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Silencing by the imprinted *Airn* macro lncRNA

Transcription is the answer

Federica Santoro and Florian M. Pauler*

CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences; Vienna, Austria

Abbreviations: ESC, embryonic stem cell; lncRNA, long non-coding RNA; polyA, polyadenylation; TSS, transcription start site

The majority of the mammalian genome is transcribed into long non-coding (lnc) RNAs, transcripts longer than 200 nucleotides and devoid of protein-coding potential. Recent genome-wide mapping efforts led to the identification of thousands novel lncRNAs, but so far only few have been functionally analyzed.[1] Fifteen years ago, our group discovered *Airn* (antisense to Igf2r RNA non-coding), the first autosomal lncRNA shown to have a silencing function.[2] *Airn* is an imprinted gene transcribed from the paternal chromosome, in antisense orientation to the imprinted but maternally expressed *Igf2r* gene. *Airn* expression controls *Igf2r* imprinted expression by silencing it in cis on the paternal chromosome,[2] but how *Airn* exerts its silencing function has long been a mystery.[3,4] Now, a study published in *Science* shows that *Airn* silences *Igf2r* through transcription alone and not via its RNA product.[5]

The road leading to this discovery was long and winding. When we first identified *Airn* and deleted its expressed, paternal promoter, we observed de-repression of the normally silent paternal *Igf2r* allele.[6] After this landmark experiment, three hypotheses were formulated to explain how *Airn* may silence *Igf2r*:[3] (1) the *Airn* promoter competes with the *Igf2r* promoter for common transcription factors or enhancers; (2) the *Airn* lncRNA coats the paternal chromosome, inducing its heterochromatization; (3) *Airn* transcription through the *Igf2r* promoter interferes with its expression. The promoter competition model was ruled out some years later by an experiment that prematurely terminated the 118 kb-long *Airn* transcript by inserting a polyadenylation (polyA) cassette 3 kb after its transcription start site (TSS).[2] Similarly to the *Airn* promoter deletion, the *Airn* truncation relieved paternal *Igf2r* silencing, indicating that either the *Airn* transcript or its transcription through the locus, but not the *Airn* promoter, is needed to silence *Igf2r*.

At that time, no examples of transcriptional interference had been reported in mammals. The lncRNA field was dominated by one exemplary cis-silencing transcript, the *Xist* lncRNA, which coats one X chromosome in female mammals and recruits chromatin-modifying repressor complexes to inactivate it. We reasoned that if *Airn* silences *Igf2r* in an *Xist*-like fashion, broad domains of allele-specific repressive chromatin should cover the silenced chromosomal region. However, when we analyzed the distribution of repressive histone marks at the *Igf2r* locus, we found no broad heterochromatic domains, but only focal enrichment at the silent paternal *Igf2r* promoter.[7] If *Airn* does not behave like *Xist*, might then mere transcription through the *Igf2r* promoter, rather than the transcript, be the answer?[4] The transcriptional interference hypothesis, by implying no role for the RNA product, would also account for some unusual, "macro" lncRNA-like features of *Airn*, which, unlike *Xist*, is a predominantly unspliced, repeat-rich and highly unstable transcript.[4]

We therefore set out to test our hypothesis, reasoning that if transcription alone is important, then no *Airn*-specific sequences should be required to silence *Igf2r*. To perform genetic manipulations of the *Airn* locus and monitor their effects on *Igf2r* imprinted expression during embryonic development, we established an embryonic stem cell (ESC) differentiation system. By inserting a polyA cassette at four different positions from the *Airn* TSS, we then truncated the *Airn* transcript at its 3' end, either before or after the *Igf2r* promoter. Interestingly, only the longer versions of *Airn*, overlapping the *Igf2r* promoter, were able to silence it (**Fig. 1**). Next, to exclude a silencing role for the *Airn* sequence located at its 5' end, we moved the *Airn* promoter immediately before the *Igf2r* promoter. This modified *Airn* allele could also silence *Igf2r* (**Fig. 1**). Having excluded *Airn* sequences located both before and after the *Igf2r* promoter, the only region that remained to test was the one overlapping the *Igf2r* promoter itself. However, we had previously shown that this sequence can be replaced with an exogenous promoter, with no sequence or structural similarity with the *Igf2r* promoter, and still undergo *Airn*-mediated silencing.[5] Together, this led us to conclude that *Igf2r* silencing does not require any part of the *Airn* lncRNA, but only transcription through the *Igf2r* promoter. Since we detected less capped *Igf2r* transcripts, indicative of transcription initiation, we propose that the *Airn*-transcribing RNA polymerase prevents *Igf2r* expression by running through its promoter and disrupting transcription initiation complex assembly.[5]

