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**H19 Antisense RNA Can Up-Regulate Igf2 Transcription by Activation of a Novel Promoter in Mouse Myoblasts**

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Abstract

It was recently shown that a long non-coding RNA (lncRNA), that we named the 91H RNA (i.e. antisense H19 transcript), is overexpressed in human breast tumours and contributes in trans to the expression of the Insulin-like Growth Factor 2 (IGF2) gene on the paternal chromosome. Our preliminary experiments suggested that an H19 antisense transcript having a similar function may also be conserved in the mouse. In the present work, we further characterise the mouse 91H RNA and, using a genetic complementation approach in H19 KO myoblast cells, we show that ectopic expression of the mouse 91H RNA can up-regulate Igf2 expression in trans despite almost complete unmethylation of the Imprinting-Control Region (ICR). We then demonstrate that this activation occurs at the transcriptional level by activation of a previously unknown Igf2 promoter which displays, in mouse tissues, a preferential mesodermic expression (Pm promoter). Finally, our experiments indicate that a large excess of the H19 RNA can transactivate 91H-mediated Igf2 activation. Our work contributes, in conjunction with other recent findings, to open new horizons to our understanding of Igf2 gene regulation and functions of the 91H/H19 RNAs in normal and pathological conditions.

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Introduction

Long non-coding RNAs (lncRNAs) are major components of the mammalian transcriptome (for a review, see ref. [1]). Recent efforts to better characterize such transcripts revealed that they play important roles in both oncogenic and tumour suppressive pathways [2]. lncRNAs display a myriad of molecular functions [3], from chromatin remodelling ([AVRIL, HOTAIR, Xist] [4,5,6,7] and modulation of alternative splicing ([Zeb2/Sip1 gene locus] [8], to RNA metabolism ([1/2-sbs and HULC RNAs] [9,10] and generation of micro- and small-RNAs ([MEG3/Gtl2 and MALAT1 transcripts]) [11,12]. They also have a great variety of forms: most of them are generated by the RNA polymerase II, but some are synthesized by the RNA Pol III ([BC200 RNA] [13]); moreover, while most are poly-adenylated, many lncRNAs remain unpoly-adenylated [14] like, for example, the natural Sense-Antisense transcripts (SAT) [15] which are known to overlap each other and are co-ordinately expressed [16].

Several lncRNA are also produced from imprinted genes, whose expression is depending on the parental origin of the chromosome. Among them, the *Aon* and *Kcnq1ot1* transcripts have been shown to “coat” the imprinted locus on the paternal chromosome from which they are expresed. Interestingly, both transcripts are known to interact with the histone H3 Lysine 9 methyltransferase G9a and to repress multiple genes in *cis* on the paternal chromosome [17]. Finally, two genes encoding imprinted lncRNAs map downstream the *Insulin-like Growth Factor 2* gene ([Igf2]) : one is the recently described *PIHit* [10] and another is the *H19* gene.

Since its discovery, twenty years ago [19], the function of the H19 gene remains enigmatic. H19 gene silencing is associated with the appearance of Wilms’ tumours in the Beckwith-Wiedemann syndrome [20,21]. Furthermore, ectopic expression of the *H19* gene in human embryonic tumour cell lines leads to loss of clonogenicity and reduced tumourigenicity in nude mice [22]. It was recently confirmed that, in the mouse, H19 acts as a tumour suppressor [23]. However, several studies have also shown that the *H19* RNA can accumulate in cancer cells and tumours [24,25,26,27,28] and it has been considered as an oncofetal RNA by some authors [29]. The gene encodes an untranslated RNA which is expressed only when maternally inherited.
Monoallelic expression of H19, like that of the neighbouring oppositely imprinted Insulin-like growth factor-2 (Igf2) gene, depends on the paternal-specific DNA methylation of an Imprinting-Control Region (ICR) located between 2 and 4 kb upstream of the H19 gene [30]. This methylation is acquired during male gametogenesis and prevents the binding of CTCF, an insulator protein. On the unmethylated maternal allele, CTCF is bound to the ICR and creates a boundary which prevents interactions between enhancers, located downstream of the H19 gene, and the Igf2 gene [31].

While the mechanisms of imprinting at the Igf2/H19 locus have focused much attention, very little is known about transcriptional regulation of the expressed Igf2 and H19 alleles. The two genes are tightly co-regulated during mouse embryonic development and are repressed shortly after birth in most tissues. Both genes belong to a network of coexpressed imprinted genes (Imprinted Gene Network, IGN) that may control embryonic growth in the mouse [32]. Recently, the non-coding H19 RNA was shown to contribute to the trans regulation of at least 9 genes of the IGN [33]. However, whether the H19 transcript acts through direct or indirect mechanisms and which step of gene expression is affected by such a regulation have not yet been investigated. Interestingly, we recently discovered in human that an antisense H19 transcript, named the H19 RNA (or H19as for “H19 opposite strand” transcript), augments in trans the paternal Igf2 expression which is known to favour tumour progression. In agreement with this notion, the H19 RNA is a large nuclear transcript which accumulates in breast tumours by RNA stabilization [34]. Preliminary experiments indicated that the antisense H19 transcript is evolutionarily conserved and expressed during the perinatal period in the mouse. In this work, we present further insights about the function of the mouse H19 and H19 transcripts. Using a genetic inactivation/complementation approach in cultured murine myoblasts, we show that ectopic expression of H19 RNA is sufficient to trans-activate Igf2 at the transcriptional level, despite hypomethylation of the H19 ICR. Interestingly, this trans-activation occurs via a novel Igf2 promoter (Igf2 Pm). Our experiments also indicate that a large excess of H19 RNA seems to counteract this effect. Globally, our work suggests that H19 sense and antisense RNAs are antagonist trans riboregulators of Igf2 transcription.

