H19 acts as a trans regulator of the imprinted gene network controlling growth in mice

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The imprinted H19 gene produces a non-coding RNA of unknown function. Mice lacking H19 show an overgrowth phenotype, due to a cis effect of the H19 locus on the adjacent Igf2 gene. To explore the function of the RNA itself, we produced transgenic mice overexpressing H19. We observed postnatal growth reduction in two independent transgenic lines and detected a decrease of Igf2 expression in embryos. An extensive analysis of several other genes from the newly described imprinted gene network (IGN) was performed in both loss- and gain-of-function animals. We found that H19 deletion leads to the upregulation of several genes of the IGN. This overexpression is restored to the wild-type level by transgenic expression of H19. We therefore propose that the H19 gene participates as a trans regulator in the fine-tuning of this IG in the mouse embryo. This is the first in vivo evidence of a functional role for the H19 RNA. Our results also bring further experimental evidence for the existence of the IGN and open new perspectives in the comprehension of the role of genomic imprinting in embryonic growth and in human imprinting pathologies.

KEY WORDS: H19, Igf2, Transgenic mouse models, Imprinted gene network (IGN), Genomic imprinting, Non-coding RNA

INTRODUCTION

The H19 gene was cloned 20 years ago (Pachnis et al., 1988) and was one of the first imprinted genes to be identified. Genomic imprinting is an epigenetic mechanism that leads to parent-of-origin specific monoallelic expression. Thus, H19 is expressed from the maternal allele in mouse and human (Bartolomei et al., 1991; Zhang and Tycko, 1992). The H19 RNA is transcribed by RNA polymerase II, capped, polyadenylated and spliced, but it lacks conserved ORFs between human and mouse (Branman et al., 1990). Its complex conserved predicted structure suggests that the functional product is a non-coding RNA (Juan et al., 2000). In addition, a microRNA (miR-675) has been recently described in the first exon of the gene (Cai and Cullen, 2007; Mineno et al., 2006). Both the full-length RNA and the miRNA have been identified in marsupials, suggesting a strong functional conservation throughout evolution (Smits et al., 2008). H19 is strongly expressed during mouse embryogenesis in mesoderm and endoderm-derived tissues. Its expression is then fully repressed after birth and is found only in skeletal muscle and heart in adults (Poirier et al., 1991). This expression pattern is similar to that of the Igf2 (insulin-like growth factor 2) gene, which is paternally expressed and encodes a major fetal growth factor (DeChiara et al., 1991).

Imprinted genes are associated with differentially methylated regions (DMRs), which are involved in the regulation of their expression. The H19 and Igf2 genes are linked on the distal part of mouse chromosome 7 and on the human 11p15.5 region (Zemel et al., 1992). The DMR located between 2- and 4-kb upstream of the H19 gene is the imprinting control region (ICR) of the locus, as its deletion affects both H19 and Igf2 expression (Thorvaldsen et al., 1998). This sequence binds the insulator protein CTCF on the unmethylated maternal allele, thereby creating a boundary between the downstream enhancers and the Igf2 maternal allele (Hark et al., 2000).

In order to decipher the role of the H19 non-coding RNA, two knock-out models were established. In the first model, referred to as H19Δ13, the 3-kb transcribed region and the 10 kb upstream of that region were deleted. In the second model, referred to as H19Δ3, the 3-kb transcription unit only was deleted. In both models, the maternal heterozygous H19mat/+ mice, which do not express the RNA, are viable, fertile and present an overgrowth phenotype. In H19Δ13 mice, the maternal Igf2 allele is totally reactivated in all expressing tissues because of the deletion of the ICR (Leighton et al., 1995). In H19Δ3 mice, expression of the maternal Igf2 allele is 25% that of the paternal allele in wild-type mice, and is only observed in mesoderm derived tissues (skeletal muscle, tongue, diaphragm and heart) (Ripoche et al., 1997; Yoshimizu et al., 2008). The reactivation of the maternal Igf2 allele in both H19 targeted models is most probably due to a cis effect of an altered chromatin structure rather than to the lack of the H19 RNA (for a review, see Gabory et al., 2006).

