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"Gene expression profile analysis of pituitary adenomas developed by HMGA transgenic mice"

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Gene expression profile analysis of pituitary adenomas developed by HMGA transgenic mice

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This dissertation is based upon the following publications:

1 **De Martino I**, Visone R, Palmieri D, Cappabianca P, Chieffi P, Forzati F, Barbieri A, Kruhoffer M, Lombardi G, Fusco A, Fedele M. The Mia/Cd-rap gene expression is downregulated by the high-mobility group A proteins in mouse pituitary adenomas. Endocr Relat Cancer. 2007;14(3):875-886.

2 Fedele M, **De Martino I**, Pivonello R, Ciarmiello A, Del Basso De Caro ML, Visone R, Palmieri D, Pierantoni GM, Arra C, Schmid HA, Hofland L, Lombardi G, Colao A, Fusco A. SOM230, a new somatostatin analogue, is highly effective in the therapy of growth hormone/prolactin-secreting pituitary adenomas. Clin Cancer Res. 2007;13(9):2738-44.

3 **De Martino I**, Fedele M, Palmieri D, Visone R, Cappabianca P, Wierinckx A, Trouillas J, Fusco A. B-RAF mutations are a rare event in pituitary adenomas. J Endocrinol Invest. 2007;30(1):RC1-3

4 Fedele M, Visone R, **De Martino I**, Troncone G, Palmieri D, Battista S, Ciarmiello A, Pallante P, Arra C, Melillo RM, Helin K, Croce CM, Fusco A. HMGA2 induces pituitary tumorigenesis by enhancing E2F1 activity. Cancer Cell. 2006;9(6):459-71.

5 Fedele M, Fidanza V, Battista S, Pentimalli F, Klein-Szanto AJ, Visone R, **De Martino I,** Curcio A, Morisco C, Del Vecchio L, Baldassarre G, Arra C, Viglietto G, Indolfi C, Croce CM, Fusco A. Haploinsufficiency of the Hmga1 gene causes cardiac hypertrophy and myelo-lymphoproliferative disorders in mice. Cancer Res. 2006;66(5):2536-43.

6 Fedele M, Pentimalli F, Baldassarre G, Battista S, Klein-Szanto AJ, Kenyon L, Visone R, **De Martino I**, Ciarmiello A, Arra C, Viglietto G, Croce CM, Fusco A. Transgenic mice overexpressing the wild-type form of the HMGA1 gene develop mixed growth hormone/prolactin cell pituitary adenomas and natural killer cell lymphomas. Oncogene 2005;24(21):3427-35.

ABSTRACT

The High Mobility Group A (HMGA) nonhistone chromatin proteins alter chromatin structure and, thereby, regulate the transcription of several genes by either enhancing or suppressing transcription factors. This protein family is implicated, through different mechanisms, in both benign and malignant neoplasias. Rearrangements of HMGA genes are a feature of most benign human mesenchymal tumors. Conversely, unrearranged HMGA overexpression is a feature of malignant tumors and is also causally related to neoplastic cell transformation. Recently, we have generated transgenic mice carrying wild type or truncated *HMGA* genes under transcriptional control of the cytomegalovirus promoter. These mice developed pituitary adenomas secreting prolactin and growth hormone. We have recently demonstrated that the mechanism of the HMGA2 induced-pituitary tumorigenesis is based on the increased E2F1 activity.

To identify other genes involved in the process of pituitary tumorigenesis induced by the *HMGA2* and *HMGA1* genes, we have analysed here the gene expression profile of HMGA2- and HMGA1-induced pituitary adenomas in comparison with a pool of ten normal pituitary glands from control mice using the Affymetrix MG MU11K oligonucleotide array representing ~ 13,000 unique genes. We have identified 82 transcripts increased and 72 transcripts decreased of at least 4-fold in all mice pituitary adenomas analyzed compared to normal pituitary gland. The microarray results have been confirmed by semi-quantitative RT-PCR on RNA extracted from different HMGA2- and HMGA1-induced mouse pituitary adenomas.

Then, we focused our attention on the *Mia/Cd-rap* and *Cyclin B2* gene, the first down-regulated and the second up-regulated in all pituitary adenomas tested by the microarray. We demonstrated that the HMGA proteins directly bind and regulate their promoters.

1. INTRODUCTION

1.1 HMG proteins

In the mammalian species the high-mobility-group HMG proteins have been grouped in three distinct families: HMGB, HMGN, and HMGA.

These HMG families have been grouped based on similarity in physical and chemical properties but they 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 (Catez et al. 2004). 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.

The HMGB (formerly HMG1/2), chromosomal proteins, are characterized by the HMG box, a DNA-binding domain that both introduces a tight bend into DNA and binds preferentially to a variety of distorted DNA structures. The HMGB proteins seem to act primarily as architectural facilitators in the formation of nucleoprotein complexes; for example, in the assembly of complexes involved in recombination and transcription. Recent genetic and biochemical evidences suggest that these proteins can facilitate nucleosome remodeling. One mechanism by which HMGB proteins could prime the nucleosome for migration is to loosen the wrapped DNA and so enhance accessibility to chromatin-remodeling complexes and possibly also to transcription factors. By constraining a tight loop of untwisted DNA at the edge of a nucleosome, an HMGB protein could induce movements in the contacts between certain core histones that would result in an overall change in nucleosome structure (Bianchi et al. 2000, Thomas et al. 2001).

The HMGN (formerly HMG14/-17) proteins alter chromatin architecture by decompacting the nucleosomal array. Studies on mini chromosomes have revealed that two HMGN molecules bind to each nucleosome via their highly conserved nucleosome-binding domains (Bustin 2001). The negatively charged C-terminal domains induce chromatin unfolding such that the mini chromosomes have a decreased sedimentation rate in a sucrose gradient and are more accessible to nucleases. The functional consequences of this change in architecture are increases in the rates of transcription and replication (Trieschmann et al. 1995; Crippa et al. 1993; Vestner et al. 1998). Functional links between HMGN proteins and transcription have also been suggested by studies on intact cells. In fact, various reports have shown that HMGNs may be preferentially associated with actively transcribed genes (Bustin

2001), and intracellular localization experiments have shown that HMGNs colocalize with nascent transcripts (Hock et al. 1998).

The 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).

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 & Thanos 2001), HMGB1 stabilizes the binding of the glucocorticoid receptor to chromatin (Bianchi & 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 (Fig. 1.1). 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 HMGA1a/b; 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).

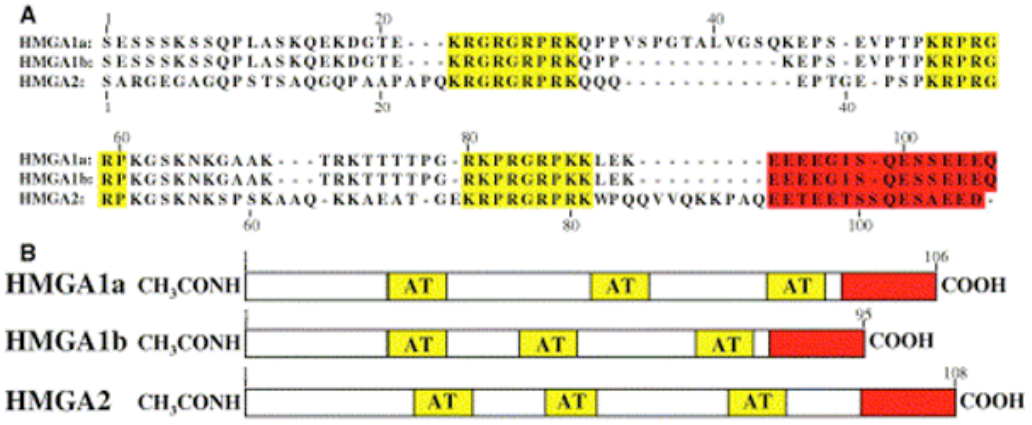


Figure 1.1: Primary structure of human HMGA proteins. (A) Amino acid residues sequences of HMGA1a, HMGA1b, and HMGA2 proteins. AT-hooks (AT) are evidenced in yellow, the C-terminus in red. (B) Scheme of the sequences of HMGA proteins pointing out to the differently spaced AT-hooks (in yellow) and C-terminal ends (in red) along the three protein molecules.

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 (Lewis et al. 2001). 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, HIPK2, SRF and Tst-1/Oct-6. Recently, other molecular partners have been 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, Pierantoni et al. 2006).

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
ATP-dependent helicase (DDX1	Q92499
hnRNP M	P52272
TAFII68	Q92804
DDX17	Q92841
DDX5	P17844
Heat shock cognate 71 kDa Protein	P11142
RNA-binding protein FUS	P35637
DRP-2	Q16555
Tubulin beta-1 chain	P07437
MA32	Q07021
hnRNP H3	P31942

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 type of promoters often involves the formation of an "enhanceosome", a stereo-specific, multi-protein complex that includes HMGA proteins and other transcription factors 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).

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 (Fig. 1.2). 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 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 through the "enhanceosome" of GCN5/PCAF that acetylates the nucleosome and also HMGA1a at K64, the latter modification resulting in the stabilization of the enhanceosome itself. Later, another acetyltransferase, called CBP, acetylates HMGA1a at K70 destabilizing the enhanceosome and, consequently, repressing transcription (Munshi et al. 1998).

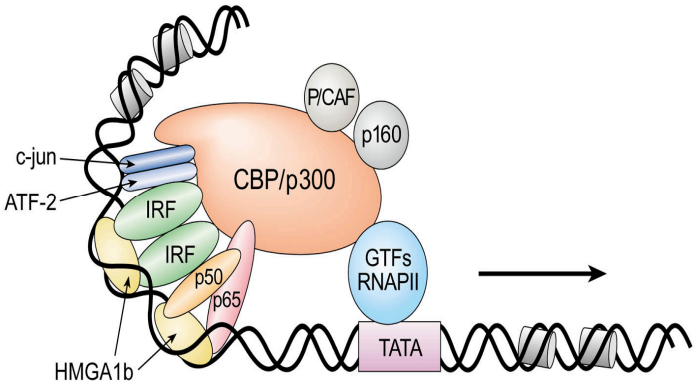


Figure 1.2 HMGA proteins facilitate the assembly of specific multiprotein complexes required for gene promoter activation: example of the human β–interferon gene.

