Abstract The developmentally regulated architectural transcription factor, high mobility group A2 (HMGA2), is involved in growth regulation and plays an important role in embryogenesis and tumorigenesis. Little is known, however, about its downstream targets. We performed a search for genes of which expression is strongly altered during embryonic development in two HMGA2-deficient mouse strains, which display a pygmy-phenotype, as compared to wild-type mice. We found that the insulin-like growth factor II mRNA-binding protein 2 gene (IMP2), but not its family members IMP1 and IMP3, was robustly downregulated in mutant E12.5 embryos. Furthermore, we show that wild-type HMGA2 and its tumor-specific truncated form have opposite effects on IMP2 expression. Our results clearly indicate that HMGA2 differentially regulates expression of IMP family members during embryogenesis.

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1. Introduction

High mobility group A2 (HMGA2) is a member of the HMGA gene family [1,2]. The HMGA proteins contain three DNA-binding domains, AT-hooks, which interact with AT-rich stretches in the narrow minor groove of DNA [3], and an acidic C-terminal tail. Although these proteins possess a classical transcription factor structure, they do not have transcriptional activation capacity by themselves. HMGA proteins play a critical role in organizing the assembly and stabilization of nucleo-protein transcriptional complexes (enhanceosomes) at the level of enhancer- or promoter-regions, resulting in induction or repression of transcription. They are classified as architectural transcription factors (reviewed in [4,5]). The best characterized example of how HMGA proteins can modulate gene expression is provided by studies describing the expression regulation of the interferon-β gene [6]. Recently, it was shown that HMGA2 modulates the promoter activity of the DNA repair gene ERCCI [7] and of the cyclin A gene [8].

In mice, HMGA2 expression is high during early development but barely detectable in adult tissues [9]. Consistent with its growth-regulatory role is the pygmy phenotype in mice in which the HMGA2 gene is homozygously inactivated [10]. HMGA2 is disrupted and aberrantly expressed in many tumor types making this gene probably the most frequently rearranged gene in human neoplasias (reviewed in [11,12]). We identified the HMGA2 gene as the gene that is disrupted by 12q15 chromosomal rearrangements observed in a variety of commonly occurring benign solid tumors [13,14]. Disruption of the gene in these tumors usually results in the expression of a truncated protein only containing the DNA-binding domains of wild-type HMGA2. In contrast to the expression of aberrant forms of HMGA2 in benign tumors, overexpression of wild-type HMGA2 has been reported in various malignant tumors, such as breast cancer [15] and pancreatic tumors [16].

Although HMGA2 has been clearly implicated in cell proliferation and tumorigenesis, the underlying molecular and biological events involved in these phenomena still need further unraveling. One way to address this issue is to search for genes of which the expression is regulated by this architectural transcription factor, such as recently described for the structurally related HMGA1 gene [17]. We have performed expression profiling experiments using embryos of the spontaneous pg/pg mouse mutant, which is HMGA2-deficient, and corresponding
wild-type mouse embryos, and report on the differential expression of HMGA2 on the expression of the genes encoding the various members of the insulin-like growth factor II (IGF-II) mRNA-binding protein (IMP) family. We have validated our results using the genetically engineered HMGA2-specific knockout mouse strain and a cell-based system.

2. Materials and methods

2.1. Mouse strains and genotyping

Mouse strains included C3W1.Cg-Hmga2\textsuperscript{\textdeg}/B6J (Jackson Laboratories), which is a spontaneous pg/pg mutant, and C57BL/129SV in which the HMGA2 allele was inactivated by gene targeting [10]. HMGA2-genotyping was performed as described in [18].

2.2. RNA extraction and Northern blot analysis

Total RNA was isolated from whole mouse embryos using the guanidine isothiocyanate method [19]. RNA from NIH/3T3 cells was isolated using the RNeasy total RNA isolation kit (Qiagen), according to the manufacturer’s conditions. Northern blot analysis was performed as described in [20]. After exposure to a PhosphoImager screen (Molecular Dynamics), signals were detected using a Typhoon 9200 scanner (Amersham Biosciences) and quantified using ImageQuant 5.2 software (Molecular Dynamics).

