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Chromosomal protein HMGN1 modulates the expression of N-cadherin

Yaffa R. Rubinstein[1], **Takashi Furusawa**[1], **Jae-Hwan Lim**[1,2], **Yuri V. Postnikov**[1], **Katherine L. West**[1,3], **Yehudit Birger**[1], **Sunmin Lee**[4], **Phuongmai Nguyen**[4], **Jane B. Trepel**[4], and **Michael Bustin**[1]

[1]Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

[2]Department of Biology, Andong National University, Gyeongsangbuk-do, South Korea

[3]Division of Cancer Sciences and Molecular Pathology, Western Infirmary (Pathology), University of Glasgow, UK

[4]Medical Oncology, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

Abstract

HMGN1 is a nuclear protein that binds to nucleosomes and alters the accessibility of regulatory factors to their chromatin targets. To elucidate its biological function and identify specific HMGN1 target genes, we generated *Hmgn1*−/− mice. DNA microarray analysis of *Hmgn1*+/+ and *Hmgn1*−/− embryonic fibroblasts identified N-cadherin as a potential HMGN1 gene target. RT-PCR and western blot analysis confirmed a linkage between HMGN1 expression and N-cadherin levels. In both transformed and primary mouse embryonic fibroblasts (MEFs), HMGN1 acted as negative regulator of *N-cadherin* expression. Likewise, the N-cadherin levels in early embryos of *Hmgn1*−/− mice were higher than those of their *Hmgn1*+/+ littermates. Loss of HMGN1 increased the adhesiveness, motility and aggregation potential of *Hmgn1*−/− MEFs, a phenotype consistent with increased levels of N-cadherin protein. Re-expression of wildtype HMGN1, but not of the mutant HMGN1 protein that does not bind to chromatin, in *Hmgn1*−/− MEFs, decreased the levels of N-cadherin and restored the *Hmgn1*+/+ phenotype. These studies demonstrate a role for HMGN1 in the regulation of specific gene expression. We suggest that in MEFs, and during early mouse development, the interaction of HMGN1 with chromatin down-regulates the expression of N-cadherin.

Keywords

HMG protein; N-cadherin; chromatin; transcription

Chromatin structure plays a key role in regulating the orderly progression of nuclear processes such as transcription, replication, recombination and repair. These DNA-related activities are associated with changes in the folding and compaction of the chromatin fiber and with rearrangements in the structure of the nucleosome. Structural reorganization of the chromatin fiber is facilitated by numerous nuclear factors including ATP-dependent nucleosome remodeling complexes [1,2], histone and DNA modifying enzymes [3], and structural proteins such as histone H1 [4,5] and high mobility group (HMG) proteins [6].

Correspondence: M. Bustin, Laboratory of Metabolism, Bldg 37, Room 3122, NCI, NIH, Bethesda, MD 20892, USA, Fax: +1 301 496 8419, Tel: +1 301 496 5234, bustin@helix.nih.gov.

HMGs are a superfamily of proteins that interact with chromatin and DNA and are known to affect a wide range of DNA-dependent activities including transcription [6]. One of the HMG families, the HMGN family, consists of a group of small, basic proteins that bind specifically to the 147 base pair nucleosome core particle, the building block of the chromatin fiber [7]. Most vertebrate nuclei contain approximately 10^5 molecules of HMGN proteins, sufficient to bind to approximately 1% of the nucleosomes. HMGNs are highly mobile molecules and their interaction with chromatin is transient [8]. Thus, although the amount of HMGN protein in the nucleus is limited, all the accessible nucleosomes form a temporary complex with HMGN proteins. HMGNs compete with histone H1 and perhaps with other proteins for chromatin binding sites [9,10]. Competitive interactions between architectural proteins may be part of the molecular mechanism that modulates the local structure and activity of the chromatin fiber [5,10]. The interaction of HMGNs with chromatin reduces the compaction of the chromatin fiber and affects the ability of DNA repair factors and histone modifying complexes to reach their chromatin targets [11].

HMGN proteins colocalize with active transcription sites and their nuclear organization is related to the cellular levels of transcriptional activity [12]. Several types of *in vitro* studies indicate that HMGNs enhance transcription from chromatin, but not from 'naked' DNA templates [7]. In spite of these and additional studies linking HMGNs to transcriptional processes, the cellular function of these proteins in living cells is still not fully understood. It is not clear yet whether the HMGN proteins act as nonspecific modulators of chromatin structure or whether they are involved in specific gene expression. Although *in vitro* they bind to chromatin with little if any sequence specificity, in living cells they could be targeted to specific sites by associating with other nuclear proteins [13]. Ectopic expression of HMGN3 in a mouse hepatoma cell line changes the expression levels of approximately 0.8% of the genes, and specifically elevates the expression of the *Glyt1* gene, suggesting that HMGN proteins could differentially regulate specific gene expression [14].

To investigate the roles of HMGN proteins in whole organisms and understand their cellular function, we generated an *Hmgn1*−/− mouse, lacking one of the major members of the HMGN protein family. The mice seem normal; however, their response to UV and other stresses is impaired [15]. In addition, the pattern of post-translational modification in histones extracted from fibroblasts prepared from *Hmgn1*−/− mice differs from that of cells prepared from their wild-type littermates [11]. Thus, potentially, the transcription profile of mice lacking HMGN1 protein may differ from that of wild-type mice.