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	

1.5 HMGA functions in development

The high expression of HMGA proteins during embryogenesis suggests that they play important roles in development. Indeed, the phenotype characterization of mice knockout for each of the *HMGA* genes revealed crucial roles of these proteins in different aspects of development. Cardiac hypertrophy and type 2 diabetes were observed in *Hmga1*-null and heterozygous mice (Fedele et al. 2006a, Foti et al. 2005) suggesting that quantitatively appropriate expression of the HMGA1 proteins are required for cardiomyocytic cell growth and function of the insulin pathway. *Hmga2*-null and heterozygous mice showed a pygmy phenotype with a decreased body size of 20% in heterozygous and 60% in homozygous mice, and a drastic reduction of the fat tissue (Zhou et al. 1995), suggesting a critical role of the *Hmga2* gene in the control of the body growth and adipocyte proliferation and differentiation.

We have previously shown that HMGA1 is present in mitotic cells (spermatogonia and primary spermatocytes), whereas HMGA2 is highly expressed in meiotic and post-meiotic cells (secondary spermatocytes and spermatids) (Chieffi et al. 2002, Di Agostino et al. 2004); in addition, we have demonstrated a specific function for HMGA2 in the regulation of spermatogenesis, because this differentiation program is dramatically hampered in HMGA2$^{-/-}$ mice in spite of the presence of HMGA1 (Chieffi et al. 2002).

Therefore, even though HMGA1 and HMGA2 may have overlapping functions, they seem to have different roles in development.

1.6 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 only observed in CD34 positive hematopoietic stem cells, and in mouse pre-adipocytic proliferating cells. The *HMGA1* gene is expressed at very low levels in adult murine and human tissues: a higher expression was observed only in testis, skeletal muscle ant thymus. Conversely, both the genes are widely expressed during embryogenesis.

Over-expression of the HMGA proteins was instead 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. 2001a). 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 (Chiappetta et al. 2001). 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).

1.7 HMGA as cellular oncogenes

Several studies, both *in vitro* and *in vivo*, clearly established the oncogenic potential of HMGA proteins. Transfection of an antisense construct for the HMGA2 cDNA into normal thyroid cells (FRTL5), followed by infection with transforming myeloproliferative sarcoma virus or Kirsten murine sarcoma virus KiMSV, generated cell lines that expressed significant levels of the retroviral transforming oncogenes v-mos or v-ras-Ki and removed the dependency on thyroid-stimulating hormones. However, in contrast with untransfected cells or cells transfected with the sense construct, those containing the antisense construct did not demonstrate the appearance of any malignant phenotypic markers (growth in soft agar and tumorigenicity in athymic mice). HMGA1 protein was also reduced by HMGA2 antisense, suggesting HMGA1 also might play a role in neoplastic transformation (Berlingieri et al. 1995).

Also the block of HMGA1 protein expression prevented thyroid cell transformation by KiMSV (Berlingieri et al. 2002), thus strongly indicating that HMGA1 protein plays a crucial role and HMGA2 an accessory role in thyroid cell transformation. Consistent with these data, the expression of an adenovirus carrying the *HMGA1* gene in an antisense orientation suppressed HMGA1 protein synthesis and induced cell death of two human thyroid anaplastic carcinoma cell lines, but not normal thyroid cells (Scala et al. 2000). Nevertheless, it is noteworthy that HMGA1 alone does not transform normal rat thyroid cells PC Cl3: the transfected cells entered the S phase earlier than normal cells, they stopped replicating at G_2-M phase and underwent programmed cell death (Fedele et al. 2001b). The different cellular context may account for the different effects of HMGA1 overexpression.

More direct evidence of the role of HMGA proteins in malignant transformation came from work by Wood et al. (Wood et al. 2000). They showed that increased expression of HMGA1 and HMGA2 leads to the neoplastic transformation of Rat 1a and CB33 cells, and induce distant metastasis when these cells are injected into athymic mice. Moreover, human breast epithelial cells harboring a tetracycline-regulated *HMGA1* transgene form both primary and metastatic tumors in nude mice only when the transgenes are actively expressed (Reeves et al. 2001). Interestingly, the HMGA1b, rather than the HMGA1a isoform, appears the most effective elicitor of both neoplastic transformation and metastatic progression *in vivo* (Reeves et al. 2001). Furthermore, expression of either antisense or dominant-negative HMGA1 constructs inhibits both the rate of proliferation of tumor cells and their ability to grow anchorage-independently in soft agar (Reeves et al. 2001).

The generation of transgenic mice overexpressing either the *HMGA1* or the *HMGA2* gene confirmed their oncogenicity *in vivo*. In fact, both the *HMGA1* and *HMGA2* transgenic mice develop GH/PRL-secreting pituitary adenomas and T/NK lymphomas (Fedele et al. 2005, Baldassarre et al. 2001, Fedele et al. 2002).

Besides the obvious role of HMGA in oncogenesis as oncogenes, an antioncogenic potential was recently attributed to HMGA1 proteins. In fact, antiproliferative activities were observed in lymphoproliferative disorders (Fedele et al. 2006a) and during cellular senescence (Narita et al. 2006), which prevent rather than promote malignant transformation. In the context of cellular senescence, HMGAs modulate global chromatin architecture by contributing to the formation of senescence-associated heterochromatin foci.

1.8 Role of HMGA proteins in neoplastic transformation

Several mechanisms have been proposed to account for the transforming ability of the HMGA proteins. According to their main function of regulating gene transcription, most of these mechanisms are based on the ability of the HMGA proteins to down- or up-regulate the expression of genes that have a critical role in the control of cell proliferation and invasion (Sgarra et al. 2004, Martinez-Hoyos et al. 2004, Fedele et al. 2005). Recently described interacting partners, such as nuclear factor (NF)-_B (Merika and Thanos 2001) and the tumor suppressors p53 (Frasca et al. 2006, Pierantoni et al. 2006) or pRB (Fedele et al. 2006b), provide a clue to how HMGA proteins could influence gene expression in cancer. However, HMGA proteins also inhibit nucleotide excision repair (Reeves and Adair 2005, Adair et al. 2005) and other DNA repair systems (Boo et al. 2005, Muller-Tidow et al. 2004), which could mean that HMGA proteins promote tumorigenesis by increasing genome instability.

1.9 HMGA in pituitary tumorigenesis

Recently, our group has shown *HMGA2* gene amplification and overexpression in human prolactinomas (Finelli et al. 2002). Consistently, trisomy of chromosome 12, which harbors *HMGA2*, represents the most frequent cytogenetic alteration in human prolactin-secreting pituitary adenomas, and structural rearrangements on chromosome 12 are recurrent in prolactinomas (Finelli et al. 2000).

HMGA2 overexpression was also found in 12 out of 18 Non Functional Pituitary Adenomas (NFPA) but, in contrast to prolactinomas, HMGA2 upregulation was associated with amplification and/or rearrangement of the *HMGA2* locus in very few cases (Pierantoni et al. 2005).

In agreement with these results transgenic mice carrying the *Hmga2* gene under the transcriptional control of the cytomegalovirus promoter developed pituitary adenomas secreting GH and prolactin (Fig. 1.3), suggesting a crucial role for HMGA2 in pituitary tumorigenesis. Interestingly, the same phenotype is also observed in mice overexpressing *Hmga1* (Fedele et al. 2005) indicating that both *HMGA* family genes could play a pivotal role in pituitary adenoma development. However, neither rearrangements nor amplification of the *HMGA1* locus has been detected in human pituitary adenomas. More recently it has been reported that HMGA2 induces pituitary adenomas in *Hmga2*-transgenic mice by binding to pRB and enhancing E2F1 activity (Fedele et al. 2006b). The mechanism by which it occurs is unique, since although HMGA2 binds to the pRB A/B pocket domain, it does not compete with the E2F1 protein, unlike proteins encoded by viral oncogenes such those produced by the E1A adenovirus. Rather, HMGA2 displaces histone deacetylase 1 (HDAC1) from the pRB/E2F1 complex, resulting in enhanced acetylation of both E2F1 and DNA-associated histones, thereby promoting E2F1 activation (Fig. 1.4). The crucial role of HMGA2-mediated E2F1 activation in pituitary tumorigenesis was confirmed by crossing *Hmga2*-overexpressing with *E2f1*-knockout mice, which resulted in the suppression of pituitary tumorigenesis in double mutant mice. Although the E2F1 activation might represent the major point for the generation of pituitary adenomas in transgenic mice, we cannot exclude that other complementary mechanisms may be envisaged for the role of HMGA2 in pituitary tumorigenesis. In fact, also in the E2F1 minus background, the HMGA2 mice develop a certain number of pituitary neoplasias, even though with a lower frequency and a minor phenotype.

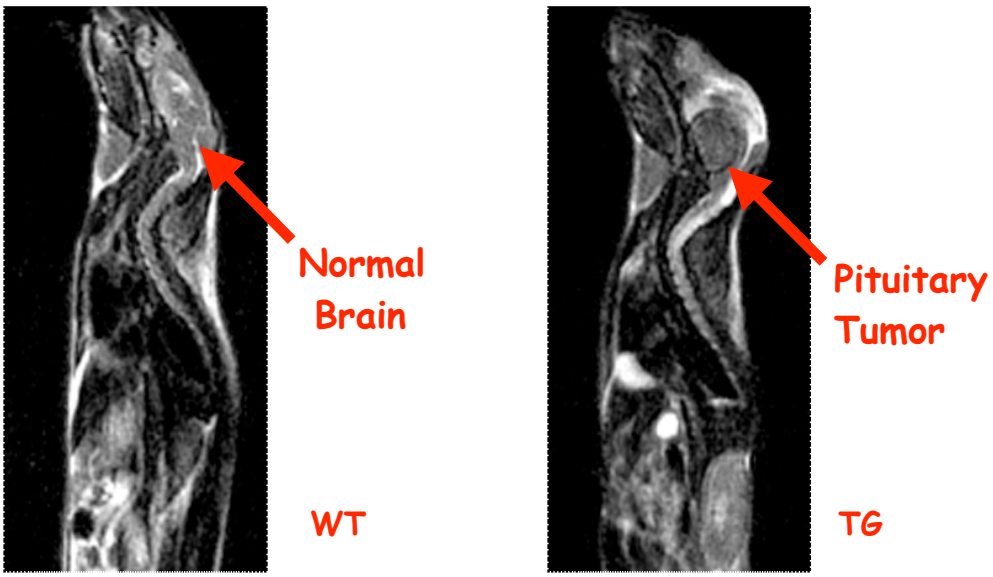


Figure 1.3 MRI analysis of a female transgenic mouse 11 months old (right) compared to a wild type control mouse (left). The arrow indicates a large tumor mass in the skull base.

