

Nrk, an X-linked Protein Kinase in the Germinal Center Kinase Family, Is Required for Placental Development and Fetoplacental Induction of Labor*[§]

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The complete mechanism of labor induction in eutherian mammals remains unclear. Although important roles for the fetus and placenta in triggering labor have been proposed, no gene has been shown to be required in the fetus/placenta for labor induction. Here we show that *Nrk*, an X-linked gene encoding a Ser/Thr kinase of the germinal center kinase family, is essential in the fetus/placenta for labor in mice. *Nrk* was specifically expressed in the spongiotrophoblast layer, a fetus-derived region of the placenta, and *Nrk* disruption caused dysregulated overgrowth of the layer. Due to preferential inactivation of the paternally derived X chromosome in placenta, *Nrk* heterozygous mutant placentas exhibited a similar defect to that in *Nrk*-null tissues when the wild-type allele was paternally derived. However, the phenotype was weaker than in *Nrk*-null placentas due to leaky *Nrk* expression from the inactivated X chromosome. Crossing of *Nrk*-null females to wild-type and *Nrk*-null males, as well as uterine transfer of *Nrk*-null fetuses to wild-type females, revealed that pregnant mice exhibit a severe defect in delivery when all fetuses/placentas are *Nrk*-null. In addition, *Nrk* was not expressed in female reproductive tissues such as the uterus and ovary, as well as the fetal amnion and yolk sac, in pregnant mice. Progesterone and estrogen levels in the maternal circulation and placenta, which control the timing of labor, were unaffected upon *Nrk* disruption. We thus provide evidence for a novel labor-inducing fetoplacental signal that depends on the X chromosome and possibly arises from the placenta.

In the gestation of eutherian mammals, the onset of labor is strictly controlled so that offspring are safely delivered at an appropriate stage of development. In humans, preterm labor, defined as labor at <37 weeks of gestation, accounts for a large proportion of the deaths and permanent disabilities among newborns (1), underscoring the physiological and medical importance of regulating the timing of labor. In many mamma-

lian species, a decline in the maternal level of circulating progesterone, referred to as progesterone withdrawal, is a prerequisite for labor initiation (2, 3). Conversely, the maternal blood level of estrogen, which induces uterine contraction, is shown to increase in late gestation in several species (2, 4). However, the complete mechanism of labor induction remains unclear.

The fetus and placenta are believed to play critical roles in labor induction. Before the Common Era, the Greek scientist Hippocrates described that a baby may decide the timing of its own birth (4). It is now suggested in several species that labor-inducing signals originate from the fetus or placenta and are transmitted to the mother (4, 5). In sheep, there is evidence that the fetal hypothalamo-pituitary-adrenal axis involving corticotropin-releasing hormone (CRH),³ adrenocorticotropin, and cortisol induces the conversion of progesterone to estrogen in the placenta near term. In primates, CRH is secreted from the placenta and eventually induces the secretion from the fetal adrenal gland of dehydroepiandrosterone sulfate, a steroid that is converted to estrogen in the placenta. In either case, placenta-derived estrogen is transported to and acts on the maternal uterus. In mice, however, *CRH* knock-out dams carrying *CRH* knock-out fetuses exhibit normal delivery (6). The lung surfactant protein-A (SP-A), which is secreted from fetal lung and activates amniotic fluid macrophages to induce an inflammatory response *in utero*, has also been proposed as a fetal hormone inducing labor in mice (7). Again, however, *SP-A* knock-out dams carrying *SP-A* knock-out fetuses deliver normally (8). These observations argue against indispensable roles for fetal CRH and SP-A in labor induction at least in mice. Although knock-out mouse studies have identified ~10 genes that are essential for labor, all of them are required in the dam and not in the fetus or placenta (9, 10). Therefore, genetic evidence for the requirement of a fetoplacental factor in labor induction is lacking.

Nrk (Nik-related kinase), also named NESK (Nik-like embryo-specific kinase), is a Ser/Thr kinase encoded in the X chromosome (Xq22.3 in humans and X 53.0 cM in mice) (11, 12). It belongs to the germinal center kinase family, which is conserved among eukaryotes and plays essential roles in various aspects of development (13, 14). In mice, *Nrk* mRNA is detected in the fetus (predominantly in skeletal muscle) but not

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³ The abbreviations used are: CRH, corticotropin-releasing hormone; dpc, days postcoitum; SP-A, surfactant protein-A; *Nrk*, Nik-related kinase.

in any adult tissues examined (11, 12). Although overexpression studies in cultured mammalian cells suggest that Nrk possibly participates in JNK signaling (12), apoptosis (15), and F-actin organization (16), its physiological role has been totally unknown. To elucidate it, we here generated and studied *Nrk* knock-out mice.

EXPERIMENTAL PROCEDURES

Animal Experiments—All animal experiments were approved by and performed under the guidelines of the Institutional Animal Care and Research Advisory Committee of Tokyo Institute of Technology. Female mice found to have copulation plugs at 9–11 a.m. were considered to be at 0.5 days postcoitum (dpc) at noon of that day.

