The Orphan Steroid Receptor Nur77 Family Member Nor-1 Is Essential for Early Mouse Embryogenesis*

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Nur77 and its family members, Nor-1 and Nurr1, are orphan steroid receptors implicated in a wide variety of biological processes, including apoptosis and dopamine neuron agenesis. Expression of these family members can be detected at low levels in many tissues but they are expressed at very high levels when cells are stimulated by outside signals, including serum, nerve growth factor, and receptor engagement. Introduction of a dominant negative Nur77 protein that blocks the activities of all family members led to inhibition of apoptosis in T cells. Nur77-deficient mice, however, exhibit no pheno-
type, and a line of Nor-1 mutant mice was reported to exhibit a mild ear development phenotype but no other gross abnormalities. Here, we report the generation of Nor-1-deficient mice with a block in early embryonic development. Nor-1 is expressed early during embryogenesis, and its loss leads to embryonic lethality around embryonic day 8.5 of gestation. The mutant embryos fail to complete gastrulation and display distinct morphological abnormalities, including a decrease in overall size, developmental delay and an accumulation of meso-
derm in the primitive streak during gastrulation. Abnormal expression of a number of early developmental markers and defects in growth or distribution of emerging mesoderm cells were also detected. These data sug-

Nor-1 (MINOR, Nr4a3) is a transcription factor that belongs to the superfamily of steroid nuclear hormone receptors. It belongs to the Nur77 subfamily that includes two other members, Nur77 (NGFI-B, TR3, Nr4a1) and Nurr1 (TINUR, NORT, Nr4a2) (1–4). All three molecules in this subfamily share greater than 97% homology in their DNA binding domains, which consist of two zinc fingers and a domain termed the A box. The homologies in the N-terminal transactivation domains and the C-terminal “ligand binding domains” are 37–53% and 53–77%, respectively. In the absence of any ligand, all three can bind and activate the NGFI-B-responsive DNA element characterized by the sequence AAAGGTCA (5). In the presence of retinoic acid, however, Nur77 and Nurr1 but not Nor-1 can heterodimerize with the retinoid X receptor and regulate a DNA element composed of direct repeats separated by five nucleotides (DR5) (6–8). The Nur77 family has been implicated in a wide variety of biological processes and is activated as an immediate early gene in response to diverse signals, including serum induction of fibroblast cells, apoptotic stimulation of T cells and neuronal differentiation of PC12 cells (9). Constitutive expression of Nurr1 can be found in the ventral midbrain in the dopaminergic neuronal region whereas Nur77 and Nor-1 can be found in the adrenal gland and thymus and at low levels in many tissues (10). Nor-1-deficient mice die immediately after birth and lack dopamine-producing neurons (11). In con-

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\[ 1 \] The abbreviations used are: E, embryonic day; ES, embryonic stem; dpc, days post coitum.

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report, we found no viable Nor-1−/− mutant mice were born. Study of embryos from Nor-1−/− inter-crosses showed that Nor-1−/− embryos die between 8.5 and 9.5 days of gestation. Analysis of these and embryos earlier in development indicated that gastrulation is impaired in the absence of Nor-1. Our study of Nor-1-deficient mice reveals that Nor-1 causes accumulation of cells within the primitive streak, preventing further development and eventually leading to early lethality. Thus, Nor-1 function is essential for mouse gastrulation.

**MATERIALS AND METHODS**

**Generation of Nor-1-deficient Mice**—Cosmid clones of the Nor-1 locus from the 129/Sv strain of mouse were isolated from a genomic library provided by M. Koshland (University of California, Berkeley, CA). The parental targeting vector was made by cloning the neomycin resistant gene flanked by loxP sites into pSF72 (pSFloxneo). The gene targeting construct was generated by subcloning a 4.5-kb KanI genomic fragment into the KpnI site of pSFloxneo, and a 2.2-kb EcoRV/NheI fragment into a blunted HindIII site. The construct was linearized and electroporated into J1 embryonic stem (ES) cells using previously described protocols (21). Drug-resistant colonies were isolated and screened by Southern blot analysis. DNA samples were digested with XbaI, then probed using a 0.7-kb genomic fragment external to the targeting vector. Three correctly targeted clones were isolated from 288 colonies screened. ES cell injection into C57BL/6 blastocysts was performed according to standard procedures at the UC Berkeley pathogen-free facility.