Different studies have lead to proposals of a role for the H19 RNA itself. First, maternal transmission of the H19Δ13 or Δ3 deletion leads to modification of the methylation pattern of the Igf2 gene DMRs in cis with hypermethylation of the maternal allele, but also in trans with hypomethylation on the paternal allele (Forne et al., 1997). Second, H19 was found to be associated with polysomes in different human and mouse cell lines (Li et al., 1998; Milligan et al., 2000). Finally, after transfection of human DiHepG2 cells, H19 was suggested to participate in Igf2 protein repression, in part through transcriptional regulation (Wilkin et al., 2000).

Another clue for the function of the H19 gene is its implication in tumorigenesis. H19 cDNA transfection into a cancer cell line results in loss of clonogenicity of the cells and reduced tumorigenicity in nude mice (Hao et al., 1993). Moreover, H19/Igf2 locus imprinting is lost in Wilms’ tumor resulting in Igf2 biallelic expression and total...
repression of H19 (Dao et al., 1999; Frevel et al., 1999). Both observations suggest a tumor suppressor effect of H19. However, other in vitro studies suggest that H19 acts as an oncogene and is activated by the c-myc transcription factor (Barsyte-Lovejoy et al., 2006; Matouk et al., 2007). Finally, we recently showed that H19 acts in vivo as a tumor suppressor in three different mouse models (Yoshimizu et al., 2008). Targets of this tumor suppressor remain to be identified.

We decided to study the potential role of the H19 RNA in vivo. We created a new H19 transgenic line (Tg24) (Fig. 1A) and used the previously described YZ8 one-copy YAC line (Fig. 1B) (Ainscough et al., 1997). These lines were bred onto an H19Δ3 background. Our aim was to rescue the H19Δ3 phenotype with the H19 transgenes in order to observe a trans effect of the RNA, independently of the cis effect observed on maternal Igf2 transcript. We found that the overgrowth phenotype observed in H19Δ3 animals compared with wild-type littermates is not present in H19Δ3 mice expressing the transgenes, thus suggesting a rescue due to transgenic expression of H19 RNA. We identified Igf2 mRNA as one of the targets of H19. We then performed an extensive analysis of the expression of genes belonging to the recently described imprinted gene network (IGN) (Varrault et al., 2006). Our results indicate that alteration of the H19 dosage modulates the expression of several other imprinted genes in this network, suggesting that H19 acts as a trans regulator of the IGN.

MATERIALS AND METHODS

Transgene construction

We created several transgenic lines expressing the H19 RNA under the control of the neclidin (Ndn) gene promoter. Ndn is expressed in some non-neural tissues (somites, tongue, axial muscles), with a more specific expression in most postmitotic neurons of the central and peripheral nervous systems (Andrieu et al., 2003). A genomic 1.5-kb SacI fragment containing the Ndn promoter (D76440) was isolated and inserted in the SacI site of the pGEM3Zf(+) vector. A 4.3-kb DraIII genomic fragment containing the H19 gene (AF04091) was further introduced in the Smad-linearized Ndn promoter-pGEM3Zf(+) vector (Fig. 1A). The Ndn promoter-H19 cassette was then excised by EcoRI and SalI digestion and introduced in an EcoRV/SalI-linearized pBS vector. The upstream 2.3-kb EcoRI Ndn genomic fragment (containing conserved motifs between mouse and human) was inserted in the EcoRI site of this latter vector (Kuo et al., 1995). Orientation of this fragment was determined by PCR amplification. The Ndn promoter region was sequenced in order to avoid any mutation in this region. The hybrid 6.5-kb Ndn-H19 mini-transgene was excised from the pBS vector by a NotI/SalI digest and agarose gel purified. The DNA was further purified on an ELUTIP-D column (Schleicher & Schuell, Germany), as described in the manufacturer’s protocol, and was resuspended in injection buffer [10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA (pH 8), 100 mM NaCl] at a final concentration of 1 μg/μl and microinjected into the pronucleus of C57BL/6×CBA fertilized eggs. Founders were identified by Southern blot analysis after HindIII digestion of tail DNA using an Ndn PCR-amplified probe (primers N8 and N28; see below; Fig. 1A). Four founders were bred with C57BL/6×CBA females to establish the transgenic lines. Ectopic H19 expression was then checked by breeding transgenic males with H19Δ3 females. Only one line, subsequently named the Tg24 line, was kept, as the others displayed low or variigated expression of H19, as detected by in situ hybridization. Expression was found not only in the central nervous system, as was expected from the expression pattern of the endogenous Ndn gene, but also to be comparatively widespread, suggesting that the promoter region did not contain all of the regulatory control sequences present in the endogenous gene (see Fig. S1 in the supplementary material). Transgene copy number was determined by Southern blot analysis to be 30 copies (Fig. 1A).

