Removal of maternal retinoic acid by embryonic CYP26 is required for correct *Nodal* expression during early embryonic patterning

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The abundance of retinoic acid (RA) is determined by the balance between its synthesis by retinaldehyde dehydrogenase (RALDH) and its degradation by CYP26. In particular, the dynamic expression of three CYP26 genes controls the regional level of RA within the body. Pregastrulation mouse embryos express CYP26 but not RALDH. We now show that mice lacking all three CYP26 genes manifest duplication of the body axis as a result of expansion of the *Nodal* expression domain throughout the epiblast. Mouse *Nodal* was found to contain an RA-responsive element in intron 1 that is highly conserved among mammals. In the absence of CYP26, maternally derived RA activates *Nodal* expression in the entire epiblast of pregastrulation embryos via this element. These observations suggest that maternal RA must be removed by embryonic CYP26 for correct *Nodal* expression during embryonic patterning.

[**Keywords:** CYP26; *Nodal*; retinoic acid; RA-responsive element; embryonic patterning]

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The abundance of retinoic acid (RA) in the body is determined mainly by the balance between its synthesis by retinaldehyde dehydrogenase (RALDH) and its degradation by the cytochrome P450 enzyme CYP26 (Ross et al. 2000; Abu-Abed et al. 2001; Sakai et al. 2001; Duester 2008; Niederreither and Dolle 2008). Mice (and humans) possess three CYP26 genes: *Cyp26a1*, *Cyp26b1*, and *Cyp26c1* (MacLean et al. 2001; Abu-Abed et al. 2002; Nebert and Russell 2002; Tahayato et al. 2003; Sirbu et al. 2005). Mutant mice lacking each of the three CYP26 genes individually have provided insight into the role of a restricted distribution of RA in various aspects of organogenesis. *Cyp26a1* is expressed in the hindbrain and tail bud, and *Cyp26a1*^{-/-} mice exhibit hindbrain patterning defects and caudal truncation, as do embryos exposed to excess RA (Abu-Abed et al. 2001; Sakai et al. 2001). *Cyp26b1* is expressed in the distal region of the developing limb, where RA signaling is absent, and the lack of CYP26B1 results in the expansion of RA signaling toward

the distal end of the developing limb and the induction of proximodistal patterning defects (Yashiro et al. 2004; Uehara et al. 2007). RA is a small molecule that can diffuse over a long distance. Transport of RA is also important as domains of RA signaling do not necessarily reflect simple patterns of synthesis and degradation.

One of the earliest roles of CYP26 isozymes in embryogenesis is apparent in anteroposterior (A–P) patterning of the developing brain (Ribes et al. 2007; Uehara et al. 2007). *Cyp26a1* and *Cyp26c1* are expressed in the anterior region of mouse embryos between embryonic day 7.5 (E7.5) and E8.5, whereas *Raldh2* and the *RARE-hsplacZ* transgene (which encodes β-galactosidase under the control of an RA-responsive element (RARE) and the *Hsp68* promoter) are expressed in the posterior region. Mouse embryos lacking both *Cyp26a1* and *Cyp26c1* exhibit expansion of RA signaling toward the anterior side as well as A–P patterning defects of the developing brain. Before E7.5, expression of *Raldh2* and *RARE-hsplacZ* was undetectable, whereas *Cyp26a1* was highly expressed (Supplemental Fig. S1), suggesting that CYP26-mediated restriction of the spatial distribution of the RA signal may also be required at earlier stages of development, during or even before gastrulation.

To determine the relevance of temporal and spatial regulation of RA signaling, we examined mutant mice

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that lack all three CYP26 genes. Our observations reveal unexpected roles of CYP26s in embryonic patterning and in regulation of *Nodal*, which encodes a transforming growth factor- β (TGF- β)-related protein that itself plays key roles in early embryogenesis.