**Genotyping—**F1 progeny were screened by Southern blotting as described above. Subsequent offspring and embryos were genotyped by PCR using primers specific for the deleted region of exon 3 (Nor1wt5p-8, 5′-TAC TCA GGC TGG TGT AGC GAT G and Nor1wt3p-8, 5′-CCC AAA TCC TGC CAG CCA ATC) to test for the presence of the wild type allele and a neo primer in conjunction with a primer 3′ of exon 3 (Nor1wt3p-2, 5′-AAG GAA GAA GGG GAG GC and MC1NEOR, 5′-GAG AGG CTT TTT GCT TCT TGC) to test for the targeted allele. The resulting products were separated on a 1.0% agarose gel and visualized with ethidium bromide staining. For genotyping of early stage embryos, either a portion of the extraembryonic region (6.5–7.5 dpc) or part of the yolk sac (8.0–9.5 dpc) was lysed in 20 µl lysis buffer (10 mM Tris, pH 8.0, 1.5 mM MgCl2, 0.05% Nonidet P-40, 0.45% Tween 20, 2 µg/ml proteinase K). A 1–µl aliquot was subjected to PCR amplification using the primers specified above.

**Histology—**Whole decidua were fixed and embedded in paraffin as described previously (22). Serial 6-µm transverse or sagittal sections were fixed onto glass slides and stained with hematoxylin and eosin as described previously (22).

**In Situ Hybridization—**Whole mount in situ hybridization was performed as described previously (23). In situ hybridization on paraffin embedded sections was performed as described previously (22).

**Plasmids—**Plasmids used in this work include pME68S (containing Brachyury cDNA), HH-16.1, Pax3 (a kind gift of A. McMahon), pKoslo2 (a kind gift of J. Rubenstein), HNF3β (a kind gift of J. Rossant), bmp4, C5mfg8, CSTbox6, mNoggin (a kind gift of R. Harland), Lim1, and Nor-1 (4). The antisense probes were prepared according to previously published sequences: Fgf8 (24); Otx2 (25); Brachyury (26); Lim1 (26); tbx6 (27); and Hnf3β (28).

**RESULTS**

**Generation of Nor-1-deficient Mice**—The Nor-1 gene in mouse ES cells was disrupted using standard techniques (21). A region containing the transcriptional domain and a portion of the DNA binding domain in the 3rd exon (the first coding exon) was deleted by homologous recombination (Fig. 1A). This corresponds to deletion of amino acids 182 to 318 (the DNA binding domain encompasses amino acids 291–380) and a loss of 37% of the transactivation region and the first zinc finger. Out of 288 colonies screened by Southern blot analysis, three G418-resistant ES cells were found to contain the targeted Nor-1 locus (Fig. 1B). They were used to generate Nor-1−/− mice. Nor-1 heterozygous mice were inter-crossed and typed by Southern blotting and PCR (Fig. 1C). The Nor-1 knockout allele was further confirmed by PCR and sequencing analysis of both 5′ and 3′ ends (data not shown). Previous work using Northern blot analysis indicated that Nor-1 is expressed as early as 7.5 dpc (17). We confirmed this by whole mount in situ hybridization. Embryos at 7.5 dpc appear to have ubiquitous expression of Nor-1, whereas by 9 and 9.5 dpc, expression is restricted to the brain (Fig. 1D).

**Targeted Disruption of the Nor-1 Gene Leads to Embryonic Lethality—**Initial observations indicated that Nor-1−/− mice were phenotypically normal and fertile and appeared indistinguishable from their wild type littermates. To our surprise, genotyping of offspring from Nor-1−/− inter-crosses indicated that no viable Nor-1−/− mice were found among 269 mice analyzed (Table I). This data indicated that homozygosity for the Nor-1 mutation caused embryonic lethality. Interestingly, these data also showed that lower than expected numbers of heterozygous offspring were produced, as the ratio of wild type to Nor-1−/− mice was close to 1:1 instead of the expected 1:2. The Chi square test showed a value of 44.07 (X2 = (141–89.67)2/89.67 + (128–179.33)2/179.33 = 44.07). This was larger than the 0.01 probability level of 6.64 (for degree of freedom, df = 1). Thus, the observed numbers of wild type to Nor-1 heterozygous offspring were statistically significantly different from the expected 1 to 2 ratio. This data suggests that partial loss of Nor-1 may also cause embryos lethality at some frequency (Table I).