**Results**

**Characterization of the Mouse 91H RNA**

Preliminary experiments showed that an H19 antisense transcript, called the 91H RNA, that controls Igf2 gene expression in human breast cancer cells is conserved in the mouse [34]. The 91H RNA is a short-lived nuclear transcript which is co-regulated with the Igf2 and H19 genes, during the perinatal period. Our first aim was to further characterize the mouse transcript before investigating the mechanisms by which it may exert its function. We previously determined in mouse liver the transcriptional orientation of the 91H RNA upstream of the H19 endodermic enhancers [34]. Since the human H19 antisense RNA is initiated further downstream, in the intron 1 of the MRPL23 gene [34], we investigated in the mouse the intergenic region located downstream of the H19 endodermic enhancers. In the heart, strand-specific RT-qPCR quantifications showed that the antisense transcript is mostly initiated downstream of the H19 enhancers (data not shown), reminiscent to the human situation [34]. However, in the mouse liver, we found only limited antisense relative to the sense transcript downstream of the enhancers (data not shown). Since, upstream the enhancers, we found substantial amounts of antisense transcript [34], we can conclude that, opposite to the situation in the heart, in liver, most of the 91H RNA is initiated within the endodermic enhancer region. As shown previously [34,35], within the H19 ICR and its upstream sequences short non-coding transcripts are initiated in both sense and antisense directions on both parental alleles, thus impairing further characterisation of the 3’ end of the 91H RNA.

3’RACE experiment from the capped fraction of non-polyadenylated d7 mouse liver RNAs then mapped the Transcription Start Site (TSS) of the 91H RNA more precisely within the endodermic enhancer 2 (position chr7: 149,755,206 or chr7: 149,755,207 on mouse July 2007/mm9 Assembly) (Figure 2).

Finally, using mice carrying a maternal deletion of the H19 transcription unit (H19KO) [36] and a primer pair specific of the icd-type allele [m] PCR amplicon, that amplifies through the 5’ end of the H19KO deletion over the H19 transcription start site, (Figure 1), we assessed by RT-qPCR that no paternal allele-specific transcript can be detected immediately upstream of the H19 gene (data not shown) while it can be detected further downstream on this allele [34]. Therefore, as previously suggested [34], the mouse 91H RNA transcription stops within the H19 gene region on the paternal allele (Figure 1).

H19 KO Myoblasts Display Low ICR Methylation and Weak Igf2 Expression Levels

We previously showed that the mouse H19 RNA is a negative trans-regulator of Igf2 mRNA levels [33] while, in the human, the 91H RNA augments paternal Igf2 expression levels [34]. Furthermore, a study by Wilkin et al. suggested that the activity linked to the 91H RNA may be restricted to the antisense strand of the H19 transcription unit [37]. To elucidate the function of the sense/antisense H19 transcripts, we generated a mouse myoblast cell line from homozygous H19KO KO mice [36] (H19 KO myoblasts +/-). These cells express an endogenous 91H RNA which is truncated within the H19 transcription unit. Therefore, we suspected that this truncated 91H RNA would not be functional and that Igf2 expression may be affected in this cell line. Remarkably, we showed that, as for the classical C2C12 myoblast line, H19 KO myoblasts can differentiate into myotubes upon 3 days of serum starvation (Figure S1). Using the mE and mF PCR amplicons (Figure 1), we then showed that the endogenous truncated 91H RNA displays a similar level of expression compared to the native 91H RNA in C2C12 myoblasts (Figure S2A). As found for tissues [34], the endogenous truncated 91H RNA levels are very low as compared to that of the sense H19 transcript (about 10^7/10^8 lower than H19 RNA levels observed in C2C12 myoblasts). Interestingly, in early passages of cell culture, H19 KO myoblasts displayed substantial Igf2 expression levels, as observed in the muscle of H19KO animals [36] (data not shown). However, in later passages, we found that Igf2 expression levels were very weak in undifferentiated cells and could only be detected by RT-qPCR (Figure S2B) but neither immunofluorescence (Figure 3A) nor in Northern-Blot (Figure 3B) experiments.

Run-on and Northern-blot experiments were also performed on myoblasts derived from mice having a paternal inheritance of the H19KO myoblasts and they were cultivated under identical experimental conditions (late passages of cell culture). However, they harbour accurate Igf2 transcription (Figure 3A) and regular steady state Igf2 mRNA levels (Figure 3B). Finally, Igf2 downregulation in H19KO myoblasts was correlated with a progressive loss of DNA methylation of the H19 ICR (CTCF site 2) observed upon
Isolation of H19 KO Myoblast Clones that Express Ectopic 91H and H19 Transcripts

To investigate the possibility that the effect of the H19 transcription unit on Igf2 expression (Figure 3) may depend on antisense sequences complementary to the H19 gene [34,37], we then transfected the H19 KO myoblasts with constructs containing this region with the H19 gene under the control of a strong promoter (CMV promoter). Unfortunately, despite intensive efforts, such mini-constructs were systematically unable to express significant levels of the transgenes in H19 KO myoblast cells. However, transfection of a 16 kb BamHI-BamHI restriction fragment encompassing the H19 endodermic enhancers and the whole H19 gene. PCR amplicons used to quantify the ectopic RNAs are indicated (H19 RNA, m1-m3, m2 and m1). DNA methylation is indicated by black lollipops and RNAs are depicted in red. Positions of restriction sites and PCR amplicons used for real-time PCRs are indicated relative to the H19 transcription start site. The mA and mB PCR amplicons have been used in a previous study [34] and are indicated here solely for clarity of our PCR nomenclature. For primer sequences see Table S1.