Here we demonstrate that HMGN1 modulates the expression of the *N-cadherin* gene during early mouse embryogenesis. We find that both in early mouse embryos and in mouse embryonic fibroblasts (MEFs) loss of HMGN1 elevates the transcription and protein levels of N-cadherin. Expression of wild-type HMGN1, but not that of a mutant that does not bind to chromatin in *Hmgn1*−/− MEFs, lowers the levels of N-cadherin. The phenotype of the *Hmgn1*−/− MEFs is consistent with elevated levels of N-cadherin protein. Thus, in these cells, HMGN1 is a negative regulator of N-cadherin. N-cadherin belongs to a family of calcium-dependent cell adhesion molecules that form and maintain adhesive contact between cells [16–18]. These proteins play an important role in the sorting of cells into tissues during embryogenesis [19–25]. The cadherin family also affects cell signaling, proliferation and differentiation, perhaps by forming a complex with members of the catenin protein family. Our findings demonstrate that HMGN1 protein may modulate the cellular levels of specific proteins and affect the cellular phenotype.

Results

N-cadherin expression is down-regulated in *Hmgn1 −/−* MEF

To test whether HMGN1 affects the cellular transcription profile we extracted RNA from *Hmgn1*−/− and *Hmgn1*+/+ MEFs and performed two color quantitative microarray analysis, with arrays containing either 2794 or 3328 cDNA spots. Loss of HMGN1 altered the expression of over 3% of the genes that could be reliably analyzed, by twofold or more. The microarray analysis identified *N-cadherin* as one of the genes whose expression was among the most significantly affected by the loss of HMGN1 protein; the expression of *N-cadherin* in *Hmgn1*−/− MEFs was over sixfold higher than in *Hmgn1*+/+ MEFs, suggesting that in these cells HMGN1 is a negative regulator of *N-cadherin* expression (Fig. 1).

N-cadherin plays an important role in cell adhesion, and in addition it may affect the gene expression profile through its association with the nuclear signaling protein *β-catenin* [20,26,27]. HMGN1 does not affect the transcription of *β-catenin* as both the microarray and the RT-PCR analysis revealed that the levels of *β-catenin* transcripts in *Hmgn1*−/− MEFs were the same as in *Hmgn1*+/+ MEFs (Fig. 1). Thus, HMGN1 affects the transcription of *N-cadherin* but not *β-catenin*.

HMGN1 down-regulates the levels of both N-cadherin and β-catenin protein

Western blot analysis of cell extracts from the MEFs (Fig. 2A) revealed that the increased levels of N-cadherin transcripts in *Hmgn1*−/− cells resulted in increased levels of N-cadherin protein. The level of N-cadherin protein in *Hmgn1*−/− cells was approximately fourfold higher than in *Hmgn1*+/+ MEFs (Fig. 2B). Surprisingly, even though HMGN1 does not affect the levels of *β-catenin* transcripts (Fig. 1) the levels of β-catenin protein were also significantly higher in *Hmgn1*−/− MEFs, as compared to *Hmgn1*+/+ MEFs. The increased cellular levels of β-catenin could be due to protein stabilization by increased levels of N-cadherin; however, we did not investigate this aspect further because it is well documented that the cellular amounts of this protein are regulated by several mechanisms [28,29].

To verify that the increased levels of N-cadherin protein and transcripts are indeed linked to the cellular levels of HMGN1, we examined the levels of N-cadherin in *Hmgn1*−/− cells that were stably transformed with plasmids expressing either the wild-type HMGN1 protein (Fig. 2C, cell line 622), or the double point mutant S20,24E HMGN1 protein (Fig. 2C, cell line M101), both under the control of the inducible tetracycline response element. Exposure of these cells to doxycycline (Dox) induced the expression of either the wild-type HMGN1, or of the mutant S20,24E HMGN1, which enters the nucleus but does not bind to chromatin [30]. Western analysis revealed that exposure to Dox induced the expression of the wild-type and mutant HMGN1 proteins to levels that were similar to the levels of HMGN1 protein in wild-type, *Hmgn1*+/+ MEFs (Fig. 2C, first row). Controls with Dox-treated, non transfected *Hmgn1*−/− cells (Fig. 2C, MEF/KO) verified that the proteins are indeed derived from the stably integrated plasmids and are not an artifact of Dox treatment.

In 622 cells, induction of HMGN1 protein by Dox exposure down-regulated the protein levels of both N-cadherin (Fig. 2C, second row) an indication that the levels of this protein is indeed linked to the cellular levels of HMGN1. In contrast, induction of the S20,24E HMGN1 double point mutant, which does not bind to chromatin, did not change the levels of N-cadherin suggesting that the interaction of HMGN1 with chromatin regulates N-cadherin expression.

Altered phenotype in *Hmgn1* −/− cells

N-cadherin plays a role in cell adhesion and motility, and misregulated N-cadherin expression or function leads to an altered cellular phenotype [31]. To test whether the HMGN1-linked changes in N-cadherin expression are functionally manifested we compared the adhesion properties of *Hmgn1*−/− and *Hmgn1*+/+ MEFs by microscopic observation and a plate-washing assay. Microscopic observation of the cells revealed significant morphological differences between the two types of MEFs at very early stages of cell adhesion. Within the first 30 min of plating, the *Hmgn1*+/+ MEFs remained rounded and were poorly attached to the plates. In contrast, within the same time, the *Hmgn1*−/− MEFs flattened on the plate, extended numerous filopodia and their borders were more diffuse (Fig. 3A, upper panels). The morphological differences between the MEFs disappeared once the cells adhered, and as soon as after 2 h there were no obvious differences in the overall appearance of the cells (Fig. 3A, lower panels).