Results

A-P patterning defects and aberrant Nodal expression in Cyp26a1b1c1^{-/-} mice

Cyp26a1b1c1^{-/-} mice exhibited defects that were more pronounced than those of *Cyp26b1^{-/-}* or *Cyp26a1c1^{-/-}* mice and proved lethal during early embryogenesis. At E9.25, 44% (11 out of 25) of *Cyp26a1b1c1^{-/-}* embryos were markedly deformed, showing complete or partial duplication of the body axis, whereas the remaining embryos exhibited only abnormal patterning in the developing brain, similar to that apparent in most *Cyp26a1c1^{-/-}* embryos (Uehara et al. 2007). Embryos showing partial or complete duplication of the body axis will hereafter be referred to as “severely affected” (Supplemental Fig. S2), and the phenotype of these embryos will be described unless indicated otherwise. Two sets of neural tubes were observed in these *Cyp26a1b1c1^{-/-}* embryos (Fig. 1B',C'). At E9.0, the expression domain of *Shh*, which marks the axial midline in wild-type embryos (Fig. 1E'), was completely (Fig. 1F') or partially (Fig. 1G') duplicated in the *Cyp26a1b1c1^{-/-}* embryos. The *Shh* expression in the midline was also duplicated at E8.25 (Supplemental Fig. S3B'). Furthermore, the expression domain of *T* (*Brachury*), which marks the primitive streak (Fig. 1I'; Supplemental Fig. S3C'), was duplicated partially (Fig. 1J'; Supplemental Fig. S3D') or completely (Fig. 1K'; Supplemental Fig. S3E') in *Cyp26a1b1c1^{-/-}* embryos. These observations thus revealed that *Cyp26a1b1c1^{-/-}* embryos manifest duplication of the primitive streak.

The patterning defects of *Cyp26a1b1c1^{-/-}* embryos resembled those of mutant mice lacking the *Nodal* antagonists *Lefty1* and *Cer1*, which also show duplication of the primitive streak as a result of the up-regulation of *Nodal* activity at the pregastrulation stage (Perea-Gomez et al. 2002). We therefore examined embryonic patterning before gastrulation in *Cyp26a1b1c1^{-/-}* embryos. Whereas expression of *Nodal* is restricted to the posterior region of wild-type embryos at E6.0 to E6.25 (Fig. 2A), the *Nodal* expression domain occupied the entire region of the epiblast in 50% (three out of six embryos) of the *Cyp26a1b1c1^{-/-}* embryos examined (Fig. 2B; Supplemental Fig. S4B,C). Similarly, whereas expression of *Cripto*, which encodes a coreceptor for *Nodal*, is most prominent in the posterior region of the epiblast in wild-type embryos (Fig. 2D), it was up-regulated and extended throughout the epiblast in 57% (four out of seven embryos) of the mutant embryos examined (Fig. 2E; Supplemental Fig. S4G,H). The aberrant expression of *Nodal* in *Cyp26a1b1c1^{-/-}* embryos was not due to impaired function of the anterior visceral endoderm (AVE), which appeared to be formed normally. Expression of various AVE markers, including *Hex*, *Dkk1*, *Cer1*, and *Lefty1*, was

Figure 1. Duplication of the body axis in *Cyp26a1b1c1^{-/-}* and *Cyp26a1c1^{-/-}* embryos. (A–D) Lateral views of wild-type (A), *Cyp26a1b1c1^{-/-}* (B,C), and *Cyp26a1c1^{-/-}* (D) embryos at E9.25. The anterior side is on the left. About two-fifths of *Cyp26a1b1c1^{-/-}* (B,C) or one-fourth of *Cyp26a1c1^{-/-}* (D) embryos were severely deformed, showing complete or partial duplication of the body axis. (A'–D') Hematoxylin-eosin (HE) staining of sections of the embryos shown in A through D, respectively. Two sets of neural tubes or grooves (red arrowheads) were apparent in the mutant embryos, compared with the single neural tube (black arrowhead) in the wild-type embryo. The planes of the sections are indicated by the horizontal lines through the embryos in A–D. The boxed region in C' is shown at higher magnification in C''. Bars, 100 μ m. (E–L) Lateral views of wild-type (E,I), *Cyp26a1b1c1^{-/-}* (F,G,J,K), and *Cyp26a1c1^{-/-}* (H,L) embryos at E9.0 (E–H) or E8.25 (I–L) that had been subjected to whole-mount in situ hybridization for analysis of the expression of *Shh* (E–H) or *T* (*Brachury*) (I–L). The anterior side is on the left. (E'–L') Ventral (E'–H') and posterior (I'–L') views of the embryos in E–H and in I–L, respectively. The expression domain of *Shh* was partially or completely duplicated in the mutant embryos (red arrowheads in F'–H'). Mutant embryos expressed *T* in two distinct primitive streaks (red arrowheads in J' and L') or ectopically at the embryonic–extraembryonic boundary (red arrowheads in K').