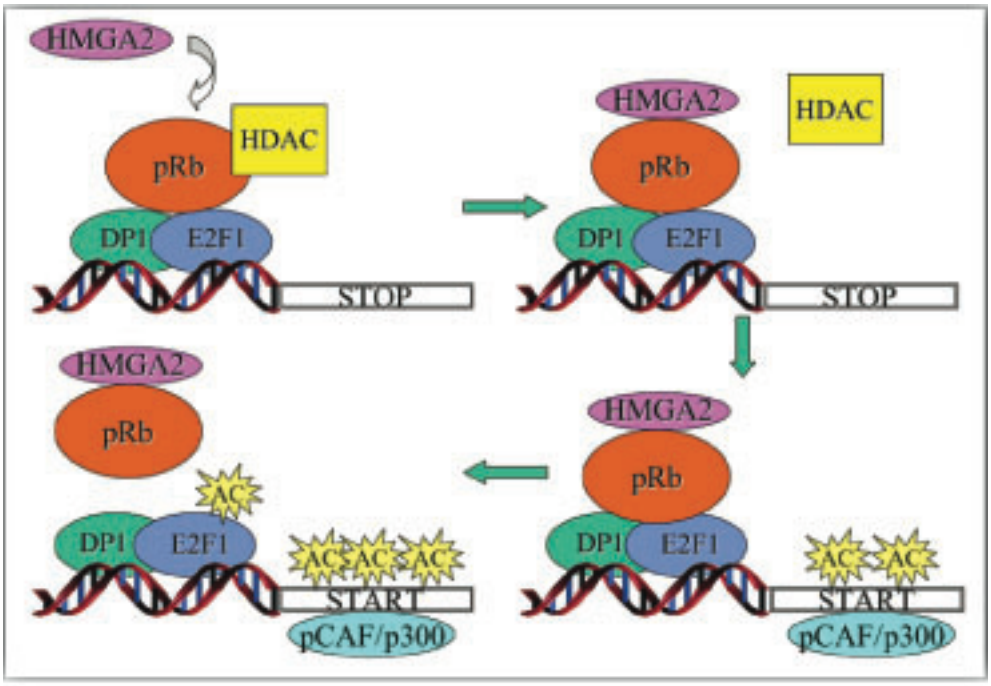


Fig. 1.4 Model of E2F1 activation by HMGA2 protein. HMGA2 protein activates E2F1 transcriptional activity, displacing HDAC1 from pRB/E2F1 complex.

1.10 Human pituitary tumors

Pituitary tumors represent 10-15% of intracranial neoplasms (Kovacs e Horvath 1986, Monson 2000), are infrequent in childhood (Mukai et al. 1986; Kane et al. 1994) and are more common in females (Kane et al. 1994).

Pituitary adenomas have been classified by various groups of investigators in different ways. A functional classification of pituitary adenomas defines these tumors based on their hormonal activity *in vivo*. This is the common clinical approach used by endocrinologists. It characterizes the tumors as GH producing adenomas associated with acromegaly and/or gigantism, adenomas causing hyperprolactinemia and its clinical sequela, ACTH-producing adenomas associated with Cushing's or Nelson's syndromes, TSH-producing tumors, the rare clinically detectable gonadotroph adenomas, and the large group of clinically nonfunctioning or "endocrinologically inactive" adenomas.

Molecular genetics studies have demonstrated that these tumors are monoclonal in origin (Hervan et al. 1990) indicating that an intrinsic defect in a single pituitary cell is the primary event in pituitary tumor. A multistep theory of carcinogenesis, involving changes in gene expression as well as hormone or growth factor

stimulation, has been implicated in their pathogenesis. Several studies have examined human pituitary tumors for genetic abnormalities in a number of known oncogenes and tumor suppressor genes, but have revealed limited information on the molecular events underlying their pathogenesis.

Pituitary oncogenes gsp, ccnd1 and PTTG are abundant in a significant number of cases (Yu and Melmed 2001). The gsp oncogene is predominantly found in a subset of GH-secreting tumors and results from a mutated, constitutively active α subunit of the heterodimeric Gs protein (Landis et al. 1990). Mutation in the *ras, p53* and *Braf* genes are also not common in human pituitary tumors (Karga et al. 1992; Herman et al. 1993, De Martino et al. 2007).

Alterations of the genes involved in the cell cycle seem to have a critical role in the onset of pituitary neoplasias. The loss of *Rb* and $p27^{Kip1}$ genes has been demonstrated as a cause of murine pituitary adenomas. In fact mice deficient in p27 (Nakayama et al. 1996) or with heterozygous Rb mutations develop pituitary tumors (Hu et al. 1994). However, the role of this tumor suppressor genes for human pituitary adenomas remains elusive (Tanaka et al. 1997).

Potential candidates for the suppressor genes are now emerging. The recently cloned multiple endocrine neoplasia type I gene is one example (Chandrasekharappa et al. 1997). MEN1A mutations, constantly found in patients affected by the MEN-1 syndrome, which includes pituitary adenomas, have never been found in sporadic pituitary adenomas (Asa et al. 1998).

Alterations of c-myc/bcl-2, and ras, although rare, appear to be an important cause of the process by which adenoma cells acquire aggressive phenotypes (Wang et al. 1996, Karga et al. 1992). Point mutations of *ras* oncogene were found in a highly aggressive prolactinoma and in metastatic pituitary carcinomas, but not in the majority of functioning and nonfunctioning pituitary tumors (Pei et al. 1994).

Aim of the work

The HMGA overexpression in many neoplastic diseases leads us to generate *HMGA* transgenic mice. High incidence of growth hormone/prolactin secreting pituitary adenomas developed from these transgenic mice demonstrated a crucial role for HMGA2 in the pathogenesis of this tumor according with the findings in human prolactinomas where HMGA2 is amplified and overexpressed. The main mechanism by which HMGA2 appears involved in pituitary tumorigensis is through E2F1 activation (Fedele et al. 2006b). Nevertheless, it is not the only one since HMGA2 transgenic mice deficient for E2F1 still develop pituitary neoplasias, even though with a minor incidence and a milder phenotype (Fedele et al. 2006b).

Therefore, the aim of the present work has been to find out other molecular changes that might contribute to the development of the HMGA-induced pituitary tumors. For this reason, using the oligonucleotide microarray hybridization technique, we first analysed the expression profile of three pituitary adenomas developed by HMGA transgenic mice in comparison with a pool of normal pituitary glands from wild-type animals. In this way we could identify the genes that are regulated, positively or negatively, by HMGA proteins in pituitary tumors.

Subsequently, we focused on two of the genes identified, *Mia/Cd-rap* and *Cyclin B2*, being the first a secreted product of malignant melanoma cells (Blesch et al. 1994), and the other an important cell cycle gene. We investigated if HMGA proteins were able to bind to the promoters of these genes, both *in vitro* and *in vivo,* and to directly regulate their transcription. Moreover, since a direct role of Mia/Cd-rap in pituitary tumorigenesis has been not identified so far, we analyzed its potential to influence pituitary adenoma cells proliferation.

2. Materials and methods

2.1 Microarray Analysis

The Affymetrix standard protocol has been described extensively elsewhere (Affymetrix GeneChip®).

Briefly, cRNA was prepared from 8 μg of total RNA, hybridized to MG MU11K Affymetrix oligonucleotide arrays (containing about 13000 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). The individual gene expression levels for each of the three pituitary adenomas arrays were divided by the expression level in the normal pituitary tissue. Thus, the data are presented as relative to the expression in normal pituitary tissue. The fold-change values, indicating the relative change in the expression levels between mutated and wild-type samples, were used to identify genes differentially expressed between these conditions

2.2 Cell cultures and transfection

All cell lines, except for aT3-1 (kindly provided by Dr P. Mellon, University of California, San Diego, CA, USA) were purchased from ATCC. They were all cultured in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT) and 50 μg/ml gentamicin (Life Technologies, Inc., Grand Island, NY) in a humidified atmosphere of 95% air and 5% CO_2. B16F0 are murine melanoma cells; AtT20 and aT3-1 are murine pituitary adenoma cells secreting ACTH and gonadotoph hormones, respectively; RC-4B/C are rat pituitary adenoma cells secreting GH, FSH, LH, GnRH, ACTH and TSHb; GH1, GH3 and GH4c1 are rat pituitary adenoma cells secreting prolactin and growth hormone.

Transfection were carried out by using Lipofectamine 2000, according with to manufacturer's instructions. Transfection efficiencies were normalized by using Renilla luciferase expression assayed with the dual luciferase system (Promega). All the assays were performed in triplicate and repeated in three independent experiments.

2.3 Plasmids

The 5' flanking region of the mouse *Mia/Cd-rap* gene spanning nucleic acid residues -1396 to -1 with respect to the ATG protein start codon was amplified by PCR and inserted into the promoterless luciferase plasmid pGL_3-basic (Promega) to obtain the MIA-luc pGL_3 plasmid. The human Mia/Cd-Rap expression plasmid, pCMV6-XL4/Mia was commercially available (TC116021 - OriGene Technologies, Rockville, MD). pBABE-puro was already described (Monaco et al. 2001).

The plasmid of B2-luci has been supplied gently by Dr. G. Piaggio from Rome (Manni et al.2001).

2.4 Luciferase and Colony Assays

For the Luciferase assay, a total of 2×10^5 B16F0 cells (or NIH3T3) were seeded into each well of six well plate and transiently transfected with 1 μg of MIA-luc pGL_3 (or with CycB2-luc) and with the indicate amounts of pCEFLHa-HMGA1 (Melillo et al. 2001) and pCEFLHa-HMGA2 (Fedele et al. 2006b), together with 0.5 μg of Renilla and various amounts of the pCEFLHa plasmid to keep the total DNA concentration constant. Transfection efficiencies were normalized by using Renilla luciferase expression assayed with the dual luciferase system (Promega). All tranfection experiments were repeated at least three times.

