HMGA1P7-pseudogene regulates H19 and Igf2 expression by a competitive endogenous RNA mechanism

Marco De Martino1, Floriana Forzati1, Marianna Marfella3, Simona Pellecchia1, Claudio Arra2, Luigi Terracciano3, Alfredo Fusco1 & Francesco Esposito1

Recent studies have revealed that pseudogene transcripts can function as competing endogenous RNAs, and thereby can also contribute to cancer when dysregulated. We have recently identified two pseudogenes, HMGA1P6 and HMGA1P7 for the HMGA1 gene whose overexpression has a critical role in cancer progression. These pseudogenes work as competitive endogenous RNA decoys for HMGA1 and other cancer related genes suggesting their role in carcinogenesis. Looking for new HMGA1 pseudogene ceRNAs, we performed RNA sequencing technology on mouse embryonic fibroblasts deriving from transgenic mice overexpressing HMGA1P7. Here, we report that HMGA1P7 mRNA sustains the H19 and Igf2 overexpression by acting as miRNA decoy. Lastly, the expression of HMGA1P7 was significantly correlated with H19 and IGF2 levels in human breast cancer thereby suggesting a role for HMGA1P7 deregulation in this neoplasia.

Pseudogenes are a subclass of long non coding RNA (lncRNA) sharing high sequence identity with protein-coding parental counterparts. As stated by the GENCODE pseudogene annotations (v.17), there are almost 15,000 human pseudogenes1. They frequently possess features, such as premature stop codons, deletions/insertions, or frame shift mutations, that impede them to produce functional proteins. There are three groups of pseudogenes: processed, duplicated, and unitary1,2. Processed pseudogenes do not have introns and are thought to arise from reverse transcription of mRNA followed by reinsertion into the genome 1,2. Duplicated pseudogenes contain introns and sometimes even upstream regulatory elements since they are produced by gene duplication. For each pseudogene belonging to these two classes there is an associated protein-coding gene that is highly similar in sequence1,2. The last type of pseudogenes are the unitary ones, which take place when protein-coding genes accumulate mutations and lose their coding potential1,2. Consequently, unitary pseudogenes do not have parental genes. From the time of their discovery in 1977, pseudogenes have commonly been thought as “biologically inconsequential” and non-functional3. However, recent studies have unveiled different mechanisms by which pseudogenes control gene expression such as the generation of siRNAs4,5, competition for RNA-binding proteins or the translation apparatus6-8, and engagement of proteins by pseudogene antisense RNAs to corresponding sites in the parental gene to modify chromatin transcription and remodeling9,10. The latest function identified for pseudogenes is post-transcriptional regulation of mRNA levels by competing for microRNAs (miRNAs). Indeed, processed pseudogenes maintain 5' and 3' untranslated region (UTR) sequences of their parental genes11. Given that miRNAs inhibit target gene expression by binding to the 3' UTR, pseudogenes can be targeted by miRNAs that modify the expression of coding genes. Definitely, pseudogene transcripts exert regulatory control of their parental gene expression levels by competing for the same miRNAs12.

We have recently characterized two processed pseudogenes, HMGA1P6 and HMGA1P7, for the HMGA1 gene that codes for the HMGA1a and HMGA1b proteins highly overexpressed in most of the human malignancies13,14.