Figures 4A,B, left panel). For comparison, in wild-type C2C12 myoblast cells, 91H RNA levels were dramatically lower than H19 RNA levels (Figure 4B, right panel). Clearly, ectopic 91H RNA is overexpressed in all transfected cells analysed. However, contrary to the situation in the wild-type C2C12 cells (Figures 4B, right panel), neither the ectopic H19 nor the ectopic 91H transcripts are up-regulated during myoblast differentiation (Figure 4B, left panel) suggesting that some regulatory elements are probably missing in the construct used for ectopic expression. 5’RACE experiment performed on total RNA from one of the clone (clone 4) mapped the same TSS found in liver for the endogenous 91H RNA (see Figure 2) as well as two minor upstream start sites (Figure S3). Finally, using actinomycin D treatments, we definitively validated our experimental system of cellular complementation by showing that, mimicking their endogenous counterparts [34,36], the ectopic H19 RNA is very stable, while the ectopic 91H transcript is much more labile (Figure S4B).

Ectopic Expressions of 91H and H19 RNAs are Oppositely Linked to Induced Igf2 Transcription

To investigate gene expression at the transcriptional level, we performed nuclear run-on experiments on undifferentiated and differentiated transfected H19 KO myoblasts corresponding to the whole cell population or to the isolated clones (Figure 5A). Using a PhosphorImager, we then quantified the relative Igf2 transcription levels from the autoradiographies shown in Figure 5A. These experiments showed that Igf2 transcription is undetectable in untransfected H19 KO myoblasts, while all transfected clones re-expressed Igf2 at significant levels (Figure 5B). No correlation was found between Igf2 transcription levels and the H19/H19...
transgene copy number ($R^2 = 0.0317$, data not shown). This demonstrates that $Igf2$ trans-activation occurs at the transcriptional level and suggests that this trans-activation depends on ectopic RNA expression.

In agreement with our previous findings in the human [33,34], $Igf2$ trans-activation is strongly correlated to the ectopic 91H RNA ($R^2 = 0.6918$) (Figure 6A). We also observed a positive correlation between $Igf2$ transcription and ectopic $H19$ RNA ($R^2 = 0.5315$) levels (Figure 6B). Both RNAs are produced from the same ectopic copies and therefore, it is not surprising that both display a positive correlation with $Igf2$ transcription levels. This indicates that at least one is truly correlated with $Igf2$ transcription. The $p$ value of 0.0025 indicates that the correlation with 91H RNA is more significant ($p = 6.10^{-7}$) than that with $H19$ RNA ($p = 10^{-5}$). This result suggests that it is essentially the ectopic 91H RNA up-regulates $Igf2$ transcription in trans. Moreover, very large amounts of ectopic $H19$ RNA, as observed in clones 4, 11 and 12ND (see Figure 4B), can counteract this effect. Indeed, by plotting $Igf2$ transcription levels versus the ratio of 91H/H19 ectopic RNA levels, we observed a clear negative effect of ectopic $H19$ RNA on $Igf2$ transcription in these clones where the 91H/H19 ratio was inferior to 0.2 (Figure 6C, black diamonds). Finally, in all other clones, where $H19$ RNA is much lower (in which the 91H/H19 ratio was superior to 0.2), the levels of the $H19$ RNA do not display any significant effect on $Igf2$ transcription levels (Figure 6C, white diamonds).

We thus propose that the relative sense/antisense ectopic $H19$ RNA levels are able to control $Igf2$ trans-activation in our complementation assay.

Figure 2. Characterisation of TSS of the endogenous mouse 91H RNA. 5′RACE experiment was performed on unpolyadenylated and capped RNA from 7 days-old mouse liver. (A) The RT Primer was designed in the mFb region (Figure 1) and a band was successfully amplified by nested PCRs. The RT primer corresponds with the forward primer of PCRa and nested PCR reactions were performed using the GeneRacer DNA oligonucleotide as reverse primer. (B) Ethidium bromide staining of an agarose gel showing PCRs product obtained from amplifications indicated above (MW: Molecular Weight). Sequencing of PCRa and PCRc products showed that these bands correspond essentially to unspecific amplifications while PCRb correspond to the TSS of the 91H RNA. (C) Electrophoregram of the sequenced 5′RACE product amplified from the capped RNA fraction (PCRb). This sequence identified a unique Cap site located in the endodermic enhancer 2 at position chr7:149,755,206 or chr7:149,755,207 on mouse July 2007/mm9 Assembly. Due to the presence of a C residue at the end of the GeneRacer RNA oligonucleotide primer and/or the possibility that the last C residue may derive from the cap of the RNA, the exact position of the TSS remains ambiguous between two consecutive C residues found in the mouse genome sequence. (D) The sequence of the endodermic enhancer 2 is indicated in bold. The position of the TSS of the 91H RNA is indicated (black arrow).

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Igf2 Trans-activation by Ectopic 91H RNA can Occur Without H19 ICR Re-methylation

We then assessed whether Igf2 up-regulation in transfected H19 KO myoblasts is accompanied by re-methylation of the H19 ICR. In addition, we also investigated the methylation levels of the other Differentially Methylated Regions (DMRs) of the locus. In order to determine DMR methylation levels at the Igf2/H19 locus, we used digests by methylation-sensitive restriction enzymes of DNA from untransfected and transfected H19 KO myoblasts as well as control myoblasts (paternal heterozygous) (Figure S6). These experiments confirm that untransfected H19 KO myoblasts are poorly methylated on

Figure 3. Transcriptional and steady state Igf2 RNA levels in control and H19 KO myoblasts. H19 KO myoblasts (−/−) have been compared to cells with a paternally inherited deletion of the H19 transcription unit (control paternal heterozygous myoblasts, +/−). These two cell lines have identical genetic background and were both harvested after identical late passage numbers (passage 40). (A) Run-on analysis of Igf2 and of the 91H/H19 transcripts. Igf2 transcriptional activity is detected only in control myoblasts (+/−). (B) Northern-blot analyses of Igf2 mRNA and H19 transcript levels showing two lanes corresponding to two separate samples for each cell type. The same membrane was sequentially hybridized with Igf2, H19 and 18S rRNA probes. The left panel shows an ethidium bromide staining of the agarose-formaldehyde gel before transfer. Note that the Igf2 transcripts are detected only in control cells (+/−). (C) Methylation levels were determined at a BceAI methylation sensitive restriction site located within the CTCF recognition site 2 of the H19 ICR in H19 KO myoblasts at the indicated passage numbers.

