding assays with total extract from wild type and *hmga1*-knockout ES cells. A specific complex with a mobility corresponding to the HMGA1 proteins was present in extracts from wild type while it was absent in extracts from homozygous *hmga1*-knockout ES cells (Figure 9B). To verify that HMGA1 proteins bind to *Hand1* promoter *in vivo* we performed experiments of chromatin immunoprecipitation. Anti-HMGA1 antibodies precipitated *Hand1* promoter from *hmga1* +/+ and +/- ES cells, but not from *hmga1* -/- ES cells (Figure 9C).

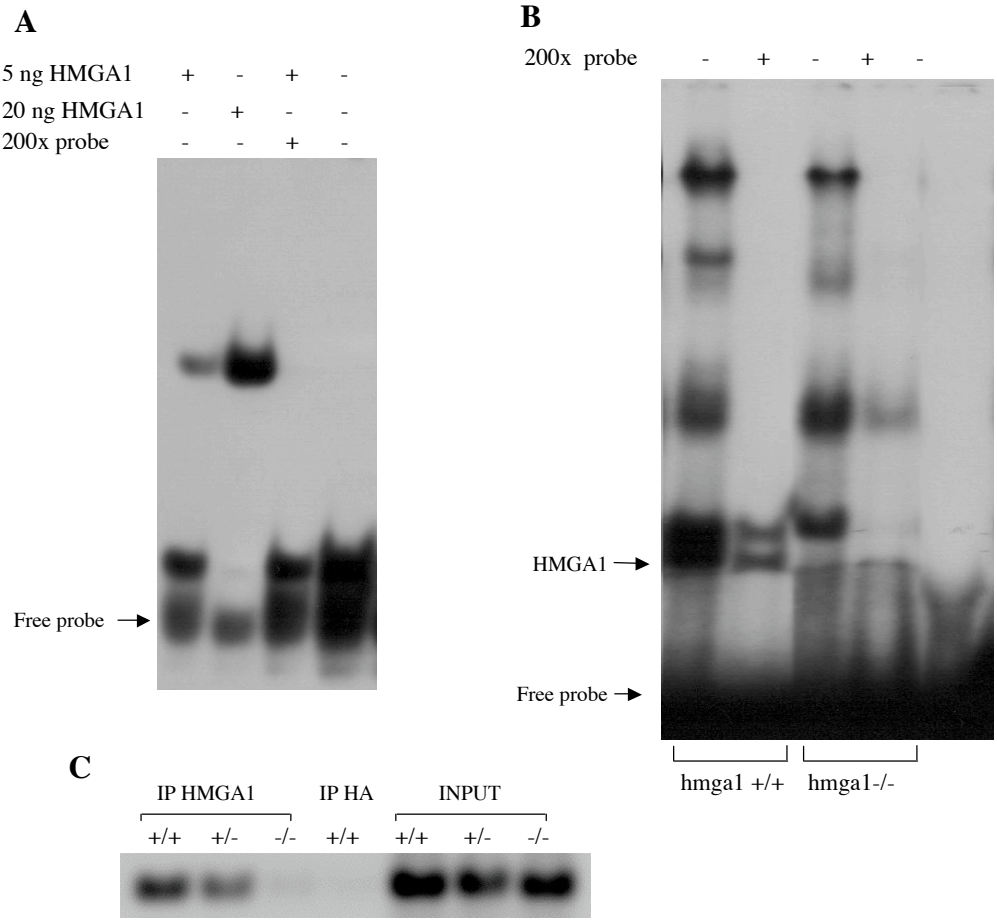


Figure 9 HMGA1 binding to Hand1 upstream regulating region

4.8 HMGA1 proteins repress the murine *Hand1* promoter

In order to investigate the functional effect of HMGA1 proteins on *Hand1* promoter, we transiently transfected the rat thyroid cells, FRTL-5, that express low levels of HMGA1 with a construct expressing the luciferase gene under the control of the mouse Hand1 promoter region -2424 – -2728. As shown in Figure 10A, when the HMGA1 expression vector was transfected, a reduction of the luciferase activity was observed in a dose-dependent manner. No decrease in *Hand1* promoter activity was obtained when the cells were transfected with a construct expressing HMGA2 the other member of the HMGA family. Then, we generated two point mutations in the putative binding site for the HMGA1 protein replacing adenine -2681 and thymidine -2682 with two guanines. Over-expression of HMGA1 was able to reduce the activity of the wild type construct but it completely failed in its inhibitory effect if the HMGA1 binding site of this construct was mutated (Figure 10B).

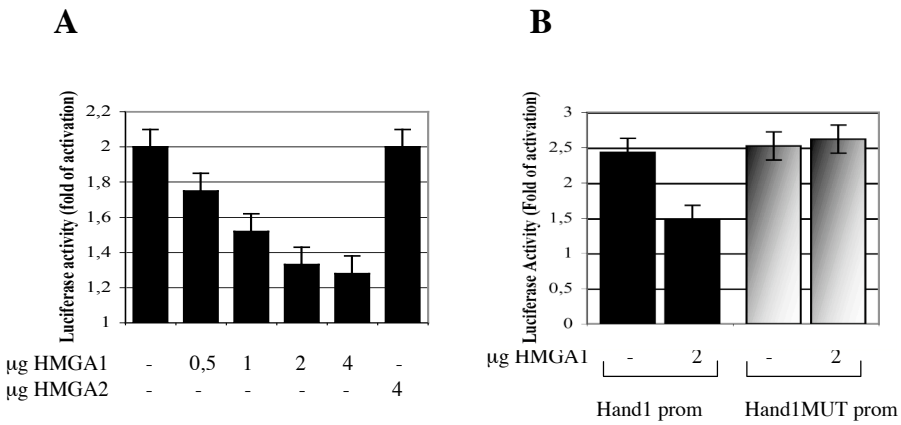


Figure 10 Repression of *Hand1* promoter activity by HMGA1 proteins

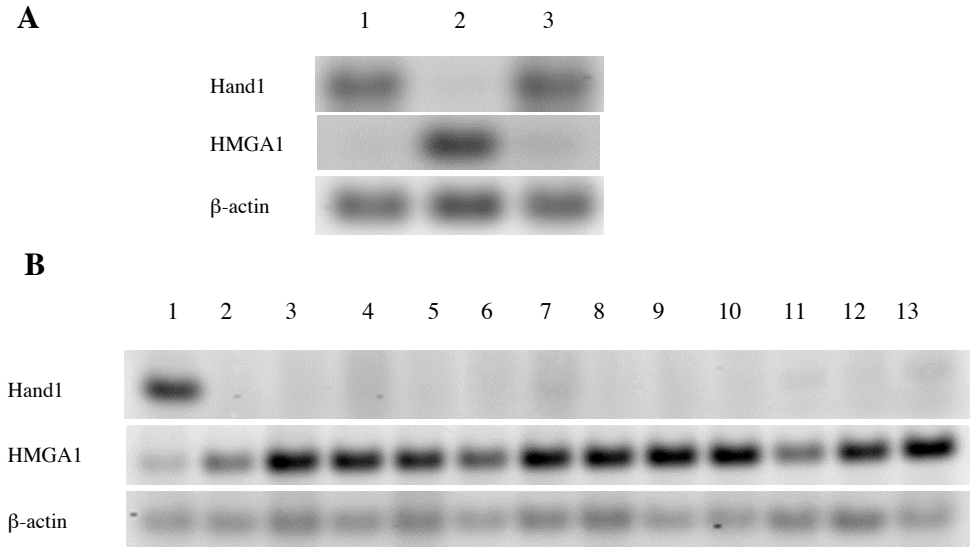
4.9 *Hand1* expression is decreased in thyroid cell transformation

The HMGA1 proteins are over-expressed in several malignant neoplasias. Then, in order to evaluate a possible role of *Hand1* regulation by HMGA1 in cell transformation, we analyzed, by RT-PCR, the expression of *Hand1* in FRTL-5 normal thyroid cells which do not express significant levels of HMGA1, in the same cells transformed by the Kirsten murine sarcoma virus, which express high levels of HMGA1 (FRTL-5 –KiMSV) and in the FRTL-5 cells which have been transfected with an *hmga1* antisense construct before being infected by Ki-MSV (FRTL-5-HMGA1as-KiMSV cells), and that do not

express the HMGA1 proteins. A significant *Hand1* expression was observed in the cells which do not express the HMGA1 proteins, such as the FRTL-5 and FRTL-5-HMGA1as-KiMSV, whereas no expression was observed in the FRTL-KiMSV cells expressing high levels of HMGA1 proteins (Figure 11A). These results confirm an inverse correlation between HMGA1 and Hand1 expression also in transformed cells.

Subsequently, we analyzed the expression of HMGA1 and Hand1 in a panel of thirteen human thyroid carcinoma-derived cell lines. An inverse correlation between HMGA1 and Hand1 expression levels was observed. In fact, HMGA1 expression was increased in all tumor-derived cell lines tested when compared to normal primary cultured cells used as a control (Figure 11B); conversely, HAND1 mRNA expression level was much lower in all of the thyroid carcinoma cell lines compared to normal cells (Figure 11B).

Finally, Hand1 and HMGA1 gene expression was examined in 20 surgically removed human thyroid carcinomas by real time PCR (Figure 11C). Again, an inverse correlation between HMGA1 and Hand1 mRNA levels was observed. In fact, HMGA1 mRNA levels were almost undetectable in normal thyroid tissue, whereas they were highly expressed in most of the tumors analyzed. In contrast, Hand1 expression was strongly diminished in all tumor samples compared to normal thyroid tissue.



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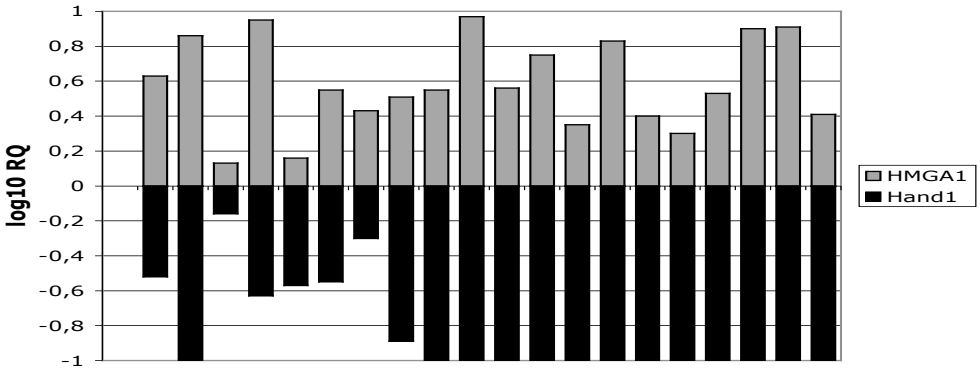

Figure 11 Hand1 expression in thyroid tumorigenesis. (A) Hand1 expression in transformed rat thyroid cells over-expressing HMGA1 by semiquantitative RT-PCR. Sources of RNA are FRTL-5, rat thyroid epithelial cell line (lane 1); FRTL-5-KiMSV, FRTL-5 infected with the Kirsten murine sarcoma virus (lane 2); FRTL-5-HMGA1as-KiMSV, FRTL-5 transfected with a construct carrying HMGA1 mRNA in an antisense orientation, and then infected with the Kirsten murine sarcoma virus (lane 3). (B) Hand1 expression in human thyroid carcinoma cell lines by semiquantitative RT-PCR (C) human thyroid tumors by real- time PCR.

4.10 Down-regulation of HAND1 expression in human thyroid carcinomas is not associated to LOH and DNA methylation

We have analyzed the methylation status of three thyroid carcinoma samples showing the lowest HAND1 expression and normal thyroid. We have analyzed the region of the 5' untranslated region of the human HAND1 gene, described under Materials and Methods. This region was unmethylated in normal and carcinoma samples indicating that the silencing of Hand1 gene in neoplastic samples is not dependent on the gene promoter hypermethylation.

Therefore, we analyzed the same three thyroid carcinoma samples for Loss of Heterozygosity (LOH). We analyzed eight Single Nucleotide Polymorphisms: however no LOH was found.

Therefore, these results would suggest that it is likely that the HMGA1-negative regulation plays a critical role in Hand1 gene suppression in human thyroid carcinomas.

4.11 Restoration of Hand1 gene expression inhibits the growth of thyroid carcinoma cell lines

To determine whether loss of *Hand1* gene expression affects thyroid carcinogenesis, we evaluated the growth rate of thyroid carcinoma cell lines in which *Hand1* expression had been restored. To this aim we carried out a colony forming assay with a cell line obtained from human thyroid carcinomas (FB-2) after transfection with the vector carrying the *Hand1* gene or the empty backbone vector. The colonies were scored after two weeks. Cells transfected with the *Hand1* gene generated a lower number of colonies than cells transfected with the backbone vector (Figure 12).

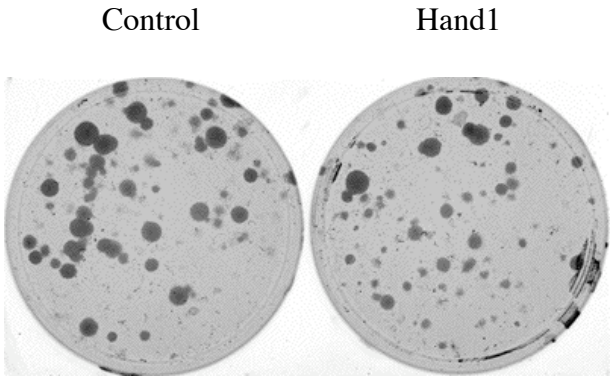

Figure 12 Restoration of Hand1 gene expression reduces clonogenic capacities of thyroid cancer cells.

5 CONCLUSIONS

- We have identified new genes regulated by HMGA1 proteins.

- HMGA1 proteins are implicated in many different pathways since we have found that the genes regulated by HMGA1 proteins belong to diverse families and have very diverse roles.

- HMGA1 exert positive and negative gene regulation since we have found some of the genes up-regulated and some down-regulated by HMGA1 proteins.

- We have found that the regulation is tissue-specific since likely depending on the multiprotein complex in which HMGA1 proteins are inserted.

- We have demonstrated that the regulation is direct in some cases, such as *Id3*, *p96* and *Hand1*.

- For Hand1, we have studied the role in transformation and we show evidence that *Hand1* down-regulation by HMGA1 proteins may account for the malignant phenotype in human thyroid carcinomas.

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Identification of the Genes Up- and Down-Regulated by the High Mobility Group A1 (HMGA1) Proteins: Tissue Specificity of the HMGA1-Dependent Gene Regulation

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ABSTRACT

High mobility group A (HMGA) proteins are chromatinic proteins that do not have transcriptional activity *per se*, however, by interacting with the transcription machinery, they regulate, negatively or positively, the expression of several genes. We searched for genes regulated by HMGA1 proteins using microarray analysis in embryonic stem (ES) cells bearing one or two disrupted *hmga1* alleles. We identified 87 transcripts increased and 163 transcripts decreased of at least 4-fold in *hmga1*−/− ES cells. For some of them, a HMGA1-dose dependency was observed, because an intermediate level was observed in the heterozygous ES cells. When the expression analysis of these genes was extended to embryonic fibroblasts and adult tissues such as heart, spleen, and liver from *hmga1*-knockout mice, contrasting results were obtained. In fact, aside some genes showing the same HMGA1 regulation observed in ES cells, there were some genes that did not modify their expression, and others showing a HMGA1-mediated regulation but in an opposite direction. These results clearly indicate that HMGA1-mediated gene regulation depends on the cellular context. Finally for a couple of analyzed HMGA1-regulated genes, electrophoretic mobility shift assay and chromatin immunoprecipitation revealed a direct binding of HMGA1 proteins to their promoters, suggesting a HMGA1-direct regulation of their expression.

INTRODUCTION

The high mobility group A (HMGA) protein family includes HMGA1a and HMGA1b, which are encoded by *hmga1* through alternative splicing (1) and the closely related HMGA2 protein (2). These proteins bind the minor groove of AT-rich DNA sequences. Their DNA-binding domain is located in the NH_2-terminal region of the protein and contains three short basic repeats, the so-called "AT-hooks." The mammalian HMGA proteins have long been known to play key roles in chromatin architecture and gene control by serving as generalized chromatin effectors, either enhancing or suppressing the ability of more usual transcriptional activators and repressors to act within the confines of chromatinized DNA (3, 4).

HMGA1 proteins seem to play their major physiological role during embryonic development (5). In fact, HMGA1 expression is very high during embryogenesis, whereas it is negligible in normal adult tissues. HMGA1 proteins has been found abundant in malignant neoplasias (6), where their expression appears critical for the acquisition of the neoplastic phenotype (7, 8).

To identify the differentiation pathways in which HMGA1 is involved and to assess the role of the HMGA1 proteins in development, we generated embryonic stem (ES) cells in which one or both *hmga1* alleles are disrupted. We reported recently that *hmga1*−/− ES cells generate less T-cell precursors than do wild-type ES cells after *in vitro*-specific differentiation. Indeed, they preferentially differentiate to B cells, probably consequent to decreased IL-2 expression and increased IL-6 expression, both of which are regulated directly by the HMGA1 proteins (9). Moreover, a lack of HMGA1 expression results in altered hemopoietic differentiation (*i.e.*, there is a reduction in the monocyte/macrophage population and an increase in megakaryocyte precursors, erythropoiesis, and globin gene expression). Re-expression of the *hmga1* gene in *hmga1*−/− ES cells restores the wild-type phenotype (9). These results indicate that drastic changes occur in the transcriptional activity of the *hmga1*−/− cells, and presumably they depend on the modification of the expression of HMGA1-regulated genes.

Using the powerful oligonucleotide microarray hybridization technique, we analyzed the expression profile of ES cells carrying two, one, and no *hmga1* functional alleles to identify the genes that are regulated, positively or negatively, by HMGA1. We screened an array in which 13,059 transcripts were represented, and we identified 87 transcripts that increased and 163 transcripts that decreased with a ≥4-fold change in hmga1−/− ES cells with respect to the wild-type ES cells. Semiquantitative and quantitative reverse transcription (RT)-PCR confirmed the differential expression between wild-type and *hmga1*-knockout ES cells. We obtained different results when we measured the expression of these genes in murine embryonic fibroblasts (MEF) and various adult tissues from *hmga1* knockout mice. The differential expression of some genes matched that found in ES cells, whereas the expression of other genes was either unchanged or opposite to that found in ES cells. Finally, electrophoretic mobility shift assay and chromatin immunoprecipitation experiments demonstrated that HMGA1 proteins bind to the promoters of some representative HMGA1-regulated genes, indicating a direct role of HMGA1 in the regulation of their transcription.