Derivation and Genotyping of Mutant Mice—The *Nrk* genomic locus was cloned from a 129/Sv mouse genomic library (Stratagene, La Jolla, CA) using a 5' region of *Nrk* cDNA as a probe. The gene targeting vector was constructed using the plasmid pPGKneolox2DTA (17) and contains a *neo* expression cassette flanked by 1.1-kb *Spe*I-BamHI and 4.0-kb *Xho*I-XbaI genomic sequences derived from the cloned *Nrk* locus. This vector replaces a 1.1-kb genomic fragment containing *Nrk* exon 1 with a *neo* cassette upon homologous recombination. The construct was electroporated into 129/S4-derived ES cells (17), and colonies were selected with G418. Homologous recombination events were screened by PCR using primers corresponding to the *neo* gene and a genomic sequence outside the targeting construct. Southern blotting, ES cell culture, and blastocyst injection were performed according to standard procedures. PCR genotyping of mice was performed on yolk sac and tail biopsy samples as described (17), using the following combination of primers: F, 5'-CTCTCCCGAACCACAAACCC-3'; R, 5'-CAGGTACCCATGCGCTAGAC-3'; and neo, 5'-GCTC-GAATCAAGCTGATCCGG-3'. PCR genotyping of the *Sry* gene was performed as described (18).

Anti-Nrk Antibody—A cDNA for an internal region of mouse *Nrk* protein (amino acid residues 776–1083), not conserved in other germinal center kinase family kinases, was cloned into the plasmid pGEX6P-2 (GE Healthcare, Piscataway, NJ) to generate a GST fusion construct. The fusion protein was purified from *Escherichia coli* cells using glutathione-Sepharose affinity beads (GE Healthcare), and 200 μ g of the protein was used to immunize each of two rabbits.

Histology and in Situ Hybridization—Placentas were dissected from pregnant mice and fixed in 4% paraformaldehyde in PBS. The tissues were gradually dehydrated in ethanol and embedded in paraffin. Sections were cut at 7 μ m and subjected to H&E staining or *in situ* hybridization by standard procedures. For *in situ* hybridization, antisense and sense RNA probes for mouse trophoblast-specific protein- α and *Nrk* mRNAs (nucleotide 169–385 and 1263–2070 from the translation initiation sites, respectively) were labeled with digoxigenin-conjugated UTP (Roche Diagnostics, Basel, Switzerland), and hybridized probes were detected by incubating the tissue sections with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics) followed by incubation with nitro blue tetrazolium chloride (Roche Diagnostics) and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics).

Immunofluorescence—After fixation in 4% paraformaldehyde in PBS for 5 h at 4 $^{\circ}$ C, placentas were cryoprotected with 30% sucrose in PBS, embedded in the Tissue-Tek OCT compound (Sakura Fine-technical, Tokyo, Japan), and sectioned at 10 μ m using a cryostat. Sections were re-fixed, permeabilized in 0.2% Triton X-100 in PBS, treated with 2 M HCl and 0.1% Triton X-100 for 1 h at 37 $^{\circ}$ C, and stained with anti-Nrk (1:100) or anti-Ki67 (1:100, Novus Biologicals, Littleton, CO) antibody together with Alexa Fluor 594-conjugated phalloidin (1:100, Invitrogen, Carlsbad, CA). The secondary antibody was Alexa Fluor 488-conjugated anti-rabbit IgG antibody (1:500, Invitrogen). Fluorescent images were captured with a laser-scanning confocal microscope (Axiovert 200M, Carl Zeiss, Oberkochen, Germany).

RT-PCR—Total RNAs from mouse placentas were isolated using an ISOGEN RNA extraction kit (Nippon Gene, Tokyo, Japan). Human placenta was obtained at delivery, and poly(A)⁺ RNA was isolated as described (19). First-strand cDNAs were synthesized from 1 μ g of RNAs using a ReverTra Ace- α kit (TOYOBO, Osaka, Japan) and used as templates for PCR. Primer sequences were: 5'-CGAAGACCATTCCGGCAGATTTC-3' and 5'-CTCAAAGCCACAGAAGACGGTATG-3' for mouse *Nrk*, and 5'-ATCTGGGCCACCTGCCGGAT-3' and 5'-CCGCTGACCAGGGGGACTCA-3' for human *Nrk*.

Immunoprecipitation and Immunoblotting—Placentas, uteri, ovaries, and fetal membranes (amnion and yolk sac) were sonicated using a Sonifier 250 (Branson, Danbury, CT) for 10–60 s in 20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A. After centrifugation at 12,000 \times g for 15 min at 4 $^{\circ}$ C, the supernatants were either directly used for immunoblotting (70 μ g of proteins) with anti-Nrk (1:500) and anti- α -tubulin (1:20,000, Sigma) antibodies or immunoprecipitated with 3 μ l of anti-Nrk antibody coupled to protein A-Sepharose beads (GE Healthcare). The immunoprecipitates were used for immunoblotting. The secondary antibodies were peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG antibodies (GE Healthcare). Blots were detected using ECL Western blotting Detection Reagents (GE Healthcare).

