**PIT1** upregulation by HMGA proteins has a role in pituitary tumorigenesis

Dario Palmieri1,2, Teresa Valentino1,2, Ivana De Martino1, Francesco Esposito1,2, Paolo Cappabianca3, Anne Wierinckx4,5, Michela Vitiello1,2, Gaetano Lombardi6, Annamaria Colo6, Jacqueline Trouillas4,5, Giovanna Maria Pierantoni1, Alfredo Fusco1,2 and Monica Fedele1,2

1Dipartimento di Biologia e Patologia Cellulare e Molecolare, 2Istituto di Endocrinologia ed Oncologia Sperimentale (IEOS) del Consiglio Nazionale delle Ricerche and 3Dipartimento di Scienze Neurologiche, Divisione di Neurochirurgia, Università degli Studi di Napoli “Federico II”, via Pansini 5, 80131 Naples, Italy
4INSERM U1028; CNRS UMR5292; Lyon Neuroscience Research Center, Neuro-oncology and Neuro-inflammation Team, Lyon F-69372, France
5University Lyon1, F-69000 Lyon, France
6Dipartimento di Endocrinologia, Università degli Studi di Napoli ‘Federico II’, 80131 Naples, Italy

(Correspondence should be addressed to A Fusco; Email: alfusco@unina.it)

**Abstract**

We have previously demonstrated that HMGA1B and HMGA2 overexpression in mice induces the development of GH and prolactin (PRL) pituitary adenomas mainly by increasing E2F1 transcriptional activity. Interestingly, these adenomas showed very high expression levels of PIT1, a transcriptional factor that regulates the gene expression of *Gh*, *Prl*, *Ghrhr* and *Pit1* itself, playing a key role in pituitary gland development and physiology. Therefore, the aim of our study was to identify the role of *Pit1* overexpression in pituitary tumour development induced by HMGA1B and HMGA2. First, we demonstrated that HMGA1B and HMGA2 directly interact with both PIT1 and its gene promoter in vivo, and that these proteins positively regulate *Pit1* promoter activity, also co-operating with PIT1 itself. Subsequently, we showed, by colony-forming assays on two different pituitary adenoma cell lines, GH3 and αT3, that *Pit1* overexpression increases pituitary cell proliferation. Finally, the expression analysis of *HMGA1*, *HMGA2* and *PIT1* in human pituitary adenomas of different histological types revealed a direct correlation between *PIT1* and HMGA expression levels. Taken together, our data indicate a role of *Pit1* upregulation by HMGA proteins in pituitary tumours.

*Endocrine-Related Cancer* (2012) **19** 123–135

**Introduction**

Pituitary adenomas are one of the most frequent intracranial tumours with a prevalence of clinically apparent tumours close to one in 1000 of the general population and are the third most common intracranial tumour type after meningiomas and gliomas (Scheithauer et al. 2006). They are mostly non-metastasising monoclonal neoplasms arising from adenohypophyseal cells in the anterior pituitary, and exhibit a wide range of hormonal and proliferative activity. The most common types (about 50%) of pituitary adenomas are prolactinomas, while GH- or ACTH-secreting adenomas account for 20 and 10% of pituitary tumours respectively, and TSH-secreting adenomas are rare (1%) (Llyod et al. 2004). About one-third of pituitary adenomas are named non-functioning adenomas because they do not exhibit signs of hypersecretion or gonadotrophin adenomas related to FSH–LH immunoreactivity (Trouillas et al. 1986). They are usually large tumours diagnosed following local compressive effects on brain structures and cranial nerves.