H19Δ3, Tg24 and YZ8 one-copy YAC lines (Fig. 1B) were all maintained on a C57BL/6×CBA background and wild-type mice used for matings were on C57BL/6×CBA. The protocol of animal handling and treatment was performed in accordance with the guidelines of the animal ethics committee of the Ministère de l’Agriculture de France.

Genotyping

DNA was extracted from tail biopsies and PCR was performed according to the manufacturer’s instructions (Invitrogen). For the Tg24 line, primers N6 and N16, hybridizing to the Ndn promoter were used; a 1.5-kb transgenic fragment and a 2-kb Ndn endogenous fragment were obtained. For the YAC line, lacZ primers were used. neo primers were used to genotype the H19Δ3 allele in heterozygous animals. Primer sequences and annealing temperature conditions are described in the section below.

Primers

Primers for genotyping

Annealing temperatures are given in parentheses.

Ndn (58°C), GCCATTTATCTGAGAGAC (N8, sense) and CTCGCT-GAGACCCAGAG (N28, antisense); Ndn (52°C), GCTCTCATCTTATTAGGTTC (N6, sense) and GCAATATT- TTCTCTACAGAGC (N16, antisense); neo (66°C), GTGTTCCGCTGTCAGCGCA (sense) and GTCTCTGA-TAGCGTCCGCGCA (antisense); lacZ (55°C), CAGTTTACCCGCTTCGCTAC (sense) and GCGTTGG-CGAATTTAACCACC (antisense).

Primers for qPCR

Annealing temperatures are given in parentheses.

H19 (60°C), GGAGACTAGGCGAGGCTCTC (sense) and GCCATAG-GTGGTTCAAGAGGC (antisense); TBP (60°C), GCAATCAACATCTACAGCAC (sense) and CGCAAGTC-GAATTTAACCGCA (antisense).

Gene expression analysis

Collected tissues were disrupted using a Mixer Mill (Qiagen) and total RNA was extracted with TRIzol reagent (Invitrogen) and then re-extracted with phenol/chloroform and chloroform before ethanol precipitation.

For the expression profile analysis, we carried out reverse transcription with SuperScriptII (Invitrogen) on 500 ng of total RNA with random hexamer oligonucleotides. Quantitative PCR was performed for H19 and TATA-binding protein (TBP) genes on 1 ng cDNA in a final volume of 10 μl with FastStart Master Mix reagent in a Light-Cycler 2.0 system (Roche). Primer sequences are given above.

For the IGAN analysis, RNA was prepared from two animals per genotype from two different litters; therefore, four animals per genotype for each mating were analyzed. We carried out three independent reverse transcription experiments for each sample on 1 μg of total RNA. qPCR was performed on 2 ng cDNA in a Light-Cycler 480 system (Roche). The primers used to amplify the imprinted genes have been described previously (Varrault et al., 2006). The selection of appropriate housekeeping genes was performed with geNorm (Vandesompele et al., 2002). The level of expression of each imprinted gene X was normalized to the geometric mean of the expression levels of several housekeeping genes (Hprt2, Trf1 and Tubb2 for the transgenic line; Gus2, Mrpl32, Hprt2 and Tubb2 for the YAC line), according to the formula:

\[
X = \frac{\text{geometric mean of } (R1, R2, R3)}{\text{arithmetic mean of } (Ct(R1), Ct(R2), Ct(R3))}
\]

where Ct is the threshold cycle, and R1, R2, R3 are the three reference genes. Data were analyzed using a non-parametric Kruskal-Wallis one-way analysis of variance with Benjamini-Hochberg correction (FDR 5%) for multiple testing, as implemented in the MeV 4.2 package (www.tm4.org), followed by post-hoc paired comparisons.