To test whether the differences between the *Hmgn1*+/+ and *Hmgn1*−/− cells are indeed linked to HMGN1 expression, we repeated these experiments in cell line 622, i.e. transformed *Hmgn1*−/− MEFs that express HMGN1 protein from the Dox-inducible tetracycline response element. The morphological appearance of the Dox-treated and nontreated 622 cells faithfully reproduced the appearance of the *Hmgn1*+/+ MEFs and *Hmgn1*−/− MEFs. Within the first 30 min of plating, the Dox-treated cells, which express HMGN1, remained rounded and relatively unattached while the untreated 622 cells, which do not express HMGN1, were flattened and generated numerous filopodia (not shown). To determine whether the change in morphology correlates with N-cadherin expression, cells were plated for 30 min, fixed and stained with antibodies to N-cadherin. As expected, N-cadherin was visible on the cell plasma membrane and its appearance correlated with the cell morphology. In the rounded, Dox-treated 622 cells, which express HMGN1, N-cadherin appeared as a tight ring surrounding the still rounded and smooth membrane boundary. In contrast, in the Dox-untreated, non-HMGN1 expressing cells, the N-cadherin staining appeared diffuse, and localized to the flattened membrane and to the numerous filapodia protruding from the attached cells (Fig. 3B, upper panels). As with the nontransformed MEFs, these early morphological differences between the Dox-treated and nontreated 622 cells were no longer visible after 3 h of plating. At this time, both the Dox-treated and the untreated cells had a typical flat, fibroblast-like appearance (Fig. 3B, lower panels).

To test further whether the HMGN1-linked changes in N-cadherin expression lead to an altered phenotype, we quantified the adhesion properties of Dox-treated and nontreated MEFs expressing either the wild-type (cell line 622) or the mutant S20,24E-HMGN1 (cell line M101). The MEFs were seeded (in triplicate) in a six-well culture dish at 4×10^5 cells per well and after various times the plates were washed, fixed, stained and the number of cells remaining attached to the plates counted. The percent of the plated cells that remained adhered to the plates after each time point is given in Fig. 4A. The change in cell adhesion can be seen within the first 30 min, a time point at which the number of attached cells expressing HMGN1 was approximately half of the number of cells that did not express HMGN1. The differences between the HMGN1-expressing and nonexpressing cells increased during the incubation period; the MEFs that did not express HMGN1 reached maximum adherence within 2 h while those expressing HMGN1 reached maximum adherence only after 4 h (Fig. 4). Thus, loss of HMGN1 enhanced the fibroblast adhesiveness, a phenotype consistent with increased levels of N-cadherin.

HMGN1 expression also affected cell motility as determined by cell migration through porous filters in response to chemotactic stimulation. The relative mobility of primary *Hmgn1*−/− MEFs was approximately four times higher than that of *Hmgn1*+/+ MEFs (Fig. 4C), suggesting that HMGN1 expression inhibits cell motility. Indeed, Dox treatment of 622

cells (expressing HMGN1) but not of M101 cells (expressing mutated HMGN1) also inhibited the rate of cell migration, suggesting that the binding of HMGN1 to chromatin affects the rate of cell migration (Fig. 4C). Thus, the expression of HMGN1, and its binding to chromatin, down-regulates cell motility, a finding that is consistent with HMGN1 acting as a negative regulator of N-cadherin expression.

As an additional test we examined the effect of HMGN1 expression on the rate of Ca^{2+}-induced cell aggregation. The rate of aggregation and the size of the aggregates is related directly to the cellular levels of N-cadherin [32–34]. We used light microscopy to determine the size and number of aggregates formed within 30 min of Ca^{2+} addition to 622 cells that were either treated, or not, with Dox (Fig. 4D). The aggregates were divided into four groups: single cells, i.e. no aggregates, two to four cell aggregates, five to 10 cell aggregates, and >10 cell aggregates. In the absence of Dox, Ca^{2+} induced the formation of large aggregates; over 30% of the cells were in the >10 cell aggregate group (Fig. 4E). In contrast, Ca^{2+} treatment of Dox-induced 622 cells formed mostly smaller (two to four cell) aggregates (Fig. 4E). Thus, induction of HMGN1 protein decreased the rate and extent of cell aggregation, a result that is consistent with decreased levels of N-cadherin in the Dox-treated 622 cells.

Reciprocal N-cadherin and HMGN1 expression during embryogenesis

N-cadherin affects cell aggregation and migration, and plays a role in the sorting of cells into specific tissues during early development [17,20,35,36]. We therefore examined the levels of *N-cadherin* and *Hmgn1* transcripts during mouse embryonic development. Quantitative RT-PCR analysis of total RNA extracted from 7, 11 and 15-day-old *Hmgn1*+/+ embryos revealed an inverse relation between *Hmgn1* and *N-cadherin* transcripts. During embryogenesis the total levels of *Hmgn1* gradually decrease while the levels of *N-cadherin* transcripts gradually increase (Fig. 5A). Consistent with the possibility that HMGN1 is a negative regulator of *N-cadherin* expression during early development, the levels of *N-cadherin* transcripts in 7.5- and 11-day-old *Hmgn1*−/− embryos were higher than in their *Hmgn1*+/+ littermates. In the later stages of embryonic development (day 15), when most tissues are already formed [19], the differences between the embryos reversed, suggesting that in mature tissue HMGN1 is not a negative regulator of N-cadherin expression. Indeed, quantitative RT-PCR of RNA extracted from adult *Hmgn1*−/− and *Hmgn1*+/+ mice indicated that loss of HMGN1 reduces the levels of *N-cadherin* transcripts (Fig. 5B). Western analysis revealed that the levels of the protein in both testis and in heart are lower in *Hmgn1*−/− mice (Fig. 5C). Our findings that the role of HMGN1 in N-cadherin transcription during embryogenesis differs from its role in adult tissue are in agreement with previous reports that the regulation of N-cadherin expression during embryogenesis is different than in adult tissues [25,37].