For the colony assay, GH3 and GH4 cells were seeded at a density of 2.5×10^6 per 10-mm dish. Two days after, cells were transfected with 10μg of pCMV6-XL4 plus 2 μg of pBabe-puro or 10μg of pCMV6-XL4/Mia plus 2μg of pBabe-puro. After about 15 days, cells were stained with 500 mg/ml of crystal violet in 20% methanol, and the resulting colonies were counted.

2.5 RNA Extraction

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

2.6 Cluster analysis by Multiexperiment viewer (MeV)

Microarray data have been elaborated by the Multiexperiment viewer (MeV) system to get gene expression signature of the samples analysed. MeV is a system of cluster analysis for genome wide expression data from DNA microarray hybridization, that

uses standard statistical algorithms to arrange genes according to similarity in pattern of gene expression. In our analysis we used a 4-fold difference in expression level between normal and tumoral samples.

2.7 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-ΔΔCT method (Livak et al. 2001). The primers sequences are:

h-MIA Real time fw: 5'-TTTCCCCAGTAGCATTGTCC-3'
h-MIA Real time rev:5'-TCTGTCTTCACATCGACTTTGC-3'
h-CyclinB2 fw: 5'-AGCTATGATCCTGCCAGTGC-3'
h-CyclinB2 rev: 5'- CAGAATGCTGTGTTCAGGTTACA-3'
h-HMGA1 fw: 5'- AAAAGGACGGCACTGAGAAG-3'
h-HMGA1 rev: 5'- CTCTTAGGTGTTGGCACTTCG-3

2.8 Protein Extraction and Western blot

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 antibody against MIA (A-20 Santa Cruz, sc-17047), or with antibody Cyclin B2 (N-20 Santa Cruz, sc-5235).

Bound antibody was detected by the appropriate secondary antibody and revealed with an enhanced chemiluminescence system (Amersham-Pharmacia Biotech).

2.9 Electrophoretic Mobility-Shift Assay

Protein/DNA-binding was determined by electrophoretic mobility shift assay (EMSA), as previously described (Battista et al. 1995). Briefly, five to 20 ng of recombinant protein were incubated in the presence of a ^{32}P-end-labeled double strande oligonucleotide (specific activity, 8,000–20,000 cpm/fmol). spanning from base -1130 to -1100 of the mouse *Mia/Cd-rap* promoter region (5'-AAACCCTGAAATAAATCTTTTTTTCCCCTT-3'), or from -229 to –189 of the mouse *Cyclin B2* promoter region (5'-AGCAAATTGACAAGCAAATACAAGCCAGCCAATCAACGTG-3').

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

2.10 Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out with an acetyl-histone H3 imunoprecipitation assay kit (Upstate Biotechnology) according to the manufacture's instruction. Approximately 3 x 10^7 cells of the NIH3T3 cell line 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 A 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-HA (sc-7392, Santa Cruz Biotechnology) 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 $NaHCO_3$, 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 *Mia/Cd-rap* or *Cyclin B2* promoters sequences. PCR reactions were performed with AmpliTaq gold DNA polymerase (Perkin-Elmer). The primers used to amplify the sequence of the promoters were:

--*Mia/Cd-rap* 5'-TTGCTGGTGCATGCCTTA-3' (forward) and 5'-TCTTAACCGCTGAGCCATCT-3' (reverse);

--*Cyclin B2* 5'- TAAGGATGATGGACCAAGAG (forward) and 5'-CCCTCGACCTAAATTACACA -3' (reverse).

PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and scanned using a Typhoon 9200 scanner.

3. Results and Discussion

3.1 Gene Expression Profile Analysis

RNAs were extracted from a pool of normal pituitary glands and from three pituitary adenomas developed in four different HMGA transgenic mice (2 HMGA2, 1 HMGA1, 1 HMGA2/T), and were hybridized to the Affymetrix MG MU11K-A oligonucleotide array, representing about 13,000 unique genes. The number of transcripts increased or decreased in all pituitary adenomas versus normal gland is shown in the Fig. 2.1. Of the 13.059 transcripts represented on the array, 1560 had a 1- to 3-fold, 290 had a 3- to 4-fold, 154 had a 4- to 10-fold, and 11 had a >10-fold change. We examined the 154 transcripts that had a fold change ≥ 4 in all HMGA-induced pituitary adenomas versus normal pituitary gland assuming that the genes altered in their expression in tumors might represent candidate genes involved in pituitary tumorigenesis.

Among these transcripts, 82 were increased and 72 were decreased, including 108 known genes, 30 expression sequence tags (ESTs) and 16 unknown genes. The relative fold-changes of these genes, grouped according to their biological function are shown in Table 1.

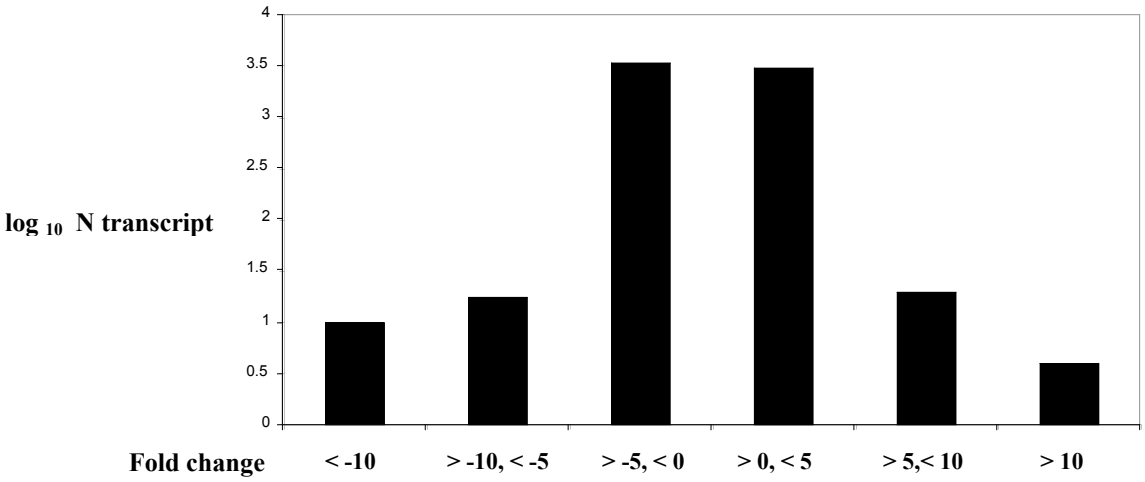


Figure 2.1 Gene expression profile of pituitary adenomas of HMGA transgenic mice compared to normal pituitary gland. Transcripts are grouped according to fold change.

A natural basis for organizing gene expression data is to group together genes with similar patterns of expression. Using the Multiexperiment Viewer (MeV), a free Java application which is used to compare datasets from microarray expression experiments (Eisen et al. 1998), we clustered the gene expression profile of HMGA-induced pituitary adenomas in comparison with normal gland. The results, illustrated in Fig. 2.2, clearly show the similar pattern of expression of several genes in all pituitary adenomas with respect to normal pituitary gland.

Table 1. Genes differentially expressed with a ≥ 4-fold change in HMGA-induced pituitary adenomas versus normal pituitary gland.

Description	GenBank accession no	FC 1	FC 2	FC 3	FC 4
Immune function					
Thy-1	X03151	4.0	1.3	5	4
Ob/Ob haptoglobin	M96827	10.2	2.2	1.2	2.3
MRP8	M83218	107	3.7	1.3	5.4
Clon B6 myeloid second. granule prot	L37297	105.1	2.2	1.7	12
Interferon inducible protein 1-8U	W12941	-2.25	-2.7	-3.3	-3.1
VCAM-1	X67783	32.6	3.7	4.1	9.8
Il-6 receptor	X51975	-3.7	-2.6	-4.3	-5.1
Ig kappa chain (HyHEL-10)	M35667	3.2	7.5	1.9	2.8
G protein γ 3 subunit	AA049022	-2.5	-1.5	-7.9	-3.8
GM-CSF	X03019	-4-.1	-4.9	-5	-6.7
Myelopeptide-2	M23236	9.2	10.6	9.5	9.3
IFN-gamma	AA015168	-7.5	-10.2	-13	-11.2
antigen pB7	M55561	5.5	-5.2	-5.6	-2.3
Cell proliferation					
β-Enolase	W33721	-1.5	-2.2	-1.3	-2.9
CAD protein	AA466758	2.1	3	5.3	4.6
dUTPase	AA110424	1.1	1.4	5.2	3.8
Galectin-3	W10936	14.3	8.2	6.3	10.1
Signal trasduction					
PKCalpha	M25811	2	1.8	6.6	3.2
FKBP65 binding protein	L07063	6.3	8.3	12.2	9.7
mR-PTPμ	X58287	2.7	2.8	4	5.7
GTP-binding protein Gb4	M63658	2.4	3.7	6.8	4.6
p36	D10024	-4	-4.4	-11	-7.7
AEBP1	X80478	-12.6	-3-3	-36.9	-15.1
AP-2	NM_011547	8.4	12.5	4.4	5.9
A-RAF	AA060840	-11.5	-11-7	-12.2	-10.8
Vitronectin	m77123	21.7	10.5	6.1	14.3
STEP61	U28217	-5.4	-9.9	-18.2	-15.9
Cell cycle					
CycD3	AA266783	4.4	6.1	3.5	5.2
CycB2	X66032	5.6	8	6.1	7.9
CycB	X58708	4.5	6.3	4.4	6.9
cdc2	U58633	4	11.2	3.6	9.8
GADD-45	l28177	-2.6	-5.9	-3.9	-6.1
Antigen					
SSAV provirus pol gene	C76668	2.1	1.6	5.1	4.3
(Emv-3) gag gene	U63133	-1.1	-1.4	-2	-3.2
Mitocondrial proteins					
Heme oxygenase	M33203	30.5	3.2	2	12.2
Fosfadilserine decarb. Proenzime	AA162205	23.9	40.8	36	27.8
Creatine kinase	W50891	8	6.8	9.3	10.1

