Knockdown High mobility nucleosomal binding proteins 2 (HMGN2) alter the histone modification H4K4me3 and H3K27me3 and regulates stem cells pluripotency.

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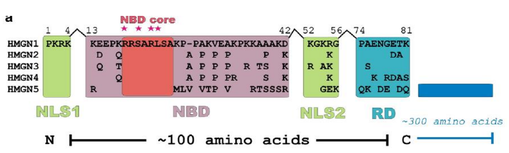
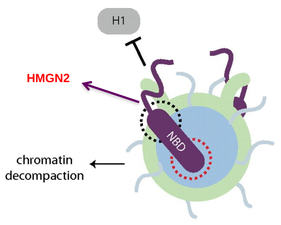
Introduction

High mobility group nucleosome binding proteins (HMGN) are ubiquitous non-histone chromosomal proteins. They play important roles in regulating chromatin architecture and transcription. There are five family members: Hmgn1, Hmgn2, Hmgn3a/b, Hmgn4 and Hmgn5.

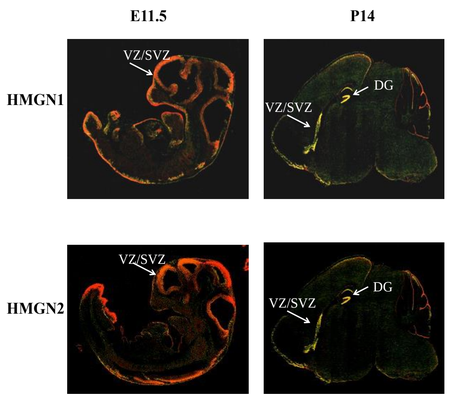


HMGN proteins bind to nucleosomes with their highly conserved nucleosome binding domain (NBD). The C-terminal regulatory domain (RD) modulates specific histone modifications and disrupts the binding of linker histones (H1) to chromatin.



Several studies have shown that HMGN1 and HMGN2 are highly expressed in tissue-specific progenitor and transit-amplifying cells, and are down-regulated in terminally differentiated cells. Functional studies have shown that they play important roles in several cellular differentiation pathways, including erythropoiesis, myogenesis, osteoblast differentiation.

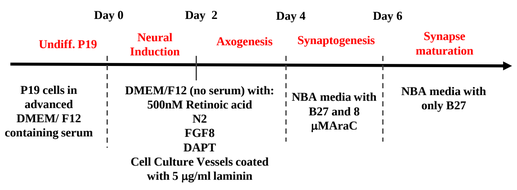
Hmgn1 and *Hmgn2* expression in the mouse brain



VZ: ventricular zone; SVZ; subventricular zone;
DG: dentate gyrus (in the hippocampus)

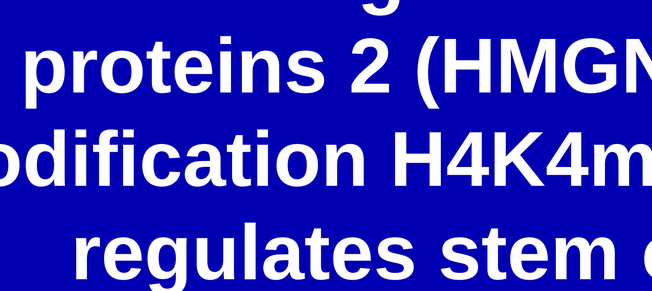
Data from the Allen Brain Atlas indicates that *Hmgn1* and *Hmgn2* mRNAs are highly expressed in active neurogenic regions of the developing and adult mouse brain, including the subventricular zone and the dentate gyrus of the hippocampus.

Consistent with the expression pattern data, a previous study[1] has shown that Hmgn1 is important for regulating the differentiation of neural stem cells, both in vitro and in vivo. Our immediate aim is to develop an in vitro model system with which to investigate the role of HMGN proteins and epigenetic processes during neuronal differentiation. Here, using an adherent culture system[2], we demonstrate that the expression of HMGN1 and HMGN2 proteins decreases during the terminal differentiation of neurons from P19 embryonic carcinoma cells.

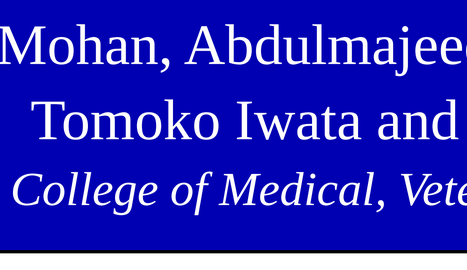


1 Nagao et al (2014) *Stem cells* 32(11):2983-97
2 Nakayama et al (2014) *Journal of Neuroscience Methods*. 227; 100–106.