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the H19 ICR (Figure S6A) and showed that Igf2 DMR1 also becomes unmethylated (Figure S6B) while Igf2 DMR2 remains highly methylated (Figure S6C). Low methylation levels are also found at the H19 promoter (Figure S7A). However, the IgDMR at the Dlk1/Gtl2 locus on chromosome 12 remains methylated (Figure S7B) indicating that the unmethylation observed at the H19 ICR is not a general phenomenon, since it is not found at another imprinted locus. In transfected clones, the ICR (Figure S6A) and Igf2 DMR1 (Figure S6B) show low DNA methylation levels while Igf2 DMR2 remains largely methylated (Figure S6C). We conclude that ectopic 91H and H19 RNA expressions do not convincingly change DNA methylation patterns observed in untransfected H19 KO myoblasts.

Finally, bisulfite-sequencing experiments confirmed that H19 KO myoblasts are indeed very poorly methylated on the H19 ICR (Figure S6E) and that the transfected H19 KO myoblasts that displays the highest Igf2 expression level (clone 4) is not re-methylated at the H19 ICR (Figure S6F). Oppositely, the H19 ICR is highly methylated in control myoblasts (+/+ and C2C12 cells) (Figure S6D and S6G). These results clearly indicate that Igf2 trans-activation by ectopic 91H RNA occurs without H19 ICR re-methylation.

Igf2 Trans-activation by Ectopic 91H RNA Occurs Through Up-regulation of a Novel Igf2 Promoter

Since Igf2 trans-activation by ectopic 91H RNA can occur without H19 ICR re-methylation, we decided to investigate in
Figure 5. Nuclear Run-on experiments. (A) Autoradiographies of nuclear Run-on experiments on transfected and untransfected H19 KO myoblasts. Nuclear Run-on experiments were performed as previously described [38] and the α32P UTP labelled transcripts were hybridized on filters to denatured plasmids containing the insert DNA of genes indicated on the figure. 91H/H19 transcription was assayed using an insert corresponding to the H19 sequence and Igf2 with a genomic 2.4 kb BamHI-BamHI DNA fragment encompassing the exon 4-exon 6 region. Such nuclear run-on experiments were performed on undifferentiated (ND) and differentiated (D) cells either on the whole hygromycin-resistant transfected H19 KO myoblast cell population ("Whole") and transfected clones. (B) The same filters as those used for the autoradiographies shown in A were used for PhosphorImager quantifications. The ectopic 91H/H19 transcription levels (open bars) were compared to the endogenous Igf2 transcription levels (black bars). For each hybridized filter, the relative transcription levels were determined for each gene by normalizing to the Gapdh transcription level. doi:10.1371/journal.pone.0037923.g005
Figure 6. Comparison between the endogenous Igf2 transcription levels and the steady state levels of the ectopic RNAs. In these graphs, we compared, for each transfected H19 KO clones, Igf2 transcription data shown in Figure 5B with the steady state 91H and H19 ectopic RNA levels shown in Figure 4B. (A) Igf2 transcription versus 91H RNA levels. (B) Igf2 transcription versus H19 RNA levels. In untransfected H19 KO myoblasts (−/−), both 91H and H19 are not expressed (RNA levels = 0) and Igf2 transcription level is below the “empty plasmid” background (see Fig. 5A) which is inferior to 0.11. (C) Igf2 transcription level versus the ratio of 91H/H19 RNA levels. Clones expressing large amount of ectopic H19 RNA (clones 4, 11 and 12ND, black diamonds) (Figure 4B) were analysed separately from the others (ratio of 91H/H19 RNA levels > 0.2; open diamonds). In clones expressing high H19 RNA levels (black diamonds), the ectopic H19 RNA level relative to the 91H RNA level (which leads to a decrease of the 91H/H19 RNA ratio) is inversely proportional to Igf2 transcription levels ($R^2 = 0.8173$).

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more detail regulation of Igf2 mRNA expression in transfected clones. Surprisingly, promoter usage analyses showed that the strongest P2/P3 Igf2 promoters were not up-regulated in transfected H19 KO myoblasts (Figure 7A, left panel). Since, the P0 and P1 promoters were not significantly expressed (data not shown), we hypothesized that Igf2 up-regulation may occur by activation of an unknown Igf2 promoter. To assess this possibility, we performed 5’ RACE experiments in clone 4 that displays the highest Igf2 transcriptional activity (see Figure 5B). We identified a novel capped Igf2 mRNA which contains a new exon which is 141 kb long and is spliced to the exon 4 common to all known Igf2 mRNAs (Figure 7B/C). This new transcript is initiated from a novel TSS located in the DMR1 (position chr17: 149,852,285 on mouse July 2007 mm9 Assembly). Using primers specific of the new Igf2 exon, we determined the levels of this novel Igf2 mRNA in different mouse tissues and showed that, while being poorly abundant, it is more expressed in mesodermic tissues (kidney, tongue and heart) than in liver or brain. We therefore called it “mesodermic” promoter (Pm) (Figure 8A).