HMG proteins					
HMG(Y)	AA290110	2.3	1.2	5.6	3.5
HMG2	Z46757	10.7	4.4	23.1	13.3
Enzymes					
Argininosuccinate synthetase	M31690	-5.6	-4.5	-6	-6.3
PAF-acetylhydrolase	U34277	-5.9	-6.7	-8.6	-9.2
TransGlutaminase Tgase	M55154	-1.1	-5.8	-5.3	-3.4
Aldehyde Dehydrogenase ALDH2	U07235	-1.3	-3.2	-5.3	-5.8
Rib-1	X60103	-16	-16.9	-18.5	-18-9
Dipeptidase	D13139	-4	-3.8	-3.9	-5.7
Aldolase 3, isoform C	W53351	-11.5	-5.5	-9.4	-7.7
Cytochrome P-450 Naphtalene Hydrox.	M77497	-13	-21	-12.2	-18.2
11 β HSDH	X83202	-5.5	-7.2	-7.9	-8.2
Ubiquitin-coniung.enzyme E2	W99019	5.6	8.6	3.1	7.2
Retinaldehyde-specific dehydrogenase	X99273	-7.2	-6.7	-6.2	-7.2
Membrane-type matrix metalloproteinase 1	U54984	-1.8	-3.1	-6	-5.5
CAMK2 β subunit	X63615	-22.6	-22.7	-31.1	-28.5
Acetylcholinesterase	X56518	18.1	4.3	9.6	12.3
DNA topoisomerase II	d12513	6	9.1	5.1	7.7
Ribonucleotide reductase M2 subunit	m14223	4.4	7.9	5	6.6
Transcriptional factor					
ID4	X75018	1.4	1.6	1.9	2.3
ID1	M31885	-4.5	-6.6	-7.5	-5.8
Zif 268	M22326	-8.3	-1.4	-14.1	-4.8
SOX-2	U31967	-8.8	-7	-10.6	-9.1
Delta-like 1 protein	X80903	4.3	6.7	6	5.8
MLZ4 zinc protein	AA015118	6.4	4.6	9.1	8.1
SOX-11	AF009414	7.4	9.3	11.2	10.0
Hormones					
FSH-β	U12932	–9.6	–5.3	–7.9	–8.8
LH-β	U25145	-14	-2	-1.2	-7.1
GH	Z46663	15.3	18.7	22.1	20.1
Pituitary glycoprotein hormone	M22992	-1.7	-1.1	-10.6	-8.9
Thyroid-stimulating hormone	M54943	-7.1	-7.7	-7.3	-7.5
Oncogenes and oncoprotein					
PTTG	AA711028	-1.2	-5.6	-2.4	-3.2
GARG-16	U43084	-3.6	-5.6	-10.4	-8.2
Bcl3	W62846	9.1	8.3	6.1	8.7
IFC oncogene	U28495	11.2	11	10.3	15.2
Lypidic metabolism					
Choline Chinase	AK078101	1.3	1.9	4.4	3.3
APOBEC-1	W29206	4.2	1.6	4.6	5.1
ALBP (Adypocite lipid bunding protein)	K02109	10.8	6.1	9.6	8.3
Allergic disease and infiammation					
IgE receptor	W41745	9.2	1.2	-1.1	-1.8
Extensin precursor	AA031158	34	12.2	3.8	6.8
Mouse complement component C3	k02782	9.6	5.8	4.6	4.3
Extracellular matrix and cellular-structure					
Laminin β-3	U43298	-3.8	-9.2	-14.3	-12.4
Decorin	X53929	-4.6	-4.6	-5.4	-3.8
Tubulin β2	AA030364	7.1	9.3	11.3	8.9
Vsm α-ACTIN	X13297	26.4	9.9	2.8	10.4
Reelin	U24703	-6.2	-12	-32.7	-18.3
E-chaderin	X06115	-5.4	-6.2	-5.1	-7.2
Tubulin β4	AA030364	9.9	4.3	11.3	10.1
Semaphorin H	Z80941	-12.1	-8.6	-7.6	-8.9
ATP-binding cassette trasp.(ABCR)	AF000149	-9.9	-10.5	-12.3	-12.5
Syntaxin 1A	D45208	-5.6	-4.9	-5.6	-6.6
Folate binding protein 1	M64782	-10.1	-14.1	-16	-12.1
Growth factors					
Igf2	U71085	8.6	3.5	2.8	7.2

	MIA/CD-RAP	X97965	-106.6	-126.6	-121	-112
	Peroxisome membrane protein (PMP22)	L28835	6.5	9.6	4.1	5.9
Development						
	Raly	L17076	-5.4	-3	-3	-4.8
	Noggin	U79163	7.5	8.3	10.3	7.7
	NFI-β	U57634	-4.5	-4.2	-7.5	-5.1
Receptor						
	Nicotinic Acetyl Choline rec. β subunit	M14537	-13.4	-15	-24.8	-12.8
	FGR receptor	M33760	-5.9	-3.1	-12.5	-9.8
	T-cell receptor beta chain	M26417	-7.2	-5.6	-6.6	-8.6
	RIP140	W85018	-6.8	-4.9	-7.1	-10.2
	Serotonin 4L receptor	W85018	-4.1	-5.6	-4.9	-6.9
	Fc gamma receptor	m14215	31.6	7.4	6	9.8
Other function						
	ANKT	AA266783	4.4	6.1	7.5	5.5
	γ -casein	D10215	1	1.1	1.1	2.4
	EN7	X53247	13.4	1.4	-1.7	-2.3
	DMR-N9 gene	Z38011	-4.3	7.3	-4.5	-5.6
	Actin-2	W50919	-7	-4.4	-6.7	-9.6
	Dihydropyrimidinase related protein 4	ab006715	-10	-10.6	-12.1	-13.4
	Hoxc-5	U28071	-5.7	-6.6	-8.5	-11.8
Prosurvival factor						
	BDNF	X55573	4.1	5.9	4.9	6.2

Abbreviations : FC, fold change, 1= HMGA2 induced PA1, 2= HMGA2 induced PA2, 3= HMGA2/T induced PA, 4=HMGA1 induced PA, PA= Pituitary adenoma.

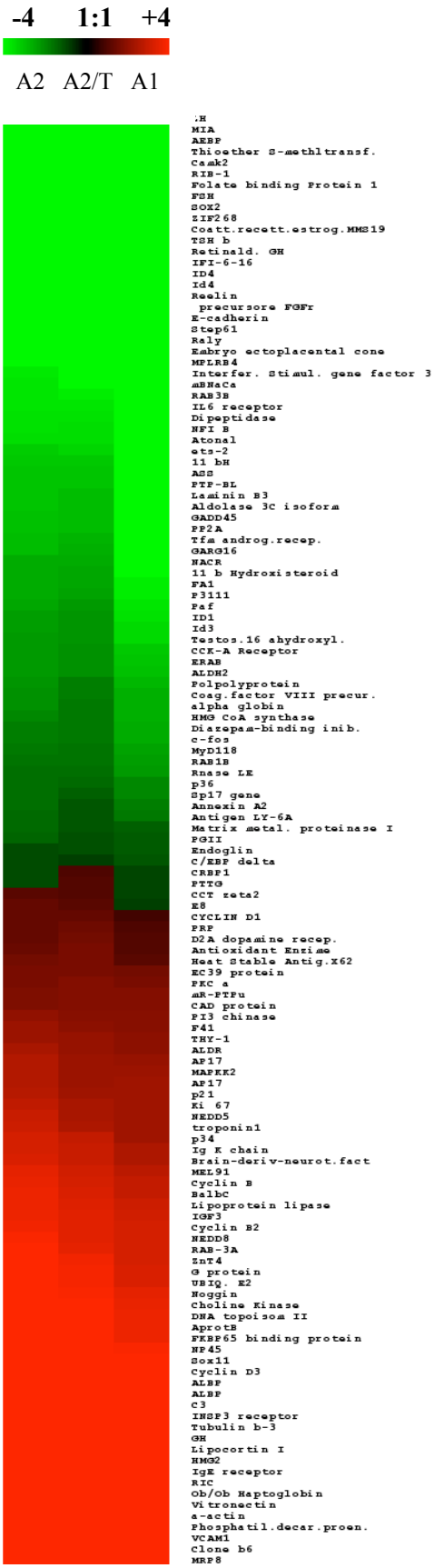


Figure 2.2. Clustered display of gene expression in three pituitary adenomas developed in HMGA2-, HMGA2/T- and HMGA1- trangenic mice compared with normal pituitary gland, using the MeV program. A 4-fold difference in expression level between adenomas and normal samples was used. The green and red colors show downregulated and upregulated genes, respectively. Each gene is represented by a single row of colored boxes; each adenoma is represented by a single column.

3.2 Validation of microarray analysis

To validate the results obtained by microarray analysis we evaluated about 100 transcripts, whose expression differed of a fold change > 4 or < -4 in all the pituitary adenomas compared to normal glands. To this aim we performed semi-quantitative RT-PCRs in normal glands and HMGA-induced pituitary adenomas derived from mice different from those used for the Gene Chip microarray. For all of these genes, we confirmed the differential expression associated with the pituitary tumors. The results of some representative RT-PCR analyses are shown in Fig. 2.3. Among these differentially-expressed genes, there are some up-regulated (e.g. Cyclin B2 and ANKT) and other down-regulated (e.g. Rib-1, CamK2 and Mia/Cd-rap) in pituitary tumors with respect to the normal gland.