Pituitary tumorigenesis is generally considered a model of the multi-step process of carcinogenesis, in which molecular genetic alterations represent the initialising event that transforms cells, and hormones and/or growth factors promote cell proliferation (Asa & Ezzat 2002). However, the molecular events leading
to pituitary tumour development are still unclear, since somatic mutations identified in other neoplasias, such as the \textit{BRAF} and \textit{RAS} genes, are rare events in pituitary adenomas (Lania \textit{et al.} 2003, De Martino \textit{et al.} 2007a). Activating mutations of \textit{Gsa} (the so-called \textit{gsp} mutations) are the most important somatic mutation in pituitary adenomas, being present in up to 40\% of GH-secreting adenomas (Lyons \textit{et al.} 1990). Mutations of \textit{MEN1A}, the gene mutated in the MEN-1 syndrome, which includes pituitary adenomas, are uncommon in sporadic tumours (Zhuang \textit{et al.} 1997, Schmidt \textit{et al.} 1999). Similarly, other genes involved in familial pituitary adenomas, such the \textit{AIP} gene, responsible for familial isolated pituitary adenomas, or the \textit{CDKN1B} gene, mutated in the MEN-1-like syndrome MEN-4, have been found to be mutated in about 3\% of sporadic GH-secreting adenoma or never in sporadic pituitary adenomas respectively (Occhi \textit{et al.} 2010). However, epigenetic events, such as hypermethylation and/or microRNA-dependent impairment of protein translation, are likely to be responsible for the downregulation of gene and/or protein expression associated with pituitary tumorigenesis (Amaral \textit{et al.} 2009, Dudley \textit{et al.} 2009, Tateno \textit{et al.} 2010). Moreover, a parental-specific methylation pattern of the \textit{Gsa} gene, responsible for a tissue-specific near-exclusive expression of \textit{Gsz} from the maternal allele, is relaxed in the majority of GH-secreting pituitary adenomas negative for \textit{gsp} (Hayward \textit{et al.} 2001). Therefore, both genetic and epigenetic alterations appear to be involved in pituitary tumorigenesis. Our recent studies have identified a crucial role for the high-mobility group A (HMGA) proteins in pituitary tumour development (Fedele \textit{et al.} 2002, 2005).

HMGA protein family includes four members, HMGA1A, HMGA1B and HMGA1C, splicing isoforms of the \textit{HMGA1} gene, and HMGA2, encoded by the \textit{HMGA2} gene (Fusco \& Fedele 2007). They are small acidic non-histone nuclear factors that bind the minor groove of AT-rich DNA sequences through their aminoterminal region containing three short basic repeats, the so-called AT-hooks (Fusco \& Fedele 2007). HMGA proteins do not have transcriptional activity \textit{per se}, but regulate gene expression interacting with other transcription factors and modifying the structure of DNA, in order to modulate the formation of stereo-specific complexes on the promoter/enhancer regions of target genes (Thanos \& Maniatis 1992, Falvo \textit{et al.} 1995).

Both HMGA genes have a critical role during embryogenesis, when they are widely expressed, whereas their expression is absent or low in normal adult tissues (Zhou \textit{et al.} 1995, Chiappetta \textit{et al.} 1996). Conversely, they are frequently overexpressed in several human cancers including thyroid (Chiappetta \textit{et al.} 1998, 2008), prostate (Tamimi \textit{et al.} 1993, Winkler \textit{et al.} 2007), cervix (Bandiera \textit{et al.} 1998), colorectum (Fedele \textit{et al.} 1996) and pancreas carcinoma (Abe \textit{et al.} 2000, 2003), and several studies indicate that HMGA proteins are causally involved in tumour development (Fusco \& Fedele 2007). In fact, overexpression of both \textit{HMGA1} and \textit{HMGA2} results in the transformation of rat\textit{a} fibroblast and human lymphoblastoid cells (Wood \textit{et al.} 2000) while inhibition of their expression prevents thyroid transformation induced by mouse transforming retroviruses (Vallone \textit{et al.} 1997) or induces apoptosis in two different thyroid anaplastic carcinoma cell lines (Scala \textit{et al.} 2000).

Several data support a critical role for \textit{HMGA2} (and probably for \textit{HMGA1}) in the generation of human pituitary adenomas (Fedele \textit{et al.} 2010). Indeed, \textit{HMGA2} was found amplified and overexpressed in a large set of human prolactinomas (Finelli \textit{et al.} 2002), and pituitary adenomas secreting prolactin (PRL) and GH developed in transgenic mice overexpressing HMGA1B or HMGA2 (Fedele \textit{et al.} 2002, 2005). Our previous studies demonstrated that HMGA2 induces pituitary tumour development by enhancing E2F1 activity (Fedele \textit{et al.} 2006). Indeed, following the interaction with the retinoblastoma protein pRB, HMGA2 displaces histone deacetylase 1 (HDAC1) from the pRB/E2F1 complex, increasing E2F1 acetylation and transcriptional activity. Consistently, functional loss of E2F1 activity (obtained by mating \textit{HmgA2} transgenic and \textit{E2F1} knockout mice) strongly reduced the incidence of pituitary tumours (Fedele \textit{et al.} 2006). However, \textit{HmgA2} mice still develop pituitary neoplasias also in an \textit{E2F1} knockout background, although with a lower frequency and a less aggressive phenotype, suggesting that other molecular pathways may be involved in pituitary tumour development induced by HMGA overexpression. Recently, using a genechip microarray approach, we have shown that HMGA proteins can contribute to pituitary cell transformation through the transcriptional modulation of target genes, such as \textit{Mia} (Cd-rap) (De Martino \textit{et al.} 2007b) and \textit{Ccnb2} (De Martino \textit{et al.} 2009).