Growth curve analysis

Newborn animals were marked at day 1 and weighed almost every day from birth to 4 weeks. Males and females were separated at 4 weeks and littermates of the same sex placed in the same cage in order to keep the same environment for the different genotypes during the rest of the experiment. The animals were then weighed twice a week up to 10 weeks. For each line, at least four litters were analyzed. Weights were plotted on growth curves and a two-factor (litter and genotype) ANOVA statistical analysis using StatView software was performed for each sex at each
point. The weight ratio of transgenic to control animals (H19^Δ3mat/+) or wild type) was calculated. This ratio was expressed as the percentage of transgenic animal weight versus control (Fig. 2B).

**RESULTS**

**Pattern of expression of H19 in transgenic animals**

We first performed a detailed analysis of H19 expression in both transgenic lines. Tg24 and YZ8 YAC mice were analyzed on the H19^Δ3 background, which meant that any resulting H19 expression was of transgenic origin only. These two genotypes were compared with wild-type mice. Northern analysis was performed on RNA from Tg24 and wild-type embryos (whole embryos or liver and limb muscle from E10.5 to 18.5, and placentas from E12.5 to 18.5; see Fig. S1A in the supplementary material). Placenta, liver and limb muscle from E14.5 embryos from both Tg24 and YZ8 transgenic lines were chosen to precisely evaluate the levels of transgenic H19 RNA by RT-qPCR (Fig. 1C). P5 neonatal pups were also dissected and brain, tongue, heart, lung, liver, kidney and muscle from posterior limbs were collected. RNA was prepared from these tissues and the level of expression of H19 was evaluated both by northern analysis (see Fig. S1B in the supplementary material) and by RT-qPCR (Fig. 1C'). In embryos (Fig. 1C), H19 was expressed in both transgenic lines and expression in posterior limb muscle was comparable to that in wild type. However, only low expression of H19 was detected in embryonic liver; this was in contrast to previously published in situ hybridization YAC data, which showed high expression in liver (Ainscough et al., 2000a). In the YAC line, expression in the placenta was similar to that in wild type, whereas the Tg24 placenta expressed only low amounts of H19 RNA.

In newborn Tg24 mice (Fig. 1C'), H19 was transcribed at a lower level than wild type in liver, tongue and muscle, and at a higher level than wild type in brain, lung, kidney and heart. The expression profile of the YAC line was consistent with previously described data (Ainscough et al., 2000b), with a notable lack of expression in lung, kidney and heart, and strong expression in liver, tongue and muscle. Based on these results, we focused on E14.5 embryonic muscle for further analyses.

**Phenotype of the transgenic lines**

As both transgenic lines expressed H19, we closely observed their phenotype. The H19^Δ3 mutant displays an overgrowth phenotype. We therefore performed a meticulous analysis of growth in both transgenic lines to observe whether transgenic expression of H19 could rescue the phenotype of the H19^Δ3 mutants. Mice were weighed for 10 weeks and weights were plotted on growth curves (Fig. 2A). At least four individual litters were followed over the 10-week period for each transgenic line. Each graph corresponds to the mean weight of these litters for each mating, females and males being separated. The ratio between transgenic and control genotypes for each sex was then calculated at each point from each litter. The average ratio for each mating is plotted on Fig. 2B. A ratio of 100% indicates that there is no difference between transgenic and control animals. We first compared the growth curves of H19^Δ3mat/+ and wild-type littermates. At birth, the H19^Δ3mat/+ mice have a significant 20% overgrowth phenotype, this overgrowth decreases to 4% at day 9, and then increases to 14% in the second week and remains stable (Fig. 2B).

H19^Δ3 females were mated with hemizygous Tg24 males in order to obtain H19^Δ3mat/+ and H19^Δ3mat+/Tg24 animals in the same litter. We observed a growth restriction of H19^Δ3mat/+;Tg24 animals compared with their control H19^Δ3mat/+ littermates (Fig. 2A). The difference in weight between the two phenotypes was evident not only in the animals after weaning (as seen in Fig. 2A), but also before, as assessed by ANOVA statistical test, with a significant
difference being observed during the first week ($P<0.05$). At birth, the $H19^{Δ3mat/+};Tg24$ mice weight was decreased by 11% compared with control $H19^{Δ3mat/+}$ littermates (Fig. 2B). Interestingly, the $H19^{Δ3mat/+};Tg24$ mice had a weight similar to that of control mice during the second week. Subsequently, the weight difference increased again and after weaning the $H19^{Δ3mat/+};Tg24$ mice presented an 11% decrease compared with control $H19^{Δ3mat/+}$ littermates. The opposite growth phenotypes of the $H19^{Δ3mat/+}$ and $H19^{Δ3mat/+};Tg24$ mice suggest that the transgenic $H19$ expression is able to rescue the $H19^{Δ3}$ overgrowth phenotype.