To determine the age of embryonic death, we first analyzed embryonic day 3.5 blastocysts from Nor-1−/− heterozygous inter-crosses. Blastocysts from Nor-1−/− inter-crosses cultured for 3, 4, and 5 days resulted in development of trophoblasts as well as inner cell mass with no differences observed between the wild type and Nor-1-deficient cells (data not shown). There-
fore loss of Nor-1 does not appear to affect pre-implantation events. We then examined embryos at later days of gestation. For PCR genotyping, genomic DNA was isolated either from a portion of the extraembryonic region of E6.5–7.5 embryos, or from the yolk sac of E8.5 embryos. At E6.5, homozygous mutant embryos were recovered at approximately Mendelian ratios and appeared morphologically normal (Table I and Fig. 2A). At E7.5, embryos with distinct morphological abnormalities were readily observed. The most severely affected class were greatly reduced in size as compared with their wild type littersmates. By E8.5, degenerating or resorbed embryos were frequently seen (Fig. 2B and data not shown). The smaller, morphologically abnormal embryos at E7.5 were found to be Nor-1−/−. Interestingly, at E8.5, in addition to the resorbing homozygous mutant embryos, there were embryos exhibiting a developmentally delayed phenotype. These embryos looked phenotypically like E7.75 embryos and were not only smaller and data not shown). The smaller, degenerating or resorbed embryos were frequently seen (Fig. 2B and data not shown). The smaller, morphologically abnormal embryos at E7.5 were found to be Nor-1−/−. Interestingly, at E8.5, in addition to the resorbing homozygous mutant embryos, there were embryos exhibiting a developmentally delayed phenotype. These embryos looked phenotypically like E7.75 embryos and were not only smaller but appeared to have abnormal morphology (Fig. 2B, broken arrows). Embryos in this category appeared to have formed neural folds but had underdeveloped trunk regions. Genotyping of these embryos indicated that they were Nor-1−/−. This is consistent with the earlier findings (Table I) that heterozygosity at the Nor-1 locus may cause lethality at some low frequency.

To confirm that the Nor-1 mutation leads to loss of Nor-1 expression, which then results in the mutant phenotype, in situ hybridization was performed on paraffin embedded sections of Nor-1−/− inter-cross E7.5 embryos using Nor-1 antisense probe. Morphologically mutant embryos showed an absence of Nor-1 staining as compared with the wild type embryos (Fig. 2C), thus deletion of the 1st coding exon of Nor-1 leads to absence of Nor-1 transcription, which correlates with the abnormal phenotype seen in E7.5 and E8.5 embryos from Nor-1−/− inter-crosses.

**Marker Analysis Reveals that Nor-1−/− Embryos Fail to Produce Anterior Mesoderm**—To further characterize the defect of the Nor-1−/− mice, embryos isolated at 6.5–8.5 dpc were subjected to whole mount in situ hybridization analysis. Hybridization probes corresponding to a number of different developmental markers were used (note: experiments with each marker were done at least three times with similar results). Brachyury is a transcription factor that belongs to the T-box family of molecules (29–32). At the onset of gastrulation, Brachyury is expressed at the junction of the embryonic/extraembryonic region and eventually moves to one side of the embryo marking the future posterior. Brachyury expression continues along the proximal-distal axis marking the migration of the primitive streak. Normal Brachyury expression in Nor-1−/− embryos indicated that primitive streak formation did occur, and thus mesoderm was being made despite the decreased size of the embryo (Fig. 3, a–c).

**Fgf8** is another gene known to be important in early gastrulation events. Fgf8 is a secreted growth factor whose expression in the mouse embryo correlates with the production and movement of mesoderm (24, 33). Fgf8 expression is seen at the onset of gastrulation at E6.5, at the prospective proximal posterior region of the embryo. Similar to Brachyury, Fgf8 expression is found in early primitive streak and is maintained as the streak moves anteriorly toward the prospective node. Our in situ analysis of Nor-1 mutant embryos shows that Fgf8 expression can be detected but appears to be expanded somewhat, indicat-
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formation of the primitive streak is one of the earliest events in gastrulation, followed by the movement of cells to the distal portion of the embryo culminating in the formation of the structure known as the node, or the organizer. Transplantation experiments in mouse have shown that the node is capable of secondary axis formation (35, 36). In our studies of Nor-1 mutant mice we looked at expression patterns of a number of molecules that are important for proper development of the embryo, such as Brachyury (Tbx6), another member of the T-box family of transcription factors. Tbx6 is known to be expressed in the primitive streak, paraxial, and axial mesoderm but not in the node (27, 34). Wild type embryos show Tbx6 staining extending along the entire length of the primitive streak toward the distal tip of the embryo. Staining of Nor-1−/− embryos, however, shows that Tbx6 expression is absent in the distal portions of the embryo as compared with the wild type littermate (Fig. 3, a–f). We also examined the expression profile of Tbx6, another member of the T-box family of transcription factors. Tbx6 is known to be expressed in the primitive streak-derived tissues but is down-regulated after E7.5 (38), with expression limited to the periphery of the node (Fig. 4a). In the retarded Nor-1-deficient embryo, there is staining of the distal tip of the embryo, but the faint staining of the lateral mesoderm seen in wild type embryo is absent (Fig. 4b). Interestingly, a broad staining pattern can be seen for Nor-1−/− embryo (Fig. 4c), suggesting a late developmental progress of these embryos or perhaps altered distribution of lateral mesoderm. Additional future studies are needed to clarify this issue.