MATERIALS AND METHODS

Cell Culture. The generation and the culture of *hmga1*+/− and *hmga1*−/− ES cells are described elsewhere (9). MEF have been established from wild-type, *hmga1*+/− and *hmga1*−/− embryos 12.5 days post-coitum following standard procedures. They were grown in DMEM medium supplemented with 10% FCS, glutamine and non-essential aminoacids (Life Technologies, Inc.) in a 7% CO_2 atmosphere. Functioning rat thyroid line (FRTL)-5, FRTL-5-KiMSV and FRTL-5–HMGA1as-KiMSV cells, and their culture conditions are reported elsewhere (8).

RNA Extraction from Tissues and Cells. Tissues were snap-frozen in liquid nitrogen and stored at −80°C until use. Total RNAs were extracted from tissues and cell culture using TRI REAGENT (Molecular Research Center, Inc.) solution, according to the manufacturer's instructions. The integrity of the

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RNA was assessed by denaturing agarose gel electrophoresis (virtual presence of sharp 28S and 18S bands) and spectrophotometry.

Microarray Analysis. Microarray analysis was performed as described previously in detail.[5] Briefly, cRNA was prepared from 8 μg of total RNA, hybridized to MG-U74 Affymetrix oligonucleotide arrays (containing 13,059 murine transcripts), scanned, and analyzed according to Affymetrix (Santa Clara, CA) protocols. Scanned image files were visually inspected for artifacts and normalized by using GENECHIP 3.3 software (Affymetrix). Comparisons were made for each mutated sample *versus* wild-type sample, taking the wild-type sample as baseline by using GENECHIP 3.3. The fold-change values, indicating the relative change in the expression levels between mutated samples and the wild-type sample, were used to identify genes differentially expressed between these conditions.

Semiquantitative and Quantitative RT-PCR. RNAs were treated with DNaseI (Invitrogen) and reverse-transcribed using random exonucleotides as primers and MuLV-reverse transcriptase (Perkin-Elmer). To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing PCR on samples that were not reversed-transcribed but otherwise identically processed.

The PCRs were performed with the same RNAs used for array analysis, and the primers sequences are available upon request. For semiquantitative 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, and scanned using a Typhoon 9200 scanner. Digitized data were analyzed using Imagequant (Molecular Dynamics).

Quantitative PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) as follows: 95°C 10 minutes and 40 cycles (95°C 15 seconds and 60°C 1 minute).

Protein Extraction, Western Blotting, and Antibodies. Tissues and cell culture were lysed in buffer 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 Diagnostic Corp.). Total proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% nonfat milk and incubated with antibodies against Id3 and tubulin. All of them were purchased from Santa Cruz Biotechnology. Bound antibody was detected by the appropriate secondary antibody and revealed with an enhanced chemiluminescence system (Amersham-Pharmacia Biotech).

Electrophoretic Mobility-Shift Assay. DNA-binding assays with the purified proteins were performed as described previously (10). Five to 20 ng of recombinant protein were incubated in the presence of radiolabeled oligonucleotide. A 200-fold excess of specific unlabeled competitor oligonucleotide was added. The double-strand oligonucleotides used were Id3 spanning from base −632 to −615 of the murine Id3 promoter region, (5′-tgatttttttttttttt-tcaaatctg-3′; ref. 11) and p96 spanning from base −901 to −872 of the 5′ untranslated region of the murine p96 gene (5′-aagaaatatttgatatttttttcttttatcc-3′; Ref. 12).

The same oligonucleotides were also used in binding assays with total extract from wild-type and *hmga1*-knockout murine spleen tissues. Eight micrograms of extracts were incubated in 20 mmol/L HEPES (pH 7.6), 40 mmol/L KCl, 0.1 mmol/L EDTA, 0.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT, and 0.1 mmol/L phenylmethylsulfonyl fluoride in a volume of 20 μl containing 1 μg of poly(dC-dG), 2 μg of BSA, and 10% glycerol, for 10 minutes at room temperature. Binding reactions were incubated for 10 minutes after the addition of 2.5 fmol of a ^{32}P-end-labeled oligonucleotide (specific activity, 8,000–20,000 cpm/fmol). For the antibody supershift analysis, the reactions were performed by preincubating extracts with 0.5 μg of antibody anti-HMGA1 (Santa Cruz Biotechnology) on ice for a minimum of 30 minutes.

The DNA-protein complexes were resolved on 6% non-denaturing acrylamide gels and visualized by exposure to autoradiographic films.

Chromatin Immunoprecipitation. Approximately 3×10^7 wild-type and *hmga1*-knockout MEF were grown on 75-cm^2 dishes and cross-linked by the addition of formaldehyde (to 1% final concentration) to attached cells. Cross-linking was allowed to proceed at room temperature for 5 minutes and was terminated with glycine (final concentration, 0.125 mol/L). Cells were collected and lysed in buffer containing 5 mmol/L PIPES (pH 8.0), 85 mmol/L KCl, 0.5% NP40, and protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin), on ice for 10 minutes. Nuclei were pelleted by centrifugation at 5,000 rpm for 5 minutes at 4°C and resuspended in buffer containing 50 mmol/L Tris-Cl (pH 8.1), 10 mmol/L EDTA, 1% SDS, the same protease inhibitors, and incubated on ice for 10 minutes. Chromatin was sonicated on ice to an average length of about 400 bp with a Branson sonicator model 250. Samples were centrifuged at 14,000 rpm for 10 minutes at 4°C. Chromatin was pre-cleared with protein G Sepharose (blocked previously with 1 mg/ml BSA) at 4°C for 2 hours. Pre-cleared chromatin of each sample was incubated with 2 μg of antibody anti-HMGA1 at 4°C overnight. An aliquot of wild-type sample was incubated also with anti-IgG antibody. Next, 60 μl of a 50% slurry of blocked protein G Sepharose was added, and immune complexes were recovered. The supernatants were saved as "input." Immunoprecipitates were washed twice with 2 mmol/L EDTA, 50 mmol/L Tris-Cl (pH 8.0) buffer and 4 times with 100 mmol/L Tris-Cl (pH 8.0), 500 mmol/L LiCl, 1% NP40, and 1% deoxycholic acid buffer. The antibody-bound chromatin was eluted from the beads with 200 μl of elution buffer (50 mmol/L NaHCO3, 1% SDS). Samples were incubated at 67°C for 5 hours in the presence of 10 μg RNase and NaCl to a final concentration of 0.3 mol/L to reverse formaldehyde cross-links. Samples were then precipitated with ethanol at −20°C overnight. Pellets were resuspended in 10 mmol/L Tris (pH 8)-1 mM EDTA and treated with proteinase K to a final concentration of 0.5 mg/ml at 45°C for 1 hour. DNA was extracted with phenol/chloroform/isoamyl alcohol, ethanol-precipitated, and resuspended in water. Input DNA and immunoprecipitated DNAs were analyzed by PCR for the presence of Id3 and p96 promoter sequences. PCR reactions were performed with AmpliTaq gold DNA polymerase (Perkin-Elmer). The primers used to amplify the sequence of the Id3 promoter were 5′-agggtttatgcagcaag-cac-3′ (forward) and 5′-atttgctgctcgtctgacct-3′ (reverse). The primers used to amplify the sequence of the p96 promoter were 5′-aactccagctgtgtcaagtt-3′ (forward) and 5′-gaaagaaagagaggggaaag-3′ (reverse). PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and scanned using a Typhoon 9200 scanner.

RESULTS

Gene Expression Profile Analysis. RNAs extracted from wild-type, hmga1+/− and hmga1−/− ES cells were hybridized to MG-U74 Affymetrix oligonucleotide arrays containing 13,059 transcripts. The expression profile of the heterozygous and homozygous ES cells was compared with that of the wild-type ES cells that were used as a common reference. The number of transcripts increased or decreased in the heterozygous and the homozygous mutant versus wild-type sample is shown in the Fig. 1. Of the 13,059 transcripts represented on the array, 1,300 had a 2- to 3-, 313 had a 3- to 4-, 227 had a 4- to 10-,

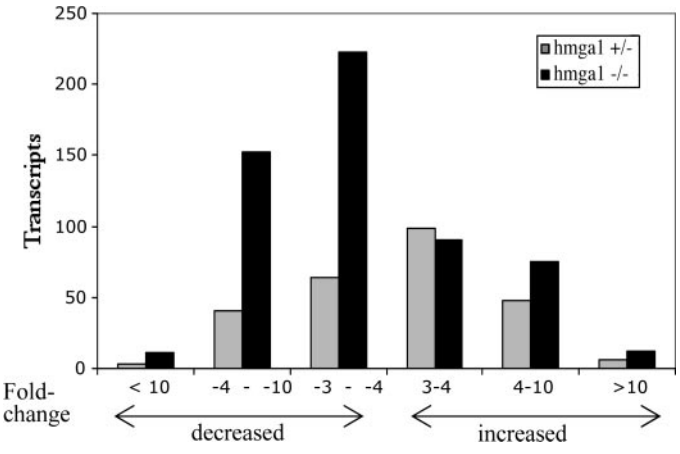

Fig. 1. Gene expression profile in ES cells carrying one or two *hmga1* disrupted alleles. The expression profile of the heterozygous and the homozygous *hmga1*-knockout ES cells was compared with that of the wild-type ES cells that were used as a common reference. Transcripts are grouped according to fold change.

[5] http://www.cancergenetics.med.ohio-state.edu/microarray.

Table 1 *The genes differentially expressed with a ≥4-fold change in heterozygous (+/−) and homozygous (−/−) hmga1-knockout ES cells were grouped in families*

Description	GenBank accession no.	FC +/−	FC −/−
A. Signal transduction			
Mus musculus parathyroid hormone/parathyroid hormone related-peptide receptor (PTHR) gene, exons 10–15, complete cds.	L34611	1.1	18.4
M. musculus mRNA for ryudocan core protein, complete cds.	D89571	3.9	10.6
M. musculus mitogen-responsive M_r 96,000 phosphoprotein p96 mRNA, alternatively spliced p67 mRNA, and alternatively spliced p93 mRNA, complete cds.	U18869	1.7	9.3
M. musculus mRNA for MAP kinase-activated protein kinase 2	BC063064	10	4.7
Mouse Wnt-6 mRNA, complete cds.	M89800	1.1	4.1
M. musculus JIP-1 (JIP-1) mRNA, complete cds.	AF003115	−1.7	−4
Murine macrophage gene, encoding bmk (B cell/myeloid kinase).	J03023	−1.4	−4.1
M. musculus TGF-β-inducible protein (TSC-36) mRNA, complete cds.	M91380	1.3	−4.2
M. musculus mRNA for retinoic acid receptor-α.	X57528	1.1	−4.3
Mouse growth factor-inducible protein (pip92) mRNA, complete cds.	M59821	−2.5	−4.4
Mouse oxytocin-neurophysin I gene, complete cds.	M88355	1.7	−4.5
M. musculus-patched mRNA, complete cds	U46155	−1.2	−4.6
Mouse G protein α subunit (GNA-15) mRNA, complete cds.	M80632	−9.4	−4.7
Mouse mRNA for NBL4, complete cds.	D28818	−2.1	−5.1
M. musculus receptor protein tyrosine phosphatase-λ (ptp-λ) mRNA, complete cds.	U55057	1.3	−5.4
M. musculus protein-serine/threonine kinase (pim-2) mRNA, complete cds.	L41495	−1.7	−5.6
Mouse (clone M1) GTPase (Ran) mRNA, complete cds	L32751	2.6	−5.7
M. musculus ShcC mRNA, complete cds.	U46854	1.3	−6.3
M. musculus syk mRNA for protein-tyrosine kinase.	Z49877	−1.3	−6.3
M. musculus c-Src kinase (Csk) mRNA, complete cds.	U05247	−1.6	−8.6
B. Transcription factors			
M. musculus Thing1 mRNA, complete cds.	U21226	3.8	14
House mouse; Musculus domesticus adult testis mRNA for truncated form of Sox17, complete cds.	D49473	1.3	13.7
M. musculus melanocyte-specific gene 1 (msg1) mRNA, complete cds.	U65091	1.4	9.7
M. musculus mRNA for fos-related antigen-2	X83971	3.1	4
M. musculus Sox4 (Sox4) mRNA, partial cds.	ET62444	−1.4	−4.1
Mouse c-fos oncogene.	V00727	−12	−4.2
Mouse c-myc gene exon 3.	L00039	−4.3	−4.3
M. musculus AP-2.2 gene.	X94694	−1.6	−4.4
Mouse mRNA for Zfp-57, complete cds.	D21850	−2.5	−4.6
M. musculus Kruppel-like factor LKLF mRNA, complete cds.	U25096	−1.4	−4.8
M. musculus transcription factor TFEB mRNA, partial cds.	U36393	−2.2	−4.9
M. musculus transcription factor NF-YC subunit mRNA, complete cds	U62297	−2.6	−5.2
M. musculus DNA binding protein NFI-X (NfiX) mRNA, partial cds	U57636	−1.9	−6.2
M. musculus mRNA for TIF1 β protein	X99644	−5.8	−6.2
M. musculus transcription factor junB (junB) gene, 5 region and complete cds.	U20735	−3.5	−6.4
M. musculus Oct-6 mRNA for octamer binding protein.	X57482	−8.1	−6.5
Mouse helix-loop-helix protein (Id related) mRNA, complete cds.	M60523	−5.3	−7.4
Mouse growth factor-induced protein (zif/268) mRNA, complete cds.	M22326	−8.2	−24.9
C. Cell proliferation			
M. musculus chop-10 mRNA.	X67083	15.1	16.9
Mouse D-type cyclin (CYL2) mRNA, complete cds.	M83749	−2.5	9
M. musculus gly96 mRNA.	X67644	4.4	8
M. musculus mRNA for zyxin.	Y07711	1.3	5.3
D. Extracellular-matrix and cellular-structure			
M. musculus laminin A chain mRNA, complete cds.	J04064	1.2	14
M. musculus α-2 type IV collagen mRNA, complete cds.	J04695	1.4	9.8
M. musculus α-1 type IV collagen (Col4a-1) mRNA, complete cds.	J04694	1.4	9.1
Mouse α-B2-crystallin gene, complete cds	M73741	1.4	7.7
Mouse laminin B1 mRNA, complete cds.	M15525	1.3	6.3
Mouse mRNA for cysteine-rich glycoprotein SPARC	NM_009242	1.2	5.5
Mouse gelsolin gene, complete cds.	J04953	1.5	4.5
M. musculus mRNA for hair keratin, mHb6.	X99143	3.5	4.1
Mouse α-B crystallin mRNA.	M63170	1	4
Mouse COL1A2 mRNA for pro-α-2(I) collagen.	X58251	−3.8	−4.1
M. musculus ρB gene.	X99963	−7.4	−4.2
M. musculus neurofibromatosis 2 (Nf-2) gene mRNA, complete cds.	L27090	1.3	−4.5
M. musculus SM22 α mRNA, complete cds.	L41154	−1.5	−4.5
Mouse COL1A2 mRNA for pro-α-2(I) collagen	NM_007743	−3.9	−4.7
M. musculus α 1 type I collagen gene, partial cds and 3 flanking region	U50767	−1.9	−5.6
M. musculus mRNA for myosin I	X97650	−4.6	−6.3
Mouse tau microtubule binding protein mRNA, complete cds.	M18776	−1.3	−6.6
M. musculus FVB/N collagen pro-α-1 type I chain mRNA, complete cds.	U08020	−2.1	−7.5
Mouse mRNA for vascular smooth muscle α-actin.	X13297	−2.1	−8.6
E. Metabolism, transport and secretion			
Mouse mRNA for mastocytoma proteoglycan core protein, serglycin.	X16133	2.6	40.9
M. musculus cathepsin H prepropeptide (ctsH) mRNA, complete cds.	U06119	1.5	17.3
M. musculus calcium binding protein D-9k mRNA, complete cds.	AF028071	2.3	9.8
Mouse serine protease inhibitor homologue (J6) mRNA, complete cds	J05609	1.7	8.7
M. musculus heparan sulfate D-glucosaminyl 3-*O*-sulfotransferase-1 precursor (3OST1) mRNA, alternatively spliced, complete cds.	AF019385	−1.4	6.5
Mouse mRNA for preproinsulin-like growth factor IA.	X04480	−1.5	6.2
M. musculus preprodipeptidyl peptidase I mRNA, complete cds.	U89269	1.2	5.9
M. musculus mRNA for cytoplasmic dynein heavy chain (partial, ID mdhc10).	ET63396	1.6	5.5
M. musculus steroid cytochrome p450 7-α hydroxylase mRNA, complete cds.	L06463	−1.1	−4
M. musculus very-long-chain acyl-CoA synthetase (VLCS) mRNA, complete cds.	AF033031	−2	−4
M. musculus (clone E31.1 in pGEM7Zf(+)) N-acetylglucosaminyltransferase I mRNA, complete cds.	L07037	2.5	−4.1
Mouse mRNA for inward rectifier K+ channel, complete cds.	D50581	1	−4.1
M. musculus preprocortistatin (Cort) mRNA, complete cds.	AF013253	2.7	−4.3
M. musculus extracellular superoxide dismutase (SOD3) mRNA, complete cds.	U38261	1.2	−4.6

Table 1 *Continued*

Description	GenBank accession no.	FC +/−	FC −/−
Mouse mRNA for a preprothyrotropin-releasing hormone.	X59387	−1.1	−4.7
M. musculus steroid sulfatase (Sts) mRNA, complete cds.	U37545	−2.3	−4.7
M. musculus mRNA for dihydropyrimidinase related protein 4, complete cds.	AB006715	5.7	−4.9
M. musculus putative chloride channel protein CLC6 (Clc6), exon 23 and complete cds.	AF030106	−1	−4.9
Mouse placental alkaline phospatase mRNA, complete cds.	J02980	−1.1	−4.9
M. musculus Balb/c cytochrome *c* oxidase subunit VIaH mRNA, complete cds.	U08439	3.1	−5
Mouse metallothionein-III gene, complete cds.	M93310	1.4	−5.3
M. musculus carboxypeptidase E (Cpe) mRNA, complete cds.	U23184	−3.5	−5.8
M. musculus hormone-sensitive lipase mRNA, complete cds.	U08188	−1.7	−8
M. musculus ADP-ribosylation factor-like protein 4 mRNA, complete cds.	U76546	−2.4	−8.7
F. Growth factors and related proteins			
M. musculus lefty gene.	AJ000083	−1.1	−4.3
M. musculus mRNA for insulin-like growth factor binding protein-3	X81581	−3.2	−6.4
M. musculus mRNA for insulin-like growth factor binding protein-4.	X81582	−1.4	−7.3
M. musculus acid labile subunit (ALS) gene, complete cds.	U66900	2.4	−7.4
Mouse Cyr61 mRNA, complete cds.	M32490	−4.2	−8.4
M. musculus follistatin-like protein (mac25) mRNA, complete cds.	L75822	−2.6	−11
G. Immune functions			
Mouse gene for M_r 47,000 heat shock protein(HSP47), exon 6.	D12907	1.1	5.7
M. musculus MHC class I B(2)-microglobulin gene (W4 allele), partial cds	M84366	1.2	5
Mouse lymphocyte differentiation antigen (Ly-6.2) mRNA, complete cds.	M18184	1.7	4.9
M. musculus anti-digoxin immunoglobulin heavy chain variable region precursor mRNA, partial cds.	ET62206	2	4.7
Mouse CD19 gene, exons 6–15.	M62553	3.3	−4.1
M. musculus (clone B6) myeloid secondary granule protein mRNA.	L37297	−2	−4.3
M. musculus putative TNF-resistance related protein mRNA, complete cds.	U90926	−1.5	−6.5
Mouse mRNA for poliovirus receptor homolog protein soluble form, complete cds.	D26107	−3.7	−12.2
H. Other functions			
Mouse surfeit locus surfeit 3 protein gene	M14689	3.3	4.5
Mouse ERA-1–993 mRNA, complete cds, and alternate ERA-1–339 mRNA, complete cds.	M22115	3.3	4.1
M. musculus imprinted in placenta and liver (Ipl) gene, complete cds.	AF002708	−1.7	−4.1
M. musculus H19 mRNA.	X58196	2.6	−5.9

Abbreviations: cds., coding sequence; FC, fold change.