Uterine Transfer of Mouse Embryos—Isolation and uterine transfer of mouse embryos were performed as described (18). Briefly, eight-cell-stage embryos were isolated from the oviducts of pregnant mice at \sim 2.5 dpc and incubated in Brinster's BMOC-3 medium (Invitrogen) for \sim 24 h at 37 $^{\circ}$ C. The embryos (blastocysts) were transferred to the uteri of pseudo-pregnant WT Slc:ICR mice (Sankyo Labo Service, Tokyo, Japan) at 2.5 dpc after mating with vasectomized mice.

Measurement of Hormone Levels—Blood was collected from the abdominal aorta of mice, and EDTA was added to a final concentration of 3 mM. The blood was centrifuged at 2,000 g for 20 min at 4 $^{\circ}$ C, and the supernatant (*i.e.* the plasma) was recovered. Placentas were sonicated in ethanol using a Sonifier 250 and centrifuged at 700 \times g for 10 min at 4 $^{\circ}$ C. The supernatants were evaporated and re-suspended in the assay buffer. The plasma concentrations of progesterone and estradiol were measured by LC-MS/MS at ASKA Pharma Medical (Kawasaki, Japan) (20, 21) and confirmed using ELISA kits (Cayman

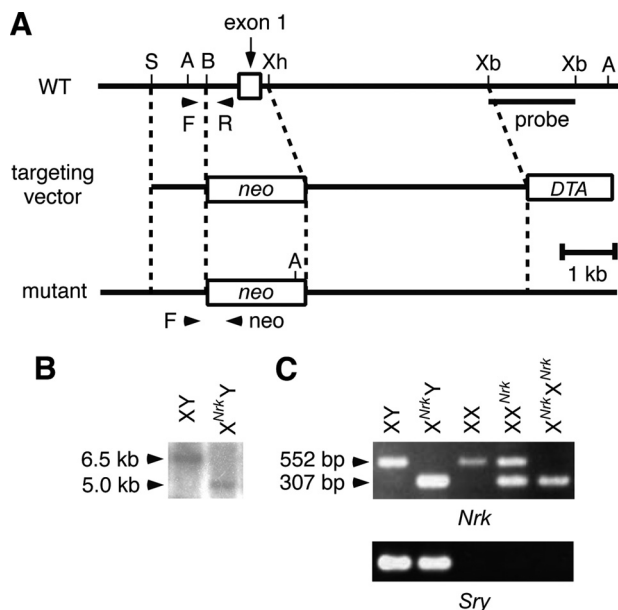


FIGURE 1. Generation of *Nrk*-null mice. *A*, schematic structure of the *Nrk* targeting vector, as well as of the WT and *Nrk*-null alleles. Arrowheads (*F*, *R*, and *neo*) indicate the positions of PCR primers used for genotyping in *C*. *DTA*, diphtheria toxin A gene; *neo*, neo-resistance gene; *A*, *Accl*; *B*, *BamHI*; *S*, *SpeI*; *Xb*, *XbaI*; *Xh*, *XhoI*. The position of the probe used for Southern blotting in *B* is also indicated. *B*, genomic DNAs from WT and *X^{Nrk}Y* mouse ES cells were digested with *Accl* and examined by Southern blotting using the *XbaI*-*XbaI* fragment within intron 1 as a probe. The WT and mutant alleles yielded 6.5- and 5.0-kb fragments, respectively. *C*, PCR genotyping using tail DNAs from XY, *X^{Nrk}Y*, XX, *XX^{Nrk}*, and *X^{Nrk}X^{Nrk}* mice as templates (top). This PCR amplified 552- and 307-bp fragments from the WT and mutant alleles, respectively. The sex of mice was confirmed by PCR of the *Sry* gene in the male-specific Y chromosome (bottom).

Chemical, Ann Arbor, MI). The placental levels of progesterone and estradiol were measured using the ELISA kits.

Statistical Analysis—The Student's *t* test was used for statistical analysis.

RESULTS

Generation of *Nrk*-null Mice—The first exon of *Nrk* was replaced by the *neo* cassette in 129/S4 mouse ES cells (Fig. 1A). Although the ES cell line was male harboring a single X chromosome, hemizygous *Nrk*-null ES cells grew normally and derived germ line chimeras when injected into mouse blastocysts. Chimeric mice were obtained from two *Nrk*-null ES cell clones, and mice derived from the two mutant clones exhibited the same phenotypes in this study.

Backcrossing of the chimeric mice to C57BL/6J mice (>11 generations) resulted in the production of healthy *X^{Nrk}Y* males and *XX^{Nrk}* females (*X^{Nrk}* designates an X chromosome carrying the *Nrk* mutation; the maternally derived X chromosome is designated first), intercrossing of which generated healthy *X^{Nrk}X^{Nrk}* females. However, although apparently normal *Nrk*-null (*X^{Nrk}Y* and *X^{Nrk}X^{Nrk}*) offspring were recovered at the Mendelian ratio at birth, their ratio was lower than expected at ~2 weeks of age (supplemental Table S1). These results suggested neonatal death of a population of *Nrk*-null mice, the reason for which is currently unknown. *Nrk*-null mice that survived the neonatal period appeared normal and were fertile, indicating that *Nrk* is not necessarily required for embryogenesis and postnatal life in mice.