2.3. Microarray analysis

Microarray analysis was performed at the VIB MicroArray Facility (www.microarrays.be). Arrays with ArrayExpress Accession Nos. A-MEXP-4 and A-MEXP-6 (www.ebi.ac.uk/arrayexpress) were hybridized with pooled RNAs from 10 E12.5 pg/pg or 10 corresponding wild-type mouse embryos, as molecular probes. Antisense RNA was amplified using a modified protocol of in vitro transcription as described in [21]. Cy3 and Cy5 labeling protocols were done as described (P-MEXP-580 and P-MEXP-582). For image analysis, ArrayVision was used (Imaging Research Inc.). Spot intensities were measured as artifact-removed total intensities (ARVol) subtracted with median intensity of the local background of each spot. For each gene, ratios of red (Cy-5) over green (Cy-3) intensities (I) were calculated and normalized via a running median of each spot. For each gene, ratios of red (Cy-5) over green (Cy-3) in-

2.4. Primers

The following primers were used: IMP1up1: 5'-GATAGAAA-GTTTGGCGGCCT-3'; IMP1up2: 5'-GACTACGAGTTCTACCATG-GAACAAGTTTACCTGCGGCAAC-3'; IMP1low1: 5'-TATCTTG-GAGCCAATGGGACC3; IMP1low2: 5'-CTGATGCGGCCGCCT-CACTTCTCTCGAGGCTTG-3'; IMP2up1: 5'-CTTCCGGAAGG-AC3; IMP2up2: 5'-GACTCAGAATTTCCGGATGAAATGAGATCCAGG-CTGTA-3'; IMP2low1: 5'-TTCAGATGCTTGAGGGGCTAC-3'; IMP2low2: 5'-CTGATGCGGCCGCCTAATCTTGCGCCTGTG-3'; IMP3up3: 5'-TACAGGCTTATCAAGTGCTG-3'; IMP3up5: 5'-GACTACAGAATTTCCGGATGAAATGAGATCCAGG-CTGTA-3'; IMP3low3: 5'-CTTCCTCTTTAGGTATCCITCTAG-3'; IMP3low5: 5'-CTGATGCGGCCGCCTAATCTTGCGCCTGTG-3'; HMGA2up3: 5'-GAATGCATTATGCAGATGAGTACGCCAGCAGCAGCAGCAG-3'; HMGA2low3: 5'-GATGCATTATGCAGATGAGTACGCCAGCAGCAGCAGCAG-3'; HMGA2low5: 5'-GATGGCGAGTTTATCAAGTGCTG-3'; IMP1: 5'-TATCTTG-GAGCCAATGGGACC3; IMP2: 5'-GACTACGAGTTCTACCATG-GAACAAGTTTACCTGCGGCAAC-3'; IMP3up3: 5'-TACAGGCTTATCAAGTGCTG-3'; IMP3low5: 5'-GAGGCCACCGGATCCACACAGA-3'.

2.5. Plasmids

Mouse IMP1 and IMP2 open reading frames, and part of the UTR of IMP1 and IMP2 were generated by nested RT-PCR from E12.5 mouse embryonic RNA using the Superscript\textsuperscript{TM} First-Strand system (Invitrogen), according to the conditions of the manufacturer. PCR primer sets: IMP1up1/IMP1low1, IMP1up2/IMP1low1 and IMP3up3/IMP3low3. Nested primer sets: IMP1up2/IMP1low2, IMP2up2/IMP2low2 and IMP3up5/IMP3low5. The IMP1 and IMP2 PCR-products were ligated into pcDNA3.1/His (Invitrogen) (plasmids pJB19 and pJB20). The IMP3-PCR-product was inserted into pcDNA3 (Invitrogen) (pJB24).

Wild-type HMGA2 and HMGA2Tr were generated by PCR from the coding sequence of human HMGA2 as template using Pwo DNA polymerase (Roche) according to the conditions of the manufacturer. Primer sets: HMGA2up3/HMGA2low3 and HMGA2up3/HMGA2low5. The amplified DNA fragments were inserted into the retroviral pMSPCvproto vector (Clontech) [23] (plasmids pJB13 and pJB15).

2.6. Hybridization probes and labeling

The IMP3 3'-UTR-specific probe (position 1063–1060 bp behind the stop codon) was obtained by digestion of AW54216. The IMP1 3'-UTR-specific probe was obtained by digestion of pJB24. The IMP1- and HMGA2 CDS-specific probes were generated by digestion of pJB19 and pJB13, respectively. The mouse β-actin probe was obtained by PCR with primers Actin-up and Actin-low on E12.5 embryonic cDNA. Probes were labeled with [α-\textsuperscript{32}P]dCTP using the megaprime DNA labeling system (Amersham) according to the manufacturer’s conditions.