Our previous findings also showed a very abundant expression of \textit{Pit1} (whose expression was not detectable in adult mouse pituitary) in pituitary adenomas from \textit{HmgA1b} and \textit{HmgA2} transgenic mice (Fedele \textit{et al.} 2002, 2005). PIT1, also named GHF1, is a member of the POU transcription factor family (Delhase \textit{et al.} 1996), and plays a key role in the specification, expansion and survival of three specific pituitary cell types (somatotropes, lactotropes and a subset of thyrotropes) during the development of the

The aim of the present study was to investigate the role of Pit1 overexpression in the generation of pituitary adenomas in Hmga1b and Hmga2 transgenic mice.

Here, we demonstrate that both HMGA1B and HMGA2 bind both PIT1 and PIT1-responsive DNA elements, thus positively modulating Pit1 promoter activity. Functional studies show that Pit1 overexpression enhances pituitary adenoma cell proliferation. Finally, a correlation was found between PIT1 and HMGA overexpression in human pituitary adenomas, further supporting a role of HMGA-mediated PIT1 overexpression in pituitary tumours.

Materials and methods

Plasmids, siRNAs, recombinant proteins and antibodies

Expression vector containing the V5-tagged full-length cDNA for Pit1 sub-cloned in the pcDNA3.1/GS vector was purchased from Invitrogen. HA-tagged HMGA1B and HMGA2 expression plasmids were previously described (Fedele et al. 2001, 2006). The PIT1 promoter construct, carrying the region −1321 to +15, related to the transcriptional start site, of the human PIT1 gene fused to the luciferase cDNA (PIT-1-Luc), was a generous gift of Dr M Delhase (Brussels, Belgium). The pBABE-puro vector was previously described (Monaco et al. 2001). The siRNA anti-HMGA1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GST- and His-HMGA1B and HMGA2 fusion proteins were expressed in Escherichia coli strain BL21 (DE3) and purified using glutathione sepharose or nickel beads as described previously (Baldassarre et al. 2001, Pierantoni et al. 2001). Full-length PIT1 protein, anti-HA (sc-805) and anti-PIT1 supershift antibodies (sc-442X) were purchased from Santa Cruz Biotechnology, whereas anti-V5 (R960-25) antibody was purchased from Invitrogen. Anti-HMGA1 and anti-HMGA2 antibodies were previously described (Fedele et al. 2006, Pierantoni et al. 2007).

Cell cultures and transfections

Human embryonic kidney (HEK) 293T, rat pituitary adenoma GH3 and mouse pituitary adenoma zT3 cells were cultured in DMEM supplemented with 10% FCS (GIBCO-BRL, Life Technologies). DNA was transfected by the calcium phosphate procedure, as described previously (Graham & Van der Eb 1973), in HEK293T, and by Lipofectamine 2000 (Invitrogen), according to the manufacturer’s instructions, in GH3 and zT3 cells.

GST pull-down assay, protein extraction and co-immunoprecipitation

For in vitro protein–protein binding, 5 μg PIT1 recombinant protein were incubated with 5 μg resin conjugated to GST, GST-HMGA1B or GST-HMGA2 recombinant proteins. Reactions and analysis of the protein–protein interactions were performed as described previously (Pierantoni et al. 2001). A similar procedure was also applied to HEK293T cells transiently transfected with the Pit1-V5 expression vector. Briefly, 500 μg total protein extracts were incubated with 5 μg resin conjugated to GST, GST-HMGA1B or GST-HMGA2 recombinant proteins. The protein–protein complexes formed on the resin were pulled down by centrifugation. The resin was washed five times at 4 °C with 1 ml cold NETN buffer containing 0.1% NP-40, 1 mM EDTA, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 20 mM piper phosphate, 0.2 μg aprotinin, 4 mM PMSF, 25 mM sodium fluoride, 10 mM activated sodium orthovanadate (Sigma) and a cocktail of protease inhibitors (Roche Applied Science).

Protein extracts were obtained by lysing cells and tissues in NETN buffer and then processed for co-immunoprecipitation as described previously (Pierantoni et al. 2001).