1Istituto di Endocrinologia ed Oncologia Sperimentale del CNR c/o Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Scuola di Medicina e Chirurgia di Napoli, Università degli Studi di Napoli “Federico II”, via Pansini 5, 80131 Naples, Italy. 2Istituto Nazionale dei Tumori, Fondazione Pascale, via Mariano Semmola, 80131 Naples, Italy. 3Institute of Pathology, Molecular Pathology Division, University of Basel, Schonbeinstrasse 40, 4003 Basel, Switzerland. Correspondence and requests for materials should be addressed to A.F. (email: alfusco@unina.it) or F.E. (email: francesco.esposito2@unina.it)
Moreover, it has been previously demonstrated an association between HMGA1 overexpression and a poor patient survival13, and that their overexpression is even required for cell transformation15,16, and is able to induce benign and malignant neoplasias in mice13. HMGA1P6 and HMGA1P7 pseudogenes, present only in human genome, have preserved seed matches for miRNAs targeting the HMGA1 oncogene. HMGA1P pseudogenes (HMGA1Ps) overexpression, working as competitive endogenous RNA (ceRNA), increases HMGA1 protein levels by blocking the suppression of HMGA1 protein synthesis exerted by miRNAs17–20. HMGA1Ps have also oncogenic activity by suppressing apoptosis and promoting cell proliferation and migration17–20. Moreover, we have previously show that HMGA1Ps are overexpressed in anaplastic thyroid carcinomas but not in the differentiated ones, indicating a critical role of them in cancer progression17. Since the HMGA1Ps contain several seed sequences for miRNAs, their overexpression derepresses the expression of different cancer-related genes, as already demonstrated for HMGA2, VEGF, EZH217–20. Therefore, the aim of this study has been to find novel ceRNA interactors differentially expressed in HMGA1P7 transgenic mouse embryonic fibroblasts (MEFs) with respect to the wild-type (WT) ones, which do not express HMGA1 pseudogenes, using a RNA sequencing (RNA-seq) approach. By this analysis, we found a set of mRNAs up- or down-regulated in HMGA1P7 overexpressing MEFs in comparison with WT cells. Among them, we focused our attention on two of the most overexpressed and HMGA1P7 miRNA-sharing genes: H19 and insulin-like growing factor 2 (Igf2).

Results

RNA-seq on HMGA1P7 transgenic MEFs. To identify the genes regulated by HMGA1P7 expression, we analyzed the whole transcriptome of WT and HMGA1P7 transgenic MEFs by RNA-seq analyses. To this aim, the entire population of RNA transcripts extracted from WT and HMGA1P7-MEFs were sequenced. The genome-wide RNA expression profiles studies reveal that about one hundred fifty transcripts (32 upregulated and 116 downregulated) were regulated by HMGA1P7 expression with a significant fold-change variation (FDR adjusted p-value of 0.05). To validate the results obtained by RNA-seq, we analyzed the expression of some upregulated genes such as Collagen Type VI Alpha 3 (Col6a3), Marker of Proliferation Ki-67 (Mki67), H19, Igf2 and downregulated genes such as Glutathione Peroxidase 3 (Gpx3), Leprecan-Like 1 (Leprel1) by Real-time PCR (qRT-PCR). As shown in Fig. 1, the quantitative qRT-PCR analyses confirmed the data obtained from the RNA-seq analyses. Interestingly, these genes have been related to several human cancers (colon, gastric, liver, breast and hematological cancers), and are considered possible therapeutic targets30–35.

Figure 1. Validation of RNA-seq analyses on HMGA1P7 MEFs. qRT-PCR analysis of selected deregulated genes from RNA-seq performed on WT and HMGA1P7 transgenic MEFs. The results are reported as the mean of values. The error bars represent mean ± SE; *P < 0.05 **P < 0.01 ***P < 0.001 (t test).
Among the differentially expressed mRNAs found in MEFs overexpressing HMGA1P7, we focused our attention on H19 and Igf2 since they, other than to be involved in carcinogenesis, showed the highest fold change among the upregulated genes, and are also targeted by several miRNAs that are able to bind to the HMGA1P7 mRNA. Western blot analysis for Igf2 confirmed the qRT-PCR data (Fig. 2). Moreover, qRT-PCR and Western blot analysis showed that H19 and Igf2 were also upregulated in heart, spleen and kidney from HMGA1P7 adult transgenic mice (Fig. 3A,B and C). As expected from previous results, qRT-PCR shows upregulation of H19 and Igf2 following HMGA1P7 pseudogene overexpression in NIH3T3 cells (Fig. 3D). Western blot confirms Igf2 upregulation also at protein level in tissues from HMGA1P7 and in the HMGA1P7-transfected NIH3T3 cells (Fig. 3C and D).

Taken together, these results strongly support the hypothesis that HMGA1P7 could act as ceRNA for H19 and Igf2.