Discussion

Several types of studies have established a link between HMGNs, chromatin structure and the overall levels of transcription [6,7]. Our main new finding is that HMGN1, a nucleosome binding protein that interacts with chromatin without any known specificity for DNA sequence, modulates the expression of a specific gene and plays a role in establishing the cellular phenotype. Several findings link HMGN1 to the expression of N-cadherin. First, the levels of N-cadherin transcripts and protein in *Hmgn1*−/− cells are higher than in wild-type *Hmgn1*+/+ cells. Second, re-expression of HMGN1, but not of the S20,24E mutant that does not bind to chromatin, in *Hmgn1*−/− cells lowers the levels of N-cadherin. Third, the phenotype of *Hmgn1*−/− cells, evaluated by cell motility, cell aggregation and adhesiveness assays, is consistent with elevated levels of N-cadherin protein. Fourth, reexpression of HMGN1 in *Hmgn1*−/− cells reverts the phenotype to one that is consistent with a reduction

in the levels of N-cadherin. Fifth, during embryogenesis, the expression of HMGN1 is down-regulated while that of N-cadherin is up-regulated.

In considering possible molecular mechanisms whereby HMGN1 down-regulates N-cadherin expression, we note that changes between *Hmgn1*−/− and *Hmgn1*+/+ cells were approximately sixfold, a value that is within the same range as observed previously for the effect of HMGN proteins on transcription from several types of *in-vitro* reconstituted chromatin systems. A sixfold difference in transcription levels between wild-type and *Hmgn1*−/− cells is lower than would be expected for a sequence specific transcription regulator and more compatible with the known function of HMGN proteins as architectural elements that modulate access to nucleosomes. HMGNs modulate chromatin accessibility at two distinct levels: first, at the higher order chromatin level they stabilize an extended, less compacted structure [6,7]; and second, at the level of the single nucleosome, they could either enhance or reduce the access to a particular nucleosomal site. Thus, by reducing the compactness of chromatin, HMGN1 increases access for the nucleotide excision repair machinery to UV-damaged DNA sites in chromatin [15], but reduces the ability of the kinase MSK1 to modify Ser10 in the tail of histone H3 [11]. It follows that HMGN1 could affect the ability of either positive or negative regulatory factors to reach their chromatin targets, and therefore may either up- or down-regulate the levels of specific transcripts, depending on the particular regulatory system of a gene.

The ability of HMGN1 to either up- or down-regulate transcription is similar to that of other chromatin architectural proteins such as histone H1 [38] and HMGN3 [14]. In several organisms including mice, loss of H1 leads to both up-and down-regulation of gene expression [38]. Interestingly, most of the genes are down-regulated rather than up-regulated as would have been expected, because H1 is considered a transcriptional repressor. Likewise, our analysis of the transcription profiles of *Hmgn1*−/− and *Hmgn1*+/+ MEFs also indicates that loss of HMGN1 leads to both increase and decrease of gene expression levels. In addition we reported recently that only part of the immediate early genes contain detectable amounts of HMGN1 [11]. These findings suggest that the effect of HMGN1 on the expression of any specific gene is dependent on additional regulatory factors, a suggestion compatible with the finding that HMGN1 is part of a larger multiprotein complex [39]. Interestingly, most of the genes affected by the ectopic expression of HMGN3 [14], another member of the HMGN protein family, are also down-regulated rather than up-regulated as would have been expected, given that HMGNs decompact chromatin. Notably, N-cadherin was not among the genes affected by misexpression of HMGN3, raising the possibility that the various members of the HMGN protein family affect the expression of discrete subsets of genes.

Several mechanisms could account for the HMGN1-mediated down-regulation of N-cadherin expression in MEFs. One possibility is that HMGN1 enhances the ability of a repressor to bind to a regulatory site in the N-cadherin gene. Conversely, the presence of HMGN1 on a target nucleosome may inhibit the ability of a positive regulator to bind to its site. Another possibility is that an HMGN1-mediated change in the pattern of post-translational modification in the histone tails reduces the level of N-cadherin transcripts. A third possibility is related to recent findings that HMGN1 functions within a network of chromatin binding proteins that compete among themselves for nucleosome binding sites [5,10]. Thus, the loss of HMGN1 may affect N-cadherin expression indirectly by altering the interaction of other members of the network (i.e. other chromatin binding proteins) to bind to their target sites. Fourth, the effects are indirect, and result from HMGN1-dependent changes in the expression of specific N-cadherin regulatory factors. We also find that in *Hmgn1*−/− MEFs the levels of β-catenin protein, but not RNA, are elevated perhaps reflecting an increase in the formation of a catenin–N-cadherin complex. These

considerations emphasize the complex role that chromatin structural proteins, including HMGN1, play in regulating gene expression. Nevertheless, our data clearly demonstrate that HMGN1 modulates the expression of N-cadherin in MEFs. The phenotype of the *Hmgn1*−/− cells is compatible with the observed changes in N-cadherin expression; however, as HMGN1 affects the expression of numerous genes it is still possible that additional changes contribute to the phenotype.

At the whole organism level, however, the phenotype of the *Hmgn1*−/− mice is not significantly different from that of their *Hmgn1*+/+ littermates. It is likely that compensatory mechanisms are more effective at the level of the entire organism than in isolated cells. Nevertheless, we have noted that the number of offspring from *Hmgn1*−/− crosses is lower than that from *Hmgn1*+/+ crosses and that loss of HMGN1 disrupts the normal hair cycle growth (M. Bustin, T. Furusawa, Y. Birger, unpublished data). Both of these phenotypes could be related to altered N-cadherin expression [40–42]. We have already noted that stress conditions amplify the phenotypic effects of loss of HMGN1 [15]. Thus, the biological consequences of loss of HMGN1 protein may be larger than those seen in mice grown unchallenged, under optimal conditions.