***Nrk* Is Required for Placental Development**—*Nrk*-null fetuses were mostly normal at 18.5 dpc (Fig. 2A), although their weight was slightly, but statistically significantly, higher than that of WT and *XX^{Nrk}* fetuses at late gestation (Fig. 2B). In contrast, their accompanied placentas were significantly larger (~2.4-fold in weight) than control placentas at 18.5 dpc (Fig. 2A). The difference in placental size was detectable at 12.5 dpc and became greater at later gestation periods (Fig. 2C). The mouse placenta is mainly composed of the decidual, spongiotrophoblast, and labyrinth layers (22, 23). Although the decidual layer is maternally derived, the other two are of fetal origin. Histological analysis showed that in *Nrk*-null placenta, the spongiotrophoblast layer is larger and invades into the labyrinth layer (Fig. 2D).

***Nrk* Terminates the Proliferation of Spongiotrophoblasts**—*In situ* hybridization analysis of placental sections for trophoblast-specific protein- α , a marker for the spongiotrophoblast layer (24), confirmed that the *Nrk*-null spongiotrophoblast layer is enlarged and protruding into the labyrinth layer at 18.5 dpc (Fig. 3A). Immunofluorescence staining for Ki67 protein, a marker for proliferating cells (25), detected Ki67-positive cells in the spongiotrophoblast layer of *Nrk*-null, but not WT, placentas at 14.5 dpc (Fig. 3B). Particularly, cells that are invading into the labyrinth layer were strongly stained. In the labyrinth layer, the proportion of Ki67-positive cells was comparable between WT and *Nrk*-null tissues (Fig. 3B). In contrast, the TUNEL assay in placental sections showed no sign of reduced apoptosis levels in *Nrk*-null tissues (data not shown). These results suggested that *Nrk* disruption leads to overproliferation of the spongiotrophoblast layer cells (*i.e.* spongiotrophoblasts as well as glycogen cells, which are believed to be a subtype of spongiotrophoblast (23)).

In cultured cells, *Nrk* overexpression is reported to induce actin polymerization (16). However, phalloidin staining showed the level of F-actin in *Nrk*-null spongiotrophoblast layers to be comparable to that in WT tissues (Fig. 3B, see also Fig. 4D below). Overexpressed *Nrk* in cultured cells is also reported to activate the JNK pathway (12). Immunoblotting of placental lysates for Thr¹⁸³/Tyr¹⁸⁵-phosphorylated JNK, however, detected no significant difference in the level of JNK phosphorylation between WT and *Nrk*-null tissues (data not shown).

***Nrk* Is Expressed in the Placenta**—Because *Nrk* expression has not been tested in the placenta, we examined it at the mRNA and protein levels. RT-PCR analysis showed *Nrk* mRNA expression in WT, but not *Nrk*-null, mouse placentas at 12.5–18.5 dpc (Fig. 4A). RT-PCR also showed that *Nrk* is expressed in human placenta obtained at delivery (Fig. 4B). Immunoprecipitation of mouse placental lysates with anti-*Nrk* antibody, followed by immunoblotting of the precipitates with the same antibody, detected five (~180-, 140-, 130-, 110-, and 90-kDa) bands in WT, but not *Nrk*-null, tissues at 12.5–18.5 dpc (Fig. 4C, open arrowheads). Because mouse *Nrk* is a 1455-amino acid protein of 167 kDa, the ~180-kDa band probably represents the full-length version. Because the germinal center kinase family kinases (26–28), including *Nrk* expressed ectopically in cultured cells (15), undergo caspase-mediated cleavage, the smaller bands possibly represent proteolytically processed forms. Immunostaining of placental sections with anti-*Nrk*