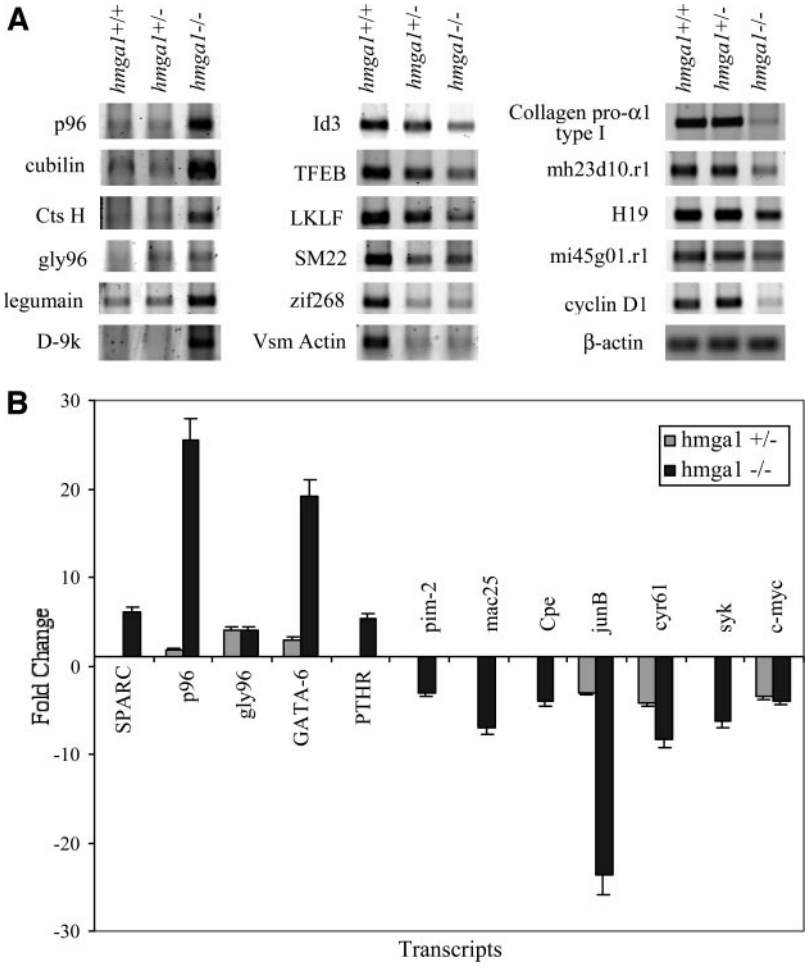

Fig. 2. Validation of microarray data by semiquantitative and quantitative RT-PCR. We confirmed some of the microarray data by semiquantitative (*A*) or quantitative (*B*) RT-PCR. Amplification of the *β*-actin gene has been evaluated as a control of the RNA amount used. *Cpe*, carboxypeptidase E.

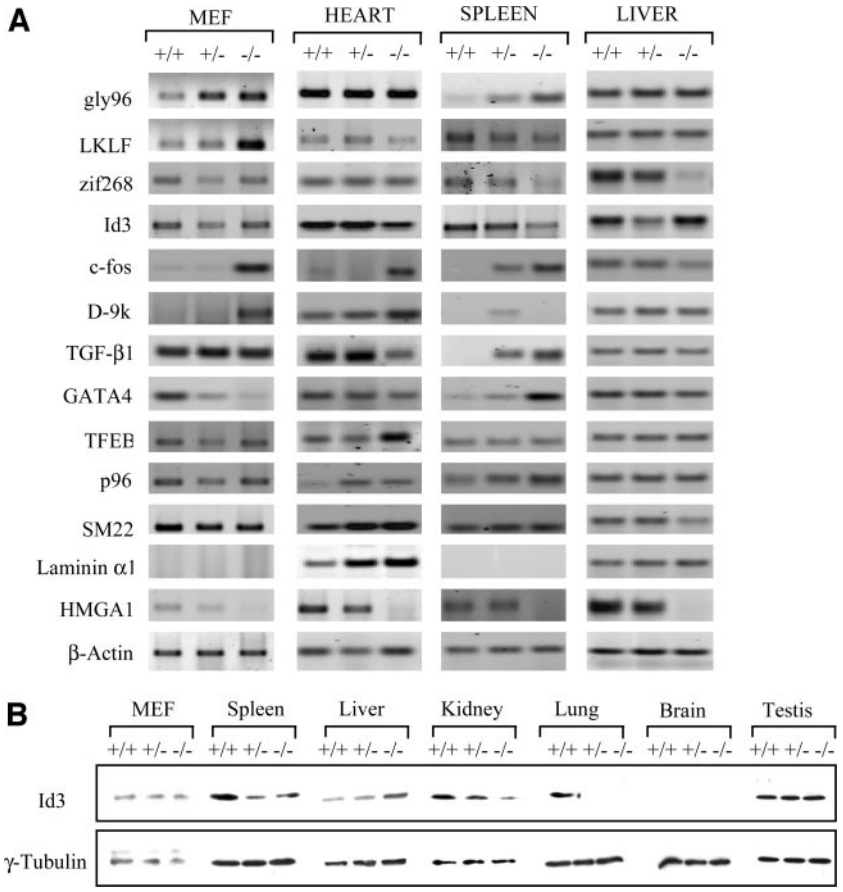

Fig. 3. Gene expression in *hmga1*-knockout cells and tissues. *A*, gene expression analysis by semiquantitative RT-PCR in *hmga1*-knockout MEFs and tissues. *β*-Actin has been used as a housekeeping gene to normalize the RNA amount used. *B*, protein expression analysis by Western blot in *hmga1*-knockout MEFs and tissues. As a control for equal protein loading, the blotted proteins were incubated with γ-tubulin-specific antibodies. The sources of proteins are indicated. *Cpe*, carboxypeptidase E.

and 23 had a >10-fold change. We examined the 250 transcripts (1.9%) that had a ≥4-fold-change in the homozygous mutant versus the wild-type sample. Among these 250 transcripts, 87 were increased and 163 were decreased, including 103 known genes (37 increased and 66 decreased), 118 expression sequence tags (ESTs) (40 increased and 78 decreased), and 29 unknown genes (10 increased and 19 decreased). As a control of microarray analysis, we verified that the HMGA1 was not expressed in hmga1−/− ES cells. The genes with ≥4-fold change in hmga1−/− ES cells were grouped according to their function: (*a*) signal-transduction pathways, (*b*) transcription factors, (*c*) cell proliferation, (*d*) extracellular-matrix and cellular-structure proteins, (*e*) metabolic pathways, transport and secretion, (*f*) growth factors and related proteins, (*g*) genes with immune functions, and (*h*) other genes. The relative fold changes in these genes, grouped as described above, are shown in Table 1. It is noteworthy that among the HMGA1-regulated genes, we found Id3 (13), lefty (14), and Wnt-6 (15) that are important in embryonic development and some oncogenes such as c-myc, junB, pim-2, and c-fos.

Validation of Microarray Analysis. To validate the results obtained by microarray analysis we evaluated the expression of 50 transcripts by semiquantitative RT-PCR in the wild-type, hmga1+/− and hmga1−/− ES cells. For all of them, RT-PCR analysis confirmed the differential expression associated with the expression of the HMGA1 proteins. Some representative RT-PCR analysis are shown in Fig. 2*A*. The expression of some genes (*i.e.*, TFEB, LKLF, and Id3) was hmga1 dose-dependent. In fact, the changes in hmga1+/− ES cells were intermediate between those found in wild-type and hmga1−/− ES cells. Conversely, the expression of other genes (*i.e.*, cubilin, p96, D-9K, legumain, and collagen) was not modified in hmga1+/− ES cells in comparison to the wild-type ES cells. We also analyzed 12 genes with quantitative RT-PCR. The results coincide with those of the microarray analysis (Fig. 2*B*). The primers used for semiquantitative and quantitative PCR are available upon request.

Some, but Not All, of the Genes Differentially Expressed in Wild-Type and *hmga1*-Knockout ES Cells Depend on HMGA1 Expression in Other Cells and Tissues. We next verified whether the genes differentially expressed in hm*ga1*-knockout ES cells showed a differential expression also in embryonic fibroblasts isolated from *hmga1*-knockout mice. A semiquantitative RT-PCR analysis showed that the differential expression of some genes (*i.e.*, D-9k, gly 96, and LKLF) in *hmga1*−/− fibroblasts matched that found in *hmga1*-knockout ES cells, whereas the expression of other genes did not (Fig. 3*A*). We next evaluated the expression of the HMGA1-regulated genes in adult heart, liver, and spleen tissue from *hmga1*+/− and *hmga1*−/− mice.

Some genes, such as Id3 and p96, showed the same expression trend as in ES cells, being down-regulated and up-regulated by HMGA1, respectively, also in heart and spleen (Fig. 3*A*). Some other genes only changed in one type of tissue. For example, gly96 and LKLF expression was different only in spleen, whereas TFEB and Laminin α1 were different only in heart. Interestingly, the regulation of some genes, such as TFEB and Laminin α1, in adult tissues was opposite to that found in ES cells. In fact, they were decreased in ES cells but increased in heart (Fig. 3*A*). These results suggest that HMGA1 function depends on the cellular context.

To verify that changes in RNA levels were associated with changes at protein levels, we analyzed by Western blot the expression of Id3 in MEF and tissues from wild-type and *hmga1*-knockout mice. As shown in the Fig. 3*B* for the Id3 protein, protein levels paralleled RNA levels and were characteristic in each tissue.

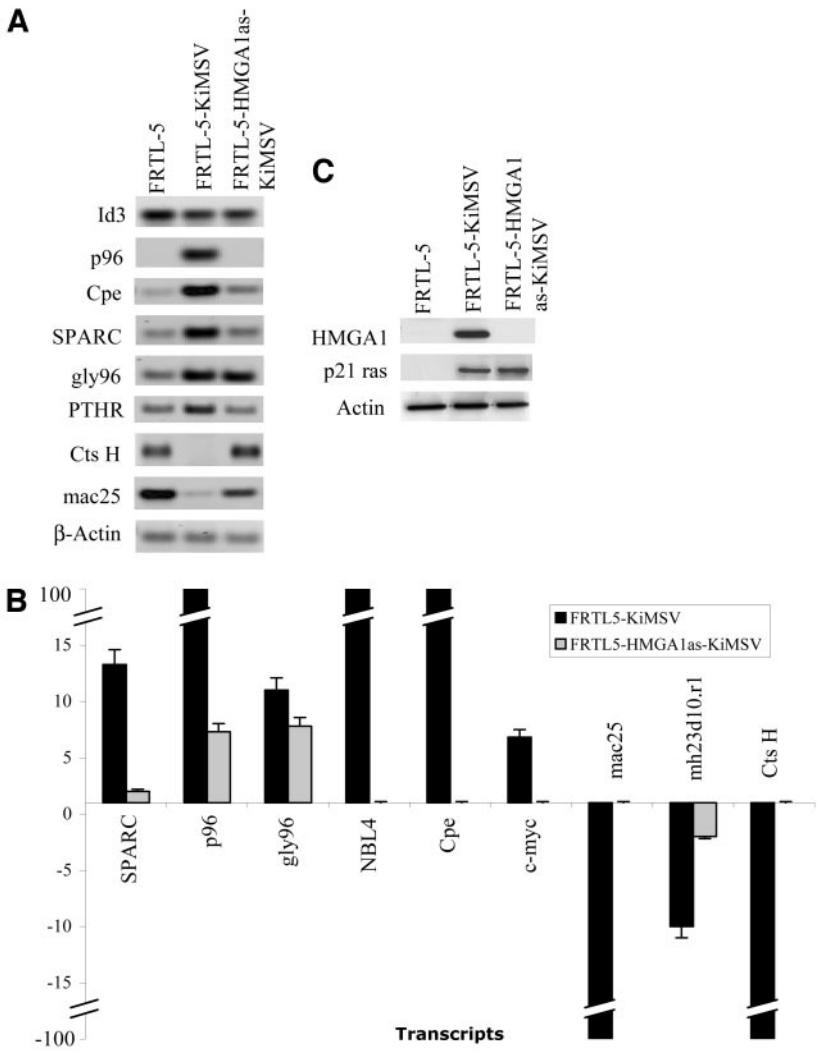

Fig. 4. Gene expression in a transformed cell system-overexpressing HMGA1. The expression of some genes has been studied by semiquantitative (*A*) or quantitative (*B*) RT-PCR in a transformed cell system overexpressing HMGA1; β-actin has been used as a housekeeping gene to normalize the RNA amount used; (*C*) Western blot analysis of v-*ras*-Ki and HMGA1 proteins. Sources of RNA or proteins are FRTL-5, rat thyroid epithelial cell line; FRTL-5-KiMSV, FRTL-5 infected with the Kirsten murine sarcoma virus; FRTL-5-HMGA1as-KiMSV, FRTL-5 transfected with a construct carrying HMGA1 mRNA in an antisense orientation, and then infected with the Kirsten murine sarcoma virus.

Analysis of the HMGA1-Dependent Genes in a Transformed Cell System. We demonstrated previously that HMGA1 overexpression is a necessary event in cell transformation. In fact, when HMGA1 expression was blocked by transfecting rat thyroid cells (FRTL-5) with an antisense *hmga1* cDNA construct and infected with the Kirsten murine sarcoma virus (KiMSV) carrying the v-*ras-Ki* oncogene, they (FRTL-5-HMGA1as-KiMSV) did not acquire the typical markers of neoplastic transformation (ability to grow in soft agar and induce tumors after injection into athymic mice), although the differentiation markers (*i.e.*, thyrotropin-dependency, ability to trap iodide, thyroglobulin synthesis, and secretion) were lost. Conversely, the neoplastic markers were shown by the untransfected rat thyroid cells infected with the same murine retrovirus (FRTL-5-KiMSV; ref. 8). Therefore, we analyzed, by semiquantitative and quantitative RT-PCR, the expression of some *hmga1*-dependent genes in FRTL-5, FRTL-5-KiMSV, and FRTL-5-HMGA1as-KiMSV cells. The experiments revealed two sets of genes. Some genes showed the same regulation observed in the ES cells, *i.e.*, carboxypeptidase E (Cpe) that decreased in *hmga1*-knockout ES cells and increased in the neoplastic cells or cathepsin H that increased in *hmga1*-knockout ES cells and decreased in the neoplastic cells compared with the wild-type controls. Other genes were regulated in an opposite direction compared with ES cells (*i.e.*, p96 and mac25), which demonstrated an increased and a decreased level, respectively, in both *hmga1*-knockout ES and the FRTL5 KiMSV cells compared with the respective controls, although the HMGA1 proteins were expressed only in the latter cells. Some representative results are shown in Fig. 4, *A* and *B*. In Fig. 4*C* we show the expression of the proteins v-*ras*-Ki and HMGA1 in the normal and neoplastic thyroid cells.

HMGA1 Proteins Bind to Id3 and p96 Promoters. We next evaluated whether the differential gene expression was a direct effect of the presence of HMGA1. We examined the Id3 and p96 genes because they were modified at RNA level in different cells and tissues and because their promoter regions contain AT-rich sequences that are a preferential-binding site for the HMGA proteins.

To investigate whether the HMGA1 proteins were able to bind the AT-rich promoter regions of both Id3 and p96, we performed an electrophoretic mobility shift assay using oligonucleotides spanning nucleotides −632 to −615 of the murine Id3 promoter region and −901 to −872 of the 5′ untranslated region of the murine p96 gene. As shown in Fig. 5*A*, a recombinant HMGA1 protein was able to bind directly to these regions. Binding specificity was demonstrated by competition experiments showing loss of binding with the addition of 200-fold molar excess of a specific unlabeled oligonucleotide. Subsequently, we performed binding assays with total extract from wild-type and *hmga1*-knockout murine spleens. Two specific complexes with mobility corresponding to the HMGA1 proteins (isoforms A1a and A1b) were present in extracts from wild-type and heterozygous (data not shown) spleens, whereas they were absent in extracts from homozygous *hmga1*-knockout mice (Fig. 5*B*). These complexes were

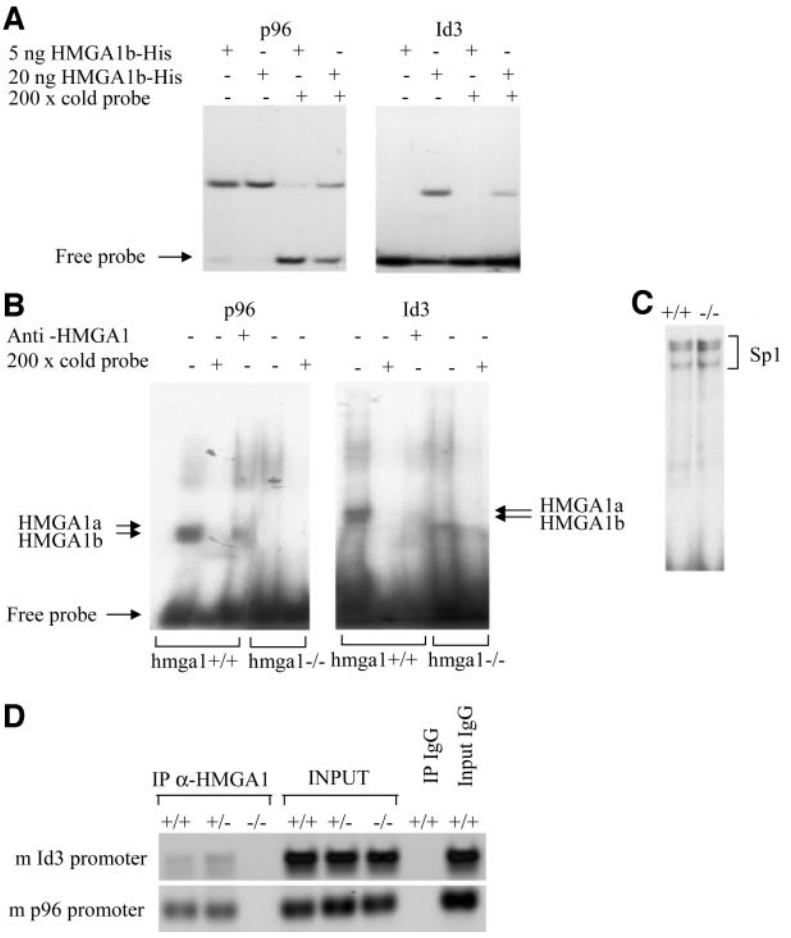

Fig. 5. HMGA1 proteins bind murine Id3 and p96 promoters *in vitro* and *in vivo*. *A*, electrophoretic mobility shift assay performed with radiolabeled oligonucleotides spanning from −209 to −169 bp of the murine Id3 promoter, spanning from −901 to −872 of the 5′ untranslated region of the murine p96 gene, and incubated with increasing amounts of recombinant HMGA1 as indicated. To assess the specificity of the binding, a 200× excess of unlabeled oligonucleotides were incubated as specific competitors. *B*, electrophoretic mobility shift assay performed with the same oligonucleotides as in *panel A*, incubated with total extracts from wild-type and hmga1-knockout murine spleens. In total extracts from wild-type (hmga1+/+) spleens there were two specific complexes, absent in total extracts from homozygous (hmga1−/−) spleen. Anti-HMGA1 antibody was used as a specific competitor. *C*, a control gel shift for Sp1 was performed to normalize the spleen extracts used. *D*, chromatin immune precipitation assay was performed on wild-type and hmga1-knockout MEFs. The recovered DNA was used as a template for PCR reactions with primers that amplify the murine Id3 and p96 promoters.

specifically displaced by the incubation with an antibody directed against the HMGA1 proteins, demonstrating that these complexes do consist of the HMGA1 proteins (Fig. 5*B*). A control gel shift for Sp1 was performed to normalize the spleen extracts used (*Panel C*).