Formation of the primitive streak is one of the earliest events in gastrulation, followed by the movement of cells to the distal portion of the embryo culminating in the formation of the structure known as the node, or the organizer. Transplantation experiments in mouse have shown that the node is capable of secondary axis formation (35, 36). In our studies of Nor-1 mutant mice we looked at expression patterns of a number of markers for the node to determine whether this structure is made. As the organizer is a derivative of the migrating mesoderm, and because the Nor-1 mutants appear to be deficient in anterior mesoderm it was important to ascertain whether the Nor-1 mutants were capable of generating this structure. We tested a number of known markers of the node, including Lim1, Hnf3b, and noggin. Lim1 is a homeodomain containing protein, loss of which leads to embryonic lethality and dramatic defects in head formation (26, 37) as well as gastrulation. In wild type embryos, lim1 antisense probe stains most of the anterior primitive streak-derived tissues but is down-regulated after E7.5 (39). Its signal is reduced in the distal tip of the embryo, and staining of HNF3β (Hnf3b) probe was used for hybridization of wild type embryo (Fig. 4g). In the retarded Nor-1-deficient embryo, there is staining of the distal tip of the embryo, but the faint staining of the lateral mesoderm seen in wild type embryo is absent (Fig. 4h). Staining with Sonic Hedgehog (Sonic hedgehog) another marker for axial mesoderm, revealed similar findings (data not shown). The anterior visceral endoderm is also important for patterning of the mouse embryo and is involved in restriction of posterior signals (42). Otx2 mutant mice are embryonic lethal at the time of gastrulation and also show defects in head formation (28, 35, 36). Otx2 expression, however, appeared unaffected in the Nor-1−/− mutant (data not shown), suggesting that Nor-1 defects are not due to loss of the anterior visceral endoderm function. These data suggest that Nor-1 function is necessary for the formation of normal anterior mesoderm and its derivatives.

Nor-1-deficient Embryos Exhibit Defects of Cell Migration during Gastrulation—Cell movement during gastrulation is very important for proper development of the embryo, such that abnormal or defective migration often leads to early lethality. The molecular aspects of this process have been investigated by a number of different groups, and several key molecules have been identified. One molecule previously mentioned is Fgf8. Loss of Fgf8 leads to lethality at E8.5. These mutants exhibit an accumulation of mesoderm at the proximal posterior region of the streak (33) and are unable to form anterior mesoderm. Another gene shown to be involved in this process is the embryonic ectoderm development gene (eed). Loss of function mutation of eed in mice shows an increase in the production of extra embryonic mesoderm at the expense of...
embryonic anterior mesoderm (43).

Data from this study suggests that the Nor-1 gene may also play a role in distribution of early mesodermal cells within the streak. Hematoxylin and eosin staining of paraffin-embedded serial transverse sections of 7.0 dpc Nor-1+/− inter-cross embryos shows that loss of Nor-1 leads to abnormal distribution of the mesoderm. Accumulation of cells at the proximal posterior of the epiblast can be seen in Nor-1-deficient embryos, leading to a narrowing of the cavity within the embryo (compare Fig. 5, a–c with d–i). Sagittal sections of Nor-1 mutant embryos also show the accumulation of mesoderm midway along the proximal-distal axis in the primitive streak (Fig. 5, j and k). In some cases, allocation of mesoderm seems irregular, such that one side of the embryo appears to preferentially have more mesoderm than the other (Fig. 5l). This accumulation of mesoderm is strikingly similar to that seen in fgf8-null embryos, whereby embryos appear to make nascent mesoderm; however, these newly formed cells are not distributed normally or not able to migrate efficiently away from the streak. Alternatively, there might be abnormal growth of the mesodermal cells.