thus maintained in the mutant (Fig. 2G,G',H,H'; Supplemental Fig. S5). The level of *Lefty1* expression was increased in *Cyp26a1b1c1^{-/-}* embryos (Fig. 2G,H), most likely as a result of increased *Nodal* activity, given that *Lefty1* expression in the AVE is regulated by *Nodal* signaling (Yamamoto et al. 2004). These results thus suggested that expansion of *Nodal* expression throughout the epiblast of the mutant embryos was not secondary to impaired AVE function but rather was due to dysregulation of *Nodal* in the epiblast.

Role of each CYP26 gene in embryonic patterning

Mice lacking any one of the three CYP26 genes do not exhibit the early patterning defects observed in

Figure 2. Epiblast patterning defects in *Cyp26a1b1c1*^{-/-} and *Cyp26a1c1*^{-/-} embryos. (A–I, G'–I') Expression of *Nodal*, *Cripto*, and *Lefty1* in wild-type (A, D, G, G'), *Cyp26a1b1c1*^{-/-} (B, E, H, H'), and *Cyp26a1c1*^{-/-} (C, F, I, I') embryos at E6.25 or E6.5, as indicated, was examined by whole-mount in situ hybridization. Lateral views with the anterior side on the left are shown, with the exception of the frontal views shown in G–I. *Nodal* was expressed in the posterior region of the wild-type embryo (A) but was expressed throughout the epiblast of *Cyp26a1b1c1*^{-/-} (B) or *Cyp26a1c1*^{-/-} (C) embryos. Whereas *Cripto* expression was most prominent in the posterior region of the epiblast of the wild-type embryo (D), it was up-regulated and extended to the entire epiblast in the mutant embryos (E, F). The expression domain of *Lefty1*, which marks the AVE in wild-type embryos (G, G'), was maintained in the mutant embryos (H, H', I, I'), although the level of *Lefty1* expression was increased in the mutants (H, I). (J–M, K', M') Expression of *Nodal* (J, K, K') and the *RARE-hsplacZ* transgene (L, M, M') in untreated (J, L) or RA-treated (K, K', M, M') wild-type embryos at E6.25 was examined by whole-mount in situ hybridization or staining with the

β -galactosidase substrate X-gal. *Nodal* was expressed in the posterior region of the epiblast in the untreated embryo (J) but was expressed in the entire epiblast of the RA-treated embryo (K, K'). Expression of *RARE-hsplacZ* was not detected in the untreated embryo (L) but was pronounced throughout the epiblast of the RA-treated embryo (M, M'). The images in K and M are shown at higher magnification in K' and M', respectively. (ep) Epiblast; (ve) visceral endoderm.

Cyp26a1b1c1^{-/-} embryos (Abu-Abed et al. 2001; Sakai et al. 2001; Yashiro et al. 2004; Uehara et al. 2007; data not shown). We examined the expression of each CYP26 gene between E5.5 and E6.5. All three genes were found to be expressed in the extraembryonic cell types that surround the embryo proper, although *Cyp26a1* was the most prominently expressed gene at these stages. At E5.5 and E6.0, *Cyp26a1* was thus highly expressed in the extraembryonic endoderm, extraembryonic ectoderm, and visceral endoderm, domains that surround the embryo proper (Fig. 3D–D'; Supplemental Fig. S1G, J). *Cyp26c1* and *Cyp26b1* were expressed in a different domain of the extraembryonic region, the ectoplacental cone, albeit at a lower level, with the expression level of *Cyp26c1* being higher than that of *Cyp26b1* (Fig. 3E, E', F, F'). These results suggested that *Cyp26a1* is the major CYP26 gene expressed at pregastrulation stages, although *Cyp26b1* and *Cyp26c1* are able to compensate for the loss of *Cyp26a1* expression. Furthermore, consistent with the relative levels of *Cyp26b1* and *Cyp26c1* expression, a substantial proportion (26%, 18 of 70 embryos) of *Cyp26a1c1*^{-/-} embryos showed gastrulation defects, including partial or complete duplication of the primitive streak (Fig. 1L'; Supplemental Fig. S3F'; Supplemental Table S1), similar to those of *Cyp26a1b1c1*^{-/-} embryos, whereas *Cyp26a1b1*^{-/-} embryos did not exhibit such defects (data not shown). Furthermore, a portion of *Cyp26a1c1*^{-/-} embryos displayed up-regulated expression of *Nodal* (two out of five embryos) and *Cripto* (three out of eight embryos) (Supplemental Fig. S4D, E, I, J).