Among the genes whose expression is related to HMGN1, as identified by the microarray screen, N-cadherin was of special interest because of its potential involvement in cellular communication and development [44]. Very little is known about the molecular mechanisms that regulate N-cadherin expression. Our studies identify HMGN1, a chromatin structural protein, as a negative modulator of N-cadherin expression during early mouse development but not in adult mouse tissues. In fact, in adult heart and in testis the loss of HMGN1 decreased N-cadherin levels, suggesting that in these adult tissues HMGN1 enhances N-cadherin expression. Analysis of additional tissues, however, revealed variability in the levels of N-cadherin between the wild-type and *Hmgn1*−/− mice, a finding that is in agreement with previous results demonstrating tissue-specific variability in N-cadherin expression. Clearly HMGN1 is only part of the molecular mechanism that regulates N-cadherin expression.

In summary, our studies demonstrate a role of HMGN1 in modulating specific gene expression and identify N-cadherin as an HMGN1 target. N-cadherin may serve as a model system for understanding the molecular mechanisms whereby structural proteins regulate specific gene expression. Given the importance of N-cadherin in cell communication and migration, an understanding of the molecular mechanisms regulating its expression may have additional biological implications.

Experimental procedures

Experimental animals and cell lines

Hmgn1−/− mice, primary MEFs, MEF-derived cell lines, and transformed MEF cells expressing wild-type or mutant HMGN1 protein under the control of the Tet promoter, were generated and characterized as described elsewhere [15]. Primary MEFs were used up to passage five, as older MEFs had an altered phenotype. The values for the expression of N-cadherin during embryogenesis were derived from three littermates at each time point indicated in Fig. 5A. Treatment of mice were done according to NIH guidelines.

Cell adhesion assay

Cells were seeded in six-well plates at a density of 4×10^5 cells per well, three wells per cell type. Cells were incubated for the indicated time at 37 °C. Following incubation, cells were washed twice with NaCl/P_i to remove nonadhered cells. Adherent cells were stained using CAMCO Quik Stain kit (Fisher Scientific, Pittsburg, PA, USA) and counted [39].

Cell motility assay

FALCON cell culture inserts with an 8-μm pore-size PET membrane (Fisher Scientific) were coated with 0.1 $mg \cdot mL^{-1}$ collagen IV (Becton-Dickinson, NJ, USA) according to the manufacturer's protocol. Inserts were placed into the wells of a 24-well plate containing 0.5 mL of DMEM medium supplemented with 10% (v/v) fetal bovine serum, 5×10^4 cells in Dulbecco's modified Eagle's medium containing 0.1% (w/v) BSA were plated into the inserts and incubated for 4 h at 37 °C. Following incubation, cells from the upper surface of the membrane were removed by scrubbing with a cotton-tipped swab. Cells that had migrated through the insert and adhered to the bottom of the membrane were Wright stained using the CAMCO Quik Stain kit (Fisher Scientific). Ten random fields per membrane were counted under low power and photographed with a cool charged-coupled-device camera (Photometrics, Tucson, AZ, USA) interfaced with OPENLAB software (Improvision, Lexington, MA, USA) and counted using NIH IMAGE [39].

Microarray analysis

Mouse expression arrays were manufactured by the Advanced Technology Center at the NCI/NIH (Gaithersburg, MD, USA). Total cellular RNA was isolated from logarithmically growing *Hmgn1*+/+ and *Hmgn1*−/− cells [15], by the Trizol method as recommended by the supplier (Invitrogen, Carlsbad, CA, USA). The integrity of the RNA samples was verified by electrophoresis on denaturing agarose gels. RNA preparations from wild-type (wt) and knockout (ko) cell lines were labeled using Cy3 and Cy5-modified dUTP (Amersham, Piscataway, NJ, USA). Arrays were hybridized and then scanned and quantified using a scanning laser microscope (GenePix 4000, Axon Instruments, Foster City, CA, USA). The raw data computations, including the spot-finding algorithm, computing ratios and quality factors, were carried out using GENEPIX PRO. The parallel two-color-reverse-labeling hybridizations to DNA microarrays were repeated three times (six hybridizations in total). The fluorescence signals have been normalized using the reference spots on the slides.

Real time PCR

Trizol-isolated RNA was treated with DNase I (Promega, Madison, WI, USA) and further purified using the RNeasy kit (Qiagen, Valencia, CA, USA). For reverse transcription, 200 ng of RNA were used in a 20 μL reaction mixture containing 1× TaqMan RT buffer (TaqMan RT-PCR kit, Applied Biosystems), 5.5 mM magnesium chloride, 500 μM of each dNTP, 2.5 μM random hexamers, 0.4 $U \cdot \mu L^{-1}$ RNase inhibitor, and 1.25 $U \cdot \mu L^{-1}$ Multiscribe Reverse Transcriptase. Reverse transcription was performed for 10 min at 25 °C, 30 min at 48 °C and 5 min at 95 °C in a PE9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The reaction mixture was diluted to 100 μL with Tris/EDTA (10 mM/1 mM) buffer. Five microliters of diluted cDNA were used for real-time PCR. Real-time PCR primers were designed using Primer Express Applications (Applied Biosystems). Quantitative real-time PCR was carried out in an ABI Prism 7700 Sequence Detector using SYBR Green PCR Master Mix reagent (Applied Biosystems). Each 25-μL amplification reaction contained 5 μL of sample, 5.5 μL dH_2O, 1 μL each of 5 μM forward primer and reverse primer, and 12.5 μL of SYBR Green PCR Master Mix. PCR cycling conditions were as follows: 10 min at 95 °C, followed by 40 two-step cycles of 95 °C for 15 s, and 60 °C for 60 s. The Ct values (the number of PCR cycles to reach the fluorescence (Rn) threshold values of each amplification reaction) were used to calculate relative RNA levels by the comparative-Ct method where ΔCt is the Ct of the gene of interest minus the Ct of the housekeeping gene β-actin. The higher the Ct, the lower the amount of the initial template. The following oligonucleotides were used in real-time PCR analysis:

N-cadherin: forward, 5′-GCACATGCAGTGGACATCA; reverse, 5′-CCTCTGGAACAGACCCATTC; β-catenin forward, 5′-

CACGCAAGAGCAAGTAGCTGAT; reverse, 5′-GCAGCTCGGACCCTCTGA; β-globin forward, 5′-TGAAGGCCCATGGCAAGA-3′; reverse: 5′-GCCCTTGAGGCTGTCCAA-3′. β-Actin: forward, 5′-ACCAACTGGGACGATATGGAGAAGA; reverse, 5′-TACGACCAGAGGCATACAGGGACAA.