Electrophoretic mobility shift assay

Recombinant proteins (5 ng) were incubated for 15 min at RT in binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 2 μg BSA, 1 μg poly-dCdG) with a 32P-end-labelled double-strand (DS) oligonucleotides (specific activity, 8000–20 000 c.p.m./fmol), corresponding to the PIT1 consensus (sc-2541; Santa Cruz Biotechnology) or to the same element mutated in the PIT1 binding site (sc-2542). Up to 400-fold excess of specific unlabelled competitor oligonucleotide was added as the control. Supershift analysis was carried out by incubating the reaction mix with 1 μg antibody for 30 min in ice. The DNA–protein complexes were resolved on 6%
were 50 used to amplify the sequence of the polymerase (Perkin-Elmer, Monza, Italy). Primers for PCR were performed with AmpliTaq gold DNA polymerase (Perkin-Elmer, Monza, Italy). Primers used to amplify the sequence of the Pit1 promoter were 5'-GCACCAACCTATCATTAC-3' (forward) and 5'-TGCTACTAACAACATTGC-3' (reverse). PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and scanned using a Typhoon 9200 scanner. The intensity of the bands was quantified by densitometric analysis using ImageQuant software (GE Healthcare, Milan, Italy).

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was carried out with acetyl-histone H3 immune precipitation assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer’s instruction, as described previously (De Martino et al. 2009). Input and immunoprecipitated chromatin were analysed by PCR for the presence of the Pit1 promoter sequence. PCR were performed with AmpliTaq gold DNA polymerase (Perkin-Elmer, Monza, Italy). Input and immunoprecipitated chromatin were analysed by PCR for the presence of the Pit1 promoter sequence. PCR were performed with AmpliTaq gold DNA polymerase (Perkin-Elmer, Monza, Italy). Primers used to amplify the sequence of the Pit1 promoter were 5'-GCACCAACCTATCATTAC-3' (forward) and 5'-TGCTACTAACAACATTGC-3' (reverse). PCR products were resolved on a 2% agarose gel, stained with ethidium bromide, and scanned using a Typhoon 9200 scanner. The intensity of the bands was quantified by densitometric analysis using ImageQuant software (GE Healthcare, Milan, Italy).

Luciferase and colony assays
For the luciferase assay, a total of 2×10^5 cells were seeded into each well of a six-well plate and transiently transfected with 1 μg PIT-1-Luc and with the indicated amounts of HA-HMGA1B and HA-HMGA2, together with 0.5 μg Renilla and various amounts of the backbone vector to keep the total DNA concentration constant. Transfection efficiency, normalised for the Renilla expression, was assayed with the dual luciferase system (Promega Corporation). All transfection experiments were repeated at least three times.

For the colony assay, GH3 and zT3 cells were seeded at a density of 2.5×10^5 per 10 mm dish. Two days after, the cells were transfected with 10 μg pcDNA3.1 or 10 μg pcDNA3.1/Pit1-V5 or 5 μg Pit1 shRNA (Santa Cruz Biotechnology) or 5 μg scrambled shRNA (Santa Cruz Biotechnology) plus 2 μg pBABE-puro. After about 15 days of positive selection in puromycin, the cells were stained with 500 mg/ml crystal violet in 20% methanol, and the resulting colonies were counted.

Tissue samples
The human pituitary adenoma samples were obtained from 46 surgical excision biopsies, including 13 GH and 33 PRL adenomas) from patients of ’Federico II’ University (Naples) and Neurosurgical Department (Pr Jouanneau E) of Hospices Civils de Lyon (France). One part of each pituitary adenoma was saved for routine histopathology evaluation, including immunohistochemistry with the systematic detection of GH, PRL, ACTH, TSH, FSH and LH, and the other one immediately frozen at −80 °C until the extraction of nucleic acids. Informed consent for the scientific use of biological material was obtained from all patients.

RNA extraction and real-time RT-PCR
Total RNA was extracted from tissues using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA) solution, according to the manufacturer’s instructions. The RNA integrity was verified by denaturing agarose gel electrophoresis (virtual presence of sharp 28S and 18S bands) and spectrophotometry. One microgram of total RNA of each sample was reverse-transcribed with the QuantiTect Reverse Transcription (Qiagen) using an optimised blend of oligo-dT and random primers according to the manufacturer’s instructions. 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 identically processed. Quantitative PCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) as follows: 95 °C for 10 min and 40 cycles (95 °C for 15 s and 60 °C for 1 min). A dissociation curve was run after each PCR in order to verify amplification specificity. Each reaction was performed in duplicate. To calculate the relative expression levels, we used the 2^-ΔΔCt method (Livak & Schmittgen 2001).