Consistent with the expression patterns of the CYP26 genes, RA signaling, as revealed by the RA-responsive transgene *RARE-hsplacZ*, was not detected in wild-type embryos at E6.25 (Figs. 2L, 3A, A') but was pronounced in

the entire epiblast of *Cyp26a1b1c1*^{-/-} (four out of eight) and *Cyp26a1c1*^{-/-} (five out of 12) embryos (Fig. 3B, B', C, C').

Expansion of *Nodal* expression in the epiblast is responsible for the embryonic patterning defects in *Cyp26a1b1c1*^{-/-} embryos

We next examined whether the expansion of *Nodal* expression in the epiblast is responsible for the observed patterning defects in *Cyp26a1b1c1*^{-/-} embryos. If such is the case, a reduction in the extent of *Nodal* expression would be expected to rescue the gastrulation defects of *Cyp26a1b1c1*^{-/-} and *Cyp26a1c1*^{-/-} embryos. We first examined the effect of the removal of one copy of *Nodal* by comparing the phenotypes of *Nodal*^{+/-}, *Cyp26a1c1*^{-/-}, and *Nodal*^{+/-lacZ}, *Cyp26a1c1*^{-/-} embryos (Fig. 4). *Nodal*^{lacZ} is a null allele, in which exon 1 of *Nodal* gene is replaced by *lacZ* (Collignon et al. 1996). Whereas 26% (18 out of 70) of the former embryos showed duplication of the body axis (Supplemental Table S1), only 6% (one out of 16) of the latter embryos did so at E8.25. We also examined the effect of a hypomorphic *Nodal* allele (*Nodal*^{neo}). *Nodal*^{neo/neo} embryos develop left–right patterning defects at E8.0 as a result of a marked reduction in the level of *Nodal* expression in the node (Saijoh et al. 2003). *Nodal* expression is also reduced in the epiblast at E6.5, but not as severely as in the node at E8.0 (Supplemental Fig. S6), which permits the *Nodal*^{neo/neo} embryo to undergo normal gastrulation. None of the *Nodal*^{neo/neo}, *Cyp26a1c1*^{-/-} embryos examined at E8.25 (zero out of seven embryos) showed the embryonic patterning defects (Fig. 4E') apparent in 35% of *Cyp26a1c1*^{-/-} embryos (Supplemental Table S1). (For these experiments, mice were maintained on a special diet CRF that contains ~30%

Figure 3. Contribution of each CYP26 isozyme to RA metabolism in pregastrulation embryos. (A–C, A'–C') Expression of the *RARE-hsplacZ* transgene in wild-type (A), *Cyp26a1b1c1*^{−/−} (B), and *Cyp26a1c1*^{−/−} (C) embryos at E6.25 as revealed by X-gal staining. Lateral views are shown with the anterior side on the left. Each image is shown at higher magnification in A'–C', respectively. Expression of the transgene was not detected in the wild-type embryo (A, A') but was pronounced in the entire epiblast (ep) of the mutants (B, B', C, C'). (D–F, D'–F', D'', D''') Expression patterns of *Cyp26a1* (D), *Cyp26c1* (E), and *Cyp26b1* (F) in wild-type embryos at E6.25 as revealed by whole-mount in situ hybridization. Lateral views are shown with the anterior side on the left. The planes of sections shown in D', D'', D''', E', and F' are indicated by the horizontal lines through the embryos in D–F. *Cyp26a1* was highly expressed in the extraembryonic endoderm (ex-en) (D', D''), the extraembryonic ectoderm (ex-ec) (D''), and the visceral endoderm (ve) (D'''). *Cyp26c1* and *Cyp26b1* were expressed in the ectoplacental cone, albeit at a lower level (E, E', F, F'). Bars, 50 μ m.

higher vitamin A than a conventional diet) (see the Materials and Methods.) Finally, three *Cyp26a1b1c1*^{−/−}, *Nodal*^{+/lacZ} embryos that were recovered at E8.75 showed normal *Shh* expression in the midline (Supplemental Fig. S7B, B'). These results thus indicated that dysregulation of *Nodal* expression in the epiblast is responsible for the abnormal patterning of *Cyp26a1b1c1*^{−/−} embryos.