We then analyzed the YZ8 YAC transgenic line. As the YAC transgene is imprinted, it must be transmitted from the mother to obtain $H19$ transgenic expression. $H19^{Δ3mat/+};YAC$ females were first obtained and mated with wild-type males in order to obtain wild-type, $H19^{Δ3mat/+}$ and $H19^{Δ3mat/+};YAC$ littermates. Unfortunately, we observed a bias in the litters and obtained only a few $H19^{Δ3mat/+}$ animals. Therefore wild-type and $H19^{Δ3mat/+};YAC$ were used for the growth study; the growth curves for the single $H19^{Δ3mat/+}$ male and female obtained were plotted on the figure as an indication of the $H19^{Δ3mat/+}$ overgrowth phenotype. $H19^{Δ3mat/+};YAC$ mice were not different from wild-type littermates at birth. The ratio of the weight
of H19Δ3mat/+;YAC versus wild-type mice clearly showed that there was no weight difference between these animals (Fig. 2B). As H19Δ3mat/+ mice are bigger than wild type and as there was no weight difference between H19Δ3mat/+;YAC and wild type, we can conclude that the YAC transgene expression on a H19Δ3mat/+ background rescues the knock-out overgrowth phenotype.

Taken together, these growth curve analyses from two independent lines show for the first time that transgenic H19 expression leads to the recovery of normal growth on a H19Δ3mat/+ background. This suggests a role for the H19 RNA itself in the control of the growth process.

As transgenic mice were smaller at birth, we also checked embryonic growth from E12.5 to E18.5 (see Fig. S1C in the supplementary material). A growth reduction trend between transgenic and control embryos was seen starting at E16.5. We therefore chose to base our expression study at the earlier E14.5 stage, in order to investigate whether a misregulation of growth controlling genes was the cause of this trend.

Control of H19 expression in transgenic animals

Our aim was to identify targets of the H19 RNA. Mice were mated to obtain the different genotypes of interest in the same litters, thereby reducing effects due to genetic background heterogeneity. H19Δ3/+ females were mated with Tg24 males and H19Δ3+;YAC females were mated with wild-type males. Wild-type, H19Δ3+/Tg24, H19Δ3mat/+ and H19Δ3mat+/Tg24 littersmates, and wild-type, H19Δ3+;YAC, H19Δ3mat/+ and H19Δ3mat+;YAC littermates, respectively, were obtained (Fig. 3A). All transgenic mice carry a hemizygous allele of the Tg24 or YAC transgene.

In our experiments, we observed a bias in transgene transmission (Fig. 3B). In the Tg24 mating, we obtained only four H19Δ3+;Tg24 mice out of 31 animals from three litters, which corresponds to 13% of H19Δ3+;Tg24 littermates and wild type, H19Δ3mat+;Tg24 and H19Δ3mat+;YAC littermates described in Fig. 3A. RNA was prepared from two embryos per genotype from two different litters (indicated by an asterisk in Fig. 3B). RT-qPCR experiments (Fig. 4) revealed the expected increase in Igf2 mRNA levels in the H19Δ3mat/+ samples. Most interestingly, a significantly reduced expression of Igf2 was detected in both the Tg24 and YAC transgenic samples, with expression being restored to the wild-type level. This suggests that the H19 RNA itself is involved in the downregulation of Igf2 mRNA expression by a trans-acting process.

Imprinted gene network (IGN) analysis

In order to investigate whether Igf2 was the only target of H19 or if other genes were also affected, hemizygous Tg24 females were mated with Igf2+/− males. We compared the weight of transgenic and non-transgenic littermates and observed that the reduction in weight H19 expression was then checked by RT-qPCR in the four genotypes. Expression of the transgene was 79% for Tg24 and 88% for YAC on the H19Δ3mat/+ background (Fig. 3B). Therefore the few transgenic animals obtained on the wild-type background (Tg24 or YAC) would be expected to express H19 at 179% and 188%, respectively. Interestingly, expression reached only 131% for H19Δ3+/Tg24 and 115% for H19Δ3+;YAC animals (Fig. 3B), suggesting a bias not only in transgene transmission but also in total H19 expression. These results are in favour of an as yet unexplained control of the H19 RNA expression level during embryogenesis.