2.7. Cell culture, generation of retrovirally transduced NIH3T3 cells

Cell lines included 293T (ATCC CRL-1573 human embryonic kidney epithelial cells expressing SV40 T-antigen) and NIH/3T3 (mouse embryo fibroblast cells; ATCC CRL-1658). Cells were grown as described [24]. Retrovirally transduced NIH3T3 cell lines were obtained as described in [25].

2.8. Antibody generation, SDS–PAGE and Western blotting

Polyclonal rabbit antiserum HMGA2lxx was raised against a mixture of three synthetic peptides: 9AREGAGQPSTSAQGGQPAAAPQQKR27, 9SPSKAACKQKAETGK25 and 80PRKWPQQVQQKQPQAEQ26, synthesized as multiple-antigen peptide conjugates (Research Genetics). Expression of HMGA2 and HMGA2Tr in recombinant retrovirus transduced NIH3T3 cells was verified by SDS-PAGE and Western blotting as described [24], using HMGA2lxx antibody.

2.9. Results and discussion

3.1. Identification of HMGA2 downstream target genes with strongly downregulated expression in HMGA2-deficient embryos

To identify highly downregulated downstream target genes of HMGA2, we initially compared by microarray analysis the gene expression profile in embryos of the spontaneous pg/pg mouse strain, which can be considered as a HMGA2 null mutant strain, to that in corresponding wild-type embryos. The developmentally regulated HMGA2 gene is only expressed during embryogenesis and since its expression is readily detected from stage E10.5 to stage E13.5 and decreases dramatically thereafter [9], we used RNA from whole embryos of stage E12.5 in microarray analysis. One of the 8600 genes on the microarray slides was HMGA2, represented by a clone with GenBank Accession No. AW550862, which was identified as an internal, positive control. The microarray hybridization was performed a second time in a color flip experiment. The results of the microarray experiments revealed many differentially expressed genes. As expected, the ratio between the measured signal for HMGA2 (AW550862) in wild-type and the spontaneous pg/pg mutant embryos was high (Table 1). To narrow down the list of differentially expressed genes in the datasets, we selected those clones that had a signal ratio comparable to that of the positive control AW550862. This selection resulted in three additional clones (Table 1), AW548319 and AW542316, and an as yet not annotated clone. All other differentially regulated genes displayed only a minor difference in expression levels and are therefore not included in this study.
After sequencing the selected clones, basic local alignment search tool (BLAST) searches revealed that the not yet annotated clone and AW548319 both corresponded to HMGA2. The nucleotide sequence of the third clone, AW542316, was similar to sequences of the 3′-UTR of the human IMP2 gene, indicating that AW542316 might correspond to the gene encoding mouse IMP2.

3.2. Downregulated clone AW542316 corresponds to mouse IMP2

To establish that AW542316 indeed corresponds to mouse IMP2, the complete mouse IMP2 cDNA sequence had to be isolated, since at the time of our investigations, the contiguous mouse IMP2 cDNA sequence was not yet available in the public databases. Using the mouse EST database of the NCBI (National Center for Biotechnology Information), a contig could be generated covering the complete putative mIMP2 cDNA sequence containing part of the 5′-UTR, the open reading frame, the complete 3′-UTR, including the AW542316 sequences, and the poly(A)-tail. Subsequently, the complete mouse IMP2 open reading frame was cloned by RT-PCR using E12.5 mouse embryonic RNA with mIMP2-specific primers, sequenced, and its nucleotide sequence was submitted to NCBI database (GenBank Accession No.: AY531659). Translation of the open reading frame revealed an amino acid sequence that indeed resembled most closely (about 94% sequence identity) the sequence of human IMP2 (Fig. 1A and B). In conclusion, results of our studies so far clearly establish that it is IMP2 that is downregulated in E12.5 embryos of the pg/pg mouse mutant strain.

IMP2 belongs to a family of three IGF-II mRNA binding proteins (IMP1, IMP2 and IMP3), which were found during a search for trans-acting factors associating with IGF-II mRNAs. Structurally, the IMP proteins are highly similar, containing the unique combination of six characteristic RNA-binding modules, two RNA recognition motifs (RRMs) [26] and four hnRNP K homology (KH) domains [27] (Fig. 1). IMP1 is orthologous to the chicken zipcode-binding protein [28] and to the mouse c-myc coding region determinant-binding protein. IMP3 is identical to the KH-domain containing protein overexpressed in cancer [29] and is orthologous to the Vera protein in Xenopus laevis oocytes [30].