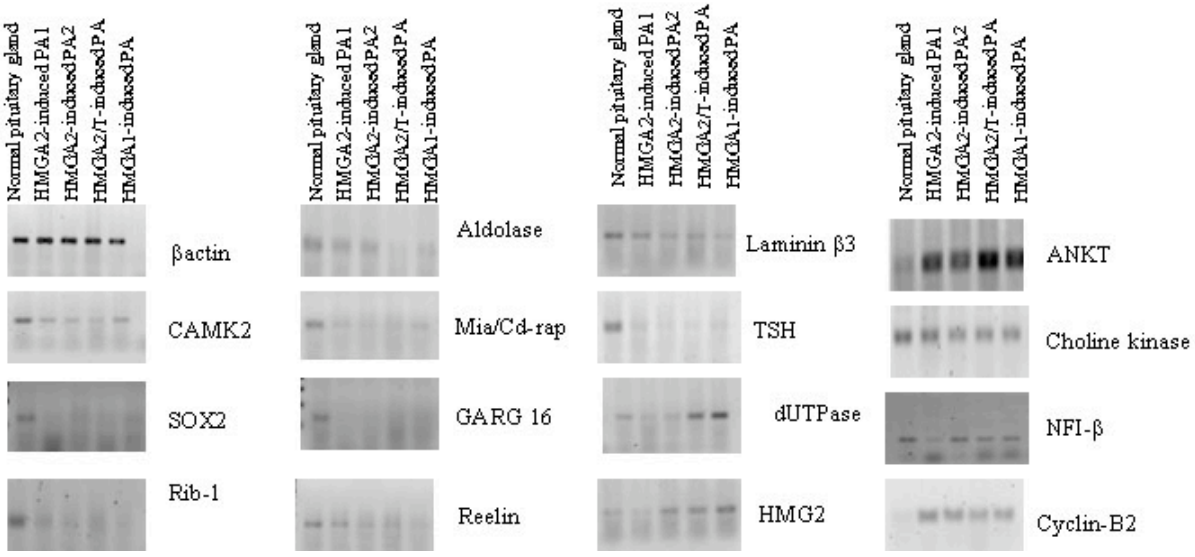


Figure 2.3 Validation of microarray data by semiquantitative RT-PCR. Representative RT-PCRs on a panel of two HMGA2- one HMGA2/T-, one HMGA1-induced pituitary adenomas and a pool of wild-type pituitary glands.

Subsequently, we also analysed by RT-PCR the expression of these genes in murine and rat cell lines derived by pituitary adenomas of different histotype in comparison with normal pituitary gland. Some representative results of these analyses are shown in the Fig. 2.4. Some genes, such as *Mia/Cd-rap* and CamK2, as for the pituitary adenomas of HMGA mice, are highly down-regulated in all the cell lines, whereas

other genes, such as FKBP65 or reelin, change their expression depending on cellular histotype.

It is known that, by interacting with different partners, the HMGA proteins are able to enhance or suppress the effect of more "traditional" transcriptional activators and repressors. The fact that partner proteins are critical for HMGA activity, and they dependent on the cellular context, may account for the cell-specific regulation exerted by the HMGA proteins.

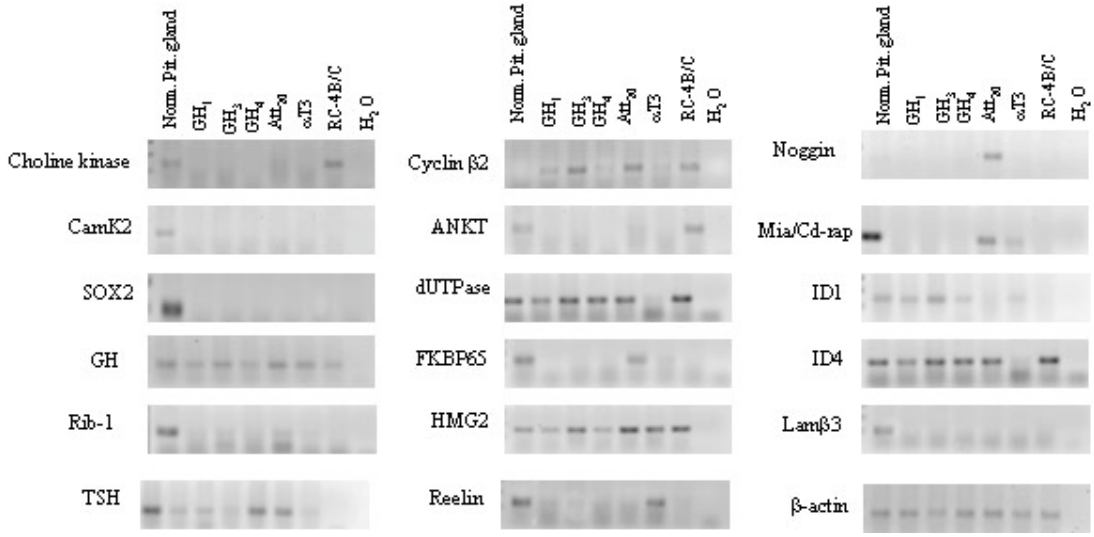


Figure 2.4 Gene expression on a panel of seven cell lines originating by rat and mouse pituitary adenomas of different histotype in comparison with a pool of wild-type pituitary glands

3.3 HMGA proteins directly regulate Mia/Cd-rap and Cyclin B2 expression

Among these genes we focused our attention on the *Mia/Cd-rap* and *Cyclin B2* genes for two main reasons: i) they were among the most changed at RNA level in all the pituitary adenomas and different mouse and rat pituitary adenoma cell lines analyzed; ii) they have been already associated to tumor development. In fact, Mia/Cd-rap is upregulated in different human neoplasias such as chondrosarcoma, melanoma, and breast cancer (Blesch et al. 1994, Chansky et al. 1998), while Cyclin B2 is overexpressed in colorectal adenocarcinomas (Park SH et al. 2007).

a) Mia/Cd-Rap protein is down-regulated and Cyclin B2 up-regulated in mouse pituitary adenomas.

To further validate the microarray data obtained for the *Mia/Cd-Rap* and *Cyclin B2* genes, we also analyzed the expression of their protein products in tissue extracts of pituitaries and pituitary adenomas from control and transgenic mice (HMGA2, HMGA2/T and HMGA1), respectively. As shown in Fig. 2.5, normal pituitary from control mice show high levels of Mia/Cd-Rap protein expression, whereas it was completely lost in pituitary adenomas from HMGA2, HMGA2/T and HMGA1 transgenic mice.

As far as concerns Cyclin B2, its expression is high in all pituitary adenomas analyzed and it is absent in normal pituitary gland (Fig. 2.5 right).

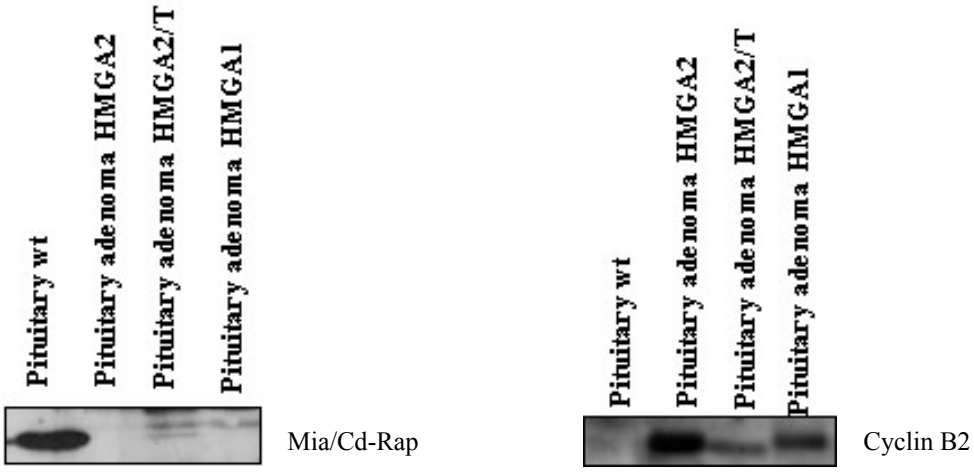


Fig 2.5 Mia/Cd-rap and Cyclin B2 protein expression in normal pituitary from wild-type animals and pituitary adenomas from HMGA2, HMGA2/T and HMGA1 transgenic mice.

b) HMGA Proteins bind to the Mia/Cd-rap and Cyclin B2 promoters

To investigate whether the HMGA proteins are directly involved in *Mia/Cd-rap* and *Cyclin B2* transcriptional regulation, we evaluated the HMGA-binding activity to the *Mia/Cd-rap*- and *Cyclin B2* promoters *in vitro* using oligonucleotides spanning from base -1130 to -1100 of the mouse *Mia/Cd-rap* promoter region (Bosserhoff et al. 1997) and -229 to – 189 of the mouse *Cyclin B2* promoter region, including AT-rich sequences that are a preferential binding site for the HMGA proteins (Bolognese et al. 1999). As shown in Fig. 2.6, panel A, lanes 1-2 and 11-13, increasing amounts (5 and 20 ng) of the recombinant HMGA2 protein were capable of binding the ^{32}P-end-

labelled double-strand oligonucleotide in Electrophoretic mobility shift assay (EMSA). The binding specificity was demonstrated by competition experiments showing loss of binding with the addition of 100-fold molar excess of the specific unlabeled oligonucleotide (lane 3 and 13). We performed the same experiment with a recombinant HMGA1 protein, and also in this case a specific binding with the Mia/Cd-rap and Cyclin B2 promoters (Fig. 2.6, lanes 5-7, 9-10 and 14) was observed.

Subsequently, we performed binding assays with total extract from pituitary adenomas developed in HMGA transgenic mice and normal pituitary glands from control mice (Fig. 2.6, panel B). For both promoters, two specific complexes with a mobility corresponding to the HMGA proteins (isoforms A1a and A1b) were present in extracts from pituitary tumors, while they were absent in extracts from normal glands.

To verify that HMGA proteins are able to bind to Mia/Cd-rap and Cyclin B2 promoters also *in vivo*, we performed experiments of chromatin immunoprecipitation in the NIH3T3 cell line transiently transfected with either the HA-HMGA2 or HA-HMGA1 expression plasmids. Chromatin prepared as described under Materials and Methods was immunoprecipitated with anti-HA or normal rabbit IgG antibody. The results, shown in Fig. 2.6, panel C, confirmed that both HMGA2 and HMGA1 proteins bind to the promoter of *Mia/Cd-rap* and Cyclin B2 genes also *in vivo*. In fact, both promoter regions were amplified from the DNA recovered with anti-HA antibody in HA-HMGA1- and HA-HMGA2- but not in mock-transfected cells. Moreover, no amplification was observed in samples immunoprecipitated with aspecific rabbit IgG.