Igf2 expression analysis

The overgrowth phenotype of H19Δ3 mice was originally attributed to an overexpression of Igf2 in mesoderm-derived tissues (Ripoche et al., 1997; Yoshimizu et al., 2008). To investigate whether Igf2 expression could be directly affected by H19, we analyzed Igf2 expression in E14.5 embryonic muscle tissue (n=4 for each genotype) from the wild-type, H19Δ3mat/+ and H19Δ3mat+/;Tg24 littersmates and wild-type, H19Δ3mat/+ and H19Δ3mat+;YAC littermates described in Fig. 3A. RNA was prepared from two embryos per genotype from two different litters (indicated by an asterisk in Fig. 3B). RT-qPCR experiments (Fig. 4) revealed the expected increase in Igf2 mRNA levels in the H19Δ3mat/+ samples. Most interestingly, a significantly reduced expression of Igf2 was detected in both the Tg24 and YAC transgenic samples, with expression being restored to the wild-type level. This suggests that the H19 RNA itself is involved in the downregulation of Igf2 mRNA expression by a trans-acting process.
could still be detected between these two genotypes (Fig. 5). This indicated that Igf2 was not the only factor affected by the transgenic expression of H19. H19 and Igf2 have recently been reported to belong to an imprinted gene network (IGN) (Varrault et al., 2006). We decided to study whether a loss of H19 function could lead to the deregulation of other genes of the IGN and whether H19 transgenic expression could rescue this deregulation. We focused on embryonic muscle tissue in which the expression of the transgenes is the strongest (Fig. 1C). For both independent transgenic matings (Fig. 3A), data were generated from four E14.5 embryos for each genotype from two different litters. The results of the RT-qPCR experiments were analyzed with a Kruskal-Wallis one-way analysis of variance test with Benjamini-Hochberg correction and histograms are shown in Fig. 4. As the effect observed was independent of the expression level of each gene, we normalized all data to wild-type expression; a complete table is available in the supplementary material (see Table S1).

We were able to show that six out of twenty genes were significantly upregulated in H19Δmat/− mice and that five were rescued to wild-type levels in both H19Δmat/−;Tg24 (Fig. 4A) and H19Δmat/−;YAC embryos (Fig. 4B). The other genes affected besides Igf2 were Cdkn1c, which is located with H19 on the distal part of mouse chromosome 7, Gnas (chromosome 2), Dlk1 and Rtl1 (chromosome 12), and Igf2r (chromosome 17). Interestingly, the expression of two genes was not modified in either transgenic line: Gatm and Rian. Moreover, three genes were modulated in the YAC line but were not studied in the Tg24 samples: Dcn, Peg3 and Slc38a4. Finally, expression of Zac1, which was one of the first genes to be associated with the IGN when it was originally described, remained constant. To summarize, we observed an upregulation of expression of six genes in H19Δmat/− embryonic muscle tissue, and five of these were restored to the wild-type expression level in the embryos of the two H19 transgenic lines. In parallel, we also analyzed E14.5 placental RNA from the YAC samples (in which transgenic H19 RNA is expressed at a level similar to the endogenous level) and found that this IGN control was not present in this extra-embryonic tissue (see Table S2 in the supplementary material).
This detailed analysis not only uncovers an effect of the H19 gene deletion on the expression of several genes, but also demonstrates a trans effect of the H19 RNA on the mRNA levels of five genes in our two independent transgenic lines. This supports the finding that a functional IGN exists, and suggests that H19 acts as a regulator of this IGN in the embryo. This is the first in vivo evidence of a functional role for H19 RNA.