Protein extracts and western blotting

Cells were washed at 4 °C in NaCl/P_i, lysed at 4 °C in lysis buffer [10mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, and protease inhibitors], centrifuged at 10,000 ***g*** for 15 min at 4 °C, and loaded on SDS polyacrylamide gels. The proteins in the gels were transferred to poly(vinylidene difluoride) membranes and probed with the appropriate antibodies.

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Abbreviations

Dox	doxycycline
HMG	high mobility group
MEF	mouse embryonic fibroblast

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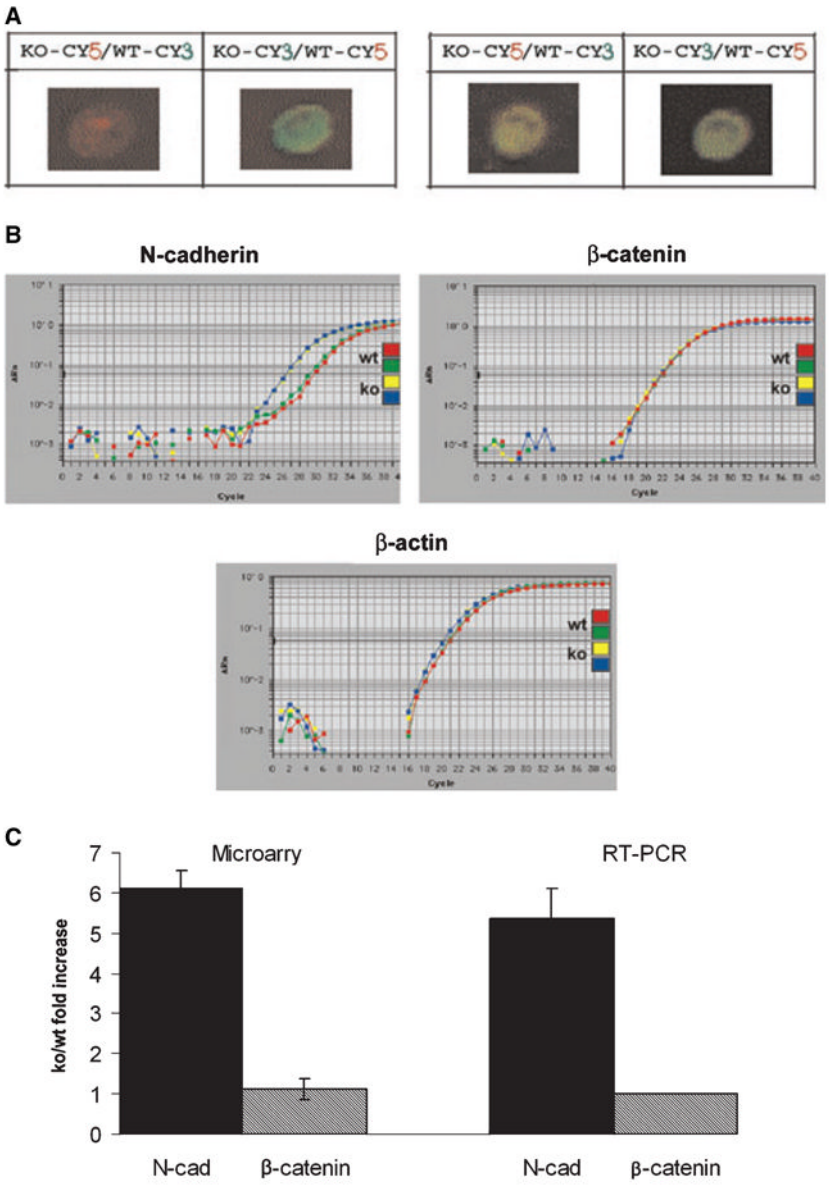

Fig. 1.

N-cadherin expression is up-regulated in Hmgn1−/− MEFs. (A) Microarray analysis of N-cadherin and beta-catenin from total cellular RNA isolated from Hmgn1−/− and Hmgn1+/+ MEFs. Arrays were scanned and quantified using a scanning laser microscope. The parallel two-color-reverse-labeling hybridizations to DNA microarrays were repeated three times and the fluorescence signals have been normalized using the reference spots on the slides. (B) Quantitative RT-PCR of N-cadherin, β-catenin and β-actin (control) in *Hmgn1*−/− and *Hmgn1*+/+ MEFs. (C) Summary and comparison of microarray and quantitative analysis N-cadherin and β-catenin expression in *Hmgn1*−/− and *Hmgn1*+/+ MEFs; wt, *Hmgn1*+/+ MEFs; Ko, *Hmgn1*−/− MEFs.

