Identification of chick Lefty2 asymmetric enhancer

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Abstract
Long before the detection of the first morphological asymmetry in the developing embryo, left-right patterning is established by a conserved feedback mechanism involving the TGF-β-like signaling molecule Nodal and its antagonist Lefty. The left-sided expression of Lefty in the lateral plate mesoderm is directly induced by Nodal signaling through the transcriptional activation of an asymmetric enhancer known as ASE, which has been found in mouse Lefty2, and in human LEFTY1 and LEFTY2 genes. Here we report the identification of a similar ASE enhancer in the cis-regulatory region of chick Lefty2 gene. This ASE sequence is able to activate reporter gene transcription in the left lateral plate mesoderm, and contains Nodal-responsive elements. Therefore, our findings suggest that Lefty2 expression may also be directly induced by Nodal signaling in the chick embryo. This hypothesis should be addressed in future functional studies.

Introduction
In vertebrates and in some higher invertebrates, the establishment of left-right patterning is directed by the Nodal signaling cascade, which involves the Transforming Growth Factor β-like molecule Nodal, its antagonists Cerberus/Dan and Lefty, and the transcription factor Pitx2 [1] [2] [3]. During early development, Nodal signaling directly activates the expression of Nodal itself, Lefty2 and Pitx2 in the left lateral plate mesoderm (LPM) [1]. This process is mediated by the transcription factor FoxH1, which recognizes conserved sequence motifs in the asymmetric enhancer (or ASE) of those genes [4] [5] [6] [7] [8]. Therefore, Nodal signaling is amplified by self-induction, but is also strictly limited in space and time due to the feedback inhibition by Lefty.

In zebrafish, mouse and human, 2 Lefty genes have arisen by independent duplications [9] [6]. In the mouse embryo, Lefty1 is expressed in the midline (floor plate and notochord), where it prevents Nodal signaling from spreading to the right side, whereas Lefty2 is expressed in the left LPM, where it leads to the downregulation of Nodal signaling [1]. In the chick, however, a single Lefty gene has been identified, Lefty2, which is expressed in both the midline and the left LPM [10] [11] [12]. Although the role of Lefty2 as an inhibitor of Nodal signaling appears to be conserved in the chick embryo [13], it is currently unclear whether the expression of chick Lefty2 is also regulated by a Nodal-responsive enhancer. In the present study, we addressed this question by investigating the presence of an ASE enhancer in the cis-regulatory region of chick Lefty2 gene.

Objective
To identify the cis-regulatory region of chick Lefty2 gene responsible for driving asymmetric expression in the left LPM.
Figure Legend

Figure 1. Characterization of chick Lefty2 left side-specific enhancer.

(A) Sequence analysis of Lefty2 cis-regulatory region. The genomic organization of chick Lefty2 gene is depicted at the top. In silico analysis using TRANSFAC Patch 1.0 and MacInspector Release professional 8.4.1 identified 3 putative binding elements for the transcription factor FoxH1 (F1, F2 and F3) in Lefty2 upstream region (3 kb). A 274 bp DNA fragment containing these elements (-1709 to -1436 bp upstream the ATG; ASE enhancer) was subcloned into a reporter vector carrying the human β-globin minimal promoter (blue box) and eGFP coding sequence (green box). FoxH1 binding sites are highlighted in the nucleotide sequence of the ASE enhancer.

(B) Chick Lefty2 expression by whole-mount in situ hybridization. In HH8 chick embryos, Lefty2 transcripts are detected in the posterior region of the left lateral plate mesoderm (LPM) (arrow) and in the notochord (arrowhead).

(C) Lefty2 ASE enhancer activity in the chick embryo. The ubiquitous reporter pCAGGS-RFP (positive control; red fluorescence) and the ASE-eGFP reporter (green fluorescence) were introduced into HH4 chick embryos by microinjection and electroporation in ex ovo culture. At HH8+, RFP expression is ubiquitously detected, whereas ASE-eGFP expression is restricted to the posterior left LPM (arrow). BF, bright field.

(B,C) Ventral views. Scale bars, 500 μm.

Results & Discussion

The asymmetric expression of mouse Lefty2 and human LEFTY1 and LEFTY2 genes is regulated by ASE enhancers that are found in their upstream regions and contain two FoxH1 binding sites each [14] [4] [6]. We therefore investigated the presence of such
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binding elements in the upstream region of chick Lefty2 gene. A 3.0 kilobases (kb) genomic sequence upstream of the coding region was analyzed using TRANSFAC Patch 1.0 [15] and MacInspector Release professional 8.4.1 [16]. This motif search identified 3 potential FoxH1 binding elements (AATC/ACACAT) closely located at -1709 to -1436 base pairs (bp) upstream of the chick Lefty2 initiation codon (Fig. 1A).