An RARE within the autoregulatory enhancer regulates Nodal expression in the epiblast

The patterning defects of *Cyp26a1b1c1*^{−/−} embryos suggested that *Nodal* expression in the epiblast might be directly controlled by RA and that an increased RA level in the epiblast of the mutant embryos may be responsible for up-regulation of *Nodal* expression. We found that such was the case. First, administration of exogenous RA to wild-type embryos at E6.0 induced expression of the *RARE-hsplacZ* transgene (Fig. 2M, M') and *Nodal* (Fig. 2K, K') throughout the epiblast, as observed for *Nodal* in *Cyp26a1b1c1*^{−/−} embryos (Fig. 2B). Second, expression of *Nodal* in the epiblast was found to be regulated by an RARE. A *Nodal*-responsive enhancer (referred to as ASE) located in intron 1 of *Nodal* contains a RARE-like sequence, TGACCCN₉GGGTCA (hereafter referred to as RARE_n), in addition to two FoxH1-binding sequences (Fig. 5A, B). While other RAREs have 1, 2, or 5 nucleotides (nt) between the two RXR-binding sites (Umesono et al. 1991), RARE_n has 9 nt. ASE regulates *Nodal* expression in the epiblast at E6.0 to E6.5 as well as in the left lateral plate at E8.0 (Adachi et al. 1999; Norris and Robertson 1999). To examine whether RARE_n responds to RA, we monitored expression of two *lacZ* transgenes: 3'–1, in which *lacZ* expression is driven by the *lacZ* promoter and a 3-kb genomic region containing ASE (Fig. 6A; Adachi et al. 1999) and 501/301 in which *lacZ* expression is driven by the heterologous *Hsp68* promoter and a 200-base-pair (bp) region of ASE (Fig. 6A; Adachi et al. 1999). The RARE_n sequence is included in both transgenes. Like *Nodal*, both *lacZ* transgenes were found to be expressed in the posterior region of the epiblast in wild-type embryos at E6.25 (Fig. 6A). However, administration of

Figure 4. Rescue of embryonic patterning defects in *Nodal*^{+/lacZ}, *Cyp26a1c1*^{−/−} and *Nodal*^{neo/neo}, *Cyp26a1c1*^{−/−} embryos. (A–E, A'–E') Expression of *T* (*Brachury*) in wild-type (A, A'), *Cyp26a1c1*^{−/−} (B, B', C, C'), *Nodal*^{+/lacZ}, *Cyp26a1c1*^{−/−} (D, D'), and *Nodal*^{neo/neo}, *Cyp26a1c1*^{−/−} (E, E') embryos at E8.25 was examined by whole-mount in situ hybridization. Lateral views with the anterior side on the left are shown in A through E, and posterior views are shown in A' through E'. Exon 1 of *Nodal* is replaced by *lacZ* in the *Nodal*^{+/lacZ} allele. Whereas ~35% of *Cyp26a1c1*^{−/−} embryos expressed *T* in two distinct primitive streaks (arrowheads in B') or ectopically at the embryonic–extraembryonic boundary (arrowheads in C'), only 6% of the *Nodal*^{+/lacZ}, *Cyp26a1c1*^{−/−} embryos (not shown) did so. (E') Furthermore, none of the *Nodal*^{neo/neo}, *Cyp26a1c1*^{−/−} embryos showed the embryonic patterning defects.

RARE_n in the ASE. A luciferase reporter assay performed with RA-responsive mouse embryonal carcinoma (P19) cells revealed that a reporter construct containing tandem repeats of TGACCCN₉GGGTCA, but not one containing the mutated sequence GATCCCN₉GGGAAT, responded to RA in a concentration-dependent manner (Fig. 5C). These results thus supported the notion that RARE_n indeed functions as an RARE.