To verify that HMGA1 proteins bind to Id3 and p96 promoters *in vivo*, we performed experiments of chromatin immunoprecipitation in MEF from wild-type and *hmga1*-knockout mice. Chromatin prepared as described under Materials and Methods was immunoprecipitated with anti-HMGA1 or normal rabbit IgG antibodies. The results shown in Fig. 5*D* demonstrate that HMGA1 proteins bind to these promoters. In fact, the Id3 and p96 promoter regions were amplified from the DNA recovered with anti-HMGA1 antibody in wild-type and *hmga1*+/− but not in *hmga1*−/− MEFs. Moreover, no amplification was observed in samples immunoprecipitated with normal rabbit IgG.

DISCUSSION

We have analyzed the expression profile of ES cells carrying two, one, and no *hmga1* functional allele by screening an "Affymetrix microarray" to identify genes that are regulated, positively or negatively, by the HMGA1 proteins. We found 87 transcripts increased and 163 decreased with a ≥4-fold change in *hmga1*−/− ES cells. The validity of these assays was confirmed by the absence of HMGA1 expression in the ES knockout cells. Semiquantitative and quantitative RT-PCR confirmed that all of these genes were differentially expressed in wild-type and *hmga1*-knockout ES cells. Several genes displayed *hmga1* dose-dependency, the phenotype of heterozygous cells was intermediate between those of wild-type and homozygous knockout cells. Thus for some genes, the level of *hmga1* expression may be critical for appropriate gene expression. In this case both alleles seem to be necessary to regulate the expression of these genes. For some other genes, the dependency on the *hmga1* expression levels was even more pronounced because the gene expression level in heterozygous ES cells was very close to that observed in homozygous cells. This type of regulation by *hmga1* expression levels may explain the appearance of pathologies, such as cardiac hypertrophy and B cell lymphomas, in mice heterozygous for *hmga1* gene disruption.[6] Several other genes showed the same expression level in wild-type and heterozygous ES cells. In this case, one *hmga1* allele is sufficient to regulate gene expression.

The genes regulated by HMGA1 in ES cells were also analyzed in MEF and in liver, spleen, and heart from wild-type, *hmga1*+/− and *hmga1*−/− mice. Different results were obtained in comparison to those observed in ES cells. In fact, the expression of some genes was either not modified by *hmga1* gene expression, or their regulation occurred in an opposite direction. It is noteworthy that the HMGA1 regulation of several genes was cell- and tissue-specific. It is known that by interacting with partner proteins, the HMGA1 proteins are able to enhance or suppress the effect of more "traditional" transcriptional activators and repressors. The fact that partner proteins are critical for HMGA1 activity may account for the cell- and tissue-specific regulation exerted by the HMGA1 proteins.

The same occurred when the HMGA1-regulated genes in ES were investigated in a cell system constituted by normal rat thyroid cells (FRTL-5) that do not express the HMGA1 proteins, the same cells

[6] M. Fedele, V. Fidanza, S. Battista, A. Fusco, manuscript in preparation.

malignantly transformed by the KiMSV (FRTL-5-KiMSV) that express high-HMGA1 levels and FRTL-5-KiMSV cells in which the synthesis of the HMGA1 protein was blocked by an antisense construct (FRTL-5-HMGA1as-KiMSV). These experiments revealed two sets of genes, those showing the same kind of regulation observed in ES and those genes showing regulation that occurred in an opposite direction.

The differential gene expression depending on the HMGA1 presence could depend on an indirect effect of the HMGA1 proteins in the sense that HMGA1 might induce some proteins that may interfere with the expression of some genes. To exclude this possibility and demonstrate a direct effect of HMGA1 on the regulation of some genes expressed differentially in *hmga1*-knockout cells, we performed electrophoretic mobility shift assay and chromatin immunoprecipitation experiments. We demonstrated the binding of the HMGA1 proteins to the promoters of Id3 and p96. We note of particular interest the finding that p96 and Id3 are regulated by the HMGA1 proteins because they are believed to have a critical role in the process of carcinogenesis. In fact, although no putative alterations on Id genes have been identified in primary human tumors to date to certify Ids as true cellular proto-oncogenes, Id proteins that are basic helix-loop-helix transcription factors have been implicated in regulating a variety of cellular processes (*i.e.*, cellular growth, senescence, differentiation, apoptosis, and angiogenesis) that regulate tumorigenesis (16). In particular, Id3 has been frequently found increased in human neoplasias (16). Equally, p96, a mitogen-responsive phosphoprotein cloned from a mouse macrophage cell expression library, is consistently down-regulated in mouse mammary carcinogenesis and in human ovarian carcinomas as compared with normal surface epithelium (17, 18). It is likely that Id3 up-regulation and p96 down-regulation in human neoplasias depend also on the HMGA1 overexpression, a feature of most of the human-malignant neoplasias (19).

When we analyzed the expression of some genes in MEF and adult tissues taken from HMGA2 knockout mice, we found that several genes do not appear to be regulated by HMGA2 (data not shown). This result could depend on the different action of these two members of the same HMGA protein family and confirms that although HMGA1 and HMGA2 have a similar structure and expression profile (high during embryogenesis and neoplastic tissue), they exert different functions. This is consistent with a body of evidence indicating that the two proteins exert different function: (*a*) the BRCA1 promoter is regulated negatively by HMGA1 but not by HMGA2 (20); (*b*) HMGA2 is critical for adipocytic cell growth (21, 22), whereas HMGA1 has negative effect on the growth of the preadipocytic cells 3T3 L1 (23); and (*c*) the phenotype of the *hmga1*- and *hmga2*-knockout mice is divergent: *i.e.*, a reduction in size and fat tissue of *hmga2*-null mice and in cardiac hypertrophy and B-cell lymphomas of *hmga1*-null mice.[7]

In conclusion, this study indicates that HMGA1 proteins are involved in the regulation of several genes. For some genes, such as Id3 and p96, we demonstrate that the regulation is direct. The positive or negative regulation appears to be tissue-specific because it likely depends on the multiprotein complex in which HMGA1 proteins are inserted.

[7] M. Fedele, V. Fidanza, S. Battista, A. Fusco, manuscript submitted for publication.

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High mobility group A1 expression correlates with the histological grade of human glial tumors

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Abstract. Glioblastoma is one of the most aggressive tumors in mankind with 50% of patients dying within the 1st year of diagnosis, and being refractory to conventional therapies. The aim of our work has been to analyse the expression of the HMGA1 proteins in human astrocytomas and glioblastomas in order to verify whether the detection of these proteins might be of some help in the diagnosis of these neoplasias. Here we report the analysis of 27 cases, including 12 astrocytomas and 15 glioblastomas, for HMGA1 expression. All the neoplastic samples showed positive staining even though the number of positive cells and the staining intensity was higher in glioblastomas compared to astrocytomas. Conversely, HMGA1 proteins were not detected in normal brain. Accordingly, expression of the *hmga1* gene, analysed by RT-PCR, was higher in glioblastomas than in astrocytomas.

Introduction

The incidence of all adult primary brain tumors is 11.8 per 100,000 person-years, with malignant primary glial tumors representing 6.5 per 100,000 person-years (20). Most adult supratentorial gliomas are forms of astrocytomas which account for >60% of all primary brain tumors.

The current WHO histologic classification of tumors of the Central Nervous System classifies among astrocytic tumors the diffusely infiltrating astrocytoma which can be divided into 3 clinicopathologic entities: diffuse astrocytomas (WHO grade II), anaplastic astrocytoma (WHO grade III) and glioblastoma multiforme (WHO grade IV). Such group of tumors is linked to a survival ranging between >5 years (diffuse astrocytoma) to <1 year (glioblastoma) with a possible progression from 'low grade' to 'high grade' tumors (17).

The majority of glioblastomas arise without clinical or histological evidence of a less malignant precursor lesion and these lesions have been designated primary glioblastoma. They manifest in older patients (mean age, 55 years) after a short clinical history of usually <3 months. These primary glioblastomas are characterized by EGFR amplification (~40% of cases) and/or overexpression (60%), PTEN mutations (30%), $p16^{INK4a}$ deletion (30-40%), MDM2 amplification (<10%) and/or overexpression (50%), and in 50-80% of cases, loss of heterozygosity (LOH) on the entire chromosome 10. In contrast, secondary glioblastomas develop more slowly by malignant progression from diffuse or anaplastic astrocytoma and manifest in younger patients (mean age, 40 years). Secondary glioblastomas contain TP53 mutations in ~60% of cases (18).

The HMGA proteins are involved in the regulation of chromatin structure and interact with the basal transcription machinery regulating the expression of several genes (19,6,12). HMGA proteins are expressed at low levels in normal adult tissues, whereas an abundant expression occurs during embryogenesis and in several human carcinomas (21). It has been also demonstrated that the expression level of the HMGA1 proteins is significantly correlated with parameters known to indicate a poor prognosis in patients with colorectal cancer (2) and also that HMGA1 could serve as a potential diagnostic molecular marker for distinguishing pancreatic malignancies from normal tissues or benign lesions (1).

Therefore, the aim of our work has been to analyse the expression of the HMGA1 proteins in human astrocytomas and

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glioblastomas in order to verify whether the detection of these proteins might be of some help in the diagnosis of these neoplasias, and whether a possible therapeutic approach based on the suppression of the HMGA1 protein synthesis might be taken in consideration, since it has been already demonstrated that the block of the synthesis of these proteins causes apoptotic death of carcinoma cells of different tissue origin (22).

Here we report the analysis of 27 cases including, 12 astrocytomas and 15 glioblastomas for HMGA1 expression. All the neoplastic samples showed positive staining even though the number of positive cells and the staining intensity was higher in glioblastomas compared to astrocytomas. Conversely, HMGA1 proteins were not detected in normal brain.

Materials and methods

Human tissues. Archival formalin-fixed and paraffin-embedded tissues from the Department of Pathology of the School of Medicine of the University of Catanzaro and from the Division of Pathology of Hospital 'A. Pugliese' of Catanzaro were used to study tumors from 27 patients (comprising 12 astrocytomas - age 24.2±5.2 years, and 15 glioblastomas - age 58.3±4.2) who underwent their surgical intervention at the Neurosurgical Clinics of the University of Catanzaro. Blocks from 3 normal brains obtained at post-mortem examination were also used as control.

Seriated deparaffinated sections (4 μm-thick) were used for staining procedures, hematoxylin and eosin and immunocytochemistry. All procedures were carried out at room temperature. The slides were treated in a microwave oven (750 W) twice for 5 min in 10 nM citrate buffer (pH 6.0) for demascation of the antigen followed by incubation in 0.3% H_2O_2 in methanol. Then, 10% goat serum was applied for 30 min followed by a polyclonal anti-HMGA1 product antibody (dilution 1:50) for 60 min (8). Biotinylated secondary antibody (1:60, goat anti-rabbit, Dako, Hamburg, Germany) and peroxidase-labeled streptavidin (1:100, Dako) were added in sequence. Diaminobenzidine (Dako) was used as chromogen. In order to assess cell proliferation, immunohistochemical staining with the monoclonal mouse anti-human anti-human Ki-67 antigen antibody (Clone: MIB-1, Dako, at a dilution of 1:100) was performed. The slides used for immunohistochemical procedures were counterstained with alum-hematoxylin. Cells were counted in tumors (5-fields) in non-overlapping microscopic fields of a light-microscope (Zeiss-axioscope) with a 40x objective, and the percentage of labeled cells was established.

RNA isolation and RT-PCR analysis of the expression of HMGA1 on astrocytoma and glioblastoma versus normal brain. RNA extraction from paraffin-embedded samples was performed following a published procedure (16). Briefly, single 6-8 μm tissue sections, cut from paraffin blocks, were stirred for 20 min in 1.5 ml tubes with 1 ml of xylene. After centrifugation, the pellet was washed with 0.5 ml of ethanol and air-dried. The dried pellet was resuspended in 200 μl of 6 mg ml^{-1} proteinase K (Sigma Chemical, St. Louis, MO), 1 M guanidinium thiocyanate, 25 mM 2-mercaptoethanol, 0.5% Sarkosyl™, 20 mM Tris-HCl, pH 7.5 and incubated at 37˚C overnight. After centrifugation, the supernatant was transferred to a new tube where 1 sample equivalent volume of 70% phenol acid/30% chloroform was added, and the aqueous supernatant was transferred to a new 1.5 ml tube. One sample equivalent volume of 25% phenol pH 7/24% chloroform/1% isoamilic acid was added and the supernatant was transferred to a new tube containing 1/10 of sample volume of sodium acetate 3 M pH 5.2, 2 sample volumes of ethanol 100% and 1 μl of glycogen and was precipitated at -20˚C overnight. After centrifugation for 15 min at 12,000 x g in an Eppendorf microcentrifuge, the pellet was washed with 70% ethanol, air-dried and resuspended in H_2O. RNA was reverse transcribed according to manufacturer's instructions (Perkin-Elmer). The PCR amplification was performed as follows: denaturation at 95˚C for 5 min, 30 cycles (95˚C, 55˚C and 72˚C for 1 min each temperature) and a final extension step at 72˚C for 10 min. The sequences of the forward primers used were for *hmga1*: 5'-AGAGACCTCGGG GCCGACCA-3', for ß-actin: 5'-TCGTGCGTGACATTAAGG AG-3' and the sequences of the reversed primers were for *hmga1*: 5'-GATGCCCTCCTCTTCCTCCTT-3', for ß-actin: 5'-GTCAGGCAGCTCGTAGCTCT-3'. To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing the PCR on samples that were not reverse-transcribed but otherwise identically processed.

The PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide. Gels were scanned using a Typhoon 9200 scanner and digitized data analyzed using Imagequant (Molecular Dynamics). *Hmga1* levels were normalized to ß-actin gene levels. The resulting values were normalized versus normal tissue, which received the value of 1. Fold induction indicates the expression ratio between glioblastoma and astrocytoma, respectively, versus normal brain tissue.

Table I. HMGA1 protein expression in human astrocytomas and glioblastomas analysed by immunohistochemistry.

Histological type of brain specimens	No. of positive cases/no. of cases analysed	Mean percentage of the stained cells
Normal brain	0/4	0
Astrocytomas	12/12	57.3±7
Glioblastomas	15/15	82.3±8

Results

Immunohistochemical analysis of HMGA1 expression in gliomas. Immunohistochemical assay was performed to evaluate the expression of the HMGA1 proteins in astrocytomas and glioblastomas compared with its expression in normal human brain tissues. The results are summarized in Table I. In astrocytomas labeling was observed in the nuclei of tumoral cells. Labeled cells ranged between 49.4-63.2%

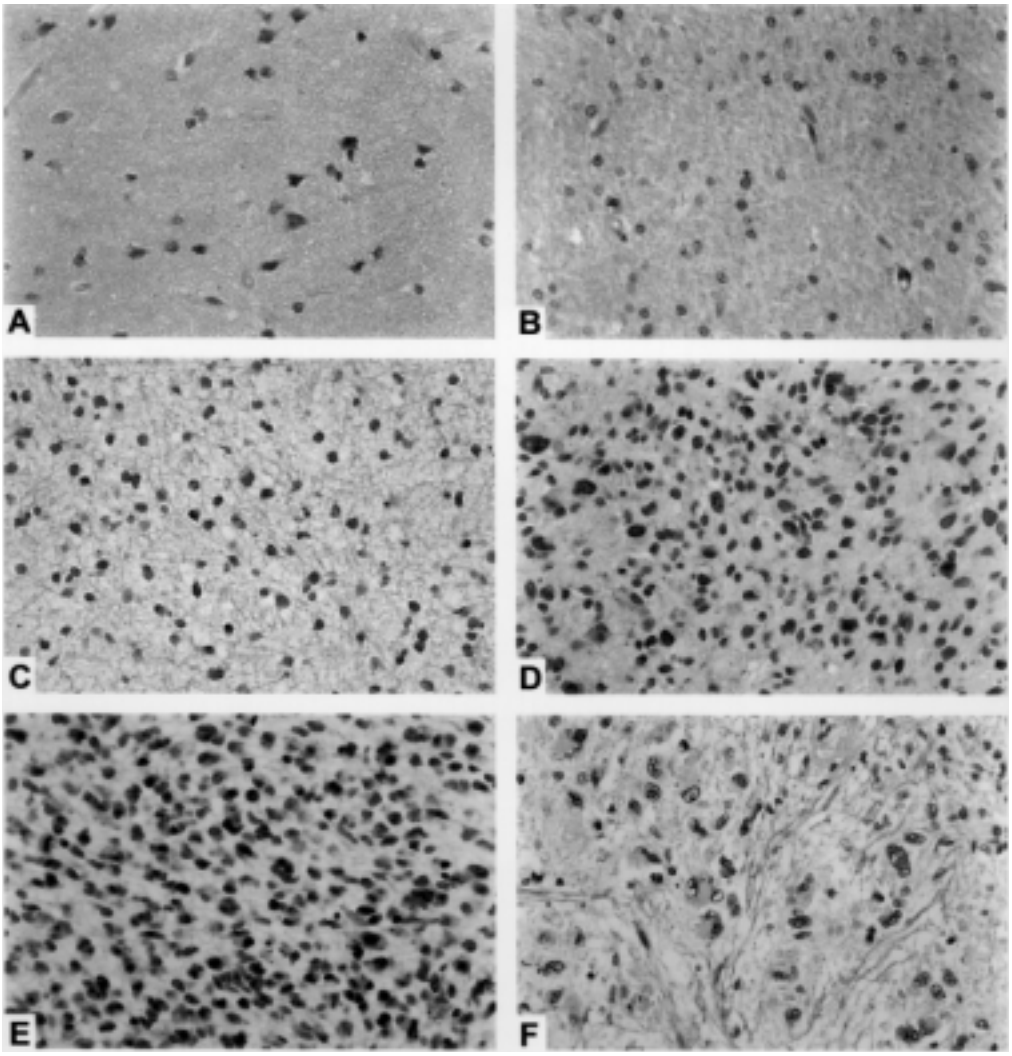

Figure 1. HMGA1 immunostaining in normal brain, astrocytoma and glioblastoma. Paraffin sections from normal brain (A,B), astrocytoma (C), and glioblastoma (D,E), were analyzed by immunohistochemistry using antibodies raised against a specific HMGA1 peptide. As a negative control (F), glioblastoma immunostained with HMGA1 antibodies pre-incubated with the peptide against which antibodies were raised, no immunoreactivity was observed. Magnification, x40.