DISCUSSION

Among the three Nur77 family members, Nur77 has been studied extensively for its role in T cell apoptosis (9). Constitutive or over-expression of Nur77 in thymocytes or several non-lymphoid cell lines leads to apoptosis. In addition, expression of a Nur77 dominant negative protein in T cells can inhibit apoptosis accompanying T cell selection (14, 15). However, Nur77-deficient mice exhibit no phenotype (12). This is in contrast to mice with Nur1 deficiency. Its absence results in the loss of dopamine-producing neurons and early post-natal lethality (11). Although Nur1 does not seem to play a role in T cells, the expression profile of Nor-1 mimics that of Nur77 during T cell activation and constitutive expression of Nor-1 in T cells leads to cell death (16). Nor-1 has also been implicated as a gene translocated in human chondrosarcomas (44). The precise role of Nor-1 in other physiological settings is not clear as in T cells but may play redundant roles with Nur77 in some situations. We show here that Nor-1 is expressed early during mouse development and is essential for the gastrulation process. Previously described mutation of the Nor-1 gene locus leads to viable homozygous animals with only a minor problem in inner ear development (13). This is in stark contrast to our Nor-1-deficient mice, where homozygous animals die in utero. Our Nor-1-deficient mice were generated by deleting a large portion of the transactivation domain, and the first zinc finger domain of Nor-1. We’ve shown that Nor-1 expression is abolished in the homozygous Nor-1 mutant embryos (Fig. 2C) and thus the loss of portion of the 1st coding exon results in a complete null phenotype. In contrast, the published Nor-1 mutant mice were generated by inserting a β-galactosidase and a promoter driving the neomycin gene into the 1st coding exon (13), deleting only 19 amino acids of the Nor-1 transactivation domain (amino acids 212–231). As Nor-1 expression was not assessed in these mice, it is not clear if this mutation resulted in a complete loss of Nor-1 expression. Exon skipping and/or utilization of cryptic splice sites in the Nor-1 mutant locus could lead to production of a cryptic mutant protein and a potentially hypomorphic Nor-1 phenotype.

The process of gastrulation marks the beginning of the regionalization of the embryo and establishes the primary axis on which the embryo will establish the foundation for organogenesis and various tissue systems. Before gastrulation, the embryo exists as a symmetrical cylinder. However, gene expression studies have revealed that prior to gastrulation, around E6.0, a number of genes exhibit asymmetrical expression marking the site of the future anterior region of the embryo, which gives rise to the head structures (reviewed in Ref. 45). Gastrulation then begins with the formation and extension of the primitive streak, which initiates the morphological changes that give rise to the third germ layer, the mesoderm. Our whole mount data as well as Northern analysis of Nor-1 gene expression (17) indicate that Nor-1 gene expression can be seen as early as 7.0 dpc, just after the onset of gastrulation. Analysis of otx2 and bmp4 gene expression at 6.0 dpc (data not shown) in Nor-1+/− embryos indicated that they are normal. Furthermore, no differences in morphology between wild type and Nor-1 mutants can be seen before 7.0 dpc, suggesting that Nor-1 deficiency has little to no impact on mouse development before the onset of gastrulation.

Our investigation of the role of Nor-1 in mouse development suggests an important function for Nor-1 in the allocation of mesoderm. Nor-1 mutant embryos are able to initiate gastrulation and form the primitive streak, but fail to complete this process due to the inability of the newly formed mesoderm to migrate away from the primitive streak. Mutant embryos appear to accumulate mesoderm midway along the proximal-distal axis. Nor-1 mutant embryos in this aspect share features similar to fgf8 null embryos (33). Fgf8 is a growth factor, and it is known that Nor-1 and its family members are immediate early response genes to growth and differentiation stimuli (1, 46). Given this information, one can speculate that Nor-1 expression might normally be induced by fgf8 and lies in a molecular signaling pathway required for the proper movement and allocation of cells as they migrate away from the primitive streak. Alternatively, as it is known that Nor-1 is involved in cell death during thymocyte development, loss of Nor-1 might cause accumulation of cells by inefficient apoptosis. However, many studies of mouse embryo gastrulation have concluded that rapid proliferation and growth are more important than apoptosis for embryogenesis at this stage in development. Nor-1 levels are also important later during development as evidenced by the morphological defects in the Nor-1−/− mice. Although normal heterozygous mice can be found, they are born in much reduced numbers. Morphologically, the affected embryos appear delayed in their development and marker expression analysis reveals that although they generate axial mesoderm, it is incomplete. Thus, haploid insufficiency of Nor-1 can also cause mouse developmental problem.

In summary, we have shown here that complete loss of Nor-1 leads to early embryonic lethality, and that Nor-1 is essential...
for either the migration of newly formed mesoderm away from the primitive streak or growth control of these mesodermal cells. A reduction in Nor-1 levels may also lead to lethality later in development. Therefore, Nor-1 plays a key role in the growth of the mouse embryo. How Nor-1 regulates genes important for early mouse development is the subject of future studies.

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