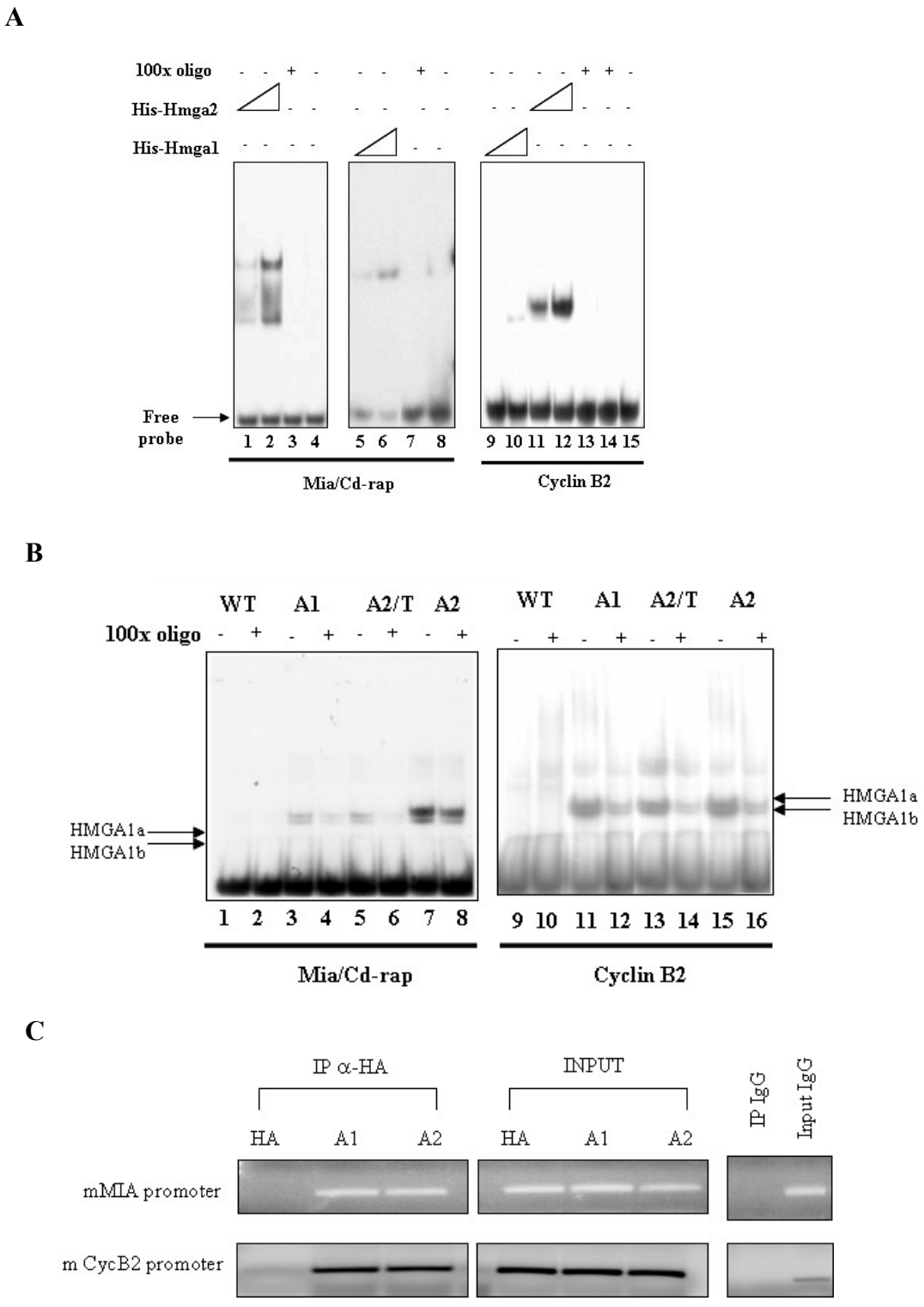


Fig 2.6 HMGA proteins bind Mia/Cd-rap and Cyclin B2 promoters *in vitro* (A e B) and *in vivo* (C).

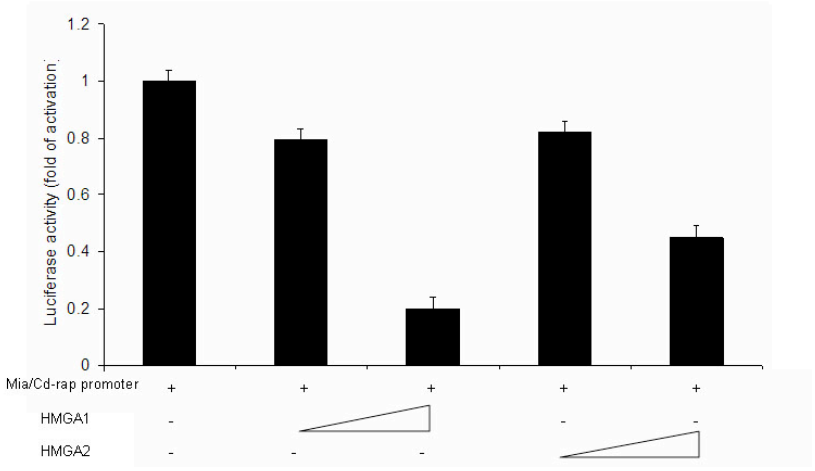
We demonstrated, by electrophoretic mobility shift assay and chromatin immunoprecipitation, that both HMGA1 and HMGA2 proteins directly bind to the promoter of these genes.

c) HMGA proteins regulate the Mia/Cd-rap and Cyclin B2 promoter activity

In order to investigate the functional effect of HMGA proteins on *Mia/Cd-rap* promoter, we transiently transfected the B16F0 cell line, a murine melanoma cell line in which Mia/Cd-rap protein is endogenously expressed, with a construct expressing the luciferase gene under the control of the mouse *Mia/Cd-rap* promoter region -1396 to +1 (mMIAprom-luc) and increasing amounts of an *HMGA2* (or *HMGA1*) expression vector. As shown in Fig. 2.8, panel A, the overexpression of HMGA2 (or HMGA1) resulted in a dose-dependent decreased activity of the *Mia/Cd-rap* promoter. Interestingly, the HMGA1 protein showed a significantly higher inhibitory effect on the Mia/Cd-rap promoter activity in comparison with the HMGA2 protein (80% with 1 μg of HMGA1 versus 55% with 1 μg of HMGA2).

As far as concerns *Cyclin B2* promoter, we transfected the NIH3T3 cells with the luciferase gene under the control of the mouse cyclin B2 promoter region -1189 to-69 (Lange-zu Dohna C.et al. 2000) (Fig. 2.8 panel B). In this case, the overexpression of HMGA2 (or HMGA1) resulted in an increased activity of the *Cyclin B2* promoter, and the HMGA1 protein showed a lower activation in comparison with the HMGA2 protein.

A



B

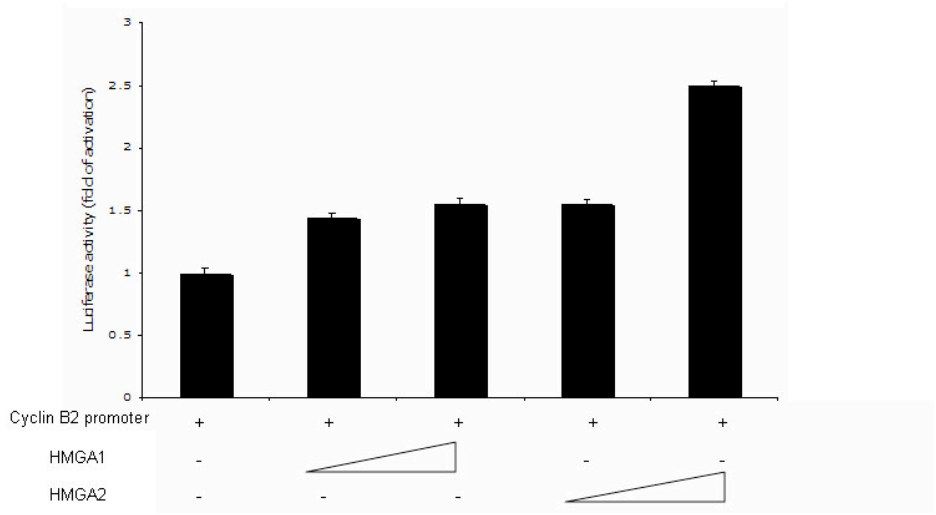


Fig. 2.8 HMGA proteins regulate the murine *Mia/Cd-rap* and *Cyclin B2* promoters. Luciferase activity (fold of activation) of the *Mia/Cd-rap* promoter in the B16F0 murine melanoma cell line and of *Cyclin B2* promoter in the NIH3T3 cell line. Where indicated 0.5 and 1 μg of either HMGA1 or HMGA2 expression vectors were co-transfected with the Mia-luc or with CycB2-luc plasmids.

3.4 Mia/Cd-rap expression inhibits growth of pituitary adenoma cells

To investigate the functional role of Mia/Cd-Rap in pituitary cell growth we performed a colony assay experiment by transfecting Mia/Cd-Rap in GH3 and GH4 rat pituitary adenoma cells. After puromicin selection, the number and growth of the colonies obtained by transfection with the Mia expression plasmid decreased dramatically compared to empty vector (Fig. 2.9). These results show that Mia/Cd-Rap inhibits cell growth of rat pituitary adenoma cells, suggesting it as negative regulator of pituitary cell proliferation.

The same experiment with the vector expressing the *Cyclin B2* gene is in progress.

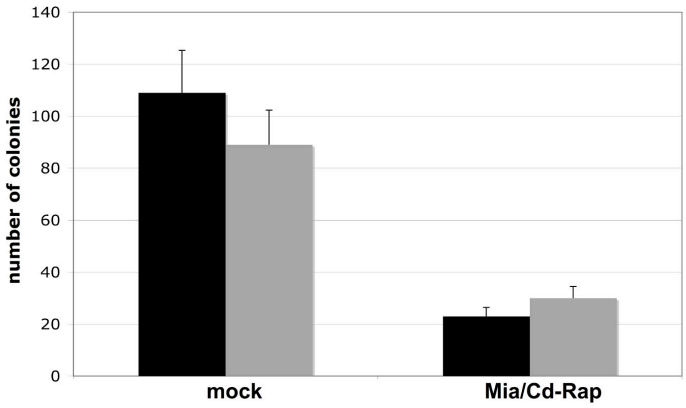


Fig 2.9 Effect of Mia/Cd-Rap expression on pituitary adenoma cell proliferation. Colony forming assay experiment performed in GH3 (black bars) and GH4 (gray bars) cells transfected with a vector expressing Mia/Cd-Rap. As a control the empty vector (mock) was used. The reported results are the mean of three experiment and error bars shows S.D.