We then analysed the Pm transcript in all our myoblastic cell lines. It turns out that this mRNA, like the other Igf2 transcripts, was down-regulated in the H19 KO myoblasts compared to control myoblasts (+/-) (Figure 8B). Remarkably, opposite to all the other Igf2 transcripts, it was trans-activated in all transfected H19 KO myoblasts except clone 11 which displays the lowest DMR2 methylation levels in addition to ICR hypomethylation (see Figure S6C and S6A respectively). Globally, the mean Pm Igf2 mRNA level showed 10 fold up-regulation in transfected clones compared to untransfected H19 KO myoblasts and reached P3 Igf2 mRNA levels (Figure 7A, right panel). Therefore, we conclude that Igf2 trans-activation by ectopic 9I1H RNA occurs essentially through up-regulation of the Pm Igf2 promoter.

Discussion

We recently contributed to show that, in the human, a large antisense H19 transcript (9I1H RNA) regulates Igf2 mRNA levels [34] whereas, in the mouse, the H19 RNA is a negative trans-regulator of Igf2 mRNA levels [33]. In the present work, we derived an H19 KO myoblast cell line from mice carrying a deletion of the H19 transcription unit (H19Δs) [36] in which the Igf2 gene is repressed. Remarkably, loss of Igf2 transcription in H19 KO myoblasts correlates with a loss of H19 ICR methylation. It is therefore very likely that the CTGF protein binds to the ICR on both parental alleles leading to an almost complete insulation of the regular P2/P3 Igf2 promoters from the enhancers. Using a genetic complementation approach (reintroduction of the H19 sequence in H19 KO myoblasts), we investigated steady-state levels and halves-lives of ectopic 9I1H and H19 RNAs, as well as endogenous Igf2 transcriptional activity, and we show (i) that strong ectopic expression of antisense H19 transcripts synthesized from the enhancer 2 region can release Igf2 silencing in mouse myoblasts (ii) that this Igf2 reactivation takes place at the transcriptional level by targeting a previously unknown Igf2 promoter and (iii) that a large amount of ectopic H19 RNA can counteract Igf2 trans-activation by ectopic 9I1H RNA. Strikingly, we show that trans-activation of this novel Pm Igf2 promoter occurs without H19 ICR re-methylation indicating that this promoter is able to by-pass the insulator function of the unmethylated ICR. It thus remains possible that Pm activity also occurs on the maternal allele. This effect may potentially rely on the activity of the DMR2 that remains methylated in our experimental system (Figure S6C) and is known in mouse to favour Igf2 transcription on the methylated paternal allele [39]. This possibility would be reminiscent to some human pancreatic tumors like insulinomas where Igf2 DMR2 is hypermethylated while the H19 ICR is monoallelically methylated and where Igf2 becomes also expressed from the unmethylated maternal allele (loss of imprinting) [40].

Altogether, our inactivation/complementation approach, in conjunction with other recent findings [33,34], reveals that the mouse H19 antisense RNA favours Igf2 transcription and activates the Igf2 Pm promoter while large amounts of the H19 transcript counteract this effect, suggesting that these two transcripts are antagonist trans riboregulators (Figure 9). Therefore, in cells like the C2C12 myoblasts where we observe very low amounts of 9I1H RNA and large amounts of H19 RNA, one can expect that the endogenous H19 RNA exerts a strong Igf2 transcriptional repression at least on the Pm promoter.

Our experiments also agrees with the pioneer work by Wilkin et al. which suggested that, in human, a partial H19 cDNA construct could activate Igf2 when expressed in the antisense orientation while H19 RNA can repress transcription from the Igf2 P3 promoter [37]. However, at that time, the endogenous antisense H19 RNA was unknown and its effect in this work remained enigmatic.

Our results reveal a functional relationship between H19 and 9I1H RNAs. Consequently, depending on the cell context, the functional relevance of the H19 transcriptional unit for Igf2 gene control will depend on the relative expression levels of the sense and antisense H19 transcripts. This finding is particularly relevant for a better understanding of the conflicting data obtained for H19 gene expression in cancer cells and tumours. Indeed, 9I1H RNA levels should be taken into consideration as this transcript is a good marker of tumourigenesis in breast cancer cells [34]. In summary, the 9I1H RNA could be assumed to be oncogenic by favouring Igf2 transcription while H19, which counteracts this effect, would act as a tumour suppressor [23]. Consistently, normal breast tissues display high H19 and very low 9I1H RNA levels, while the opposite is observed in cancerous breast tissues [34]. Interestingly, the effect of the 9I1H RNA on Igf2 derepression observed here in complementation studies, may explain the Igf2 derepression occurring in many tumours where 9I1H RNA was found to accumulate while the H19 gene is maintained in a repressed state [34]. One can note that an H19 antisense transcript called H19 opposite tumor suppressor (HOTS) was recently found in human [41]. It extends from 2.8 kb downstream of H19 to 1 kb upstream and is encoding a nucleolar protein which is not conserved in the mouse. An evolutionarily conserved microRNA miR675 has been also described in the H19 exon 1 [42,43]. Recently, this H19-derived miR-675 was shown to regulate tumor suppressor RB in human colorectal cancer favoring its progression [34]. An interesting possibility is that this miRNA may also be directly involved in controlling levels of 9I1H RNA. An open question is why, in vivo, so much H19 RNA would be required to produce this miRNA and to control such small amounts of 9I1H RNA? This may be due to the fact that the H19 RNA is mainly cytoplasmic while the 9I1H transcript is nuclear [34]. Therefore, only a small sub-set of nuclear H19 RNA may be involved in this process. Furthermore, the miRNA production does not appear to significantly affect H19 RNA levels and therefore this process should not interfere with other functions that the H19 RNA may have in the cytoplasm where it is known to localize with the polyosomes [38,45]. Alternatively, the opposite effects of the 9I1H and H19 RNAs on Igf2 transcription may occur through more indirect mechanisms involving for example the Igf2 DMRs.