**DISCUSSION**

Our purpose was to use mouse models to explore in vivo the function of the H19 non-coding RNA. Targeted deletions of the H19 gene (H19<sup>Δ3</sup> and H19<sup>Δ3</sup>) have lead to overgrowth phenotypes, which can be explained by a cis effect of the H19 locus on the Igf2 gene, which becomes biallelically expressed (Leighton et al., 1995; Ripoche et al., 1997). It has, however, been difficult to separate chromatin configuration effects on the locus and a role for the H19 RNA itself. We therefore decided to produce transgenic animals to express the RNA outside of its own genomic context. We created several transgenic lines expressing the H19 RNA under the control of the Ndn gene promoter. We focused our study on one line, the Tg24 line, as the others showed either low expression of H19 or mosaicism. We added to our study the previously described YZ8 one-copy YAC transgenic line, with H19 being expressed under its own promoter (Ainscough et al., 1997). Because these two totally independent lines produced almost identical results, we can exclude any insertional effect due to the transgene. Furthermore, our genetic approach was designed to compare the different genotypes of interest in the same litter, thus limiting the variability of genetic background and maternal effect.

Our first observations lead to the conclusion that, on an H19<sup>Δ3/Δ3</sup> background, the H19 RNA was expressed correctly in both Tg24 and YAC lines, at a level similar to the endogenous level. However, on a wild-type background, overexpression of H19 only reached 30% at the most, suggesting a control of the total level of expression of H19. In addition, a strong bias was found in the transmission of the H19<sup>Δ3/Δ3;Tg24</sup> and H19<sup>Δ3/Δ3;YAC</sup> genotypes. Taking these data together with previous observations suggesting a possible deleterious control of the level of transgenic H19 expression (Ainscough et al., 2000a; Yoo-Warren et al., 1988), we postulate that one of the first targets of H19 RNA is the H19 gene itself.

Careful growth curve analysis clearly shows that the H19 transgenic expression on an H19<sup>Δ3mat/Δ3</sup> background is able, in both transgenic lines, to rescue the H19<sup>Δ3mat/Δ3</sup> overgrowth phenotype, and results in a phenotypical mirror effect between presence and absence of H19. Upon close analysis, we observed that Igf2 expression was reduced in both H19<sup>Δ3mat/+</sup>;Tg24 and H19<sup>Δ3mat/+</sup>;YAC embryos compared with H19<sup>Δ3mat/−</sup> embryos. This is the first direct evidence in vivo of an effect of the H19 RNA on Igf2 mRNA expression in trans.

Another clue for the implication of H19 in growth control is that it belongs to the IGN that controls embryonic and postnatal growth (Varrault et al., 2006). This IGN is composed of at least 16 co-expressed imprinted genes, with almost all of these inducing growth phenotypes upon targeted deletion (Fig. 6). A recent study suggests that some of these genes, and among them H19 and Igf2, are important for fetal and early postnatal growth, and that their downregulation contributes to growth deceleration in order to limit body size (Lui et al., 2008). We observed an upregulation of at least six imprinted genes in H19<sup>Δ3mat/+</sup> animals and a recovery of normal expression, comparable to that of wild type, for five of these genes in transgenic H19-expressing animals on a H19<sup>Δ3mat/+</sup> background. Interestingly, among the regulated genes, some are maternally expressed (Cdkn1c, Igf2r and Dcn) and others are paternally expressed (Igf2, Dlk1, Rtl1, Gnas, Peg3 and Slc38a4; see Fig. 6A).

Fig. 6. The imprinted gene network. (A) Maternally expressed genes are shown in red and paternally expressed genes in black. Grey lines represent connections between genes as predicted by the original meta-analysis and dotted black lines represent the link between genes found in the Zac1 study (Varrault et al., 2006). Black lines represent links from the present study. (B) H19 regulated genes from the IGN and their effect on growth according to targeted deletion experiments. Six of the regulated genes are paternally expressed and four of them have a positive growth effect. Three are maternally expressed and one has a negative effect on growth.
In agreement with the parental conflict theory (Moore and Haig, 1991), most of the paternally expressed genes have a growth-promoting effect, whereas most of the maternally expressed genes inhibit growth, as shown by their targeted deletion phenotype (Fig. 6B). Our mouse models support the functional existence of the IGN and are in favour of a role for H19 in its fine-tuned regulation. We hypothesize that by affecting one of the genes of the network, a fully coordinated up or downregulation of oppositely imprinted genes is triggered. This would produce a fine-tuned equilibrium between the interconnected genes of the network, resulting in a compensated growth control and harmonious embryonic development. In fact, our data show that the deregulation of the genes is modest in magnitude, with a maximum significant increase of ~35% for Igf2 and ~15% for Gnas, for example. This hypothesis could also explain the relatively mild phenotype of the H19Δ mice: although H19 is highly expressed during embryogenesis, targeted deletion of the gene does not result in lethality. This could be due to the triggering of a balance between growth-activating and growth-repressing genes belonging to the IGN.