(mean 57.3%) in all grade II tumors. Labeling index by MIB-1 antibody against the Ki-67 antigen, an index of cell proliferation, was <1%. In glioblastomas, the number of cells stained by the HMGA1 antibody ranged from 73.2-98.0% (mean 82.3%) in all tumors. The growth fraction, as determined by the antibody MIB-1, ranged between 12.3-32.7% with a mean value of 20.2%. Conversely no HMGA1-immunostaining at all was observed in normal brain.

Some representative results of these analyses are shown in Fig. 1. In glioblastomas, nuclei of neoplastic cells showed a diffuse HMGA1 expression (Fig. 1D and E). The intensity of the staining was higher compared to that detected in astrocytomas (Fig. 1C). In these tumors, also the number of the stained cells was lower in the neoplastic cells. No staining was observed in normal brain (Fig. 1A and B). The specificity of the reaction was confirmed by the absence of staining when glioblastoma samples were stained with antibodies pre-incubated with the peptide against which antibodies were raised (Fig. 1F). There was no staining in the absence of the primary antibodies (data not shown). Conversely, a positive nuclear staining was present in the same glioblastoma immunostained with the HMGA1 antibody (Fig. 1E).

RT-PCR analysis of the hmga1 gene expression. To validate the immunohistochemical data and to objectively compare the level of *hmga1* expression, 3 representative normal brain tissues, 3 astrocytomas and 3 glioblastomas were also analysed by RT-PCR using *hmga1* specific primers. The relative expression levels of *hmga1* mRNA to ß-actin which was used as an internal control were calculated. The resulting values were normalized versus normal tissue, which received the value of 1. The results are shown in Fig. 2. The RT-PCR data essentially confirmed the immunohistochemical results. In fact, the relative expression level of *hmga1* was increased (2.24-fold) in astrocytomas in comparison to the normal brain. The increase in *hmga1* expression level was even higher (5.58-fold) in glioblastoma, thus supporting the immunohistochemical findings that suggest that an increased *hmga1* expression correlates with the tumour aggressiveness.

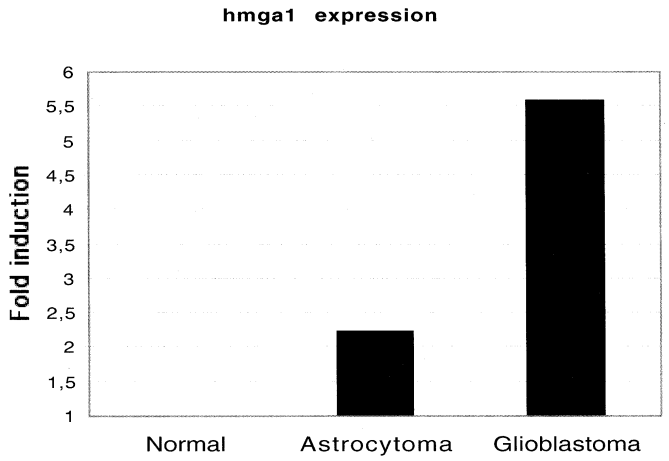

Figure 2. RT-PCR analysis of the *hmga1* gene in normal brain, astrocytoma and glioblastoma. *Hmga1* expression was evaluated in 3 normal brain tissues, 3 astrocytomas and 3 glioblastomas by RT-PCR as described in Materials and methods. *Hmga1* levels were normalized to ß-actin gene levels. The resulting values were normalized versus normal tissue, which received the value of 1. Fold induction indicates the ratio in *hmga1* expression between glioblastoma and astrocytoma, respectively, versus the normal brain tissue.

Discussion

Glioblastoma is one of the most aggressive tumors in mankind with 50% of patients dying within the 1st year of diagnosis, and being refractory to conventional therapies (17,18,20). Therefore, it may represent an excellent target for new therapeutic approaches, and the search for new molecular targets might be a useful means to approach an innovative therapy for this kind of neoplasia.

HMGA1 expression, abundant during embryonic development, is low or absent in normal adult tissues (7,24), whereas its induction represents a common feature of malignant tumors. Indeed, in the 1980s elevated expression of HMGA1 protein was found to be associated with the acquisition of a highly malignant phenothype in rat thyroid transformed cells and in thyroid and skin experimental tumors (13-15). Subsequently, HMGA1 proteins were detected in human thyroid (8,10), colon (2,9,11), prostate (23), cervix (3), and pancreatic (1) carcinomas, but not in normal counterpart tissues.

Therefore, the present study tested whether HMGA1 product is expressed in astrocytic tumors and whether there is a correlation between tumoral progression and expression of this gene. Here we report the analysis of the HMGA1 expression in 12 astrocytomas and 15 glioblastomas. Positive staining was observed in all the tumors, however the number of the stained cells and intensity of staining was more elevated in glioblastomas. RT-PCR results confirmed the immunohistochemical data.

Therefore, given the fact that increased expression level of HMGA1 protein is closely associated with the malignant phenotype, determination of the HMGA1 expression level could be used as a diagnostic marker in the pathology of these tumors. These results are consistent with previous data in colon and cervical carcinomas where it has been shown that an increase in HMGA1 levels correlate with malignancy grade (2,3,9).

It has been demonstrated that overexpression of the HMGA1 proteins is causally associated with both neoplastic transformation and metastatic progression. In fact, the block of HMGA1 synthesis prevents rat thyroid cell transformation by murine transforming retroviruses and an adenovirus carrying the *hmga1* gene in antisense orientation induced cell death in thyroid anaplastic carcinoma cells (4,5). Therefore, we could envisage a therapy based on the suppression of the HMGA1 protein function also in glioblastoma, expecially if associated with surgical therapy. Studies are in progress in our laboratory to evaluate this possibility.

In conclusion, on the basis of these findings, we propose that HMGA1 proteins could play an important role in a multistage process of carcinogenesis of astrocytic tumors and that the HMGA1 protein level could serve as a potential diagnostic marker, which may enable the identification of tumor cells with potential to be biologically malignant, and as a potential target for gene therapy of human glioblastoma tumors.

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High-mobility-group A1 (HMGA1) proteins down-regulate the expression of the recombination activating gene 2 (RAG2)

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HMGA1 (high-mobility-group A1) proteins are architectural transcription factors that are found overexpressed in embryogenesis and malignant tumours. We have shown previously that they have a role in lymphopoiesis, since the loss of HMGA1 expression leads to an impairment of T-cell development and to an increase in B-cell population. Since RAGs (recombination activating genes) are key regulators of lymphoid differentiation, in the present study we investigate whether RAG2 expression is dependent on HMGA1 activity. We show that RAG2 gene expression is up-regulated in Hmga1−/− ES (embryonic stem) cells and EBs (embryoid bodies) as well as in yolk sacs and fibroblasts from Hmga1−/− mice, suggesting that HMGA1 proteins control RAG2 gene expression both *in vitro* and *in vivo*. We show that the effect of HMGA1 on RAG2 expression is direct, identify the responsible region in the RAG2 promoter and demonstrate binding to the promoter *in vivo* using chromatin immunoprecipitation. Since RAG2 is necessary for lymphoid cell development, our results suggest a novel mechanism by which HMGA1 might regulate lymphoid differentiation.

Key words: electrophoretic mobility-shift assay (EMSA), embryonic stem cell, high-mobility-group A1 (HMGA1), lymphopoiesis, MEF, recombination activating gene 2 (RAG2).

INTRODUCTION

HMGA1 (high-mobility-group A1) proteins are 'architectural transcription factors' capable of binding the minor groove of AT-rich DNA sequences and inducing the bending of DNA intermediates. HMGA1 proteins have been found overexpressed in many kinds of human malignancies and rearranged in benign tumours [1]. They seem to play a major physiological role during development and cell differentiation [1]. We showed previously that HMGA1 proteins play a pivotal role in lymphocyte differentiation [2]. In particular, we suggested that the loss of Hmga1 gene expression might force the B-cell/T-cell common lymphoid precursor to differentiate to B-lymphocytes rather than to T-lymphocytes, probably by regulating the expression levels of cytokines involved in B- and T-cell proliferation/differentiation. In fact, the loss of HMGA1 induces a decrease in interleukin-2 expression and an increase in interleukin-6 expression both *in vitro* and *in vivo* [2] (M. Fedele, V. Fidanza, S. Battista, F. Pentimalli, A. J. P. Klein-Szanto, R. Visone, I. De Martino, A. Curcio, C. Morisco, L. Del Vecchio, G. Baldassarre, C. Arra, G. Viglietto, C. Indolfi, C. M. Croce and A. Fusco, unpublished work). More strikingly, the lack of HMGA1 in homozygous knockout mice leads to the development of different B-cell neoplasias (M. Fedele et al., unpublished work), probably due to the alteration in B-cells/T-cells balance.

To investigate further the molecular mechanisms involved in HMGA1 regulation of lymphopoiesis, we analysed the expression of RAGs (recombination activating genes). RAG1 and RAG2 are key performers of the V(D)J recombination, through which the specific antigen receptors in lymphocytes are generated [3,4]. In particular, they initiate the process of recombination, introducing double-strand breaks in target sequences of the Ig and T-cell receptor genes. In the absence of either RAG1 or RAG2 gene product, the development of mature lymphocytes is completely abrogated, leading to immunodeficiency, both in mouse and humans [5–7]. On the other hand, to limit recombinase activity, their expression is tightly regulated both at the transcriptional and post-transcriptional levels [8,9]. At the transcriptional level, the alteration of chromatin structure in the 5′-region of RAG1 and RAG2 genes has been shown to be responsible for their tissue- and stage-specific regulation [10,11]. The RAG2 promoter is differently regulated in B- and T-cells [12]. Moreover, in the RAG2 promoter region, a 300 bp 5′-upstream region from the major transcription initiation site is conserved between mice and humans [12], indicating that this region is important for the promoter activity. Human RAG2 promoter has been shown to be activated both in lymphoid and non-lymphoid lineages [13]. A core promoter of mouse RAG2 confers lymphoid specificity and may be regulated by distinct transcription factors in B- (Pax-5) and T- (GATA-3 or c-Myb) cells [8,12,14]. It has been shown that the LEF-1−β-catenin complex regulates the RAG2 promoter activation, together with c-Myb and Pax-5 in immature B-cells [9]. In the present study, we report that lack of HMGA1 proteins, which are known to regulate lympho-specific genes, is associated with increased RAG2 expression in mouse ES (embryonic stem) cells. RAG2 up-regulation is also found in yolk sacs and MEFs (mouse embryonic fibroblasts) from Hmga1-null embryos. Conversely, introduction of an Hmga1-expressing construct into Hmga1−/− ES cells restores RAG2 gene expression at levels comparable with wild-type ES cells. Functional assays demonstrate that HMGA1 proteins are capable of repressing the RAG2 promoter in 293T cells and that the HMGA1 repressive activity is noticeably

Abbreviations used: c/EBP-β, CAAT/enhancer-binding protein β; CMV, cytomegalovirus; dpc, days post-coitum; EB, embryoid body; EMSA, electrophoretic mobility-shift assay; ES cell, embryonic stem cell; FBS, fetal bovine serum; HA, haemagglutinin; HMGA1, high-mobility-group A1; MEF, mouse embryonic fibroblast; MTG, monothioglycerol; RAG, recombination activating gene; RT, reverse transcriptase.

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increased by co-expression of c/EBP-β (CAAT/enhancer-binding protein β). Finally, we show that the repressive effect of HMGA1 on RAG2 promoter is due to a direct specific interaction of the architectural factor with the RAG2 promoter *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell cultures

Wild-type, Hmga1+/− and Hmga1−/− AB2.1 ES cells have been described in [2]. Hmga1−/−R and Hmga1−/−CMV (where CMV stands for cytomegalovirus) clones were generated by electroporating Hmga1−/− ES cells with 20 μg of pc-*Hmga1*/Hygro construct or the empty vector respectively [2]. Transgene expression was detected by Northern blotting and RT (reverse transcriptase)–PCR. ES cells were cultured on a layer of mytomicin D-inactivated fibroblasts. Before RNA extraction, fibroblasts were removed by three passages in 0.1 % gelatin-treated plates and the maintenance of the undifferentiated state was ensured by the addition of leukaemia inhibiting factor (10^3 units/ml; Chemicon, Temecula, CA, U.S.A.). MEFs were obtained from 12.5-day-old embryos. Cells were cultured at 37 °C (5 % CO_2) in Dulbecco's modified Eagle's medium containing 10 % (v/v) FBS (fetal bovine serum) supplemented with penicillin and streptomycin. The human embryonic kidney 293T cell line [14] was cultured in Dulbecco's modified Eagle's medium + 10 % FBS.

Differentiation of ES cells

Differentiation of ES cells in a methylcellulose-based medium has been described in [2]. Briefly, 48 h before differentiation, 2×10^5 ES cells were plated on gelatin-coated plates in Iscove's modified Dulbecco's medium supplemented with 15 % FBS, sodium pyruvate (1 mM), L-glutamine (2 mM) and non-essential amino acids (0.1 mM; Gibco BRL, Life Technologies, Gaithersburg, MD, U.S.A.), MTG (monothioglycerol; 100 μM; Sigma), leukaemia inhibiting factor (10 ng/ml; Chemicon), penicillin G and streptomycin. Materials for differentiation were purchased from Stem Cell Technologies (Vancouver, BC, U.S.A.) unless otherwise specified. To obtain EBs (embryoid bodies), 2×10^3 ES cells were plated on low-adherence 35 mm Petri dishes as a single cell suspension in 'primary differentiation medium', constituted by 0.9 % methylcellulose in Iscove's modified Dulbecco's medium, 15 % FBS, L-glutamine (2 mM), MTG (150 μM) and murine stem cell factor (40 ng/μl). Feeding medium (0.5 % primary differentiation medium, 15 % FBS, 150 μM MTG and 160 ng/ml murine stem cell factor) was added after 7 days in culture and subsequently every 3–4 days.

Generation of Hmga1+/− and Hmga1−/− mice

Hmga1+/− and Hmga1−/− mice have been described in [2]. Briefly, Hmga1+/− ES cell clones were microinjected into 3.5 dpc (days post-coitum) C57BL/6J blastocysts and reimplanted into foster mothers (the Animal Facility in Thomas Jefferson University). Chimaeric mice were crossed with wild-type and some of them gave germline transmission. Single knockout mice were then intercrossed to obtain double knockout mice. Pregnant mothers were killed at 14.5 dpc and the embryo genotype was evaluated [2].

RT–PCR analyses of embryos, MEFs and ES cell cultures

Tissues from mice were rapidly dissected, frozen on solid CO_2 and stored at − 80 °C. Total RNA from embryos and cell cultures was extracted with TRI Reagent solution (Molecular Research Center, Cincinnati, OH, U.S.A.) according to the manufacturer's instructions and treated with DNase I (GenHunter Corporation, Nashville, TN, U.S.A.). RNA (1 μg) was reverse-transcribed using a mixture of poly-dT and random exonucleotides as primers and MuLV RT (PerkinElmer, Boston, MA, U.S.A.). PCR amplifications were performed as described in [15] in a GeneAmp PCR System 9600. Primers for RAG2, Hmga1 and Gapdh have been described in [2,16]. Non-reverse-transcribed RNA was amplified (results not shown) to rule out the possibility of DNA amplification. The PCR products were separated on 2 % (w/v) agarose gel and, if necessary, blotted and hybridized with specific probes.

Plasmids

The pc-Hmga1/Hygro and pCEFL/HA-HMGA1 constructs (where HA stands for haemagglutinin) have been described in [2,17]. For the RAG2prom-luc construct, the region − 279/+ 21 of the mouse RAG2 gene [12] was amplified using the following primers: forward primer, 5′-ACGCGTAAGCTTAAGACAGTC-ATT-3′, containing an MluI restriction site, and reverse primer, 5′-CTCGAGCTGAAGGCTGCAGGGTAG-3′, containing an XhoI restriction site. The resulting fragment was subcloned into the pGL3 vector (Promega, Madison, WI, U.S.A.). C/EBP-β expression vector has been described in [17].

Production of recombinant proteins

Production of the recombinant HMGA1b–His protein has been described in [18]. Recombinant HMGA1b(1–53) is constituted by the first 53 amino acids spanning the first two AT-hook domains, whereas HMGA1b(54–96) contains the spacer region between the second and third AT-hook, the third AT-hook domain and the C-terminal region of the protein. The recombinant HMGA1 proteins were generated by cloning the full-length or truncated Hmga1b cDNAs in the pET2c (Novagen, Madison, WI, U.S.A.). BL21/DE3 cells transformed with each vector were grown in Luria–Bertani medium, induced with isopropyl β-D-thiogalactoside, sonicated and purified by using the His-Trap purification kit (Amersham Biosciences) according to the manufacturer's instructions. The proteins were dialysed and analysed by SDS/12.5 % PAGE.