A search of the ~70-kb genomic region encompassing mouse *Nodal* for the RARE-like sequence TGA/C/A/C/C/

Figure 6. *Nodal* as a target of RA signaling in pregastrulation embryos. (A) Structure of three *lacZ* constructs and their expression in mouse embryos. (E) EcoRI; (No) NotI; (S) SalI; (Sm) SmaI. The open black and closed blue circles indicate the proximal promoter of *Nodal* and the *Hsp68* promoter, respectively. Staining with X-gal revealed that both 3'-1 and 501/301 transgenes were expressed in the posterior region of the epiblast (at E6.25) and in the left lateral plate (at E8.0) of untreated [RA(-)] wild-type embryos, whereas they were expressed in the entire epiblast of RA-treated wild-type embryos at E6.25. In contrast, the expression of 501/301-Rm in wild-type embryos was not affected by RA treatment. (B) Expression of the 501/301 transgene in wild-type, *Cyp26a1b1c1*^{-/-}, and *Cyp26a1c1*^{-/-} embryos at E6.25 as revealed by X-gal staining. Lateral views are shown for each embryo with the anterior side on the left. The transgene was expressed in the posterior region of the wild-type embryo but was expressed in the entire epiblast of the mutant embryos.

T)N_x(G/A)G(G/T)TCA (Ross et al. 2000) revealed RARE_n to be the only copy, although half sites consisting of TGACCC, AGGTCA, or TGACCT were detected in the upstream region or 3' untranslated region of the gene (Fig. 7A). To test whether RARE_n in intron 1 is the only RA-responding element of *Nodal*, we generated a *lacZ* reporter construct [*Nodal-lacZ* (BAC)] from a bacterial artificial chromosome (BAC) clone containing an ~200-kb genomic region including *Nodal*. The expression pattern of the *Nodal-lacZ* (BAC) construct in transgenic embryos mimicked that of endogenous *Nodal* (Fig. 7B; data not shown), and the transgene responded to administration of exogenous RA at E6.0 (Fig. 7B). However, a corresponding reporter construct [*Nodal-ΔRARE-lacZ* (BAC)] with a 25-bp deletion including RARE_n failed to respond to exogenous RA (Fig. 7B), indicating that RARE_n in intron 1 is the only functional RARE of mouse *Nodal*.

Finally, we searched *Nodal* genes from 25 different vertebrates for RARE-like sequences as well as for the ASE and FoxH1-binding sequences (Fig. 7C). FoxH1-binding sequences act as an autoregulatory element that is required to amplify *Nodal* expression (Saijoh et al. 2000; Brennan et al. 2001). The ASE was found in intron 1 of all vertebrate *Nodal* genes examined. Two copies of the FoxH1-binding sequence, AAT(C/A)(C/A)ACA, were also detected in most of the vertebrate genes, whereas only one copy was found in the *Nodal* genes of pika, rabbit, and shrew. As suggested by mutational analysis of mouse *Nodal* ASE (Saijoh et al. 2000), a single FoxH1-binding site may be sufficient for autoregulation of the *Nodal* gene. A RARE-like sequence was found in the corresponding region (~100 bp downstream from the FoxH1-binding sequences) of *Nodal* genes from all the mammals exam-

ined with the exception of opossum and platypus. Whereas rat, rabbit, and squirrel, like mouse, possess two half sites separated by 8 or 9 bp, the remaining mammalian species have three half sites each separated by 8, 9, or 10 bp. A RARE-like sequence was not detected in the corresponding region of *Nodal* or *Nodal*-related genes from nonmammalian vertebrates such as chicken, *Xenopus*, and zebrafish. RARE_n is thus an RA-responsive transcriptional *cis*-element that is highly conserved among mammals, while FoxH1-binding sites are conserved among vertebrates.