To determine if this region could be the ASE enhancer of chick Lefty2, we assessed its ability to drive transcription specifically in the left LPM, where chick Lefty2 is asymmetrically expressed (Fig. 1B) [10] [12]. For this, the 274 bp DNA fragment was subcloned into an enhancer-less vector containing the human \( \beta \)-globin minimal promoter upstream of the eGFP coding sequence (i.e., ASE-eGFP; Fig. 1A), and introduced into chick embryos by electroporation in New culture [17]. Together with the ASE-eGFP construct, embryos were co-transfected with the ubiquitous reporter pCAGGS-RFP to control for targeted area and electroporation efficiency. Our results showed that eGFP expression is specifically restricted to the posterior LPM on the left side (Fig. 1C; 17/18 embryos), mirroring the asymmetric expression pattern of chick Lefty2 (Fig. 1B). eGFP fluorescence starts to be detected approximately 2–3 h after the initial detection of Lefty2 transcripts in this region (e.g., stages HH8+ vs. HH8) [18], which corresponds to the time required for eGFP gene transcription and protein synthesis. ASE-eGFP expression remains in the posterior region of the left LPM until the last stage analyzed (HH11; data not shown), in a similar pattern to Lefty2 asymmetric expression [10] [12]. However, eGFP expression was not clearly detected in the notochord domain of Lefty2 expression at any of the developmental stages tested (HH7-11). These observations suggest that chick Lefty2 ASE regulatory region is indeed a typical ASE enhancer, being able to specifically drive expression in the left LPM. Moreover, the presence of 3 FoxH1 binding elements indicates that chick Lefty2 expression may be directly induced by Nodal signaling in the left LPM, as previously shown for mouse Lefty2 [4] [19] [20]. In addition, our results indicate that chick Lefty2 expression in the midline is not induced by the ASE enhancer. Similarly to the transcriptional regulation of mouse Lefty1 [6], midline expression may be under the control of regulatory sequences that do not contain FoxH1 motifs.

Unlike mouse Lefty2, the expression of chick Lefty2 is not detected in the left LPM at early stages. Based on their similar expression patterns and functions, it was proposed that the role of mouse Lefty2 has been taken by the Nodal antagonist Cerberus (Cer1) in chick left-right patterning [21] [22]. Indeed, chick Cer1 is expressed in the left LPM at early stages (HH7-9) [10] [11], and its transcription is also regulated by a Nodal-responsive enhancer containing two essential FoxH1 binding elements [22]. Taken together, our results suggest that chick Lefty2 may replace Cer1 in the left LPM at later developmental stages (HH8-11) as a feedback inhibitor of Nodal signaling.

Conclusions

We have identified the asymmetric enhancer (ASE) of chick Lefty2 gene. This ASE sequence contains three conserved Nodal-responsive elements and is able to drive transcription specifically in the left lateral plate mesoderm, thus reproducing the asymmetric pattern of chick Lefty2 expression.

Limitations

Although the identification of FoxH1 binding sites in the ASE enhancer suggests that chick Lefty2 transcription is regulated by Nodal signaling, further experimental evidence is required. Namely, functional studies are needed to assess if Nodal misexpression on the right side ectopically induces chick Lefty2 expression, whereas Nodal inhibition on the left side represses it. Moreover, as in the study of chick Cer1 transcriptional regulation [22], a mutagenesis analysis of the ASE enhancer would reveal whether the FoxH1 sites are indeed essential for the induction of left LPM expression. Taken together, these experiments would bring support to the hypothesis that, as in other chordates, Lefty2 is a direct target of Nodal signaling in the chick embryo. Despite having similar asymmetric enhancers, the patterns of chick Lefty2 and Cer1 in the left LPM are slightly different, with Lefty2 expression starting at later developmental
stages [10]. Therefore, the question arises as to how Nodal signaling is able to differently regulate the transcription of Cer1 and Lefty2. One possible explanation may rely on the number of FoxH1 binding sites in their ASE enhancers: 2 in Cer1 [22] and 3 in Lefty2 regulatory region (this study). In fact, the number of transcription factor binding sites in homotypic clusters is known to influence gene regulation [23]. In particular, if transcription is activated only when all binding sites are occupied, having a higher number of sites would generate a time delay in gene expression. A simple way to address this possibility would be to evaluate the effect of mutating each of the FoxH1 binding elements in the chick Lefty2 enhancer in ASE-eGFP reporter assays.

Additional Information

Methods and Supplementary Material
Please see https://sciencematters.io/articles/20180700006.

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Ethics Statement
Not Applicable.

Citations

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