Primer sequences are available upon request.

Statistical analyses
For the comparison between two groups of experiments, Student’s t-test was used. Three or more groups of experiments were compared using the one-way ANOVA followed by Tukey’s multiple comparison test. All results are expressed as mean ± s.d. The statistical significant difference was considered when P value was <0.05. Linear regression analysis was performed to determine the association of PIT1 with HMGA1 or HMGA2 expression levels in human pituitary adenomas. The square of correlation coefficient (R^2) close to 1 was considered to be indicative of a significant direct correlation.

Results
HMGA proteins interact with PIT1
To investigate the role of HMGA proteins in the modulation of PIT1 function, we first hypothesised that HMGA proteins directly bind PIT1 protein. The finding
that other members of the POU transcription factor family, such as Oct-6 and Oct-2A, interact with HMGA proteins through their POU domain supports this hypothesis (Abdulkadir et al. 1995, Leger et al. 1995, Zwilling et al. 1995). Therefore, we performed a GST pull-down assay incubating the PIT1 recombinant protein with GST-HMG1B or GST-HMG2 fusion proteins. As shown in Fig. 1A, PIT1 was able to directly interact with both GST-HMG1B and GST-HMG2, but not with GST alone. To confirm this interaction in a cellular context, we transfected HEK293T cells with expression vectors containing the full-length cDNAs for PIT1, HMG1B or HMG2, fused to the V5 (Pit-1-V5) and HA (HA-HMG1B and HA-HMG2) tags respectively. Total cell extracts were immunoprecipitated with anti-V5 antibody and analysed by immunoblot with anti-HA antibody. As shown in Fig. 1B (left panels), HA-HMG1B and HA-HMG2 were immunoprecipitated by the anti-V5 antibody only when transfected along with Pit-1-V5. This result was confirmed by reverse co-immunoprecipitation carried out by immunoprecipitating with anti-HA antibody and analysing with anti-V5 antibody (Fig. 1B, right panels). The negative result obtained by blotting for the unrelated and endogenous E2F1 protein confirmed the specificity of the PIT1/HMGA interactions. Ethidium bromide was added to the immunoprecipitation reaction to prevent DNA-mediated interaction between proteins. Interestingly, cells co-transfected with PIT1 and each of the HMGA proteins show more abundant levels of HMGA proteins than those transfected with HMGA1b or HMGA2, fused to the V5 (Pit-1-V5) and HA (HA-HMG1B and HA-HMG2) tags respectively. Total cell extracts were immunoprecipitated with anti-V5 antibody and analysed by immunoblot with anti-HA antibodies. As shown in Fig. 1B (left panels), HA-HMG1B and HA-HMG2 were immunoprecipitated by the anti-V5 antibody only when transfected along with Pit-1-V5. This result was confirmed by reverse co-immunoprecipitation carried out by immunoprecipitating with anti-HA antibody and analysing with anti-V5 antibody (Fig. 1B, right panels). The negative result obtained by blotting for the unrelated and endogenous E2F1 protein confirmed the specificity of the PIT1/HMGA interactions. Ethidium bromide was added to the immunoprecipitation reaction to prevent DNA-mediated interaction between proteins. Interestingly, cells co-transfected with PIT1 and each of the HMGA proteins show more abundant levels of HMGA proteins than those transfected with HMG1B or HMG2 alone (input in Fig. 1B, middle panels), suggesting that PIT1 can positively influence their expression. Western blot anti-V5 or anti-HA antibody, for samples immunoprecipitated with anti-V5 or anti-HA antibody respectively was performed to control the successful immunoprecipitation reactions (Fig. 1B).

Finally, to validate the HMGA/PIT1 interaction in the context of the pituitary tumours, we pulled down pituitary adenoma extracts from Hmg1b or Hmg2 transgenic mice, where PIT1 is abundantly expressed (Fedele et al. 2002, 2005), from GST-HMG1B or GST-HMG2 beads. Figure 1C shows that both GST-HMG1B and GST-HMG2, but not GST, interacted with endogenous PIT1 protein in transgenic mouse tumours. These data demonstrate that HMGA proteins are direct molecular partners of PIT1 both in vitro and in vivo.