Transcriptional interference models predict a strong promoter interfering with activity of a weak promoter. In accordance with this model, in another recent study we report that, although *Airn* transcription can repress *Igf2r* at any time, it does

*Correspondence to: Florian M. Pauler; Email: fpauler@cemm.oeaw.ac.at
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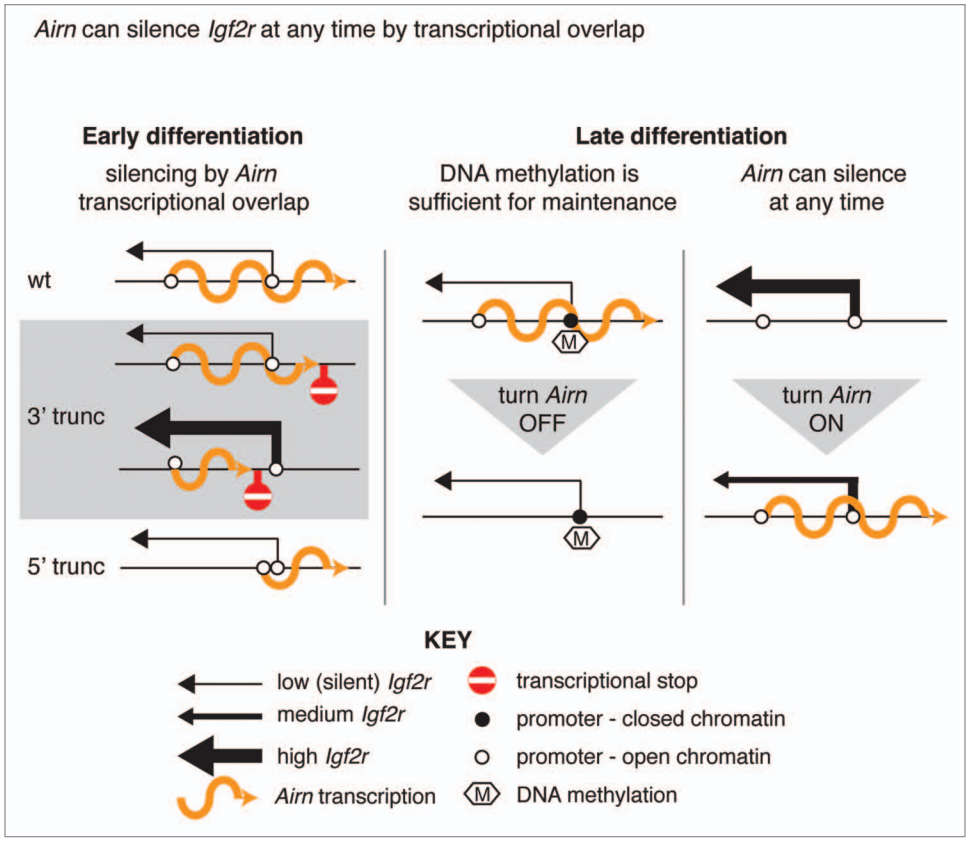

Figure 1. *Airn* can silence *Igf2r* at any time by transcriptional overlap. Left: early embryonic stem cell (ESC) differentiation. *Airn* silences *Igf2r* on the paternal allele and the *Igf2r* promoter is in an open chromatin conformation (top). Truncation of *Airn* from the 3' end (3' trunc) after but not before the *Igf2r* promoter maintains *Airn*-mediated silencing (middle). To truncate *Airn* from the 5' end (5' trunc), the *Airn* promoter was moved close to the *Igf2r* promoter: *Igf2r* was silenced in this case too (bottom). Together, these truncations show that *Airn* only needs to overlap the *Igf2r* promoter to silence it and that all 3' and 5' sequences are not necessary. Right: late ESC differentiation. *Airn*-mediated silencing causes the late acquisition of closed chromatin and DNA methylation on the *Igf2r* promoter, which maintains the silent state when *Airn* is turned off (left). In the absence of *Airn*, *Igf2r* is expressed at high levels and can only be partially silenced by turning *Airn* on at this developmental time point (right). Note that only the paternal allele is shown.

so less efficiently when paternal *Igf2r* expression is high, as in late ESC differentiation (**Fig. 1**).[8] Once silenced, the paternal *Igf2r* promoter normally gains DNA methylation. Interestingly, the same study shows that *Igf2r* silencing requires continuous *Airn* expression, but only until DNA methylation is established, at which point *Airn* becomes dispensable, indicating a role for DNA methylation in safeguarding lncRNA-mediated silencing (**Fig. 1**).[8]

In conclusion, 15 years after the first report of the *Airn* lncRNA, the cumulated evidence indicates that transcription is indeed the answer.[5] We hope our work will act as a "wake-up call" for the entire lncRNA field, as many more lncRNAs may act via their transcription alone.

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