3.5 Mia/Cd-rap and Cyclin B2 expression in human pituitary adenomas

Subsequently, we analyzed the expression of Mia/Cd-rap and Cyclin B2 in a panel of human pituitary adenomas of different histotype by Real-time PCR (Fig. 2.10). We found that Mia/Cd-rap expression was increased in all human pituitary adenomas tested compared to normal gland used as a control.

As far as concerns Cyclin B2, we found that the mRNA expression level was much higher in all human pituitary adenomas analyzed compared to normal pituitary tissue. Interestingly, a direct correlation between HMGA1, Mia/Cd-rap and Cyclin B2 mRNA levels was observed.

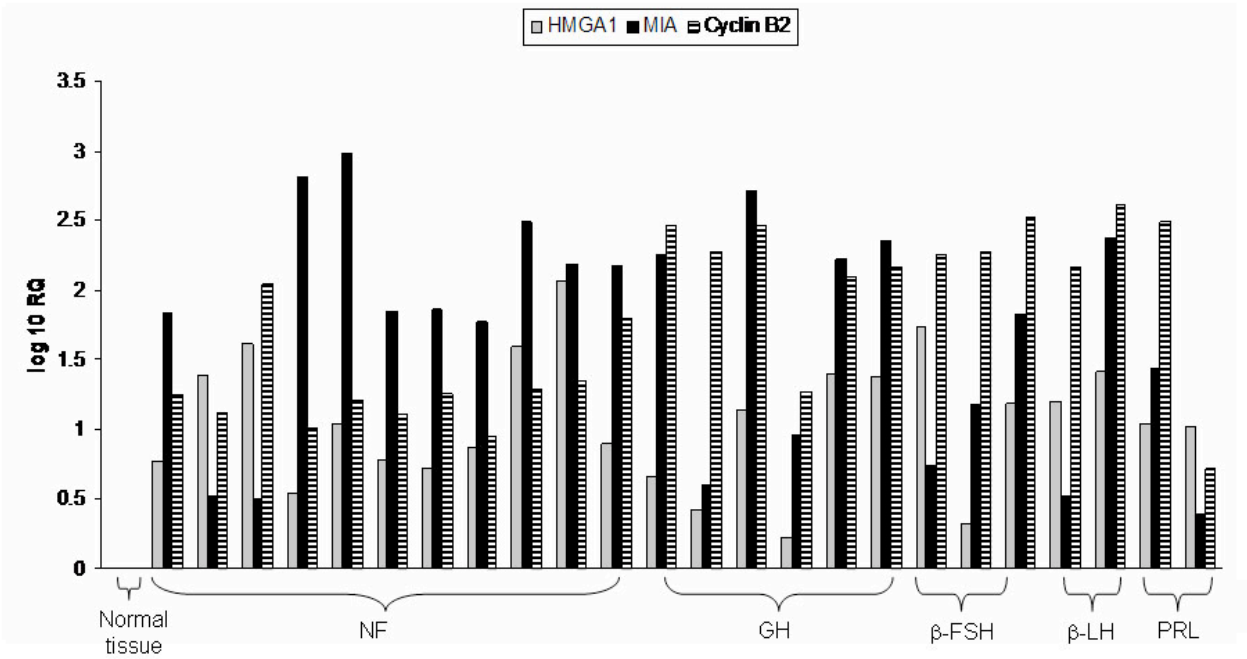


Fig. 2.10 Mia/Cd-rap, Cyclin B2 and HMGA1 expression in human pituitary tumors by real-time PCR. NF = non functioning; GH = GH-secreting; β-FSH = β-FSH-secreting; β-LH = β-LH-secreting; PRL = prolactin-secreting.

4 DISCUSSION

Pituitary tumors are among the most common human neoplasms. Despite extensive research, the pathogenesis of the majority of pituitary adenomas remains unsolved. It is not surprising that studies into many of the common oncogenes or tumor-suppressor genes involved in human cancer have been unrevealing when applied to these tumors.

A crucial role for the *HMGA* family genes in the process leading to the generation of human pituitary adenomas was first suggested by the phenotype of transgenic mice overexpressing a truncated or a wild-type *HMGA2* gene, or wild-type *HMGA1* gene, under the transcriptional control of the strong and ubiquitous promoter of the cytomegalovirus. Most of these mice (85% of females and 40% of males), in fact, developed pituitary adenomas secreting prolactin and growth hormone (Fedele *et al.* 2002, Fedele *et al*. 2005).

The strong correlation between the phenotype of the *HMGA2* transgenic mice and the *HMGA2* overexpression in human prolactinomas suggested that this animal model might represent a unique tool for studying the molecular mechanisms underlying the generation of pituitary adenomas induced by the *HMGA2* overexpression. In fact these mice allowed us to demonstrate that the mechanism of the HMGA2 induced-pituitary adenoma development is based on the increased E2F1 activity (Fedele *et al.* 2006b). Since other additional mechanisms can be envisaged on the base of a minimal residual tumoral phenotype showed by some HMGA2 transgenic mice lacking a functional E2F1 gene (Fedele et al. 2006B), in the present study we have analysed the gene expression profile of HMGA-pituitary adenomas in comparison with a pool of ten normal pituitary glands in order to identify other genes involved in the process of pituitary tumorigenesis induced by the *HMGA1* and *HMGA2* genes. The results of our analysis led to the identification of 82 transcripts increased and 72 transcripts decreased of at least 4-fold in all mice pituitary adenomas analyzed compared with normal pituitary gland. Among these genes we focused our attention on the *Mia/Cd-rap* gene and *Cyclin B2* genes because they were among the most changed at RNA level in all the pituitary adenomas.

It is noteworthy to observe that both genes have a critical role in the process of carcinogenesis. In fact, overexpression of the human *Mia/Cd-rap* gene has been associated with progression of melanocity tumors (Bosserhoff et al. 1999). The biological function of Mia/Cd-rap in adult organism is still unclear, but evidence is growing that it might be involved in cellular motility, metastasis, and modulation of immune responses.

Cyclin B2 is a mitotic B-type cyclin that plays a role in entering the G2-M phase in association with cdc2. It appears to be dispensable, in fact cyclin B2 knockout mice develop normally but are smaller than normal mice and have reduced litter sizes, which suggest that cyclin B2 has some growth advantage (Brandeis et al. 1998). The overexpression of cyclin B2 contributes to the chromosomal instability observed in cancer cells through an alteration of the spindle checkpoint (Soria et al. 2000). Very

recently it has been demonstrated that Cyclin B2 expression is strongly associated with colorectal adenocarcinoma (Park et al. 2007).

Cyclin B2 expression, similarly to what has been published for other neoplasias (Park et al. 2007) is overexpressed in all pituitary adenomas analyzed compared to normal pituitary gland.

We demonstrated, by electrophoretic mobility shift assay and chromatin immunoprecipitation, that both HMGA1 and HMGA2 proteins directly bind to the Cyclin B2 and Mia/Cd-rap promoters and regulate their expression in transcription assays. We retain of particular interest this finding because, in this way, another mechanism of HMGA-induced pituitary tumorigenesis, other than the E2F1-dependent mechanism, can be envisaged.

Even though the expression of Mia/Cd-rap has been already associated with a neoplastic phenotype (Blesch et al 1994, Chansky et al 1998), it has been never described in pituitary tumorigenesis so far. Yet, there are no data that could explain us a possible mechanism by which Mia/Cd-rap exhibits its anti-tumourigenic effect in mouse. Moreover the exact role of this protein in this model has not been estabilished.

Interestingly, a recent study proves that HMG1, another member of the HMG family, is an important factor in MIA regulation and melanoma progression. In fact, a sequence-specific DNA motif in the Highly Conserved Region of the Mia/Cd-rap promoter is recognized by HMG1 (Golob et al. 2000). Besides, recent data showed that HMG1 is upregulated in malignant melanoma cell lines compared to Normal Human Epidermal Melanocytes (NHEM) cell line and that it plays a pivotal role in Mia/Cd-rap transcriptional activation (Poser et al. 2003). Consistently, the human promoter sequence of *Mia/Cd-rap* contains many potential regulatory domains including an AT-rich domain that are known DNA-binding sites for the HMGA proteins (Bosserhoff et al. 1997).

The results of our analysis led to a role of tumor suppressor of Mia/Cd-rap in HMGA-induced pituitary adenomas. Its expression appears inversely correlated with the proliferative potential of pituitary adenoma cells, therefore suggesting a relevant role for its downregulation in the generation of pituitary adenomas. Obviously, the unsuspected tumor suppressor role of Mia/Cd-rap protein is in contrast with its oncogenic potential that several studies, described above, have clearly estabilished.

Nevertheless, it is not the first time that a gene has been shown to have both oncogenic and antioncogenic properties (Evan et al. 1995). In the case of Mia/Cd-rap this effect could be explained by a hypothetic mechanism of action of Mia/Cd-rap, which could regulate the expression of several genes by interacting with diverse partners. Because their protein(s) partner might change depending on the cell-type, Mia/Cd-rap-dependent gene regulation may also depend on the cellular context, which could account for the dual role of this protein in pituitary tumorigenesis.

In further studies, potential interactor(s) of Mia/Cd-rap protein, as well as downstream signaling pathways, in pituitary adenomas, have to be determined.

Yet, further investigation is required to shed light on the functional relevance of *Mia/Cd-rap* and *Cyclin B2* gene regulation in the generation of pituitary adenomas.

5 CONCLUSIONS

- We have identified new genes regulated by HMGA proteins in HMGA-induced pituitary adenomas.

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

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

- We have demonstrated that the regulation is direct in some cases, such as *Mia/Cd-rap* and *Cyclin B2*.

- The expression of *Mia/Cd-rap* appears inversely correlated with the growth potential of rat pituitary adenoma cells mouse pituitary adenomas.

- The expression of Mia/Cd-rap and Cyclin B2 is increased in human pituitary adenomas compared to normal tissue and is directly correlated with the expression of HMGA1.

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whereas cyclin B1-null mice die in utero. Proc Natl Acad Sci U S A 1998;95(8):4344-4349.

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