**HMGA1P7 act as decoy for H19 and Igf2 targeting miRNAs.** To test whether the effect of the HMGA1P7 pseudogene on H19 and Igf2 expression is dependent on sharing targeting-miRNAs, we evaluated the ability of HMGA1P7-targeting miRNAs17 to bind to H19 and Igf2. To this aim, we transfected miR-15, miR-16, miR-214 and miR-761 (already reported to target HMGA1P7)17 into NIH3T3 cells, and analyzed H19 and Igf2 mRNA levels by qRT-PCR. As presented in Fig. 4A, the transfection of the HMGA1P7-targeting miRNAs yield a significant reduction of H19 and Igf2 mRNA levels. Western blot confirms Igf2 downregulation also at protein level following the transfection of the HMGA1P7-targeting miRNAs (Fig. 4A). To define whether the HMGA1P7-targeting miRNAs straightly interacted with Igf2 mRNA, we cloned the Igf2 3' UTR downstream of the luciferase open reading frame. This reporter vector was transfected into NIH3T3 cells together with miRNA precursors and a control non-targeting scrambled oligonucleotide. The luciferase signal was considerably lower (Fig. 3C and D).

To test whether the effect of the HMGA1P7-targeting miRNAs strictly interacted with Igf2 mRNA, we cloned the Igf2 3' UTR downstream of the luciferase open reading frame. This reporter vector was transfected into NIH3T3 cells together with miRNA precursors and a control non-targeting scrambled oligonucleotide. The luciferase signal was considerably lower (Fig. 3C and D).

**HMGA1P7, H19 and IGF2 expression positively correlates in human breast cancer.** Then, we investigated whether HMGA1P7 functions as ceRNA through, or partially through H19, IGF2 and HMGA1 in breast cancer human cells. As expected, we found upregulation of H19, IGF2 and HMGA1 following HMGA1P7 overexpression in MCF7 cells (human breast adenocarcinoma cell line) (Fig. 5A). Moreover, MCF7-HMGA1P7 cells grow faster than the control transfected cells as consequence of HMGA1P7 ceRNA pathway activation (Fig. 5B).

To confirm whether HMGA1P7 works as miRNA sponge for the regulation of H19 and IGF2 expression levels also in human cancer, we evaluated the expression of H19, IGF2 and HMGA1P7 in a panel of breast carcinoma samples by qRT-PCR, since H19 and IGF2 have been reported to be overexpressed in this type of tumor36,37. As shown in Fig. 5C, HMGA1P7 was overexpressed in most of the carcinoma samples as well as H19 and IGF2. Moreover, the direct correlation between HMGA1P7 and H19 expression (Spearman r = 0.8656; p < 0.001) and between HMGA1P7 and IGF2 expression (Spearman r = 0.7958; p < 0.001) underlines that these genes are co-regulated (Fig. 5D). Altogether, these results strongly support the idea that HMGA1P7 could act as ceRNAs in human breast cancer and represent a novel potential mechanism accounting for H19 and IGF2 upregulation in these tumors.
Discussion

lncRNAs are involved in regulating the complexity of biological processes with specific regulatory mechanisms, thereby, attracting considerable research interest. We have previously isolated and characterized two pseudogenes, HMGA1P6 and HMGA1P7, for the HMGA1 gene and demonstrated that they act as decoys for...
HMGA1-targeting miRNAs. In fact, their overexpression enhances HMGA1 protein levels whereas their knocking down results in the reduction of HMGA1 mRNA and protein amounts. Moreover, their decoy activity protected the synthesis of other proteins involved in carcinogenesis. In this study, we used RNA-seq technology to identify additional mRNAs differentially expressed in MEFs transgenic for HMGA1P7.