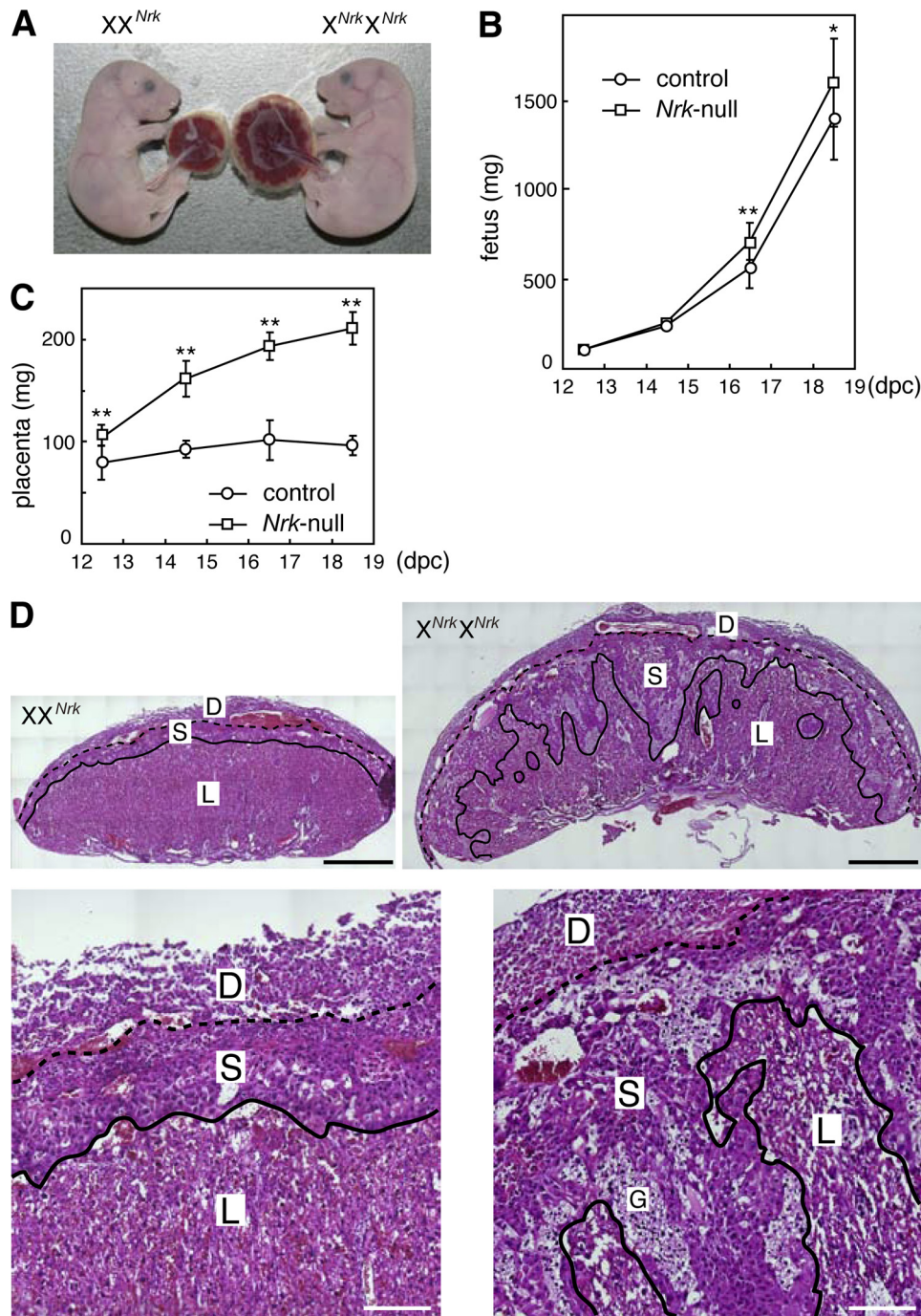


FIGURE 2. NrK is required for placental development. A, control (XX^{NrK}) and *Nrk*-null ($X^{NrK}X^{NrK}$) fetuses and placentas at 18.5 dpc. B and C, the weight of control (XY , XX , or XX^{NrK}) and *Nrk*-null ($X^{NrK}Y$ or $X^{NrK}X^{NrK}$) fetuses (B) and placentas (C) at 12.5–18.5 dpc. The mean \pm S.D. are shown ($n = 5$ for 12.5–14.5 dpc, $n > 15$ for 14.5–18.5 dpc). $p < 0.05$ (*) and 0.005 (**). D, H&E staining of control (XX^{NrK}) and *Nrk*-null ($X^{NrK}X^{NrK}$) placentas at 18.5 dpc at low (top) and high (bottom) magnification. D, decidual layer; S, spongiotrophoblast layer; L, labyrinth layer; G, glycogen cells in the spongiotrophoblast layer. The three layers are divided by broken and solid lines. Bars, 1 mm (top) and 200 μ m (bottom).

antibody (Fig. 4D), but not a pre-immune serum (data not shown), detected NrK protein in the spongiotrophoblast layer of WT, but not *Nrk*-null, placentas. This indicated that specific NrK expression occurs in the spongiotrophoblast layer. This expression pattern was confirmed at the mRNA level by *in situ* hybridization (see Fig. 5C below).

Paternally Derived NrK Is Preferentially but Incompletely Inactivated in the Female Placenta—In the female mouse placenta, the paternally derived X chromosome undergoes prefer-

ential inactivation (29, 30). We therefore examined whether paternally derived *Nrk* is functionally inactivated in $X^{NrK}X$ placentas obtained by crossing XX^{NrK} females to WT males. When isolated at 18.5 dpc, $X^{NrK}X$ placentas harboring paternal WT *Nrk* were larger (~ 1.7 -fold heavier) than WT littermate tissues (Fig. 5A; see also supplemental Fig. S1A), with overgrowth of the spongiotrophoblast layer being observed in *Nrk*-null tissues (supplemental Fig. S1, B and C). These results suggested that paternal *Nrk* is preferentially inactivated in the female placenta.