**HMGA proteins bind to and activate the Pit1 promoter**

PIT1 is able to directly regulate the expression of several genes with a key role in pituitary gland physiology such as PRL, GH, GHRHR and PIT1 itself (Lefevre et al. 1987, Nelson et al. 1988, Chen et al. 1990, Li et al. 1990, McCormick et al. 1990). Since HMGA proteins physically interact with PIT1, we investigated whether this interaction may affect PIT1 activity. HMG1B or HMG2 recombinant proteins were incubated with a 32P-end-labelled DS oligonucleotide corresponding to the consensus site recognised by PIT1 in an electrophoretic mobility shift assay (EMSA). As shown in Fig. 2A, both HMG1B and HMG2 were able to bind the PIT1 responsive element (Pit-1-RE) in vitro. The specificity of the binding was assessed using a 100- and 400-fold molar excess of the specific unlabelled DS oligonucleotide or a 100-fold molar excess of the same unlabelled, but single-strand (SS) oligonucleotide as specific and non-specific competitors respectively. Moreover, the
binding was also abolished pre-incubating the reaction mix with anti-HMGA1 and anti-HMGA2 antibodies, which, as reported previously (Martinez Hoyos et al. 2009), specifically displace HMGA proteins from their target DNA (data not shown). As shown in Fig. 2B, the binding of HMGA proteins to the Pit-1-RE does not interfere with the binding of PIT1 to the same oligonucleotide. Moreover, as shown by the absence of a slower migrating spot when both HMGA and PIT1 proteins are incubated with the probe, it appears that they do not form a unique complex, but independently bind the same DNA response element. We also used, as a control of specificity of the PIT1 binding, an oligonucleotide mutated in a key residue within the PIT1 consensus site, which was incapable of binding PIT1 (Fig. 2C, lane 1). Interestingly, this mutant oligonucleotide still binds HMGA1B with the same efficiency of the wild-type Pit-1-RE, whereas the binding to HMGA2 was highly compromised (Fig. 2C, lanes 2 and 3). Therefore, it is likely that HMGA1B and HMGA2 do not bind exactly to the same residues nearby the PIT1 consensus site.

Next, since one of the PIT1 targets is Pit1 gene itself, we focused on the potential role of HMGA in PIT1-dependent Pit1 gene regulation in pituitary adenomas. For this purpose, we first performed a ChIP assay in pituitary adenomas from Hmga1b or Hmga2 transgenic mice. Chromatin was immunoprecipitated using specific anti-HMGA1 or anti-HMGA2 antibody, or IgG as the negative control, and analysed by PCR using primers specific for the mouse Pit1 promoter. Figure 3A shows the in vivo binding of both Hmga1 and Hmga2 to the Pit1 promoter, while no amplification was obtained in the negative control. Then, we investigated the functional effect of the physical interaction between HMGA proteins and PIT1 on the PIT1 promoter activity by luciferase assays. HEK293T cells were transiently transfected with a reporter vector (PIT1-1-Luc), containing the luciferase gene under the control of the PIT1 promoter, along with vectors coding for HA-HMGA1B, HA-HMGA2 or Pit-1-V5 proteins. As shown in Fig. 3B, only HA-HMGA2, but not HA-HMGA1B, was able to positively regulate the activity of the PIT1 promoter. Moreover, a strong and significant cooperation between HMGA2 and PIT1 was observed ($P<0.001$), while HMGA1 only slightly but significantly increased the positive transcriptional effect of PIT1 on its promoter ($P<0.05$). To confirm these data in a pituitary context, we transiently transfected with the radiolabelled PIT1 consensus site incubated with recombinant His-HMGA1B and His-HMGA2 as indicated. To assess the specificity of the binding, a 100- and 400-fold excess of unlabelled double-strand (DS) oligonucleotide was added as a specific competitor, and a 100-fold excess of unlabelled single-strand (SS) oligonucleotide was added as a non-specific competitor. (B) EMSA performed with the same oligonucleotide as in (A), incubated with recombinant PIT1, His-HMGA1B and His-HMGA2 as indicated. Supershift assay was performed with anti-PIT1 antibody where indicated. Two different autoradiographic exposure times were needed to allow a good view of both the binding of HMGA proteins and that of PIT1: upper panel, 18 h; lower panel, 1 h. (C) The same EMSA as in (B), but with an oligonucleotide mutated in the PIT1 consensus site.