Discussion

RA in the pregastrulation mouse embryo is likely of maternal origin, given that RALDH genes are not expressed within the embryo at this stage (Supplemental Fig. S1; Duester 2008; Niederreither and Dolle 2008). *Raldh2* is highly expressed in the deciduas (Vermot et al. 2000), and RA is present in mouse serum at a concentration of ~5 nM (Fig. 8A; Kane et al. 2008a,b). However, expression of CYP26 genes in the extraembryonic regions of the embryo prevents the epiblast from being exposed to RA (Fig. 8B), with the result that *Nodal* expression in the epiblast is maintained without activation of RARE_n. Indeed, RA signaling as visualized with the *RARE-hsplacZ* transgene was not detected in embryos at E5.5 to E6.5 (Fig. 8B; Supplemental Fig. S1I,L), even though the epiblast expresses genes for intracellular components (including RA receptors [RARs] and retinoid X receptors [RXRs]) necessary for RA signaling (Supplemental Fig. S8) and is competent to respond to the RA signal (Fig. 2M). In the absence of CYP26s, however, maternally derived

Figure 7. Role of RARE_n in regulation of *Nodal* expression in the epiblast and its structural conservation among mammals. (A) Relative positions of RARE_n (red) and three RARE-like half sites (blue) in mouse *Nodal*. (B) X-gal staining of E8.0 and E6.25 embryos harboring the indicated *lacZ* (BAC) constructs. The expression pattern of *Nodal-lacZ* (BAC) mimicked that of endogenous *Nodal* and responded to the administration of exogenous RA at E6.0. Expression of *Nodal-ΔRARE-lacZ* (BAC) failed to respond to exogenous RA. (C, left panel) Evolutionary conservation of RARE-like and FoxH1-binding sequences in intron 1 of vertebrate *Nodal* genes; numbers in parentheses indicate nucleotide spacing. The vertebrate evolutionary tree is shown in the right panel.

RA is able to reach the embryo proper and induces *Nodal* expression in the entire epiblast via RARE_n (Fig. 8B). CYP26s thus act in a non-cell-autonomous manner to maintain the epiblast free of RA.

We found that the level of vitamin A in the diet influences the phenotype of *Cyp26a1c1*^{-/-} and *Cyp26a1b1c1*^{-/-} embryos (Supplemental Table S1). The conventional diet on which pregnant mice were maintained for the present experiments contains 3222 IU of vitamin A per 100 g of

chow. An increase in the amount of vitamin A in chow to 4250 or 6000 IU/100 g resulted in a concentration-dependent increase in the frequency of the severe phenotype for both *Cyp26a1c1*^{-/-} and *Cyp26a1b1c1*^{-/-} embryos, consistent with the notion that the phenotypic variation among *Cyp26a1c1*^{-/-} and *Cyp26a1b1c1*^{-/-} embryos reflects a variable level of maternal RA. We also examined how much RA would be sufficient to induce early patterning defects in wild-type embryos by administering

Figure 8. Model for the role of CYP26 genes in early embryonic patterning. (A) In the pregastrulation mouse embryo, RA appears to be of maternal origin. *Raldh2* is expressed in the decidua and RA is present in serum. (VE) Visceral endoderm. (B) The expression domains of the three CYP26 genes in the wild-type embryo at E6.25 are shown in the top panels. (Bottom panels) In mutant (MT) embryos lacking all three CYP26 genes, maternally derived RA activates *Nodal* expression in the entire epiblast via *RARE_n*. Red arrows indicate the route of maternally derived RA. The removal of maternal RA by embryonic CYP26 is thus required for correct *Nodal* expression during embryonic patterning. See the text for details.

various doses of exogenous RA to pregnant wild-type mice at E5.5. The defects were not induced by oral administration of RA at 10, 20, or 40 mg per kilogram of body weight but were apparent in 57% (eight out of 14) of embryos exposed to RA at a dose of 50 mg/kg (Supplemental Fig. S9). Given that a dose of 50 mg/kg represents an amount of RA much higher than that contained in a conventional diet, the embryo is effectively protected by CYP26s under normal conditions. However, the administration of RA-containing drugs (such as tretinoin and isotretinoin for acne) or agents that inhibit the activity or expression of CYP26s (such as triazole antifungal agents) (Pautus et al. 2006) during early pregnancy might increase the risk of embryonic patterning defects.