We found that the expression of several genes were influenced by HMGA1P7 including also genes involved in cancer progression such as Col6a3, Mki67, H19, Igf2, Gpx3 and Leprel1. Indeed, oncomine analyses and tissue-microarray immunohistochemistry showed overexpression of COL6A3 in colorectal carcinomas that was significantly and directly correlated with Dukes stage, T stage, stage, recurrence and smoking status and then with a poor prognosis. The MKi-67 protein (also known as Ki67) is a cellular marker for proliferation. Ki-67 protein is expressed during all active phases of the cell cycle (G1, S, G2, and mitosis), but is absent from resting cells (G0). GPX3 gene codes for the Glutathione peroxidase 3, also known as plasma glutathione peroxidase (GPx-P), the variations in activity of GPX1, GPX2, and GPX3 isoforms may be associated with the development of cancers, for example, prostate cancer or even colon cancer. Leprecan-like 1 is a potential tumor suppressor gene since it has been demonstrated to be downregulated in the hepatocarcinoma tissues and its overexpression inhibits cancer cell proliferation and colony formation through regulation of the cell cycle by downregulation of cyclins.

Figure 4. H19 and Igf2 are targeted by HMGA1P7-targeting miRNAs. (A) qRT-PCR analysis of H19 (left panel) and Igf2 (middle panel) mRNA from the NIH3T3 cells transfected with scrambled-oligonucleotide, miR-15, miR-16, miR-214 and miR-761. Right panel, Western blot analysis of Igf2 in the same samples as in the middle panel. (B) Igf2 was cloned into the pGL3 control vector. Relative luciferase activity in HEK293 cells transiently transfected with miR-15, miR-16, miR-214, miR-761 and a control scrambled oligonucleotide. (C) H19 and Igf2 mRNA levels 24 h after the transfection of HMGA1P7 in scrambled oligonucleotide or siRNA-Dicer NIH3T3 transfected cells. (D) qRT-PCR analysis of H19 mRNA levels from the NIH3T3 cells transfected with siRNA-control, siRNA-Igf2 alone or in combination with the Anti miR-16 oligonucleotide.
Deregulation of H19 noncoding gene was found in many tumors such as hepatocellular and bladder cancer. Finally, IGF2 overexpression is widely reported in pediatric and adult tumors, and several studies involve IGF2 as a key factor leading to cancerogenesis.

Among the most deregulated genes, we selected and studied H19 non-coding gene and Igf2, that share several miRNAs with HMGA1P7. Here, we report the ceRNA relationship between HMGA1P7, H19 and Igf2. We demonstrate that HMGA1P7 overexpression increases H19 and Igf2 levels inhibiting their mRNA suppression by miRNAs that target HMGA1P7 gene, namely, miR-15, miR-16, miR-214, and miR-761. Interestingly, preliminary results show an analogous ceRNA connection between H19, Igf2 and HMGA1P6, supporting the oncogenic role of the both HMGA1 pseudogenes.

Figure 5. H19 and IGF2 expression positively correlates with HMGA1P7 in breast cancer. (A) Left Panel, Western blot analysis of HMGA1 and IGF2 protein levels in control and HMGA1P7-overexpressing MCF7 cells. Right Panel, qRT-PCR analysis of H19 expression of control and HMGA1P7-overexpressing MCF7 cells. (B) MCF7 cell proliferation of control and HMGA1P7-overexpressing cells (C) qRT-PCR analysis in tumor and normal breast tissues. The fold change indicates the relative change in expression levels between tumor samples and normal samples, assuming that the value of normal sample is equal to 1. (B) Correlation analysis of HMGA1P7 versus H19 and HMGA1P7 versus IGF2 are shown. The Spearman’s rank correlation coefficient is shown.
Finally, we show that expression of HMGA1P7 significantly correlates with H19 and IGF2 levels in human breast cancer, suggesting the upregulation of HMGA1P7 may increase H19 and IGF2 expression by a ceRNA mechanism then contributing to cancer progression. Interestingly, the oncogenic role of HMGA1P7 is also supported by the development of malignant hematological neoplasias in HMGA1P7 transgenic mice (manuscript in preparation).

Then, the data reported here confirm the oncogenic role of the HMGA1P7 pseudogene that is exerted by the increased expression through a ceRNA mechanism of HMGA1 and other cancer-related genes. Future studies need, however, to characterize other genes regulated by the HMGA1 pseudogenes and thereby better define the mechanisms by which they can contribute to cancer progression.