**Figure 2** In vitro binding of HMGA proteins to the PIT1 consensus site. (A) Electrophoretic mobility shift assay (EMSA) performed with the radiolabelled PIT1 consensus site incubated with recombinant His-HMGA1B and His-HMGA2 as indicated. To assess the specificity of the binding, a 100- and 400-fold excess of unlabelled double-strand (DS) oligonucleotide was added as a specific competitor, and a 100-fold excess of unlabelled single-strand (SS) oligonucleotide was added as a non-specific competitor. (B) EMSA performed with the same oligonucleotide as in (A), incubated with recombinant PIT1, His-HMGA1B and His-HMGA2 as indicated. Supershift assay was performed with anti-PIT1 antibody where indicated. Two different autoradiographic exposure times were needed to allow a good view of both the binding of HMGA proteins and that of PIT1: upper panel, 18 h; lower panel, 1 h. (C) The same EMSA as in (B), but with an oligonucleotide mutated in the PIT1 consensus site.
vector. As shown in Fig. 3C, HA-HMGA2 expression led to a significant increase in Pit1 promoter activity, while only a slight but not significant increase was observed after the transfection of the HA-HMGA1B construct. These data clearly demonstrate that HMGA2 is able to positively regulate PIT1 promoter activity in co-operation with PIT1, whereas they suggest that HMGA1 shows only a very weak effect on the regulation of PIT1 gene expression. The abundant expression of HMGA1 in GH3 cells, in contrast to the total absence of HMGA2 expression (Fig. 3D), could probably account for the lack of a significant effect of HMGA1 transfection on PIT1 promoter activity.

To further evaluate the role of endogenous HMGA1 on Pit1 expression in pituitary cells, Pit1 expression was analysed in GH3 cells interfered for HMGA1, through an anti-HMGA1 siRNA, in comparison with GH3 cells treated with a scrambled siRNA. As shown in Fig. 3E, Pit1 mRNA levels were significantly decreased in cells knocked down for HMGA1 compared with their scrambled-treated controls. Therefore, both HMGA1 and HMGA2 play a crucial role in the regulation of Pit1 expression in GH3 cells.

**Overexpression of Pit1 increases the proliferation rate of pituitary adenoma cells**

To evaluate the role of Pit1 overexpression in cell proliferation in a pituitary context, we performed a colony-forming assay in GH3 cells. As shown in Fig. 4A, the number of colonies obtained, after puromycin selection, by transfection of a Pit1 expression vector, was significantly higher (a fourfold increase) compared with that obtained by transfecting the empty vector. Consistently, the knock-down of the endogenous PIT1 in GH3 cells caused a significant decrease in their growth in a colony-forming assay (data not shown). Similar results were obtained using a different pituitary cell type, such as the mouse gonadotroph cell line αT3. Indeed, as shown in Fig. 4B, Pit1 overexpression caused a twofold increase in the number of colonies with respect to the backbone vector. Since αT3 cells do not express Pit1 normally, we asked whether the exogenous expression of Pit1 upregulates the classical PIT1 targets, such as Gh and Ghrhr. To answer this question, we performed RT-PCR analysis in αT3 cell clones stably expressing Pit1, with the result that Gh was not expressed in these...
cells (data not shown), and Ghrhr expression did not change significantly between parental and Pit1-transfected cells (Fig. 4C). Similarly, the expression of Pit1 in these cell clones does not lead to a different expression, compared with the parental cells, of genes, such as Ccnb2 and Mia (Cd-rap) (Fig. 4C), that are directly regulated by HMGA proteins in pituitary adenomas (De Martino et al. 2007a, b, 2009). Conversely, as reported for other cell systems (Gaiddon et al. 1999), the expression of Pit1 in αT3 cells, but not in GH3 cells, leads to the upregulation of c-Fos (Fig. 4C and D). Surprisingly, overexpression of Pit1 in GH3 cells inhibits the expression of Mia (Cd-rap) (Fig. 4D). These findings indicate that Pit1 overexpression positively regulates pituitary cell proliferation through different mechanisms depending on the specific pituitary cellular context.