X-linked Placental Development and Labor Induction

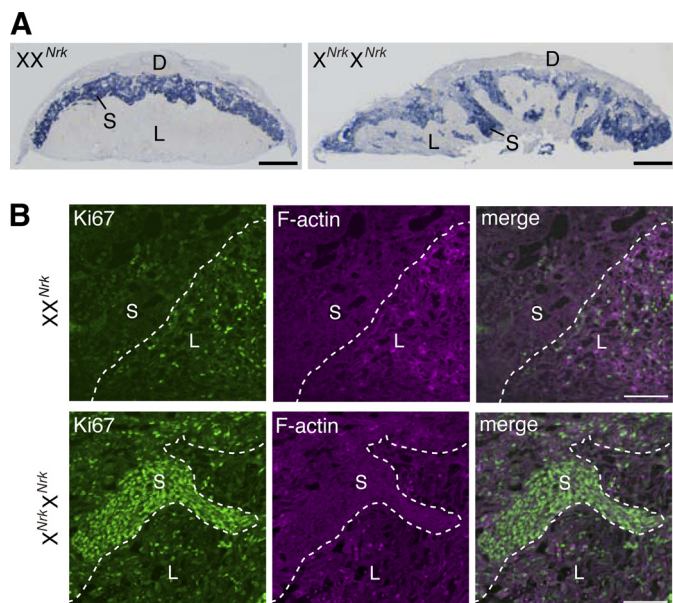


FIGURE 3. *Nrk* terminates the proliferation of spongiotrophoblasts. *A*, *in situ* hybridization analysis of trophoblast-specific protein- α mRNA in control (XX^{Nrk}) and *Nrk*-null ($X^{Nrk}X^{Nrk}$) placentas at 18.5 dpc. *B*, immunofluorescence staining of Ki67 protein in control (XX^{Nrk}) and *Nrk*-null ($X^{Nrk}X^{Nrk}$) placentas at 14.5 dpc. Cryosections were stained with anti-Ki67 antibody (green) and phalloidin (magenta). Merged images are also shown (right). D, decidua layer; S, spongiotrophoblast layer; L, labyrinth layer. Bars, 1 mm (A) and 100 μ m (B).

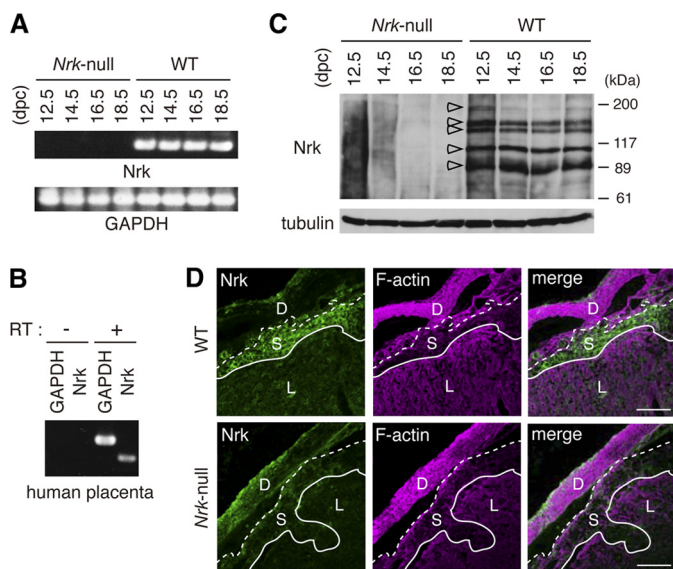


FIGURE 4. *Nrk* is expressed in placenta. *A*, RT-PCR analysis of *Nrk* mRNA in mouse placenta at indicated gestation periods (top). GAPDH mRNA was also examined (bottom). *B*, RT-PCR analysis of *Nrk* mRNA in human placenta, using RT-treated (+) or untreated (–) human placenta RNAs as templates. *C*, immunoprecipitation/immunoblot analysis of *Nrk* protein with anti-*Nrk* antibody in mouse placenta at indicated gestation periods (top). Open arrowheads indicate bands representing *Nrk* proteins. Placental lysates were also blotted with anti- α -tubulin antibody (bottom). *D*, immunofluorescence staining of *Nrk* protein in mouse placenta. Cryosections of WT and *Nrk*-null ($X^{Nrk}X^{Nrk}$) placentas were stained with anti-*Nrk* antibody (green) and phalloidin (magenta). Merged images are also shown (right). D, decidua layer; S, spongiotrophoblast layer; L, labyrinth layer. Bars, 200 μ m.

However, $X^{Nrk}X$ placentas were not as large as *Nrk*-null placentas, which were ~ 2.2 -fold heavier than WT tissues (Fig. 5A, see also Fig. 2C).

Since some X-linked genes have been shown to partially escape silencing in the inactive X chromosome (31, 32), *Nrk*