EMSA (electrophoretic mobility-shift assay)

DNA-binding assays with the recombinant proteins were performed as described previously [19]. Briefly, 5 (14 nM) to 50 ng of wild-type recombinant protein or 5 ng of truncated proteins were incubated with radiolabelled double-strand oligonucleotides, corresponding to the region spanning bases 14–53 of the murine RAG2 promoter region (RAG2pr) (NCBI accession no. AF159439). The full-length protein was also assayed with truncated or mutated oligonucleotides (Figure 2B) representing different AT-rich segments of the RAG2pr region. For EMSA on mouse spleens, 8 μg of protein extracts from wild-type, Hmga1+/− and −/− adult spleens were incubated in a solution made up of 20 mM Hepes (pH 7.6), 40 mM KCl, 0.1 mM EDTA, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM PMSF, 1 μg of poly(dC-dG), 2 μg of BSA and 10 % (v/v) glycerol to a final volume of 20 μl for 10 min at room temperature (25 °C). The samples were incubated for 10 min after the addition of 2.5 fmol of a ^{32}P-end-labelled oligonucleotide (specific activity, 8000–20000 c.p.m./fmol). In some experiments, a 100-fold molar excess of unlabelled oligonucleotide was added as a specific competitor. For antibody competition analyses, extracts were preincubated with 0.5 μg of

anti-HMGA1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or an unrelated antibody (anti-Pit-1; Santa Cruz Biotechnology) on ice for at least 30 min. The Sp1 oligonucleotide was purchased from Santa Cruz Biotechnology. The DNA–protein complexes were resolved on 6 % (w/v) non-denaturing acrylamide gels and visualized by exposure to autoradiographic films.

Chromatin immunoprecipitation

Briefly, approx. 3×10^7 wild-type, Hmga1+/− and Hmga1−/− ES cells were grown on 75 cm^2 dishes. Chromatin immunoprecipitation was performed as described in [2,20] using antibodies binding to the N-terminal region of the HMGA1 proteins [21] and not reactive to other members of the HMGA family. Input DNA (500 ng) and immunoprecipitated DNAs were analysed by PCR for the presence of RAG2 promoter sequences or the prolipase promoter region, as negative control. PCRs were performed with AmpliTaq gold DNA polymerase (PerkinElmer). The primers used to amplify the sequence of RAG2 promoter were: forward, 5′-AAGCTTAAGACAGTCATT-3′; and reverse, 5′-CTGAAGGCTGCAGGGTAG-3′. Primers for prolipase promoter were: forward, 5′-ACCAAAGTGTCAAGGGCAAC-3′; and reverse, 5′-ATTCCCTAAACCCAGCATCC-3′. PCR products were resolved on a 2 % agarose gel, stained with ethidium bromide and scanned using a Typhoon 9200 scanner.

Transient transfections

Before transient transfections in ES cells, feeder fibroblasts were removed as described above. A total of 4×10^5 wild-type or double knockout ES cells or 293T cells were plated on 6-well plates and transfected after 48 h with 1 μg of reporter plasmid (either RAG2prom-luc or pGL3), by FuGene6 (Roche, Indianapolis, IN, U.S.A.). Where indicated, 3 μg of HA–Hmga1 and/or 3 μg of c/EBP-β were co-transfected. Cells were harvested 48 h post-transfection and lysates were analysed for luciferase activity. Transfection efficiency was normalized using the β-galactosidase activity, and fold activation was calculated by dividing by pGL3 luciferase activity. All the assays were performed in triplicate and repeated in three independent experiments.

RESULTS

Loss of HMGA1 is correlated with an increased RAG2 expression in ES cells

We previously generated Hmga1−/− mouse ES cells and showed that their ability to differentiate in lymphohaematopoietic lineages is greatly compromised [2]. In particular, we showed that the T-cell population is decreased, whereas the B-cell population is increased in Hmga1−/− ES cells, yolk sacs and fetal livers compared with wild-type. To investigate further the role that HMGA1 proteins play in B-cell/T-cell differentiation, we analysed the expression of RAG2, a lymphoid-specific gene, by RT–PCR analyses. As shown in Figure 1(A), we detected an 8-fold increase in RAG2 expression in Hmga1−/− ES cells compared with wild-type and single knockout ES cells and a 2.5-fold increase in ES cell-derived Hmga1−/− EBs compared with wild-type (lanes 6 and 7). No band was detected when the RNA was not reverse-transcribed before amplification (results not shown).

To verify that the lack of HMGA1 was responsible for RAG2 up-regulation, we transfected the pc-Hmga1/Hygro construct in double knockout ES cells [2]. We verified the rescue of Hmga1 expression in some clones (−/−R1) by RT–PCR (Figure 1A). A decreased RAG2 expression was observed in Hmga1-transfected cells (−/−R1) compared with Hmga1 null cells and

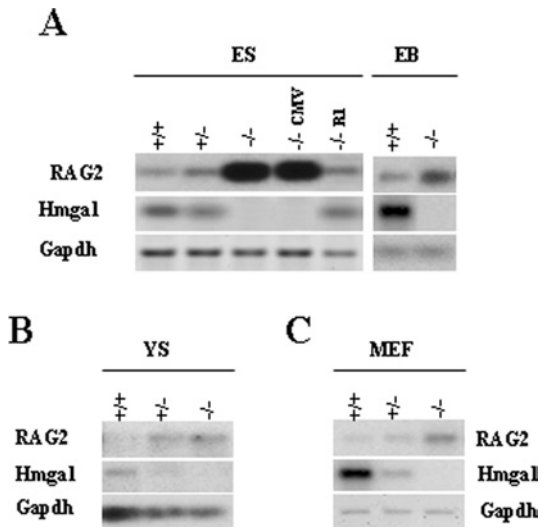

Figure 1 RAG2 expression in wild-type and Hmga1 knockout ES cells, EBs, yolk sacs and MEFs

RT–PCR analyses for RAG2 and Hmga1 expression were performed on RNA extracted from (**A**) ES cells and EBs; (**B**) yolk sacs (YS); and (**C**) MEF. Gapdh expression was evaluated as internal control. −/− CMV and −/− R1 indicate Hmga1−/− ES cells transfected with the empty vector and with the pc-Hmga1-expressing construct respectively.

Hmga1−/− cells transfected with the empty vector (−/−CMV) (compare lanes 3, 4 and 5 in Figure 1A). These results suggest that HMGA1 proteins regulate RAG2 gene expression in ES cells and in ES cell-derived EBs. Moreover, when the mammary epithelial cells MCF-7 were stably transfected with the Hmga1/Hygro construct (results not shown), the expression of RAG2 was down-regulated, suggesting that HMGA1 proteins induce RAG2 down-regulation in different cell types.

Loss of HMGA1 determines up-regulation of RAG2 gene expression *in vivo*

We next investigated RAG2 expression *in vivo*. We analysed RAG2 expression in 14.5 dpc yolk sacs from wild-type, Hmga1+/− and −/− embryos. As shown in Figure 1(B), RAG2 expression was sensibly higher in Hmga1-null and heterozygous yolk sacs, compared with wild-type. RT–PCR analyses for Hmga1 expression were performed as a control of the genotype of the tissues analysed (Figure 1B, middle panel). Moreover, RAG2 overexpression was observed also in the spleen from Hmga1−/− adult mice, compared with wild-type (M. Fedele et al., unpublished work), indicating that HMGA1 affects RAG2 expression both *in vitro* and *in vivo*. Next, we investigated whether lack of HMGA1 affects RAG2 expression in non-lymphoid cells. Again, RAG2 expression in MEFs from wild-type, Hmga1+/− and −/− embryos was inversely related to HMGA1 expression (Figure 1C).

These results indicate that RAG2 expression is inversely related to Hmga1 expression both *in vitro* and *in vivo*, suggesting a suppressive role for the HMGA1 proteins in the regulation of RAG2 transcription.

HMGA1 proteins directly bind the RAG2 promoter

To investigate whether HMGA1 proteins are directly involved in RAG2 transcriptional regulation, we evaluated the HMGA1 DNA-binding activity to the RAG2 promoter. In particular, we analysed a region spanning nt 14–53 of the murine RAG2 promoter

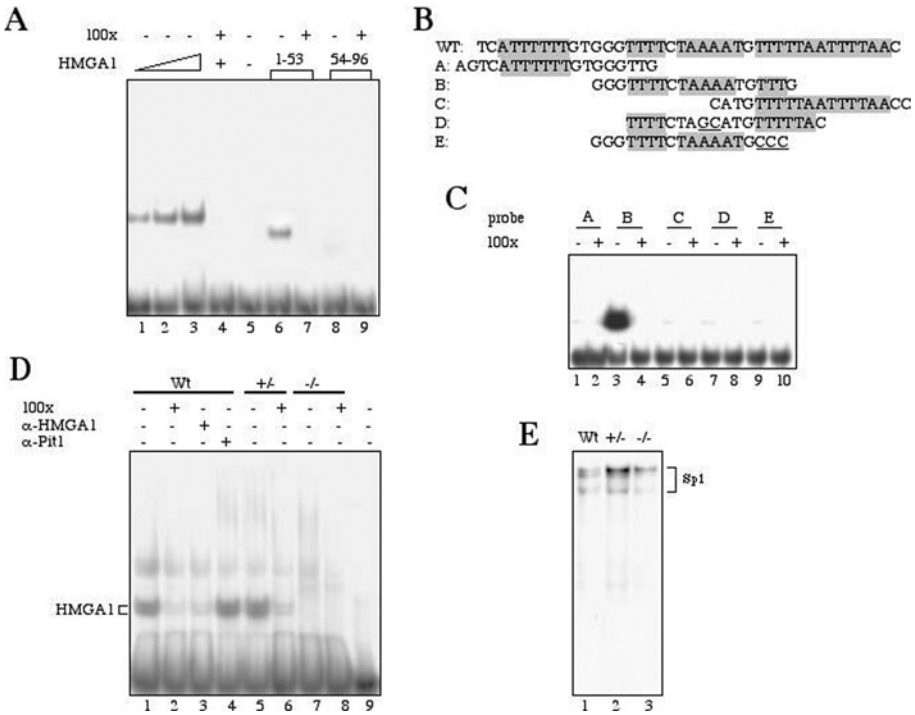

Figure 2 HMGA1 binding to RAG2 upstream regulating region

(**A**) EMSA was performed by incubating the radiolabelled wild-type RAG2 promoter oligonucleotide (RAG2pr) with 5 ng (lanes 1 and 4), 20 ng (lane 2) or 50 ng (lane 3) of the recombinant full-length HMGA1b–His protein or with 5 ng of truncated HMGA1b(1–53) (lanes 6 and 7) or HMGA1b(54–96) (lanes 8 and 9) proteins. Where indicated, a 100 times molar excess of unlabelled RAG2pr oligonucleotide was incubated as a specific competitor. (**B**) Sequences of RAG2 promoter oligonucleotides used in electrophoretic binding assays. Hypothetical HMGA1-binding sites are boxed in grey. WT, the wild-type sequence. Oligonucleotides A–C are shorter wild-type sequences, encompassing a few hypothetical HMGA1-binding regions. In oligonucleotides D and E, some nucleotides were mutated (underlined). (**C**) EMSA was performed with deleted or mutated oligonucleotides. The probes used (A–E) are the same as in (**B**). Full-length HMGA1b–His protein (5 ng) was incubated with the indicated probes (A–E) in the presence (+) or absence (–) of a 100 times molar excess of the corresponding unlabelled oligonucleotides, as specific competitor. (**D**) EMSAs were performed by incubating 8 μg of protein extracts from wild-type (wt), Hmga1+/– and –/– mouse spleens with the RAG2pr probe. Where indicated, the samples were preincubated either with anti-HMGA1 (α-HMGA1) or unrelated antibodies [anti-Pit-1 (α-Pit-1)] or with a 100 times molar excess of unlabelled RAG2pr. Two main specific complexes, corresponding to isoforms a an b of HMGA1, were observed in wt and Hmga1+/– extracts, whereas no binding activity was detected in Hmga1–/– extracts. (**E**) EMSA was performed with the same extracts as in (**D**), incubated with a probe corresponding to the Sp1 consensus sequence to normalize the amount of protein extracts.

[12] (Rag2pr) and containing four AT-rich putative HMGA1-binding sites (Figure 2B). As shown in Figure 2(A), increasing amounts (5, 20 and 50 ng) of a recombinant HMGA1 protein [18] were capable of binding the ^{32}P-end-labelled double-strand oligonucleotide in EMSA. This binding was specific, as demonstrated by competition with 100-fold molar excess of unlabelled RAG2pr oligonucleotide (lane 4) and by lack of competition when an unrelated unlabelled oligonucleotide was used as competitor (results not shown). To evaluate the regions of HMGA1 proteins involved in binding to the RAG2 promoter, we performed EMSAs by incubating 5 ng of truncated recombinant HMGA1 proteins with the RAG2pr probe. We demonstrated that the HMGA1 DNA-binding activity is due to the first two AT-hook domains, since HMGA1b(1–53), containing the first two AT-hook domains, was capable of binding RAG2pr, whereas HMGA1b(54–96), containing the spacer region between the second and third AT-hook, the third AT-hook domain and the C-terminal region of the protein was not (compare lanes 6 and 8). To map better the preferential HMGA1-binding sites on the RAG2 promoter, we assayed three shorter oligonucleotides, oligos A–C (Figure 2B), representing three different AT-rich segments of the RAG2 promoter. As shown in Figure 2(C), HMGA1 binds oligo B with high affinity, whereas the binding to oligos A and C is almost undetectable (lanes 1 and 5). Binding specificity was demonstrated by competition experiments after the addition of a 100-fold molar excess of unlabelled oligo B (lane 4). These results restrict the HMGA1-binding region to the central part of the sequence, which retains three of four putative HMGA1-binding sites (TTTT, AAAA and TTT). To identify which site is responsible for the binding, we mutated the A-stretch region (oligo D): we found that the HMGA1 protein was no longer able to bind the sequence (lane 7), demonstrating that the A-stretch is important for the binding. On the other hand, when the downstream T-stretch was mutated (oligo E), the A-stretch was not sufficient to carry on the binding (lane 9), showing that both the A-stretch and the downstream T-stretch are necessary for the binding (Figure 2C). Conversely, the upstream T-stretch does not consistently co-operate with the A-stretch for the binding, since oligo E contains both the upstream T- and A-stretches, but does not show a significant binding to HMGA1.

To verify the binding of HMGA1 to RAG2 promoter also in mouse tissues, we assayed the DNA-binding activity of total protein extracts from spleens of wild-type, heterozygous and knock-out mice to the RAG2pr probe. As shown in Figure 2(D), two specific complexes, with a mobility corresponding to isoforms a and b of HMGA1 proteins, were present in extracts from both wild-type and heterozygous spleens, whereas they were absent from extracts derived from homozygous Hmga1 knockout spleens. These complexes were specifically displaced by incubation with an antibody directed against the HMGA1 proteins (compare lanes 3 and 1), but not by an unrelated antibody (lane 4), showing that they do consist of HMGA1 proteins. Binding activity was normalized using an oligonucleotide probe for the ubiquitous Sp1 transcription factor (Figure 2E).

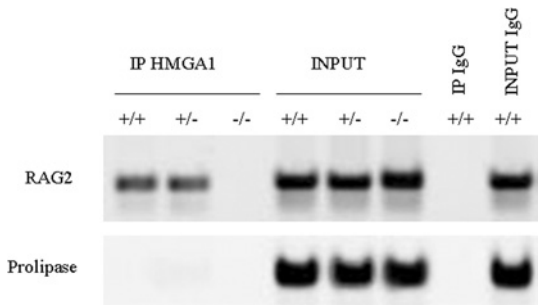

Figure 3 ***In vivo*** **binding of HMGA1 proteins to the RAG2 promoter region**

Chromosomes and nuclear proteins from Hmga1+/+, +/− and −/− ES cells were cross-linked and immunoprecipitated (IP) with anti-HMGA1 antibodies. The presence of the −279/+21 sequence of the RAG2 promoter was detected by PCR. INPUT indicates PCR products with chromosomal DNA without immunoprecipitation. As an immunoprecipitation control, IgG was used (lane 7). The lower panel shows PCR amplification of the immunoprecipitated DNA using primers for the prolipase gene promoter.

In conclusion, these results indicate that HMGA1 proteins are capable of binding directly a specific sequence in the RAG2 promoter region and that the normal spleens contain a binding activity that is lost in Hmga1−/− spleens.

To verify whether HMGA1 proteins bind the −297/+21 RAG2 promoter region *in vivo*, we performed chromatin immunoprecipitation experiments in Hmga1+/+, +/− and −/− ES cells (Figure 3). Anti-HMGA1 antibodies precipitated the −297/+21 RAG2 promoter region from Hmga1+/+ and +/− ES cells, but not from Hmga1−/− ES cells (Figure 3). The RAG2 promoter was immunoprecipitated by anti-HMGA1 antibodies, whereas no precipitation was observed with normal rabbit IgGs (lane 7); moreover, when primers for a control promoter (prolipase) were used, no band was detected (Figure 3, lower panel), suggesting that the reaction is specific for the RAG2 promoter. The results indicate that HMGA1 proteins bind the RAG2 promoter region *in vivo*.

HMGA1 proteins repress RAG2 promoter activity in functional assays

To investigate the effect of HMGA1 proteins on RAG2 promoter, we transiently transfected wild-type and double knockout ES cells with a construct (RAG2prom-luc) expressing the luciferase gene under the control of the mouse RAG2 promoter region, −279 to +21. The region spanning −279 to +123, conserved between mice and humans, has been shown to be necessary for maximal activity of RAG2 promoter [12]. As shown in Figure 4(A), Hmga1−/− ES cells showed a 2-fold increase in RAG2 promoter activity compared with wild-type.

We next transfected 293T cells with the RAG2prom-luc reporter construct. As observed previously [12], the promoter showed extremely low but reproducible activity in non-lymphoid cell lines, such as 293T. However, when the HMGA1 expression vector was transfected, a decrease in the luciferase activity was observed (Figure 4B). We showed previously that c/EBP-β co-operates with HMGA1 in activating the leptin gene promoter [17] by direct interaction with HMGA1. At least two binding motifs for c/EBP-β are present in the promoter region of the human RAG2 gene (at −146 to −138 and at −137 to −129) and mutations in the −137 to −129 region abrogate promoter activity [22]. In the mouse RAG2 promoter, a c/EBP-β consensus sequence is located at −154 to −146. To evaluate whether c/EBP-β and HMGA1 co-operate in regulating RAG2 promoter activity, we co-transfected a construct expressing c/EBP-β together with the HMGA1 expression construct: the co-transfection of c/EBP-β, together with Hmga1, induced a further decrease of luciferase activity (Figure 4B), whereas c/EBP-β by itself induced just a slight decrease.

These results indicate that HMGA1 proteins negatively regulate the RAG2 promoter in different cell types and that c/EBP-β is capable of co-operating with HMGA1 in repressing RAG2 expression.

DISCUSSION

The aim of the present study was to investigate the role of HMGA1 proteins in the regulation of RAG2 gene expression. The rationale for this study was our previous observation that disruption of the Hmga1 gene leads to alterations of lymphopoiesis [2].