Positive correlation between HMGA and PIT1 expression in human pituitary adenomas

Overexpression of PIT1 is a common feature of GH-, PRL- and TSH-, but not of ACTH-, FSH-, LH- or non-functioning human pituitary adenomas (Pellegrini-Bouiller et al. 1997). Moreover, we have previously demonstrated that HMGA1 and HMGA2 expression levels are significantly increased in human pituitary adenomas compared with normal gland (De Martino et al. 2009). To evaluate whether there is a direct correlation between HMGA1/2 and PIT1 mRNA levels, we analysed a panel of 46 human pituitary adenomas (including 13 GH and 33 PRL adenomas) for the expression of PIT1, HMGA1 and HMGA2 mRNAs by quantitative RT-PCR. As shown in Fig. 5, a direct correlation between PIT1 and HMGA1 or HMGA2 mRNA levels was observed. In fact, the correlation coefficients for the fold changes between adenomas and normal gland, calculated in both PIT1 and HMGA1, as well as PIT1 and HMGA2 expression levels, were $R^2 = 0.82$ ($P < 0.001$) and $R^2 = 0.61$ ($P < 0.001$) respectively.

Discussion

Various studies support a critical role of HMGA proteins in the development of human pituitary adenomas (Finelli et al. 2002, De Martino et al. 2009, Qian et al. 2009, Wang et al. 2010). However, the mechanism by which they act in pituitary tumour development is still not completely known. We have previously demonstrated, using mouse models overexpressing HMGA2 and knockout for E2F1, that...
show that HMG proteins bind both PIT1 and PIT1-responsive DNA elements, thus positively modulating the PIT1 promoter activity, also synergistically co-operating with Pit1. Moreover, we demonstrated that Pit1 overexpression drastically enhances (up to fourfold) pituitary cell proliferation by inhibiting the expression of c-Fos in gonadotroph cells or by inhibiting the expression of Mia (Cd-rap) in GH/PRL-secreting cells. Therefore, these results indicate a potential causal role of the aberrant Pit1 expression in the cell biology of pituitary tumour.

We can envisage two different, but not mutually excluding, mechanisms by which HMGA-mediated Pit1 upregulation may contribute to pituitary cell transformation:

a) HMGA overexpression may upregulate Pit1 levels in pituitary adenoma cells of the Pit1 lineage, enhancing their proliferation.
b) The enhancement of Pit1 expression by HMGA during development might lead to abnormal growth of the embryonic cells secreting GH and PRL, which results in pituitary adenoma during adult life.

Interestingly, high expression levels of PIT1 represent a constant feature of human pituitary GH, PRL and TSH adenomas (Asa et al. 1993, Delhase et al. 1993, Friend et al. 1993, Pellegrini et al. 1994, Pellegrini-Bouiller et al. 1997), and several previous studies suggested a potential role for PIT1 in cell proliferation, the prevention of apoptotic death and the pathogenesis of pituitary tumours (Castrillo et al. 1991, Gaiddon et al. 1999, Salvatori et al. 2002, Pellegrini et al. 2006). In fact, microinjection of Pit1 antisense sequences blocks cell growth in the GC somatotroph cell line (Castrillo et al. 1991) and dominant-negative mutants of Pit1 reduce cell viability by decreasing the growth rate and inducing apoptosis via a caspase-independent pathway (Pellegrini et al. 2006). Moreover, PIT1 can also upregulate the expression of genes, such as c-Fos (Gaiddon et al. 1999) and Ghrhr (Salvatori et al. 2002), involved in cell proliferation. Interestingly, recent studies have identified an increased expression of PIT1 also in breast tumours (Ben-Batalla et al. 2010), suggesting a potential role of PIT1 in the proliferation of different cell types.

In conclusion, our data demonstrate that the high expression of Pit1 in the pituitary adenomas of Hmga transgenic mice is induced by a positive regulation by HMGA proteins of Pit1 transcription and support a role for Pit1 overexpression in pituitary tumour.
Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by grants from Associazione Italiana Ricerca sul Cancro (AIRC), CNR (DG.RSTL. 030.001) and MIUR (PRIN 2008). D Palmieri and F Esposito are recipients of fellowships from Fondazione Italiana Ricerca sul Cancro (FIRC).

Acknowledgements
We thank Mireille Delhase for providing us the Pit-1 promoter plasmids, Angelo Ferraro for performing real-time PCR on human tissues and Luigi Di Guida for animal care.

References


www.endocrinology-journals.org


Vallone D, Battista S, Pierantoni GM, Fedele M, Casalino L, Santoro M, Viglietto G, Fusco A & Verde P 2004 Neoplastic transformation of rat thyroid cells requires the junB and fra-1 gene induction which is dependent on the pituitary-specific transcription factor. Molecular Endocrinology 18 2116665


Received in final form 9 December 2011
Accepted 23 December 2011
Made available online as an Accepted Preprint 23 December 2011