Here, upon isolation of H19 KO mouse myoblasts, the Igf2 gene expression was strongly decreased after passages in cell culture probably due to the observed loss of H19 ICR DNA
Trans-Activation of Igf2 Transcription by 91H RNA

A

B

C

D

21751 5' cap-ATAGACCATTTGCTTGGTCTCTTCTTGTTGTGGAGTTCCCTAT
21801 GTGTTGCTCAAGACCAGCCAGCCAGTTGAGTGCTCTTCTTGGCAGGCAT
21851 CCAGCAACGAGCTGGAAGAGGCTGTTGGGAGCTCGAGAGAGAAGTAG
21901 G-GTACCAATGGGGAGCCGTCACCCCTTCAGCTACAC...

Splice Site Exon 4

SD SA

D

DMR1

P0 Pm P1 P2 P3

DMR2

Pgf2as C Pgf2as B

10 kb
methylation. It is formally possible that methylation levels have changed as a consequence of cell culture. Alternatively, this may also result from the deletion of the \(H19\) transcriptional unit. In the mouse mesoderm-derived tissues, and more particularly in the postnatal muscle, maternal inheritance of the \(H19\) deletion is known to lead to loss of \(Igf2\) imprinting and re-expression of this gene from the maternal allele [23,36]. In the physiological context of this tissue, the presence of other cell types, such as for example satellite cells, may strongly contribute to maintain normal \(Igf2\) levels by signalling through intercellular pathways which may control myoblast cell differentiation [46].

In the present study, we reactivated \(Igf2\) transcription in \(H19\) KO myoblasts by ectopic \(91H\) RNA expression without \(H19\) re-methylation. Although we could not investigate whether this reactivation is monoallelic or biallelic, both parental alleles are largely unmethylated since methylation levels are very low (Figure S6) indicating that \(Igf2\) reactivation could occur on both parental alleles.

It now would be of interest to modify in the animal the \(91H\) RNA levels independently of \(H19\) RNA levels, as performed above.
in the myoblast H19 KO cell line. Unfortunately, such an experiment is tricky to do in vivo since both RNAs possess identical expression patterns and are both produced from the maternal allele. Furthermore, the investigations should be performed at the transcriptional level (nuclear run-on assays) to exclude any potential post-transcriptional effects. Finally, a transgenic line that would display high ectopic 91H RNA but low ectopic H19 RNA has not yet been produced. Such a transgenic mouse strain would most probably in the sequence inverted in 91H mouse mutant. Indeed, Davis et al. [47] have shown that the H19 RNA is initiated downstream of the endodermic enhancers, most probably in the sequence inverted in mouse. However, we cannot rule out that the H19 antisense RNA expression may persist in Mnt mice by activation of some propitious transcription start sites. Therefore, it may be interesting to investigate 91H and H19 Pm transcript levels in this mouse mutant.

Since we previously demonstrated that the 91H RNA acts in trans on H19 mRNA levels in human cells, one could hypothesize that this RNA may act exclusively at the post-transcriptional level. The present work clearly demonstrates that this is not the case, and that the 91H RNA augments H19 expression by acting at the transcriptional level. This finding raises the question about the mechanisms involved in such a regulation. H19 transcriptional regulation is known to be controlled through long-range interactions between regulatory elements, such as the Differentially Methylated Regions (DMRs) and the enhancers located downstream of H19 [18,48,49]. We could therefore propose that the 91H RNA can up-regulate tissue-specific H19 transcription by contributing, directly or indirectly, to higher-order chromatin architecture of this locus. For example, it may favor interactions between the H19 gene and specific enhancers since our experiments show that, in myoblastic cells, the 91H RNA can reactivate the Pm H19 promoter which is used in mesodermic tissues. Alternatively, the 91H RNA could also titrate factors such as transcriptional repressors, targeting H19 as well as some other genes of the Imprinted Gene Network (IGN) [32]. Finally, the 91H RNA is produced in liver from the endodermic enhancers (Figure 2) that themselves control the H19 expression levels in cis [50]. It is also able to act in trans to control H19 RNA (Figure 5) and to up-regulate the Pm transcript (Figure 8). Therefore, this lncRNA appears as a novel important player for co-regulation of genes at the H19/H19 locus.

Materials and Methods

Ethics Statement

All experimental designs and procedures are in agreement with the guidelines of the animal ethics committee of the French “Ministère de l’Agriculture”. Our animal unit has been registered at the departmental office for population protections (Direction départementale de la protection des populations) at the “Hérault préfecture” (Agreement N°34-172-16). All the experimental protocols (mouse dissections) have been specifically approved by the inspector in charge of the veterinary public health from the same office at the “Hérault préfecture” (Agreement N°34-31).

Isolation of H19 KO Myoblasts

Primary cultures were prepared from the thigh muscles of H19+/− mice as previously described [51]. Primary cells (H19 KO and control paternal heterozygous myoblasts) were serially passaged for analysis. Using qPCR on genomic DNA we checked that, as expected, the isolated H19 KO myoblasts were devoid of the H19 transcription unit and that the Igf2 RNA was in an identical copy number in H19 KO myoblasts as in C2C12 myoblast cells, suggesting that no aberrant loss or duplication of chromosome 7 occurred in the H19 KO myoblast cell line (data not shown).