The results presented here demonstrate that Hmga1−/− ES cells express higher levels of RAG2 and that increased RAG2 expression also occurs in MEFs and yolk sacs of Hmga1−/− mice. The increase in RAG2 expression is due to the lack of HMGA1 proteins, since the introduction of an Hmga1-expressing construct brings RAG2 expression to levels comparable with wild-type ES

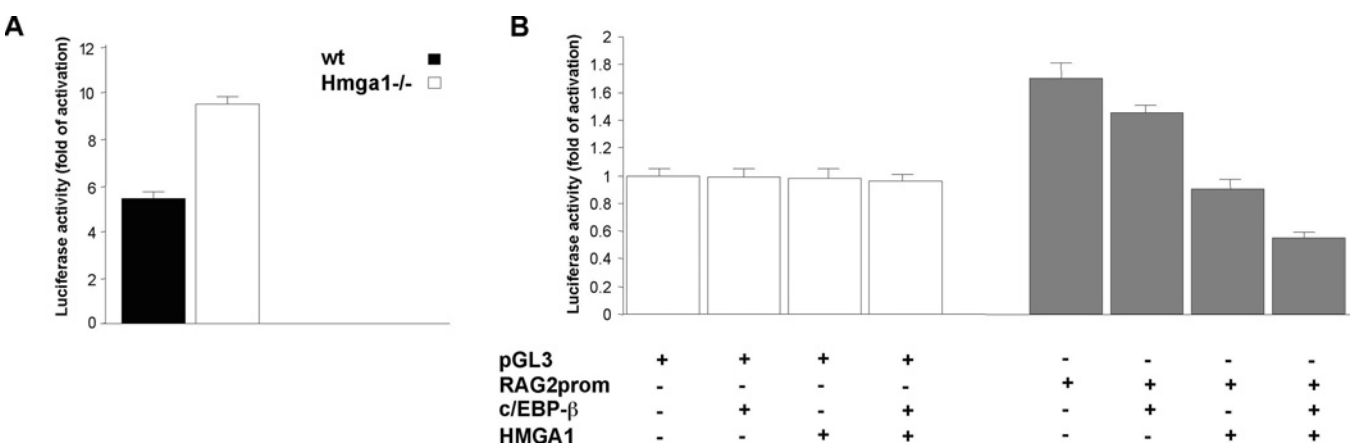

Figure 4 Functional assays of RAG2 promoter activity

Luciferase activity (fold activation) of RAG2 promoter in Hmga1+/+ and −/− ES cells (**A**) and in 293T cells (**B**). Where indicated, 3 μg of HMGA1 and/or 1 μg of c/EBP-β expression vectors were co-transfected.

cells. In support of this statement, we show that the HMGA1 proteins bind to the RAG2 promoter, *in vitro* and *in vivo*, by EMSA and chromatin immunopreciptiation assays respectively. This suggests a direct regulation of RAG2 by HMGA1. We conclude that the binding is mediated by two specific A and T adjacent stretches on the RAG2 promoter and by the first two AT-hook domains of the HMGA1 proteins. Moreover, we demonstrate that an HMGA1-expressing construct is capable of repressing the activity of a RAG2 promoter-driven luciferase gene in functional assays. We also show that the HMGA1 repressive activity is increased by the co-expression of c/EBP-β, a transcription factor already known to co-operate with HMGA1 in the regulation of other promoters [17] and possibly affecting human RAG2 promoter activity [22]. The slight decrease in gene expression, exerted by HMGA1 in transient transfections, matches with transient assays on other HMGA1-responsive promoters, where the light stimulating effect of HMGA1 is potentiated by co-operating transcription factors [23,24]. Together, these results demonstrate that HMGA1 proteins down-regulate RAG2 expression. On the other hand, the repressive activity of HMGA1 proteins on RAG2 promoter does not seem to be exerted through the down-regulation of other factors involved in RAG2 transcription such as Pax-5, c-Myb and GATA-3, since their expression is unchanged in Hmga1−/− ES cells compared with wild-type (results not shown). Since RAG2 plays a major role in B-cell/T-cell differentiation, the HMGA1-mediated down-regulation of RAG2 expression suggests a possible mechanism by which loss of HMGA1 expression may lead to an impairment of lymphoid differentiation *in vitro*.

Given the role of architectural transcription factor ascribed to HMGA1 (capable of recognizing the DNA structure rather than sequence), a probable mechanism by which HMGA1 proteins exert their action on the RAG2 promoter might be in opening the chromatin structure of RAG2 promoter region and improving its accessibility to appropriate transcriptional factors. Similar mechanisms have been already described for transcriptional regulation of the RAG1 gene [10]. Consistent with our findings, it has been recently reported that another member of the HMG-box family, LEF-1 (lymphoid enhancer-binding factor-1), binds the −41/−17 RAG2 promoter region and, together with c-Myb and Pax-5, is capable of activating the RAG2 promoter [9].

The negative regulation of RAG2 by HMGA1 might have quite important implications since an increased RAG2 expression may induce a higher V(D)J recombination and, in the absence of an appropriate cell-cycle checkpoint, lead to increased susceptibility to develop neoplasias of the lymphoid tissues. RAG proteins may also mediate the insertion of cleaved recombination signals into new DNA sites [25] and this mechanism has been proposed to be responsible for certain types of DNA translocation associated with lymphatic tumours. Interestingly, Hmga1−/− mice develop B-cell lymphomas, characterized by a high frequency of aberrant V(D)J rearrangements in the *IgH* gene (M. Fedele et al., unpublished work).

Since RAG2 activates double-strand breaks, our results suggest that HMGA1 proteins may have an indirect role in regulating processes such as double-strand breaks and recombination. We previously showed that HMGA1 is inherently involved in the down-regulation of the DNA repair protein BRCA-1 [20] and that this function is converted into a greater ability of Hmga1−/− ES cells to repair cisplatinum-induced DNA breaks [26]. On the other hand, HMGA1 can bind a fourway (Holliday) junction DNA, an intermediate structure formed by DNA during recombination processes [27,28], competing for its binding with histone H1 and HMG1 [29]. Consistently, a possible involvement of HMGA1 in *in vivo* processes such as genetic recombination, DNA repair and chromosome rearrangements have been proposed [28]. Together, these observations suggest that HMGA1 might have a specific and pleiotropic role at different steps of DNA break repair and recombination. Other than an indirect role (such as down-regulation of BRCA1 and RAG2), it might play a direct role in binding and bending of DNA, allowing the apposition of sequences to be recombined.

It has been shown that inactivating mutations in either RAG1 or RAG2 are responsible for the so-called 'Omen syndrome', in which no circulating mature B-cells are found, while a large number of poorly functional T-lymphocytes could be detected [30]. Conversely, overexpression of RAG proteins may contribute to some cases of human immunodeficiency [31] and lymphocytic leukaemia. Interestingly, Hmga1-null mice develop B-cell lymphomas (M. Fedele et al., unpublished work), whereas those overexpressing the full-length construct develop T-cell lymphomas [18]. Therefore it can be hypothesized that the impairment of the HMGA1 function might be responsible for some cases of human immunodeficiency.

In conclusion, the results presented here demonstrate that HMGA1 down-regulates the RAG2 promoter and suggest an additional mechanism for the modulation of lymphopoiesis by HMGA1 proteins.

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Hand1 gene expression is negatively regulated by the High Mobility Group A1 (HMGA1) proteins and is drastically reduced in human in thyroid carcinomas

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ABSTRACT

HMGA1 proteins play their major physiological role during embryonic development and have a critical role in neoplastic transformation. Here, we show that the *Hand1* gene, which codes for a transcription factor crucial for differentiation of trophoblast giant cells and heart development, is up-regulated in *hmga1* minus Embryonic Stem cells. Consistently with these results, we were able to demonstrate that HMGA1 proteins bind directly to *Hand1* promoter *in vitro* and *in vivo* resulting in the inhibition of the *Hand1* promoter activity. Since the HMGA1 proteins are over-expressed in malignant neoplastic tissues, we have also investigated *Hand1* expression in human thyroid carcinoma cell lines and tissues: an inverse correlation was found between HMGA1 and *Hand1* expression. Since the down-regulation of the *Hand1* gene expression was not associated neither to loss of heterozygosity nor to alteration of the methylation pattern, it is reasonable to hypothesize that HMGA1 over-expression may play a critical role in *Hand1* silencing. Finally, we show that the restoration of the *Hand1* induces a significant reduction in the growth rate of two thyroid carcinoma cell lines suggesting a role of the *Hand1* down-regulation in the process of thyroid carcinogenesis.

INTRODUCTION

The high-mobility group A (HMGA) protein family includes HMGA1a and HMGA1b, which are encoded by *hmga1* through alternative splicing (1 Johnson), and the closely related HMGA2 protein (2 Manfioletti). These proteins are non-histone architectural nuclear factors, that 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. The involvement of HMGA proteins in embryogenesis, cell proliferation, differentiation, apoptosis and, above all, cancer development has been extensively demonstrated (3 Reeves, Fedele carcinogenesis). HMGA1 proteins seem to play their major physiological role during embryonic development (4 Thanos), in fact, HMGA1 expression is very high during embryogenesis, whereas it is negligible in normal adult tissues. The generation of *hmga1* null mice unveiled a critical role of these proteins on cardiomyocytic cell growth: in fact, both heterozygous and homozygous mice for the *hmga1*-null allele showed cardiac hypertrophy, moreover, these mice also developed hematologic malignancies, including B cell lymphoma and myeloid granuloerythroblastic leukemia (6 Fedele 06).

HMGA1 proteins has been found abundant in several tumor cells, including colorectal, prostate, thyroid, cervical, lung, glioma (Donato), and thyroid, and the protein level is correlated to the increasing degree of malignancy or metastatic potential (3).

We identified HMGA1-regulated genes analyzing by microarrays the expression profile of ES cells carrying two, one and no *hmga1* functional alleles (5 Martinez). Among the genes showing an increased expression with a higher fold change in the ES *hmga1* minus cells in comparison to the

wild type, therefore negatively regulated by the HMGA1, we focused our attention on the *Hand1* gene, because of its role in heart development.

Hand1 (also named eHand/Hxt/Thing1) and *Hand2* (dHand/Hed/Thing2) belong to the Twist subfamily of Class B bHLH transcription factors (8 Cross et al., 1995; Cserjesi et al., 1995; Hollenberg et al., 1995; Srivastava et al., 1995 SCEGLIERE UNA DI QUESTE REFERENCES). *Hand* genes are expressed in numerous tissues, including the heart, lateral mesoderm and neural crest derivatives. In addition, *Hand1* is expressed at high levels in extra-embryonic membranes, whereas *Hand2* is expressed at high levels in the deciduum and at lower levels in extra-embryonic membranes. Both *Hand* genes are expressed in similar embryonic tissues during development but often with complementary instead of overlapping expression within the tissues (9 Dev & Disease 04 Cserjesi). Hand1 promotes giant cell differentiation and, when ectopically expressed in trophoblast stem cells, is sufficient to promote giant cell differentiation. This implies that suppressing Hand1 in the trophoblast stem cell compartment is essential for normal placental development (Cross Placenta 2005). Embryos carrying a homozygous mutation of *Hand1* arrest at E7.5 with severe abnormalities in differentiation of trophoblast giant cells (10 Riley et al., 1998). Additionally, *Hand1* has important functions in cardiac looping and differentiation since failures in these processes were observed upon rescue of the placental defect (11 Riley et al., 1998; Firulli et al., 1998). In humans, *Hand1* expression has been detected in trophoblast-like cells, the amniotic epithelium and in adult heart tissue, suggesting that the protein may fulfill similar functions (12 Gene 03 Knofler). Besides the importance of *Hand1* in cardiac function and development, a role of *Hand1* in cell proliferation and neoplastic transformation has been envisaged. In fact, the *Hand1* gene has

been reported down-regulated in human gastric, (13 CR Ushijima), pancreatic (14 Oncogene Ushijima) and ovarian carcinomas (15 …): hypermethylation of some genes, including *Hand1*, seems account for gene silencing in these neoplasias.

Here, we report that *Hand1* is up-regulated in *hmga1* minus Embryonic Stem cells, and that HMGA1 proteins bind directly to *Hand1* promoter *in vitro* and *in vivo* resulting in the inhibition of the *Hand1* promoter activity. Moreover, we report that *Hand1* gene expression is down-regulated, in absence of gene hypermethylation, in human thyroid carcinoma cell lines and tissues where the HMGA1 proteins are over-expressed. Finally, the restoration of *Hand1* expression inhibits the growth of thyroid carcinoma cell lines suggesting a critical role of the down-regulation of *Hand1* expression in thyroid carcinogenesis.

MATERIALS AND METHODS

Plasmids

For the Hand1prom-luc construct, the region -2424 – -2728 of the mouse *Hand1* gene was amplified using as primers gggatacacgaaggtcagtttt (forward) and ctgagatcccagatcactca (reversed), cloned in TA Cloning Vector (Invitrogen) and subcloned in pGL3 (Promega) KpnI-XhoI cloning site. The point mutations in the HMGA binding site of the *Hand1* promoter were generated using the QuikChange Mutagenesis Kit (Stratagene) in accordance with the manufacture's protocols. The primers used were tattttaactaattaGGtaataacagagtctcctcctgcc (forward) and ggcaggaggagactctgttattaCCtaattagttaaaata (reversed). Point mutations are shown in uppercase type. Hand1, HMGA1 and HMGA2 expression plasmids were constructed by cloning the murine full-length cDNAs of *Hand1* or *Hmga1b* or *Hmga2* into the mammalian expression vector pcDNA3.1 (Invitrogen).

Cell culture and transfections

The generation and the culture of *hmga1*+/- and *hmga1*-/- Embryonic Stem cells are described elsewhere (15 Battista). FRTL-5, FRTL-5-KiMSV and FRTL-5–HMGA1as-KiMSV cells, and their culture conditions are reported elsewhere (16 Berlingieri). Five x 10^5 FRTL-5 cells were plated in 6- well plates and transfected after 48 hours with 1 μg of reporter plasmid (either

*Hand1*prom-luc, *Hand1*promMUT-luc or pGL3), by Lipofectamine 2000 (Invitrogen). Where indicated, Hmga1 or Hmga2 were transfected. Cells were harvested 24 hours post-transfection and lysates were analysed for luciferase activity. Transfection efficiency was normalized using the β-galactosidase activity and fold of activation were calculated by dividing for pGL3 luciferase activity. All the assays were performed in triplicate and repeated in three independent experiments.

Human thyroid primary culture and human thyroid carcinoma cell lines (TPC-1, WRO, NPA, ARO, FRO, NIM 1, B-CPAP, FB-1, FB-2, Kat-4 and Kat-18) are described elsewhere (17). For cloning efficiency assays N-PA, FB-2 cells were either transfected with equal amounts of pCDNA3-Hand1 or the corresponding empty vector. 48 h post-transfection of the plasmids $5x10^5$ cells were plated on 10-cm dishes and cultured with medium supplemented with G418 (Invitrogen) for selection of transfected cells. Colonies were stained (1.5% glutaraldehyde and 0.06% methylene blue in Hanks' balanced salt solution (Invitrogen) and photographed.

Tissue samples

Neoplastic human thyroid tissues and normal adjacent tissue or the controlateral normal thyroid lobe were obtained from surgical specimens and immediately frozen in liquid nitrogen. Thyroid tumours were collected at the Service d'Anatomo-Pathologie, Centre Hospitalier Lyon Sud, Pierre Benite, France. The tumour samples were stored frozen until RNA extractions were performed. Tissues from *hmga1* +/- and *hmga1* -/- mice have been described elsewhere (15).

RNA extraction from tissues and cells

Tissues were snap-frozen in liquid nitrogen and stored at –80°C until use. Total RNAs were extracted from tissues and cell culture using TRI REAGENT® (Molecular Research Center INC) solution, according to the manufacture's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis (virtual presence of sharp 28S and 18S bands) and spectrophotometry.

Semiquantitative and quantitative RT-PCR

RNAs were treated with DnaseI (Invitrogen) and reverse-transcribed using random exonucleotides as primers and MuLV reverse transcriptase (Perkin Elmer). To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing the PCR on samples that were not reversed-transcribed but otherwise identically processed. For semiquantitative 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 and scanned using a Typhoon 9200 scanner. Digitized data were analyzed using Imagequant (Molecular Dynamics). Quantitative PCR was performed with SYBR® Green PCR Master Mix (Applied Biosystems) as follows: 95 °C 10 min and 40 cycles (95 °C 15 s and 60 °C 1 min). Each reaction was performed in duplicate. To calculate the relative expression levels we used the 2-DDCT method (18 Kenneth et al 2001). The primers sequences are: murine F gatgccttctcgagttaaaa and R aagtgtagcgacaagaagga, rat F gttcaggacccaaaaagg and R gcagagtcttgatcttggag, human F ctggctctttctctcttgtc and R cgtctggttctctttctcag.

Protein Extraction, Western Blotting and Antibodies

Tissues and cell culture were lysed in buffer 1% NP40, 1mM EDTA, 50mM Tris-HCl pH 7.5, 150 mM NaCl, supplemented with CompleteTM protease inhibitors cocktail (Roche Diagnostic Corp.). Total proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Biorad). Membranes were blocked with 5% non-fat milk and incubated with antibodies against Hand1 and Tubulin. Hand1 antibody was a generous gift from Dr. Peter Cserjesi (New Orleans, USA). Bound antibody was detected by the appropriate secondary antibody and revealed with an enhanced chemiluminescence system (Amersham-Pharmacia Biotech).

Electrophoretic Mobility Shift Assay

The production of recombinant proteins was previously described (20). Five or 20 ng of recombinant protein were incubated with radiolabeled double-strand oligonucleotides, corresponding to the region spanning bases -2658 to -2688 of the 5' untranslated region of the murine *Hand1* promoter (5'-atttatttttattttaactaattaattaataa-3'). A 200-fold excess of specific unlabeled competitor oligonucleotide was added. The same oligonucleotides were also used in binding assays with total extract from wild type and *hmga1*-knockout murine ES cells. 8 µg of extracts were incubated in 20 mM Hepes pH 7.6, 40 mM KCl, 0.1 mM EDTA, 0.5 mM MgCl2, 0.5 mM DTT, 0.1 mM PMSF in a volume of 20 µl containing 1 µg of poly(dC-dG), 2 µg BSA and 10% glycerol, for 10 min at room temperature. Binding reactions were incubated for 10 min after addition of 2.5 fmol of ^{32}P-end labeled oligonucleotides (specific activity, 8,000 to 20,000 cpm/fmol). The DNA-

protein complexes were resolved on 6% non-denaturing acrylamide gels and visualized by exposure to autoradiographic films.

Chromatin immunoprecipitation

Approximately $3x10^7$ wild type, *hmga1* +/- and -/- ES cells were grown on 75 cm^2 dishes. Chromatin immunoprecipitation was performed as previously described (21). Input and immunoprecipitated DNA were analyzed by PCR for the presence of *Hand1* promoter sequence spanning –2424 to -2927. The primers used were 5'- cttggtgacaagcacctt-3' (forward) and 5'- ctgagatcccagatcactca-3' (reverse).