A splice variant of human IMP2, p62, missing 43 amino acids located between KH-domains 2 and 3, was identified as an autoantigen in human hepatocellular carcinoma [31]. RT-PCR analysis (data not shown) indicated that the observed downregulation of IMP2 expression in pg/pg mutant embryos concerns only the IMP2 transcript, since the p62 transcript could not be detected in wild-type E12.5 mouse embryos.

3.3. Differential downregulation of IMP family members in E12.5 pg/pg mouse embryos

To confirm the microarray results, we performed Northern blot analysis of RNA isolated from pg/pg and corresponding wild-type mouse embryos. As expected, HMGA2 expression was detected in wild-type but not in the pg/pg embryos (Fig. 2A). Furthermore, IMP2 expression appeared to be beyond the detection level in the pg/pg embryos, whereas it was clearly detectable in the wild-type embryos (Fig. 2A). Similar Northern blot analysis using IMP1- or IMP3-specific probes showed no detectable differences in IMP1 or IMP3 expression levels in pg/pg embryos as compared to wild-type embryos (Fig. 2B), establishing that only the expression of the IMP2 member of this gene family is affected in pg/pg embryos. Finally, Northern blot analysis of RNA isolated from pg/pg and corresponding wild-type newborn mice of 10 days old revealed expression of low, but comparable, levels of IMP2 transcripts in both strains. HMGA2 expression was clearly reduced in the wild-type pups (data not shown).

3.4. IMP2 is a downstream target of HMGA2

A remaining issue pertains to the question whether IMP2 is a downstream target of HMGA2. Genetically, the pg/pg mouse is only partially characterized. A genomic region on chromosome 10 of more than 56 kb, encompassing the 5′ sequences and the first two exons of HMGA2, is deleted [10]. To exclude the possibility that the reason for IMP2 expression not being detectable in Northern blot analyses is due to the fact that IMP2 maps closely to HMGA2, and might therefore also be deleted in pg/pg mice, the chromosomal mapping position of IMP2 was determined. Mouse BLAST-Like Alignment Tool search of the open reading frame of IMP2 against the UCSC (University of California, Santa Cruz) Genome Browser revealed, however, that the mouse IMP2 gene is located on chromosome 16. The possibility still cannot be excluded that IMP2 expression is regulated by another gene mapping to the deleted region in pg/pg embryos. To resolve this issue, we studied IMP2 expression in HMGA2 null mutant mice generated via targeted disruption of the HMGA2 gene [10]. Northern blot analysis of RNA isolated from E12.5 HMGA2−/−, HMGA2+/−, and HMGA2+/+ embryos revealed that no IMP2 expression was clearly reduced as compared to the levels observed in HMGA2+/− embryos (Fig. 3). No differences in expression levels of the IMP2 family members, IMP1 and IMP3, could be observed by performing similar Northern blot analysis (Fig. 3), clearly revealing a differential regulation control within the IMP family during embryogenesis.

These results point towards a prominent role for HMGA2 in the regulation of IMP2 expression. Expression of IMP2 is bi-phasic with an early expression in oocytes and in the zygote, and a sharp increase in expression around embryonic day E12.5, which declines towards birth [30]. In this respect, HMGA2 and IMP2 display a somewhat similar expression profile during mouse embryogenesis. Our data, showing that

Table 1

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IMP2 expression was drastically downregulated in E12.5 HMGA2<sup>−/−</sup> mice, indicating that HMGA2 is very likely to be required for the sharp increase in expression of IMP2 at E12.5. We have to note here that a very faint IMP2 signal was still detected by Northern blot analysis of RNA isolated from E12.5 HMGA2<sup>−/−</sup> mice (Fig. 3), indicating that very low expression levels of IMP2 are still present in E12.5 HMGA2<sup>−/−</sup> mice. These data indicate that, although HMGA2 plays a prominent role in the regulation of IMP2-expression, other factors must be involved too.

Functionally, the IMP proteins are capable of strong and specific RNA-binding, and because of this, they have been

Fig. 1. Amino acid alignment of mouse IMP2 with all human IMP-family members. (A) Protein sequence alignment of hIMP1, hIMP2, hIMP3 and mIMP2. The alignment was made using AlignX, a component of Vector NTI suite 8.0 software (InforMax, Inc). Amino acids identical to the ones in mIMP2 are shown in gray. The two RRM's and four KH-domains are underlined [30]. (B) % Amino acid (AA) identity between all human and mouse IMP-family members. Identity was calculated with AlignX.
implicated in post-transcriptional processes such as mRNA localization, turnover and translational control. The three human IMP proteins have been shown to specifically bind to IGF-II leader 3 mRNA (5' UTR), where they have an influence on the translation of the mRNA into protein [30], and to the 3' region of H19 RNA. To look whether Imp2 is involved in the turnover of these RNAs, we looked for the expression levels of Igf2 and H19 in the microarray dataset. However, microarray analysis did not reveal a significant change in expression levels of these genes.