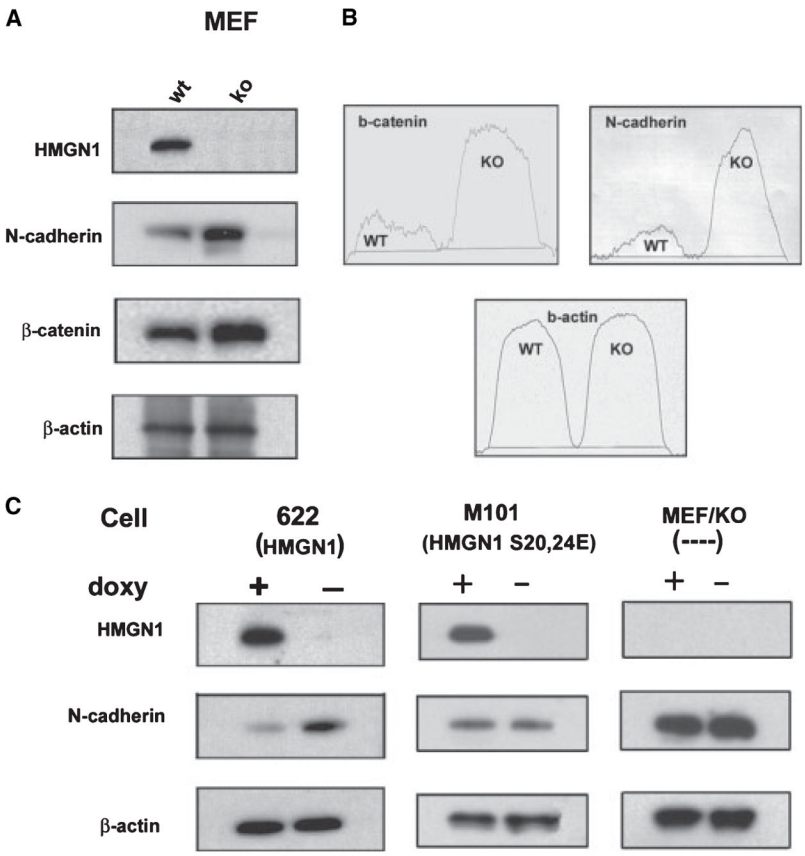

Fig. 2.

HMGN1 binds to the N-cadherin gene and suppress its expression. (A) Western blot analysis of HMGN1, N-cadherin, β-catenin and β-actin (loading control) expression in Hmgn1−/− and Hmgn1+/+MEFs. (B) Western blot quantification of N-cadherin, β-catenin and β-actin expression in Hmgn1−/− and Hmgn1+/+ MEFs. (C) Western blot analysis of stably transfected Hmgn1−/− MEFs expressing either wildtype (622 cells) or the mutant (M101) HMGN1 protein under the control of the Tet promoter. Note that induction of protein expression by the addition of Dox leads to down-regulation of N-cadherin in 622 but not in M101 cells. MEF/KO denotes control, nontransformed Hmgn1−/− cells.

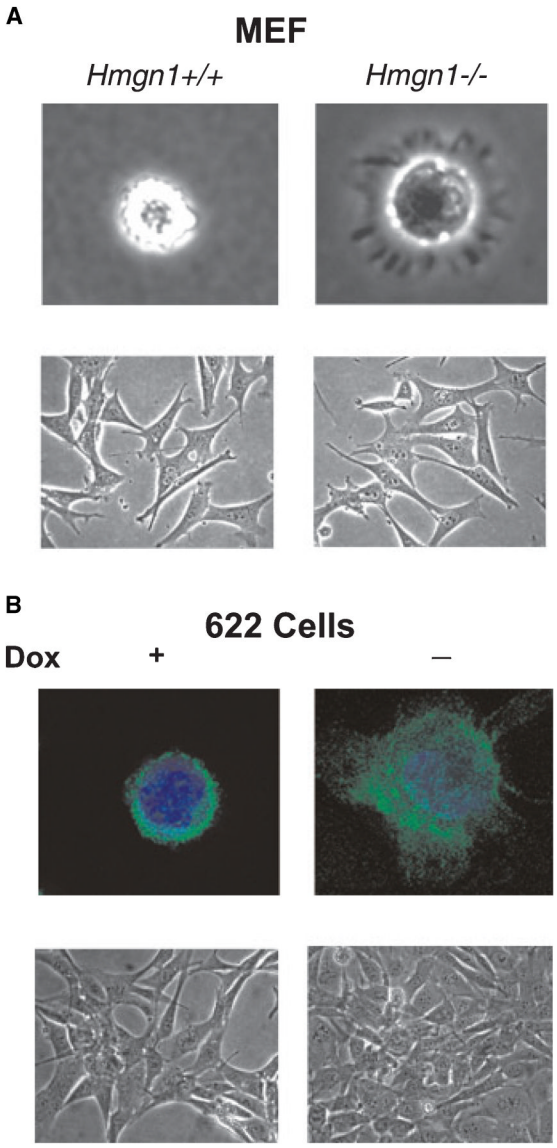

Fig. 3.

Changes in the distribution of N-cadherin during the attachment of MEF cells. (A) Enhanced rate of cell adhesion in nontransformed Hmgn1−/− MEFs. Shown are photomicrographs of wt and ko MEFs 0.5 h (upper panels) and 4 h (lower panels) after seeding. (B) HMGN1 affects N-cadherin distribution. Upper panels are confocal images of 622 cells, that either do (+ Dox) or do not (− Dox) express HMGN1, fixed 30 min after plating (upper panels) and stained with anti-mouse cadherin Ig followed by fluorescein isothiocyanate- conjugated secondary antibody and counter-stained with 4′,6-diaminidino-2-phenylindole (DAPI). Note that cells that do not express HMGN1 contain higher levels of N-cadherin, with spike-like-structures protruding from the membrane. In the Dox-treated cells that do not express HMGN1 and are not attached the N-cadherin remains a tight ring confined to the cell membrane. Lower panels, cells 4 h after plating. The differences in N-cadherin distribution disappear when cells are growing for longer time.