Materials and Methods

Cell culture and transfections. MEFs and MCF7 were cultured in DMEM supplemented with 10% foetal calf serum (Thermo Fisher Scientific Inc). NIH3T3 cells were maintained in DMEM supplemented with 10% calf serum (Thermo Fisher Scientific Inc), glutamine and antibiotics. MycoAlert (Lonza) was regularly used to test that cells were not infected by mycoplasma. Lipofectamine plus reagent was used to transfect the cells (Thermo Fisher Scientific Inc) according to the manufacturer’s instructions. The transfected cells were selected in a medium containing geneticin (Sigma). Transfection efficiency was tested for each experiment by assessing GFP signal. To inhibit Dicer and Igf2 expression, small interfering RNAs and corresponding scramble small interfering RNAs were designed and used as suggested by the manufacturer (RIBOXX).

RNA-sequencing. RNA samples were initially checked for quality and quantity using a Bioanalyzer with the total RNA Pico chip (Agilent Technologies, Inc) and a Qubit® with RNA Assay Kit (Thermo Fisher Scientific Inc) respectively.

Spike-In Mix 1 and Spike-In Mix 2, each containing the full complement of 92 polyadenylated transcripts from the ERCC plasmid reference library, were added to samples.

mRNA was selected from total RNA preparation using MicroPolyA Purist kit (Ambion, Inc).

SOLiD™ Total RNA-Seq Kit (Life Technologies Corporation) was used to convert RNA transcripts into a cDNA library, starting from low input amounts of poly(A) RNA, for analysis on the 5500 Genetic Analysis System. First of all, mRNA was incubated in a thermal cycler at 95 °C for 10 minutes to fragment the RNA by chemical hydrolysis. The RNA digested was hybridized and ligated with Solid specific adaptors. Two rounds of size selection using Agencourt® AMPure® XP Reagent were performed to increase the percentage of library inserts that were in the desired size range, >150 bp. The purified DNA was amplified and barcoded by 18 PCR cycles to enable sequencing of all the samples in a single multiplexed SOLiD System sequencing run.

The yield and size distribution of the amplified DNA libraries was assessed running the samples on an Agilent® 2100 Bioanalyzer™ Instrument with the DNA HS Kit following the manufacturer’s instructions and Qubit® dsDNA HS kit.

Ninety nanograms of each library was pooled together and one E80 emulsion was prepared following SOLiD® EZ Bead™ System. About 400 millions of pooled templated beads were deposited on a 4 lanes of a 6 lanes slide and the sequencing was performed up to a read length of 50 bp, based on 5500 Genetic Analysis System Run sequencer protocol.

Bioinformatic Analysis. Four samples were analysed: two from WT and two from HMGA1P7 transgenic MEFs. The comparison performed was WT versus transgenic, two biological replicates for condition.

Sequencing reads in SOLiD “xsq” format were mapped against the reference genome (UCSC GRC38/mm10); reference gene structure was Refseq from the refGene.txt file of the UCSC genome browser FTP site; the mapping software was the Whole Transcriptome Analysis module from the Lifescope 2.5.1 Genomic Analysis Software suite from Applied Biosystems/ThermoFisher Scientific.

A filter, containing 6415 sequences (sequencing adaptors; barcodes; tRNAs; rRNAs; rRNA fragments; repetitive sequences; ERCC RNA sequences) was used (1) to filter the transcripts for non-significant reads and (2) to quantify the absolute expression using the External RNA Controls Consortium (ERCC) RNA Spike-In Mix.

The genome-mapped reads were then correlated with Refseq genes and the resulting gene-associated read counts were analysed with a Genomia proprietary procedure based on the Bioconductor library edgeR41. The chosen limit for evaluating differential expression was 5 counts per millions in at least half of the examined samples. The normalization procedure used was the standard for edgeR (TMM). Genes were called differentially expressed when the comparison was evaluated with a FDR < 0.05. Absolute gene expression was evaluated from the read counts in RPKM (Reads per kilo base per million mapped reads).