Different studies implicated the IMP proteins in temporal and spatial control of gene expression at the level of mRNA rather than at the level of gene transcription. In this regard, it is tempting to speculate that the effect of HMGA2 on cell proliferation during embryonic development and tumorigenesis possibly involves IMP2. This also suggests that strong downregulation of IMP2 expression at E12.5 in mice may contribute to a pygmy phenotype.

Since IMP2 is drastically downregulated to nearly undetectable levels in the E12.5 pygmy and HMGA2+/− mice, an IMP2 knockout mouse would further clarify the importance of IMP2 deficiency in the HMGA2+/− phenotype. Because of the drastic downregulation of IMP2 in the HMGA2 knockout models, we could predict a similar growth retarded phenotype for the IMP2−/− mouse, pointing to a very important role of the HMGA2-IMP2 pathway in embryonic development. In this regard, it is interesting to note that the recently published Imp1-deficient mice were on an average 40% smaller than wild-type and heterozygous sex-matched littermates [32].

At present, not much is known about possible differences in the function of the three IMP proteins. They are expressed in the same time frame during embryonic development and since it was shown that their major functions are carried out by the phylogenetically conserved KH-domains [33], one could assume that there is functional redundancy between the IMP family members. However, from the observed differential regulation during embryogenesis reported here, a first indication seems to emerge, pointing towards functional differentiation.

3.5. Effect of a tumor-related aberrant form of HMGA2 on IMP2 expression

With respect to tumorigenesis, the IMP proteins share various characteristics with HMGA2. They all are oncofetal proteins that are highly expressed during embryonic development, silenced during adult life, and misexpressed in different kinds of neoplasias [34]. Certain benign tumors are characterized by chromosomal aberrations involving the HMGA2 gene [13,14]. Many of these tumors express a truncated form of HMGA2 (HMGA2Tr), only containing the three DNA binding domains and lacking the acidic and spacer domains (Fig. 4A). Such a HMGA2Tr induces neoplastic transformation of NIH/3T3 murine fibroblasts [35]. Transgenic mice aberrantly expressing this truncated HMGA2 protein present gigantism that is associated with lipomatosis [36], develop adiposity, and display an abnormal high prevalence of lipomas [37]. To study the effect of HMGA2Tr on IMP2, NIH/3T3 cell lines overexpressing HMGA2 or HMGA2Tr were made by retroviral transduction. Expression of HMGA2 and HMGA2Tr was assayed by Western blot analysis with an HMGA2-specific antibody (Fig. 4B, lanes 2 and 3). IMP2 expression levels were determined by Northern blot analysis reported here, a first indication seems to emerge, pointing towards functional differentiation.
downregulate IMP2 expression to about 36% of the level in the control cells (Mock). Similar results were obtained by comparing mRNA expression levels between HMGA2Tr-overexpressing and control NIH/3T3 cells by microarray analysis (our unpublished observations). Similar Northern blot analysis with probes specific for IMP1 and IMP3 revealed no difference in the expression levels of these genes (results not shown).

These experiments indicate that the HMGA2Tr that is frequently expressed in benign solid tumors exerts a differential, negative effect on expression of members of the IMP gene family and point to the potential significance of the HMGA2-IMP2 pathway being deregulated in these tumors. Our results also suggest that the acidic tail of HMGA2 plays a role in the transcriptional regulation of target genes. Recently, Noro et al. [38] showed that the acidic tail of HMGA2 is not involved in determining the HMGA2 DNA-binding specificity, but can alter HMGA2/DNA complexes. Their results suggested that wild-type HMGA2 and HMGA2Tr could have the same target genes, but a differential regulating effect, in agreement with the results presented here. To resolve the question as to whether the HMGA2-IMP2 pathways are involved in tumor development, IMP2 expression levels can be assessed in tumors with aberrant expression of HMGA2.

In any case, the differential effect of wild-type HMGA2 on IMP gene expression during mouse embryogenesis constitutes a new opening for the functional dissection of the latter gene family.

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