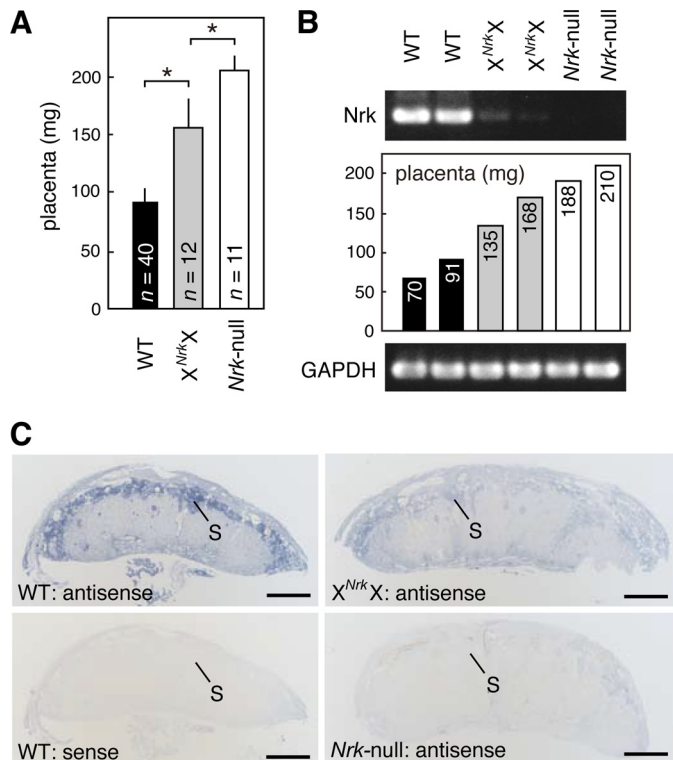


FIGURE 5. Paternally derived *Nrk* is preferentially but incompletely inactivated in placenta. *A*, the weight of WT (XY or XX), $X^{Nrk}X$, and *Nrk*-null ($X^{Nrk}Y$) placentas at 18.5 dpc. The mean \pm S.D. values are shown. Numbers (n) of placentas examined are indicated. *, $p < 0.0001$. *B*, RT-PCR analysis of *Nrk* mRNA in $X^{Nrk}X$ placentas at 18.5 dpc (top). The weight of each placenta is shown (middle). *C*, *in situ* hybridization analysis of *Nrk* mRNA in WT (XX), $X^{Nrk}X$, and *Nrk*-null ($X^{Nrk}Y$) placentas at 18.5 dpc. S, spongiotrophoblast layer. Bars, 1 mm.

mRNA expression was examined in $X^{Nrk}X$ placentas at 18.5 dpc. RT-PCR analysis detected a significantly lower level of *Nrk* expression in $X^{Nrk}X$ placentas ($n = 7$ from five litters) than in WT placentas ($n = 8$ from five litters) (Fig. 5B and data not shown). By *in situ* hybridization, the spongiotrophoblast layer was specifically stained for *Nrk* mRNA in WT, but not *Nrk*-null, placentas (Fig. 5C). Consistent with the results of the RT-PCR experiments in Fig. 5B, a low level of *Nrk* staining was detected in the spongiotrophoblast layer in $X^{Nrk}X$ placentas (Fig. 5C). These results suggested that the weak phenotype in $X^{Nrk}X$ placentas is due to limited *Nrk* expression from the paternally derived WT X chromosome.

Fetoplacental *Nrk* Is Required for Labor—When XX^{Nrk} females were mated to $X^{Nrk}Y$ males, both normal (XY and XX^{Nrk}) and *Nrk*-null offspring were delivered at term (~ 19 dpc) (supplemental Table S1), indicating that when accompanied by normal littermates, *Nrk*-null offspring were delivered normally. Therefore, the large size of *Nrk*-null placentas does not physically interfere with delivery. In the *Nrk*-null intercross ($X^{Nrk}Y \times X^{Nrk}X^{Nrk}$), however, normal labor was observed only once in 56 cases (Fig. 6A). In 19 cases, *Nrk*-null dams died without delivery, but with dead fetuses *in utero*, at 20–22 dpc. In 29 cases, they exhibited delayed delivery of all dead offspring at 20–22 dpc. In seven cases, they initiated delivery of 1–3 live offspring at ~ 20 –21 dpc, but the rest of the offspring were delivered dead subsequently. The death of the fetuses was probably secondary

A

phenotype	mating		
	$X^{Nrky} \times X^{Nrky}X^{Nrky}$	$XY \times X^{Nrky}X^{Nrky}$	$X^{Nrky} \times XX$
normal delivery	1	11	33
death of dam without delivery	19	1	1
delayed delivery	offspring all dead	29	5
	offspring live and dead	7	0

B

recipient: WT fetus: WT	timing of delivery	number of offspring	recipient: WT fetus: <i>Nrk</i> -null	timing of delivery	number of offspring
case 1	~19.25 dpc	6 (live)	case 1	~19.25 dpc	7 (live)
2	~19.5 dpc	3 (live)	2	~19.75 dpc	7 (live) + 1 (dead)
3	~20.0 dpc	5 (live)	3	~20.5 dpc	7 (dead)
			4	~20.75 dpc	1 (live) + 6 (dead)
			5	~21.25 dpc	5 (dead)
			6	~21.25 dpc	4 (dead)
			7	*no delivery	2 (dead <i>in utero</i>)
			8	*no delivery	2 (dead <i>in utero</i>)

FIGURE 6. **Fetoplacental *Nrk* is required for labor.** *A*, labor phenotype in WT and *Nrk*-null dams mated to WT or *Nrk*-null male mice. Numbers of cases of normal (blue) and failed (red) labor are indicated. *B*, timing of delivery and number of offspring in recipient WT dams transplanted with WT or *Nrk*-null blastocysts. *, dams did not deliver offspring and were dissected at 22.75 dpc to examine fetuses *in utero*.

to delayed delivery, because all the *Nrk*-null fetuses were alive and appeared normal at 19.5 dpc in the *Nrk*-null intercross (data not shown). In this experiment, not only the dams but also all the fetuses and placentas were *Nrk*-null, indicating that either maternal or fetoplacental *Nrk* is required for labor. We then mated *Nrk*-null ($X^{Nrky}X^{Nrky}$) females to WT males, giving rise to X^{Nrky} and $X^{Nrky}X^{Nrky}$ fetuses/placentas *in utero*. As shown in Fig. 6*A*, *Nrk*-null dams successfully gave birth in 11 of 17 cases and failed in six cases. This partial defect suggested that maternal *Nrk* is not essential, whereas placental rather than fetal *Nrk* is responsible, for labor induction (see "Discussion").