Cell Culture and Transfections

H19 KO (−/−) and control paternal heterozygous (+/−) myoblasts were cultured in DMEM/MCDB 1:1, containing 20% FCS and 2% Ultroser (Gibco). Cells were differentiated into myotubes upon 3 days of serum starvation. The 16 kb BamHI-BamH1I fragment corresponding to the H19 gene locus (Figure 1) was cloned into the pNot site of the pBluescript plasmid using appropriate linkers. The construct was digested with NotI and the insert was gel-purified before being co-transfected with a hygromycin-resistance plasmid into H19 KO myoblast cells using lipofectamin (Gibco) according to the recommendations of the manufacturer. Actinomycin D at a final concentration of 3 µg/ml was added to the cell culture medium for the times indicated in figure legends.

RNA Isolation, Northern-blot and RT-qPCR Analyses

Total RNA was isolated from mouse tissue samples or from myoblastic cells by the guanidinium thiocyanate procedure as previously described [30]. Non-polyadenylated RNAs were prepared by using the PolyA Tract mRNA isolation system III® (Promega). The Igf2 and H19 RNAs were analysed in Northern-blot as previously described [52]. Reverse transcriptions and real-time quantitative PCRs were performed as previously described [34,33] using a qPCR mix described in Lutftalla et al. [54] with some modifications given in Court et al. [55]. The Igf2 steady-state mRNA levels were quantified using a PCR amplicon which targeted the messenger RNA (for primer sequences see Table S1). The ectopic H19 RNA levels were quantified using primers

**Figure 9. Model of regulation of Igf2 transcription by 91H and H19 RNAs in myoblastic cells.** This model is based on the three critical parameters that we have quantified in this work: the Igf2 transcription levels, the 91H and H19 steady state RNA levels. 91H and H19 RNAs are direct and/or indirect antagonists riboregulators of Igf2 transcription. H19 appears as having a negative effect on Igf2 transcription while 91H RNA has an opposite effect. Interestingly, 91H RNA stimulates a new Igf2 promoter (Pm) located within the Igf2 Differentially Methylated Region 1 (DMR1).

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located at H19 exon-exon junctions while the ectopic 91H transcript was quantified either in the intergenic region between the endodermic enhancers and the H19 gene (mC, mD, mE, mF and mj PCR amplicons) or within the H19 introns (mI1, mI2 and mI3 PCR amplicons) (Figure 1 and Table S1). Indeed, since the levels and the half-lives of the RNAs quantified by the intronic PCR amplicons are similar to those quantified by the mC PCR amplicon, we assume that the H19 intron sequences essentially account for the ectopic 91H RNA in transfected H19 KO myoblasts (Figure S3). Throughout this work RNA levels determined by RT-qPCR were expressed relative to Gapdh mRNA levels. Igf2 promoter usage was assessed by quantifying transcripts on each promoter-specific exon (first exons).

5’RACE

Rapid Amplification of 5′ complementary DNA Ends (5’RACE) was performed on non-polyadenylated d7 mouse liver RNAs (endogenous 91H RNA) or transfected H19 KO myoblast RNAs (clone 4) (ectopic 91H RNA and endogenous Igf2 Pm transcript) according to manufacturer’s instructions (GeneRacer Kit from Invitrogen ref. L1502). RT and nested PCR primer sequences are given in Table S1.

Transgene Copy-number Determination

Transgene copy-numbers were determined by qPCR relative to the endogenous Igf2 gene.

Nuclear Run-on

Isolation of nuclei and nuclear run-on experiments were performed as previously described [38,53].

DNA Methylation Analyses

Each sample was digested by the StyI restriction enzyme (20 units) to eliminate potential PCR bias due to the reduced accessibility of primers on undigested genomic DNA [56]. For H19 ICR methylation analyses (CTCF site 2), half of each samples was then additionally digested by the BceAI methylation-sensitive enzyme (4 Units/reaction) and qPCR quantifications were performed on BceAI-digested and undigested fractions after normalization against a loading control (242C19 primer pair). A similar approach was followed using the methylation-dependent McrBC enzyme to determine methylation levels in the Ig-DMR (Dlk1/Gtl2 locus on mouse chromosome 12) [57] or methylation-sensitive enzymes (NaeI for Igf2 DM1 and HpaII for Igf2 DM2). Methylation levels of the CpG residues studied for the H19 ICR and Igf2 DM2s are known to be representative of DNA methylation levels of the whole DMRs [52]. Primer sequences are available in Table S1.

Bisulfite Treatments

Genomic DNA was prepared from myoblastic cells and conversion with sodium bisulfite was performed with the Epitect kit (Qiagen) following the manufacturer’s instructions. PCR fragments were cloned using a PCR cloning Kit from Qiagen. Clones with strictly identical patterns of conversion were removed from the results (since they are likely to represent identical molecules). We used the MethPrimer software to design primers on bisulfite treated DNA. Primer sequences are available in Table S1.

Supporting Information

Figure S1 Differentiation of H19 KO myoblast cells. The figure shows pictures of the H19 KO myoblasts under the optical microscope (20× enhancement) during the myogenic differentiation process (ND = undifferentiated; d1, d2 and d3 correspond to 1, 2 or 3 days of differentiation). The transcriptional levels of the myogenin, a myogenic marker, are up-regulated during differentiation of H19 KO myoblast cells with the same amplitude (6–7 fold) as observed in C2C12 myoblasts (data not shown).

(TIF)

Figure S2 Quantifications of the intact or truncated endogenous 91H RNA levels (A) and of Igf2 mRNA (B) relative to gapdh mRNA levels in myoblast cell lines. (A) Comparison between the intact (black bars) and truncated (grey bars) endogenous 91H RNA levels determined by RT-qPCR in C2C12 myoblasts and H19 KO myoblasts respectively. (B) Quantification of Igf2 mRNA levels during differentiation of H19 KO myoblasts (late passage cells) (ND = undifferentiated; D = differentiated). One can note that, as observed for the endogenous truncated 91H RNA (Figure S2A), the low Igf2 levels observed in H19 KO myoblasts were strongly up-regulated (by at least 20-fold) during myogenic differentiation (Figure S2B). This suggests that the H19 transcription unit is dispensable to Igf2 up-regulation processes observed during myogenic differentiation.