Methylation analysis using bisulphite genomic sequencing

The promoter region of human *HAND1* gene was analyzed for CG content; a CpG island was determined based on a 200-bp length of DNA with a CG content of >50% and a CpG/GpC ratio of >0.5, using CpGplot program, available at http://www.ebi.ac.uk/emboss/cpgplot/. Bisulphite genomic sequencing was used to analyze the methylation patterns of individual DNA molecules. Sodium bisulphite conversion of genomic DNA (about 200 ng for each conversion) was obtained using EZ DNA Methylation Kit™ (ZYMO Research) following the manufacturer's instructions. The CpG islands identified as described previously were then PCR amplified using the following primers: *Pre-Nested PCR* 3FHand (-461 to -430)

gtagagtagttggagttygggattgggaattg, 3RHand (+180 to +211) ctccatacrccccaaaaactaccraaaaccac. *Nested PCR* 3FnHand (- 275 to -249) ggaggggtggtagtaatagtttaggg, 3RnHand (+170 to +201) ccccaaaaactaccraaaaccacctataaactc. PCR reactions were carried out using FastStart Taq DNA polymerase (Roche) under the following conditions: 1) Pre-nested PCRs were normally carried out on 10 ng of bisulphite treated DNA in a final reaction volume of 50 ml, using standard conditions with 1,5 min at 95°C, followed by 5 cycles of 30 sec at 95°C, 30 sec at 59°C, and 40 sec at 72°C, then 25 cycles of 30 sec at 95°C, 30 sec at 57°C, and 40 sec at 72°C, then a final elongation of 6 min at 72°C before holding at 4°C. 2) Nested PCRs were performed in the same conditions, using 5 ml of the corresponding pre-nested PCRs in a final reaction volume of 50 ml. PCR final products (477 bp) were then cloned into the pGEM-Teasy vector provided by Promega pGEM®-T Easy Vector System II, following the supplier's procedures. The positive screened colonies contained the unique sequence of one individual DNA molecule. The plasmidic DNA from the selected positive colonies containing vectors with the insert was purified using the Qiagen plasmid Mini Kit. The purified plasmids were sequenced in both directions using T7 and Sp6 primers. 20 independent clones for each genomic preparation and fragment of interest were sequenced to determine

the methylation pattern of individual molecules. Sequencing was performed at the CEINGE Sequencing Core Facility.

SNP-based Loss of Heterozygosity analysis

We performed LOH analysis using Single Nucleotide Polymorphisms (SNP). To identify the SNPs scattered in the genetic locus of *HAND1,* we input *HAND1* human gene name in the SNP database of NCBI and we found eight SNPs. The primers, SNP reference and alleles are: F-cgaaataggcaaacaggctc and R- aaagctcatccagggacga for rs924581 (A/G); F-gaagacccgatctgttttacct and R- cttcaaggctgaactcaagaa for rs4370323 (A/G), rs1846966 (C/G), rs11748765 (A/T); F- cgctgttaatgctctcagt and R-gtaaaacctgggatagcca for rs6880185 (A/G), rs13171812 (C/T), rs993098 (A/C), rs3822714 (A/G). The primers used for PCR was also used for sequencing assays. PCR was performed using HotMaster Taq DNA Polimerase (Eppendorf AG, Germany) in a final volume of 25 μl. For amplification reaction we used 50 ng of genomic DNA, 0,5 unit of Hotmaster Taq DNA polymerase, a final concentration of each primer of 0.2 μM and 0.2mM of dNTPs and 2,5 μl of 10x HotMaster Taq DNA Polimerase Buffer with Mg2+. The conditions used for PCR was an initial denaturation at 94°C for 2 min, 35 cycles of 94°C for 20 sec, 58°C for 10 sec, 70°C for 40 sec and a final extension of 5 min at 70°C. After amplification the size and quality of amplicons was checked loading 5 μl of reactions on agarose gel. 20 μl of each PCR was sequenced with specific forward and reverse primers used for amplification reaction.

RESULTS

Loss of HMGA1 correlates with an increased *Hand1* expression in murine ES cells, heart and thyroid tissues

Microarray analysis of the expression profile of embryonic stem (ES) cells bearing one or two disrupted *hmga1* alleles revealed the *Hand1* gene as a gene likely negatively regulated by HMGA1. In fact, the chip showed a 3,8 fold-change for heterozygous cells and a 14 fold-change for homozygous cells (Martinez et al. 04). Therefore, our first aim was to validate the results obtained by microarray analysis by semiquantitative and quantitative RT-PCR (Figure 1A and 1B). These analyses confirmed the differential expression between wild type and *hmga1*-knockout ES cells. They clearly showed that regulation of *Hand1* expression was HMGA1-dose dependent since an intermediate level of *Hand1* expression was observed in the heterozygous ES cells.

The analysis of the *Hand1* expression in heart and thyroid tissues derived from *hmga1*-knockout mice revealed the same kind of regulation by HMGA1 (Figure 1C). Conversely, no changes in *Hand1* expression were observed depending on the *Hmga1* expression when embryonic fibroblasts, brain, spleen, liver, and thymus from *hmga1* minus mice were analyzed (data not shown) This confirmed that HMGA1-mediated gene regulation depends on the cellular context.

Interestingly, when we analyzed same tissues from the *Hmga2* minus mice, no changes in *Hand1* expression were observed indicating that *Hand1* regulation was HMGA1 specific.

HMGA1 proteins bind to murine *Hand1* promoter *in vitro* and *in vivo*

To evaluate whether the differential *Hand1* gene expression was a direct effect of HMGA1 we performed an electrophoretic mobility shift assay (EMSA). In particular, we analyzed a region spanning nucleotides -2658 to -2688 of the 5' untranslated region of the murine *Hand1* gene containing AT-rich putative HMGA1 binding sites. As shown in Figure 2A, a recombinant HMGA1 protein was able to bind directly to this region. Binding specificity was demonstrated by competition experiments showing loss of binding with the addition of 200-fold molar excess of specific, unlabeled oligonucleotides. Subsequently, we performed binding assays with total extract from wild type and *hmga1*-knockout ES cells. A specific complex with a mobility corresponding to the HMGA1 proteins was present in extracts from wild type mice while it was absent in extracts from homozygous *hmga1*-knockout ES cells (Figure 2 B). To verify that HMGA1 proteins bind to *Hand1* promoter *in vivo* we performed experiments of chromatin immunoprecipitation. Anti-HMGA1 antibodies precipitated *Hand1* promoter from *hmga1* +/+ and +/- ES

cells, but not from *hmga1* -/- ES cells (Figure 2C). Moreover, no amplification was observed in samples immunoprecipitated with a not related antibody.

HMGA1 proteins repress murine *Hand1* promoter

In order to investigate the functional effect of HMGA1 proteins on *Hand1* promoter, we transiently transfected the rat thyroid cells, FRTL-5, that express low levels of HMGA1 with a construct expressing the luciferase gene under the control of the mouse *Hand1* promoter region -2424 – -2728. As shown in Figure 3A, when the HMGA1 expression vector was transfected, a reduction of the luciferase activity was observed in a dose-dependent manner. No decrease in *Hand1* promoter activity was obtained when the cells were transfected with a construct expressing HMGA2 the other member of the HMGA family. Then, we generated two point mutations in the putative binding site for the HMGA1 protein replacing adenine -2681 and thymidine -2682 with two guanines. Over-expression of HMGA1 was able to reduce the activity of the wild type construct, but it completely failed in its inhibitory effect if the HMGA1 binding site was mutated (Figure 3B).

***Hand1* expression is drastically decreased in thyroid cell transformation**

The HMGA1 proteins are over-expressed in several malignant neoplasias. Then, in order to evaluate a possible role of *Hand1* regulation by HMGA1 in

cell transformation, we analyzed, by RT-PCR, the expression of *Hand1* in FRTL-5 normal thyroid cells which do not express significant levels of HMGA1, in the same cells transformed by the Kirsten murine sarcoma virus, which express high levels of HMGA1 (FRTL-5 –KiMSV) and in the FRTL-5 cells which have been transfected with an *hmga1* antisense construct before being infected by Ki-MSV (FRTL-5-HMGA1as-KiMSV cells), and then do not express the HMGA1 proteins. A significant *Hand1* expression was observed in the cells which do not express the HMGA1 proteins, such as the FRTL-5 and FRTL-5-HMGA1as-KiMSV), whereas no expression was observed in the FRTL-KiMSV cells expressing high HMGA1 levels (Figure 4A).

These results confirm an inverse correlation between HMGA1 and Hand1 expression also in transformed cells.

Subsequently, we analyzed the expression of HMGA1 and Hand1 in a panel of 13 human thyroid carcinoma-derived cell lines. An inverse correlation between HMGA1 and Hand1 expression levels was observed. In fact, HMGA1 expression was increased in all tumor-derived cell lines tested when compared to normal primary cultured cells used as a control (Figure 4B); conversely, HAND1 mRNA expression level was much lower in all of the thyroid carcinoma cell lines compared to normal cells (Figure 4B). **Therefore,** **Finally,** Hand1 and HMGA1 gene expression was examined in 20 surgically removed human thyroid carcinomas by real time PCR (Figure 4C). Again, an inverse correlation between HMGA1 and Hand1 mRNA levels was observed. In fact, HMGA1 mRNA levels were almost undetectable in normal thyroid tissue, whereas they were highly expressed in most of the tumors analyzed. In contrast, Hand1 expression was strongly

diminished in almost all of the tumor samples compared to normal thyroid tissue.

Down-regulation of HAND1 expression in human thyroid carcinomas is not associated to LOH and DNA methylation

We have analyzed the methylation status of thyroid carcinoma samples showing the lowest HAND1 expression and in normal thyroid. We have analyzed two regions of the 5' untranslated region of the human *HAND1* gene, described under Materials and Methods.

These regions were unmethylated in normal and carcinoma samples indicating that the silencing of *Hand1* gene in neoplastic samples is not dependent on the gene hypermethylation.

Therefore, we analyzed thyroid carcinoma samples for Loss of Heterozygosity (LOH). We analyzed eight Single Nucleotide Polymorphisms: however no LOH was found.

Therefore, these results would suggest that it is likely that the HMGA1-negative regulation plays a critical role in *Hand1* gene suppression in human thyroid carcinomas.

Restoration of *Hand1* gene expression inhibits the growth of thyroid carcinoma cell lines.

To determine whether loss of *Hand1* gene expression affects thyroid carcinogenesis, we evaluated the growth rate of thyroid carcinoma cell lines in which *Hand1* expression had been restored. To this aim we carried out a

colony forming assay with cell lines obtained from human thyroid carcinomas (NPA, FB-2) after transfection with the vector carrying the *Hand1* gene or the empty backbone vector. The colonies were scored after two weeks. Cells transfected with the *Hand1* gene generated a lower number of colonies than did cells transfected with the backbone vector (Figure 6).

DISCUSSION

In our previous work we identified HMGA1-regulated genes analyzing by microarrays the expression profile of embryonic stem (ES) cells bearing one or two disrupted *hmga1* alleles. We found the *Hand1* gene, a transcription factor crucial for differentiation of trophoblast giant cells and heart development, negatively regulated by HMGA1 proteins with a high fold change in the ES *hmga1* minus cells in comparison to the wild type (Martinez). Moreover, we examined the consequences of disrupting the *Hmga1* gene in mice and we reported that both heterozygous and homozygous *Hmga1*-null mice show cardiac hypertrophy due to the direct role of HMGA1 on cardiomyocytic cell growth regulation (Fedele).

Here, we focused our attention on *Hand1* gene regulation by HMGA1 proteins. We validated the microarray results by semiquantitative and quantitative RT-PCR in ES cells. As in microarrays, we found that *Hand1* expression displayed dose-dependency, the phenotype of heterozygous cells was intermediate between those of wild type and homozygous knockout cells. When we extended the analysis to embryonic fibroblasts and diverse tissues we found an increased *Hand1* expression only in heart and thyroid from *hmga1*-knockout mice respect to wild type. This results indicated that HMGA1-mediated *Hand1* regulation depends on the cellular context.

When we analyzed by RT-PCR the expression of *Hand1* in MEF and adult tissues taken from *hmga2*-knockout mice, we found no changes between wild type and knockout cells and tissues. This result indicates that *hand1* is probably not regulated by HMGA2. In fact, by Luciferase assay, we did not found an alteration in promoter activity when we included an *Hmga2*

expression construct. This specific responsiveness to HMGA1 and not HMGA2 confirms that even though HMGA1 and HMGA2 have a similar structure and expression profile (high during embryogenesis and neoplastic tissue), they exert different functions. This is consistent with our previous findings: the phenotype of the *hmga1*- and *hmga2*-knockout mice is divergent: a reduction in size and fat tissue in *hmga2*-null mice, and cardiac hypertrophy and B-cell lymphomas in *hmga1*-null mice (Fedele).

We wanted to know if HMGA proteins could regulate also *Hand2*, a gene that belongs to the Twist subfamily of class B bHLH transcription factors as *Hand1*, and shares 87% homology in bHLH region. We did not find altered expression of *Hand2* neither in ES cells, MEF, nor in adult tissues from *hmga1*- and *hmga2*-knockout mice (data not shown).

To demonstrate a direct effect of HMGA1 on *Hand1* regulation, we performed mobility shift assay and chromatin immunoprecipitation experiments. We show that the HMGA1 proteins bind to the *Hand1* upstream regulating region, *in vitro* and *in vivo*. We performed Luciferase assay to demonstrate a reduction in *Hand1* promoter activity caused by HMGA1 expression. Since *Hand1* plays a major role in heart development, the HMGA1-mediated regulation of *Hand1* suggests a possible mechanism by which loss of HMGA1 expression may lead to cardiac hypertrophy.

Our next aim was study the role of *Hand1* in thyroid transformation. The rationale for this study was that HMGA1 are over-expressed in several malignant neoplasias and have a major role in transformation, since we have demonstrated that *Hand1* is regulated by HMGA1 proteins, we suspected that *Hand1* could have a role in transformation. Moreover, in literature it has

been described *Hand1* silencing due to promoter methylation in pancreas, stomach and ovarian carcinomas (Ushijima).

First of all, we study by RT-PCR *Hand1* expression in a cell system constituted by normal rat thyroid cells (FRTL-5) that do not express HMGA1 proteins, the same cells malignantly transformed by the KiMSV (FRTL-5-KiMSV) that express high HMGA1 levels and FRTL-5-KiMSV cells in which the synthesis of the HMGA1 protein was blocked by an antisense construct (FRTL-5-HMGA1as-KiMSV). We found Hand1 expression in cells that do not express HMGA1 and vice versa, confirming an inverse correlation between HMGA1 and Hand1 expression. We found same result when we analyzed by RT-PCR 12 human thyroid carcinoma-derived cell lines and by real time PCR 20 human thyroid carcinomas surgically removed. IHC …

We did not find alterations of DNA methylation of *HAND1* gene in human thyroid carcinomas, suggesting that *Hand1* down-regulation is not mediated by this phenomenon. We analyzed two regions of the 5'untranslated region of the human *HAND1* gene, one has been already described to be methylated in some tumors and cancer cell lines (Ushijima). This discrepancy can be explained by the fact that HMGA1-mediated gene regulation depends on the cellular context, we have demonstrated that *Hand1* is regulated by HMGA1 in some cell and tissues but not in others. Maybe, what is important is *Hand1*-silencing, in some tumors this is accomplished by promoter methylation and in another tumors this is accomplished by HMGA1-down-regulation. Therefore we analyzed thyroid carcinoma samples for loss of heterozygosity (LOH) however, no LOH was found. In literature, no micro RNA targeting *HAND1* have been found altered in human thyroid

carcinomas (Croce 2005 e Fusco 2006), so we exclude that the down-regulation of *Hand1* is due to micro RNA altered expression. For all these reasons we hypothesize that direct *Hand1* down-regulation exerted by HMGA1 proteins may account for the malignant phenotype in human thyroid carcinomas.

To confirm Hand1 role in transformation, we have performed Colony assay. Upon re-expression of Hand1 in human thyroid carcinoma cell lines lacking endogenous *Hand1* expression, colony formation was significantly compromised in two cell lines studied. ……

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21.

LEGEND TO FIGURES

Figure 1

***Hand1* expression in *hmga1*-knockout cells and tissues.**

Semiquantitative (**A**,**C**) and quantitative (**B**) RT-PCR analyses for *Hand1* expression were performed on RNA extracted from wild type (+/+), *hmga1*-single knockout (+/-) and *hmga1*-double knockout (-/-) ES cells and tissues. β-actine expression was evaluated as internal control.

Figure 2

HMGA1 binding to *Hand1* upstream regulating region.

A) Electrophoretic mobility shift assay performed incubating radiolabeled oligonucleotides spanning from -2658 to -2688 of the 5' untranslated region of the murine *Hand1* gene with 5 or 20 ng of the recombinant HMGA1 as indicated. To assess the specificity of the binding, a 200x molar excess of unlabeled oligonucleotides were incubated as specific competitor. **B**) Electrophoretic mobility shift assay performed with the same oligonucleotides as in panel A, incubated with total extracts from wild type (+/+) and *hmga1*-double knockout (-/-) ES cells. **C**) Chromatin immunoprecipitation assay was performed on wild type, *hmga1*-single knockout (+/-) and *hmga1*-double knockout (-/-) ES cells. The presence of -2424 – -2927 sequence of the murine *Hand1* promoter was detected by PCR. Anti-HA was used as a negative immunoprecipitation control.

Figure 3

Repression of *Hand1* promoter activity by HMGA1 proteins.

(**A**) Effect of HMGA1 expression on the activity of murine *Hand1* promoter transfected in FRTL-5 cells. (**B**) the same promoter in which A -2681 and T -2682 were replaced with G.

Figure 4

Hand1 expression in thyroid tumorigenesis.

Hand1 expression in transformed rat thyroid cells over-expressing HMGA1 (**A**). Sources of RNA are FRTL-5, rat thyroid epithelial cell line; FRTL-5-KiMSV, FRTL-5 infected with the Kirsten murine sarcoma virus; FRTL-5-HMGA1as-KiMSV, FRTL-5 transfected with a construct carrying HMGA1 mRNA in an antisense orientation, and then infected with the Kirsten murine sarcoma virus. Hand1 expression in human thyroid carcinoma cell lines (**B**) and human thyroid tumors (**C**). β-actine expression was used as internal control of RNA quantity.

Figure 5

Analysis of Hand1 expression in normal and neoplastic thyroid tissues by immunohistochemistry.

Figure 6

Restoration of Hand1 gene expression reduces clonogenic capacities of thyroid cancer cells.

N-PA and FB-2 thyroid cancer cell lines were transfected with a Hand1 expression vector (**A**,**C,**) or control vector (**B**,**D,**) and selected for neomycin resistance. Three weeks after transfection plates were stained, counted, and photographed.