Primary gene annotation was performed using the Bioconductor libraries biomaRt and GOstats, while functional clustering of the genes was performed using the DAVID functional annotation web site (https://david.ncifcrf.gov/).

Mouse embryo and tissue samples. The use of mouse embryos and tissues and the experiments performed in this study were approved by the Ministero della Salute; the methods and experiments were carried out in accordance with the approved guidelines by the Ministero della Salute.

Human breast tissue samples. Normal and neoplastic human breast tissues were obtained from surgical specimens and immediately frozen in liquid nitrogen. 32 breast samples were collected at the Institute of Pathology, University of Basel, Switzerland. The tumor samples were frozen until required for RNA extraction. The use of human tissues and the experiments performed in this study were approved by the Institute of Pathology, Molecular Pathology Division, University of Basel; the methods and experiments were carried out
in accordance with the approved guidelines by the University of Basel. We declare that informed consent for the scientific use of biological material was obtained from all patients.

**RNA extraction and quantitative reverse transcription PCR.**  Total RNA was extracted from cells or tissues with TRIzol (Aurogene) according to the manufacturer’s instructions. For mRNA transcripts detection, we reverse transcribed total RNA from samples by using the QuantiTect Reverse Transcription Kit (Qiagen), and then Real-time PCR was performed by using Power SYBR Green PCR Master Mix (Bio-Rad) and the following primers:

For growth curve assay,

For dual-luciferase reporter assay, 3 × 10^5 NIH3T3 cells were co-transfected in 6-well plates with the pGL3-IGF2 or the pGL3-H19 luciferase reporter vectors, together with the Renilla luciferase plasmid and miRNA precursors or a control no-targeting scrambled oligonucleotides (Thermo Fisher Scientific Inc), using siPORT neoFX Transfection Agent (Thermo Fisher Scientific Inc). The pRL-TK control vector expressing Renilla luciferase (Promega) was used for normalization of cell number and transfection efficiency. Luciferase activity was measured 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega) with a Lumat LB 9507 apparatus (Berthold Technologies).

**Protein extraction, western blotting and antibodies.**  Protein extraction and Western blotting were performed as previously described. The primary antibodies used were anti-IGF2 (#32592) from Sabbiotech; anti-GAPDH (sc-32233) and anti-γ-Tubulin (sc-17787) from Santa Cruz Biotechnology. Blots were visualized by using the Western blotting detection reagents (Thermo Fisher Scientific Inc).

**Plasmid and miRNA oligonucleotides.**  For transfection of miRNA oligonucleotides, cells were transfected with 50 nmol/ml of miRNA precursors or with a control no-targeting scrambled oligonucleotides (Thermo Fisher Scientific Inc) using siPORT neoFX Transfection Agent (Thermo Fisher Scientific Inc). For transfection of Anti miR-16 oligonucleotides, cells were transfected with 50 nmol/ml of Anti miR-16 or with a control no-targeting scrambled oligonucleotides (Thermo Fisher Scientific Inc).

For IGF2 luciferase reporter construct (pGL3-IGF2), the miRNA seed sequence containing fragment of IGF2 gene (ENSMUST000000000033) was amplified by using the primers:

The amplified fragment was cloned into pGL3-Control luciferase reporter vector (Promega).

All the generated vectors were confirmed by sequencing. The Renilla luciferase vector (pRL-CMV), for transient transfection efficiency, was purchased from Promega.

**Data were analyzed using a two-sided unpaired t test (GraphPad Prism, GraphPad Software, Inc.). Values of P < 0.05 were considered statistically significant. Regression analysis, correlation coefficients and statistical analysis were generated using GraphPad Prism, GraphPad Software, Inc.**

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Author Contributions
F.E. and A.F. conceived and designed the study. M.D.M. with help from F.E., F.F., M.M. and S.P. performed all experiments, and F.E., M.D.M., C.A., L.T. and A.F. analyzed most data. F.E. and A.F. wrote the manuscript with contributions from all authors.

Additional Information
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