(TIF)

Figure S3 Characterisation of TSS of the ectopic mouse 91H RNAs. 5’RACE experiments were performed on total capped RNA from transfected H19 KO myoblasts (clone 4). (A) Map of the enhancer region showing the primers used for RT and PCR reactions. The RT was initiated from the forward primer of PCRa. (B) Ethidium bromide staining of an agarose gel showing PCR products obtained from amplifications as indicated in Figure 2B (MW: Molecular Weight). Sequencing of PCRs product showed that the band corresponds essentially to unspliced amplification while PCRb correspond to the major TSS of the 91H RNA (position chr7:149,755,206 or chr7:149,755,207 on mouse July 2007 mm9 Assembly) and PCRe contains two minor TSS initiated within the endodermic enhancer 2 sequence upstream of the major TSS. These minor TSS could be identified in this experiment probably because ectopic 91H RNA is overexpressed compared to its endogenous counterpart. (C) The sequence of the endodermic enhancer 2 is indicated in bold. The positions of the minor and major TSS are indicated by black arrows. Due to the presence identical nucleotidic sequences at the end of the GeneRacer RNA oligonucleotide primer and at the TSS, the exact position of the major and one minor TSS remain ambiguous.

(TIF)

Figure S4 Endogenous vs ectopic 91H/H19 RNA half-lives. (A) Stability of the endogenous 91H and H19 RNAs in C2C12 myoblasts. C2C12 myoblast cells were treated with Actinomycin D and relative RNA levels were determined by real time RT-qPCR at the indicated times (in hours). Data were normalized to Gapdh expression levels. H19 (H19 RNA PCR amplicon), 91H (RT-qPCR quantifications with the mC PCR amplicon) and H19 precursor (intron 2, mI2 PCR amplicon) RNA levels are shown. Note that the half-life of the 91H RNA (middle panel) is similar to that of an unspliced H19 precursor RNA (right panel). (B) Stability of the ectopic 91H and H19 RNAs were determined in transfected H19 KO myoblasts using the same PCR amplicons as above. The whole hygromycin-resistant transfected H19 KO myoblast cell population was treated with Actinomycin D as described above and the ectopic H19 and ectopic 91H RNA levels were quantified as indicated above. Note that the ectopic 91H RNA appears to be more stable than the endogenous 91H transcript in C2C12 cells (compare Figure S4A with Figure S4B). This may be due to the
1000-overexpression of the ectopic 9I1H RNA found in transfected H19 KO myoblasts relative to the endogenous levels observed in C2C12 cells (Figure 4B, compare right and left panels). Since, in transfected KO myoblasts, the ectopic 9I1H RNA is found in similar amounts as the ectopic H19 RNA (Figure 4B, left panel) despite its low stability (Figure S4B), we should conclude that ectopic H19 transcription is much higher than that of the ectopic H19.

**Figure S7** RT-qPCR quantifications of 9I1H RNAs. Note that, in C2C12 cells, quantifications for the m1-m3 PCR amplimers (blue bars) account for the endogenous H19 precursor RNA level but not the endogenous 9I1H transcript which is much lower as shown using the mC’ PCR amplicon (red bar). In the opposite, in transfected H19 KO myoblasts, quantifications using the m1-m3 PCR amplimers, as well as with the mC’ PCR amplicon, account for the ectopic 9I1H RNA level which is very high. The 9I1H RNA levels shown in Figure 4B corresponds to the mean of quantifications using mC’ and m1-m3 PCR amplimers.

**Figure S5** DNA methylation patterns of H19 ICR and Igf2 DMRs. Methylation patterns were analysed in control (+/−) and H19 KO (−/−) myoblasts after 40 passages and in transfected clones, 3 passages after clonal isolation. The methylation pattern of the H19 ICR (A), the Igf2 DMR1 (B) and Igf2 DMR2 (C) were estimated by digestion of the genomic DNA with methylation-sensitive restriction enzymes (BceAI, NacI and HpaII for ICR, DMR1 and DMR2 respectively) and quantifications by qPCR. Noteworthy, this BceAI site encompasses CpG dinucleotides from DMR1 and DMR2 respectively) and quantifications by qPCR. Levels shown in Figure 4B corresponds to the mean of quantifications using mC’ and m1-m3 PCR amplimers.

**Figure S6** DNA methylation patterns of H19 ICR and Igf2 DMRs. Methylation patterns were analysed in control (+/−) and H19 KO (−/−) myoblasts after 40 passages and in transfected clones, 3 passages after clonal isolation. The methylation pattern of the H19 ICR (A), the Igf2 DMR1 (B) and Igf2 DMR2 (C) were estimated by digestion of the genomic DNA with methylation-sensitive restriction enzymes (BceAI, NacI and HpaII for ICR, DMR1 and DMR2 respectively) and quantifications by qPCR. Noteworthy, this BceAI site encompasses CpG dinucleotides from CTCF site 2 of the H19. Error bars represent s.e.m. of independent digestions. Methylation patterns of the H19 ICR around CTCF site 2 was determined by bisulfitesequencing in control (+/−) D, H19 KO (E), clone 4 (F) and C2C12 (G) myoblasts. Black and white circles indicate methylated and unmethylated CpGs respectively.

**References**


42. Cai X, Cullen B (2007) The imprinted H19 noncoding RNA is a primary