Interestingly, we did not detect this regulation of the IGN in the placenta of the corresponding embryos, which suggests that regulatory mechanisms differ between extra-embryonic and embryonic tissues, as has been previously shown for X-inactivation or for the Cdkn1c locus (Wagschal and Feil, 2006). Further studies of the IGN in the placenta at different times in development might highlight other regulatory aspects of imprinting.

The functional evidence of this IGN raises the question of the mechanism of regulation between these genes. The transcription factor Zac1 is able to bind common enhancers of the H19 and Igf2 genes to promote their expression (Varrault et al., 2006). Because Zac1 is not affected in samples from our study, we can assume it acts upstream from H19. Whether several genes of the IGN are direct downstream targets for H19, or whether H19 acts on one gene of the network which then triggers a cascade of events that act on other targets remains to be elucidated. Interactions between imprinted genes have been previously reported, although mechanisms are unclear. A link between Igf2 and Cdkn1c has been described, because an increased circulating Igf2 peptide level in Igf2Δ mice leads to a decrease of Cdkn1c gene expression (Grandjean et al., 2000). In addition, Igf2r is a negative regulator of the insulin and IGF signalling pathway (Smith et al., 2006; Wang et al., 1994).

We could therefore propose that, in H19Δ mice, the increased Igf2 expression might be compensated by Igf2r overexpression as a response to maintain overall homeostasis.

Whether a similar IGN exists in human might be of interest to understand some imprinting pathologies. The H19/IGF2 locus is implicated in both Beckwith-Wiedemann syndrome and Silver-Russell syndrome (SRS) (Gicquel et al., 2005). In 60% of SRS patients, hypomethylation of the ICR is observed (Netchine et al., 2007) and could lead to a loss of imprinting of the locus, with loss of IGF2 expression and H19 overexpression. However circulating IGF2 peptide levels are found within normal values. Therefore, H19 overexpression itself could be involved in this syndrome. Other SRS (10%) patients show maternal disomy of chromosome 7, which carries two imprinted loci with Grb10 and Sgec, and Peg10, Mest and Cyp2 genes, respectively (Eggermann et al., 1997; Preece et al., 1997). Although our study did not show a significant modulation of Grb10 or Mest mRNA levels, exploring H19 RNA and IGN expression in SRS patients might help to gain an understanding of how these three loci, which contain functionally different genes, can be linked to the same SRS syndrome.

In our models, we show that H19 can act in trans to regulate genes of the IGN, which suggests an effect of the RNA itself. This regulation could occur at either the transcriptional or the post-transcriptional level. Two major features arise from an evolutionary conservation study of the H19 gene: the exon-intron structure of the gene and the precursor of the microRNA miR-675 are conserved, suggesting a role for both the full-length spliced transcript and for the processed miRNA (Smits et al., 2008). The full-length RNA could possibly induce a transcriptional or post-transcriptional control of IGN genes through interactions with chromatin-modifying proteins or with as yet unknown RNA-binding proteins. The post-transcriptional control could also be mediated by miR-675. By binding their mRNA targets in an miRISC complex with an imperfect match, microRNAs are known as translational repressors but they can also lead to mRNA degradation or sequestration in P-bodies (Chu and Rana, 2007). MiR-675 could therefore act directly on the microRNA levels found to be regulated in our mouse models or could indirectly control mRNA stability or the translation of a common regulator. Further experiments will be required to distinguish between the effects of the full-length H19 RNA and miR-675.

In summary, we have shown with two independent transgenic lines that the H19 non-coding RNA can act as a trans regulator not only on Igf2, but also on several other imprinted genes belonging to an interconnected network involved in the control of growth and survival of mouse embryos. Although the direct or indirect mechanisms involved in this regulation are not yet understood, this opens up new perspectives on the regulatory aspects of genomic imprinting and its function in development and in human pathologies.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/20/3413/DC1

References

H19 is a regulator of IGN

RESEARCH ARTICLE