Adherent monolayer culture efficiently drives production of NPCs and neurons from P19 ECs

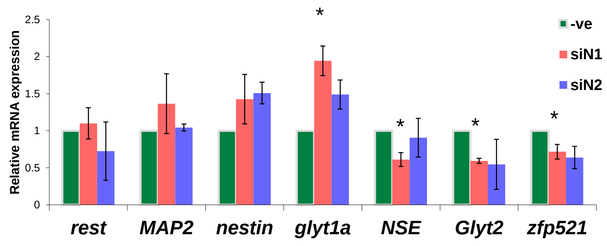


Hmgn1 and *Hmgn2* expression is reduced during neuronal differentiation *in vitro*



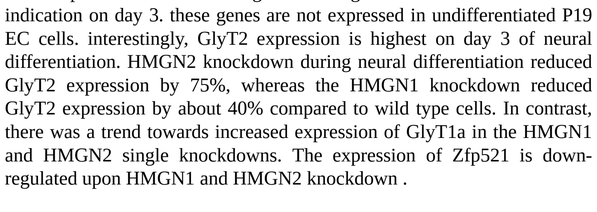
Western blot data from two different experiments indicate a reduction in the expression of *Hmgn1* and *Hmgn2* after neural induction. Both proteins are found at high levels in undifferentiated ECCs, and are nearly undetectable in terminal stages of differentiation. The *Hmgn2* mRNA levels are consistent with the western blot results, decreasing during the differentiation process. However, *Hmgn1* mRNA seems to be fluctuating through the different time points analyzed.

Hmgn1 and Hmgn2 regulate gene expression in day 3 neuronal cells



Gene expression data from Hmgn1 and Hmgn2 knockdown after neuronal indication on day 3. these genes are not expressed in undifferentiated P19 EC cells. interestingly, GlyT2 expression is highest on day 3 of neural differentiation. HMGN2 knockdown during neural differentiation reduced GlyT2 expression by 75%, whereas the HMGN1 knockdown reduced GlyT2 expression by about 40% compared to wild type cells. In contrast, there was a trend towards increased expression of GlyT1a in the HMGN1 and HMGN2 single knockdowns. The expression of Zfp521 is down-regulated upon HMGN1 and HMGN2 knockdown .

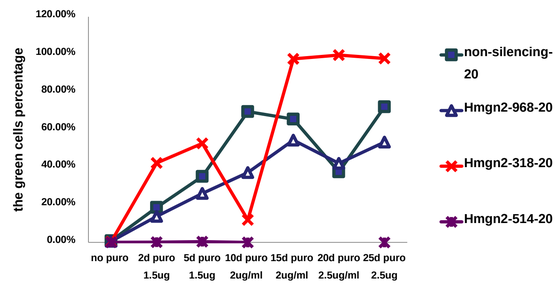
siRNA knockdown of *Hmgn1* or *Hmgn2* reduces the expression of pluripotency markers in pluripotent P19 EC cells.



PCR array analyses reveal that several markers of early differentiation are upregulated in Hmgn-knockdown EC cells. The expression of several genes required for ES self renewal and pluripotency is also disrupted.

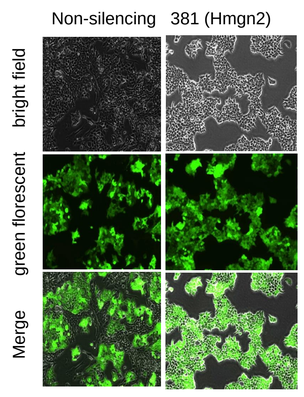
Knockdown of *Hmgn2* using a lentiviral system (*pGIPZ*) in P19 cell lines

In order to verify the results from the transient siRNA experiments, HMGN2 was knocked down using shRNAmiR expressed from lentiviral vectors.



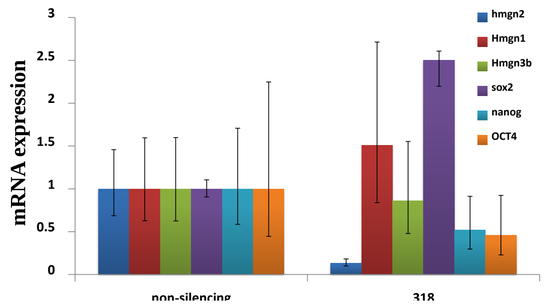
P19 cells were transduced with lentiviruses expressing three different shRNAmir triggers against Hmgn2 or a control. Cells were selected with puromycin, and FACS analysis shows the percentage of positive transduced cells.

Transduced cells express GFP



Fluorescent microscopy on day 20 for P19 cells transduced with the 381 shRNAmiR trigger against Hmgn2.