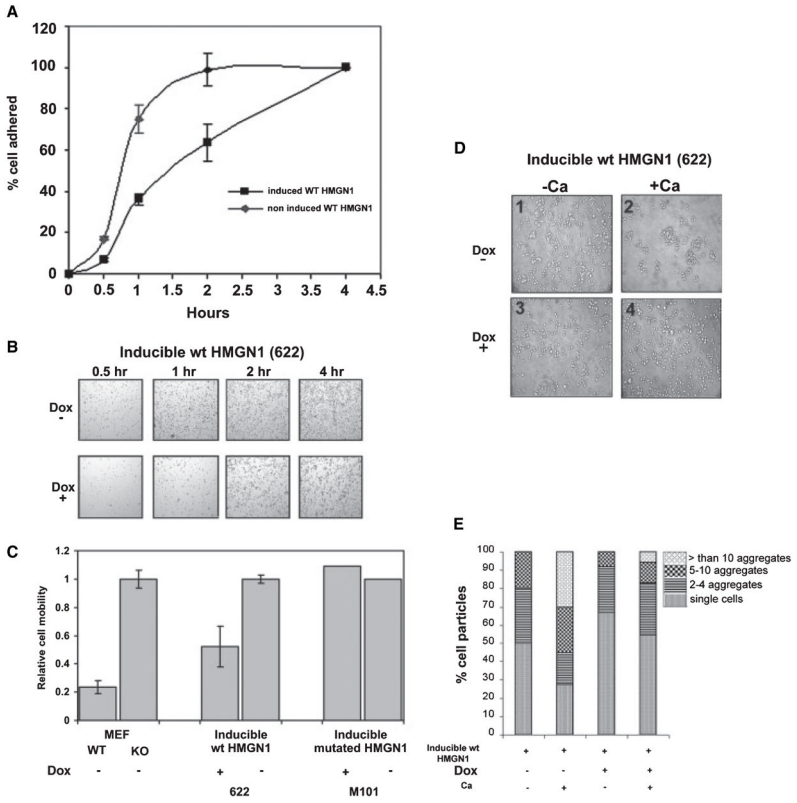

Fig. 4.

Loss of HMGN1 alters the cellular phenotype. (A) Cell adhesion. Shown are the cell adhesion assays with cell line 622 that was either treated (■) or not (◆) with Dox. Dox treatment induces the expression of HMGN1. Note that induction of HMGN1 lowered adhesion. Treatment of control, nontransfected cells with Dox did not affect adhesion (not shown). (B) Photographs of representative fields from the adhesion assays. Note that Dox treatment (i.e. HMGN1 expression) lowered the # of adhering cells. (C) Motility assay of nontransformed wt and ko MEF cells, transformed cells expressing wild-type HMGN1 and transformed cells expressing a mutated HMGN1 that does not bind to chromatin. Dox treatment induced the expression of the respective protein. Note that the expression of the wild-type, but not mutated protein lowers the rate of cell motility. Likewise the wild-type cells migrate slower than the ko cells lacking HMGN1 protein. In each set of cells, the migration was normalized to the cells that do not express HMGN1. (D) Photomicrographs showing the aggregation properties of MEF cells expressing (Dox +) or not-expressing (Dox −) wt HMGN1 protein, in the presence of 2 mM Ca^{2+}. (E) Distribution of aggregates in cells that either do, or do not express HMGN1, in the presence or absence of Ca. Note that induction of HMGN1 expression decreases the relative number of large aggregates (containing more than 10 cells per aggregate).

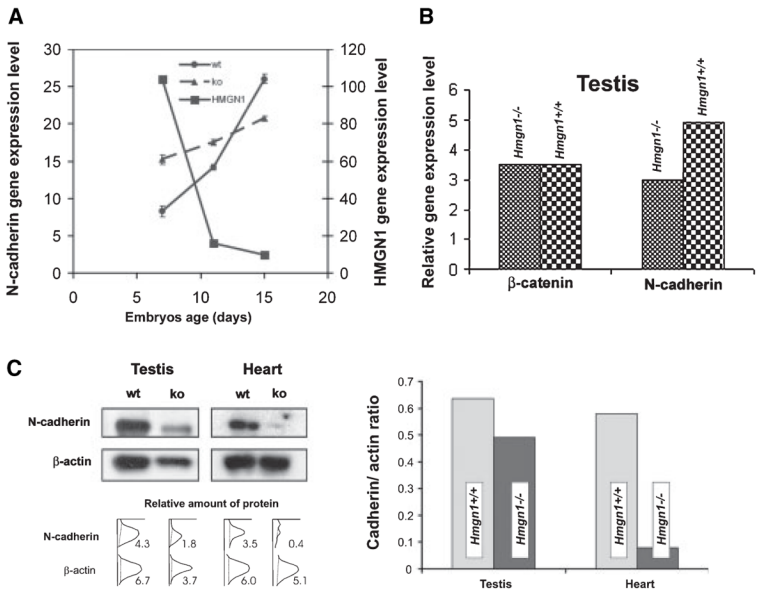

Fig. 5.

HMGN1 down-regulates N-cadherin gene expression only during early development. (A) Expression of HMGN1 (■), and N-cadherin in *Hmgn1*+/+ (●) or *Hmgn1*−/− (▲) embryos at age 7, 11 and 15 days, measured by quantitative RT-PCR. (B) N-cadherin and β-catenin expression in *Hmgn1*+/+ and *Hmgn1*−/− adult testis. (C) Western blot analysis of N-cadherin in adult testis and heart of *Hmgn1*−/− and Hmgn1+/+ mice. The scans of the signals are shown under the westerns and the ratios of the N-cadherin to actin, derived from the scans are shown in the bar graph on the right.