With regard to the physiological role of *RARE_n*, a transcriptional regulatory element of *Nodal* that is highly conserved among mammals, we found that the *Nodal-lacZ-ΔRARE* (BAC) transgene, which lacks *RARE_n*, was expressed in mouse embryos in a manner similar to that

of endogenous *Nodal* at least at E6.25 and E8.0. Consistent with this, loss of RA synthesis in the *Raldh2* mutant mouse does not affect early *Nodal* expression (Niederreither et al. 2001). Although it may thus appear that the presence of *RARE_n* only increases the risk of embryonic patterning defects, *Nodal* plays essential roles in other events, such as left-right patterning, at later stages of development. Given the highly conserved nature of *RARE_n*, *RARE_n*-mediated regulation of *Nodal* may play a key role in such events. Although the expression and function of *Nodal* have been studied extensively during early embryogenesis, they have not been examined in detail at later developmental stages or in the adult. It will be important to search for *RARE_n*-dependent expression domains of *Nodal* at later developmental stages and to study their role in development and physiology.

Materials and methods

Generation of *Cyp26a1b1c1*^{-/-} mice

Cyp26a1b1c1^{-/-} mice were obtained by intercrossing *Cyp26a1b1c1*^{+/-} mice, which were generated by mating *Cyp26a1c1*^{+/-} mice (Uehara et al. 2007) with *Cyp26b1*^{+/-} mice (Yashiro et al. 2004). All mice were maintained on a conventional diet (NMF; Oriental Yeast Co.) containing 3222 IU of vitamin A per 100 g of chow, unless indicated otherwise. Where indicated, mice were fed a special diet containing higher concentrations (4250 or 6000 IU/100 g) of vitamin A (CRF and NMF2, respectively; Oriental Yeast Co.).

RA treatment

RA treatment was performed as described previously (Yashiro et al. 2004) with minor modifications. Pregnant ICR mice at E6.0 or the indicated embryonic stages were thus administered *all-trans* RA (10 mg per kilogram of body weight unless indicated otherwise; Sigma) in sesame oil (Sigma) by oral gavage. Control females received sesame oil only.

Histological analysis

Mouse embryos were staged on the basis of their morphology. In situ hybridization was performed with whole-mount preparations as described previously (Sakai et al. 2001). To detect the RA signal in embryos, we crossed *Cyp26a1b1c1*^{+/-} mice harboring the *RARE-hsplacZ* transgene (Rossant et al. 1991) with *Cyp26a1b1c1*^{+/-} mice and subjected the resulting embryos to genotyping and staining with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

Transgenic reporter assay

Permanent transgenic lines harboring the 3'-1 or 501-301 *lacZ* transgenes have been described previously (Adachi et al. 1999). The *Nodal-lacZ* (BAC) transgene consists of the *Nodal* BAC clone RP23-55A6, with exon 1 of *Nodal* replaced by *lacZ*. The recombinant BAC clone was constructed with the use of the highly efficient recombination system in *Escherichia coli* (Copeland et al. 2001). To generate the *Nodal-ΔRARE-lacZ* (BAC) transgene, we replaced a 25-bp region including the TGACCCN₉GGGTCA sequence with the *FRT* sequence. BAC DNA was prepared for microinjection (Gong et al. 2003), and transgenic mice were generated (Saijoh et al. 1999) as described previously.

Luciferase assay

The reporter assay was performed as described previously (Loudig et al. 2005). P19 (mouse teratocarcinoma) cells were cultured under 5% CO₂ at 37°C in minimal essential medium (pH 7.2). The cells were seeded at a density of 2×10^5 cells per well in six-well plates 12 h before transfection with a total of 0.5 µg of DNA with the use of the Lipofectamine 2000 reagent (Invitrogen). After transfection for 24 h, the cells were exposed to *all-trans* RA in dimethyl sulfoxide for 24 h in the dark. The cells were then washed twice with phosphate-buffered saline and lysed in 100 µL of Passive Lysis Buffer (Promega) for 15 min, after which the cell lysates were assayed for luciferase activity with a Luciferase Assay System (Promega). All experiments were repeated three times.

Genome information from various vertebrates

The nucleotide sequence of intron 1 of *Nodal* was recovered for various vertebrates from NCBI (<http://www.ncbi.nlm.nih.gov/genomes>) and was searched for the FoxH1-binding sequence AAT[C/A]C[A]ACA and the RARE-like sequences TGACCC and GGGTCA.

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Removal of maternal retinoic acid by embryonic CYP26 is required for correct *Nodal* expression during early embryonic patterning

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