HMGN2 KD cells show reductions in Oct4 and Nanog expression

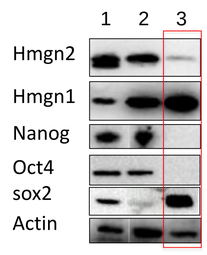


Gene expression data from transduced cells, 20 days after transduction. The data show a knockdown in HMGN2 levels, no change in HMGN3, and an increase in HMGN1.

Consistent with the data from siRNA transfections, HMGN2 knockdown was associated with reductions in Nanog and Oct4 expression. However, Sox2 expression was increased in the lentiviral experiment rather than decreased.

Pluripotency gene expression is altered in *Hmgn2* knockdown cells

Western blotting of whole cell extracts confirms Hmgn2 knockdown and loss of Nanog and Oct4 expression.

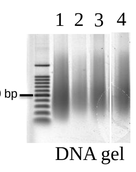


1. **WT P19**
2. **NON-SILENCING**
3. **shRNA 381**

In order to study the levels of histone modifications and DNA bound factors, chromatin was prepared from transduced cells. After reversing the crosslinks, samples were run on SDS-PAGE and analysed by western blotting.

ChIP-qPCR analysis in *Hmgn2* knockdown cells

1. **WT P19**
2. **Mouse ES cells**
3. **381 (Hmgn2 trigger)**
4. **Non-silencing trigger**

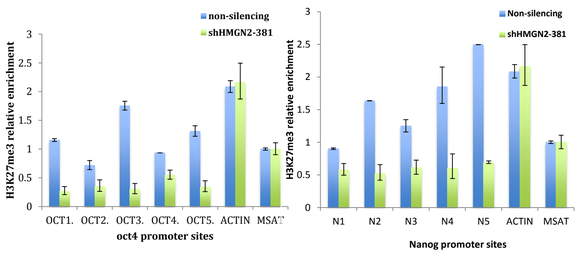


Consistent with the data from qRT-PCR and western blotting of whole cell extracts, reductions in Hmgn2, Oct4 and Nanog were observed in chromatin from cells transduced with the 318 trigger.

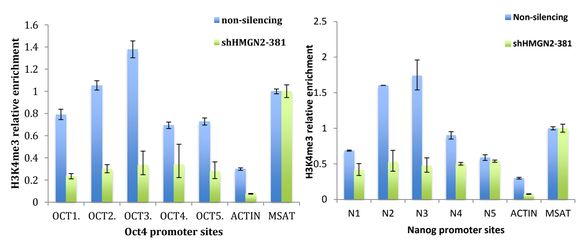
Notably, Sox2 and c-Myc were also downregulated, whereas Klf4 was upregulated.

A slower migrating form of TBP was observed in the 318 knockdown cells

QPCR analysis of Oct4 and Nanog around transcription stating sites (TSS) after Hmgn1 and Hmgn2 ChIP. Global loss of Hmgn2 and enrichment of Hmgn1 around Oct4 and Nanog TSS in Hmgn2 knockdown P19 EC cells.



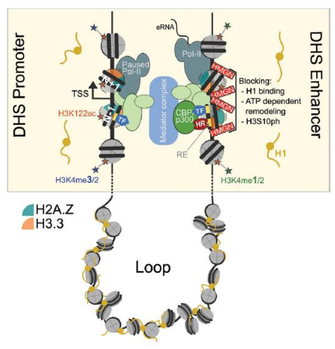
Repression of *Oct4* and *Nanog* in *Hmgn2* knockdown cells is accompanied by increases in the repressive mark H3K27me3



Repression of *Oct4* and *Nanog* in *Hmgn2* knockdown cells is accompanied by a reduction in the active mark H3K4me3

Hmgn2 binds at the enhancer of active genes

We have presented data from two independent approaches indicating that Hmgn2 plays a role in regulating key pluripotency genes such as Oct4, Nanog and c-Myc in mouse embryonal carcinoma cells.
Moreover we found that the loss of Hmgn2 compensate with the Hmgn1 at the same location in mRNA , western blot and ChIP-QPCR analysis.
Enhancer is marked by Histone post translational modification (H3K4me3). this indicate that Hmgn2 may affect the enhancer region at the active genes.



Schematic representation of the main chromatin structural elements of enhancers (Martinez de Paz and Ausio, 2015)

Work is currently ongoing to investigate whether Hmgn2 regulates expression of pluripotency genes in mouse ES cells as well as in EC cells.

Acknowledgments

We would like to thank Dr. Adam West, and all his lab members, for all advice and scientific discussions

Funding for this project was provided by the Saudi Arabian Ministry of Education (Albah University). the Ministry of Education in Saudi Arabia (King Abdullah scholarship programme), the Malaysia government, and CONACyT.