To further examine the requirement for fetoplacental *Nrk* in labor, we transplanted *Nrk*-null fetuses into WT dams. Eight-cell-stage *Nrk*-null embryos, derived from the *Nrk*-null intercross, were isolated at ~2.5 dpc, cultured for ~24 h *in vitro* to allow development to blastocysts, and transferred to the uteri of pseudo-pregnant WT mice (9–11 blastocysts per recipient). As a control, recipients that received WT blastocysts delivered live offspring normally at term (by 20.0 dpc for pseudo-pregnant mice) (Fig. 6*B*). By contrast, six of eight recipients that received *Nrk*-null blastocysts exhibited a delayed delivery of dead offspring (Fig. 6*B*), indicating that fetoplacental *Nrk* is mainly responsible for labor induction. However, two recipients delivered live *Nrk*-null offspring at term (Fig. 6*B*). The reason for this is unclear, but it is unlikely that maternal *Nrk* compensated for the loss-of-function of fetoplacental *Nrk*, because *Nrk* is not expressed in any adult tissues tested in mice (11, 12) (see also Fig. 7 below).

Nrk Is Not Expressed in Reproductive Tissues Other Than the Placenta—*Nrk* expression in female reproductive tissues has not been tested. We therefore performed immunoblot analysis of *Nrk* protein in the WT and *Nrk*-null uterus and ovary, as well as fetal membranes, including the amnion and yolk sac, in pregnant mice at 18.5 dpc. When lysates of these tissues, containing the same amount of total proteins, were blotted with anti-*Nrk*

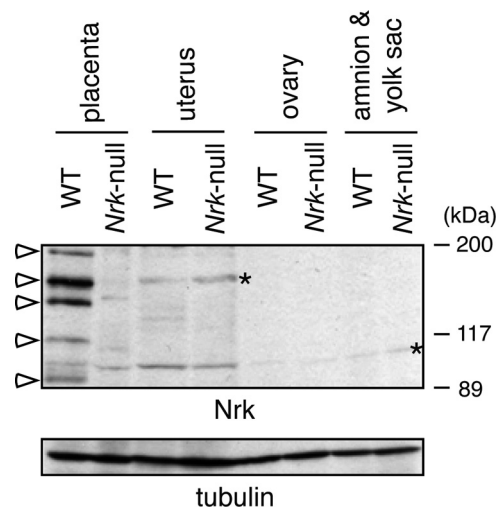


FIGURE 7. ***Nrk* is not expressed in reproductive tissues other than placenta.** Immunoblot analysis of *Nrk* protein with anti-*Nrk* antibody in the lysates of WT and *Nrk*-null mouse reproductive tissues at 18.5 dpc (top) is shown. The lysates were also blotted with anti- α -tubulin antibody (bottom). Open arrowheads and asterisks indicate *Nrk* and nonspecific bands, respectively.

antibody, only nonspecific bands, which were also detected in *Nrk*-null tissues, were detected in the WT uterus, ovary, and fetal membranes (Fig. 7, asterisks) in an experimental condition where placental *Nrk* was clearly detected (Fig. 7, open arrowheads).

Nrk Controls Neither Progesterone Withdrawal nor Estrogen Drive—To examine whether the labor defect in *Nrk*-null mice is due to a dysregulated level of progesterone or estrogen, we measured the concentration of these hormones in the plasma of *Nrk*-null dams mated to *Nrk*-null males. Measurements using LC-MS/MS (20, 21) (Fig. 8*A*) and ELISA (data not shown) both showed that similar in WT dams carrying WT fetuses, a drastic decrease in the progesterone level was observed in *Nrk*-null dams carrying *Nrk*-null fetuses: whereas the level ranged between ~20 and ~100 ng/ml at 17.5 dpc, it ranged between ~1 and ~2 ng/ml in five of seven mice at 19.5 dpc. In two cases, the level was irregularly high but not as high as that at 17.5 dpc. These progesterone levels were consistent with those previously reported in pregnant mice (33, 34). The plasma estradiol level was also comparable between the WT and *Nrk*-null conditions at 17.5 and 19.5 dpc (ranging from ~3 to ~50 pg/ml) (Fig. 8*B*). The estrogen levels were consistent with those reported in mice (33, 34) and, as shown previously (34), not elevated at 19.5 dpc compared with that at 17.5 dpc. We also measured the levels of these hormones in the placenta using ELISA. The levels of progesterone (Fig. 8*C*) and estradiol (Fig. 8*D*) were similar between control (XY and XX^{Nrky}) and *Nrk*-null placentas at 19.5 dpc. These results suggested that the labor defect in the absence of *Nrk* is not primarily due to a failure in progesterone withdrawal or estrogen drive.

DISCUSSION

In this study, we show that *Nrk* is expressed in the spongiotrophoblast layer, a fetus-derived region in the placenta, and is required for its development. We also show that fetoplacental

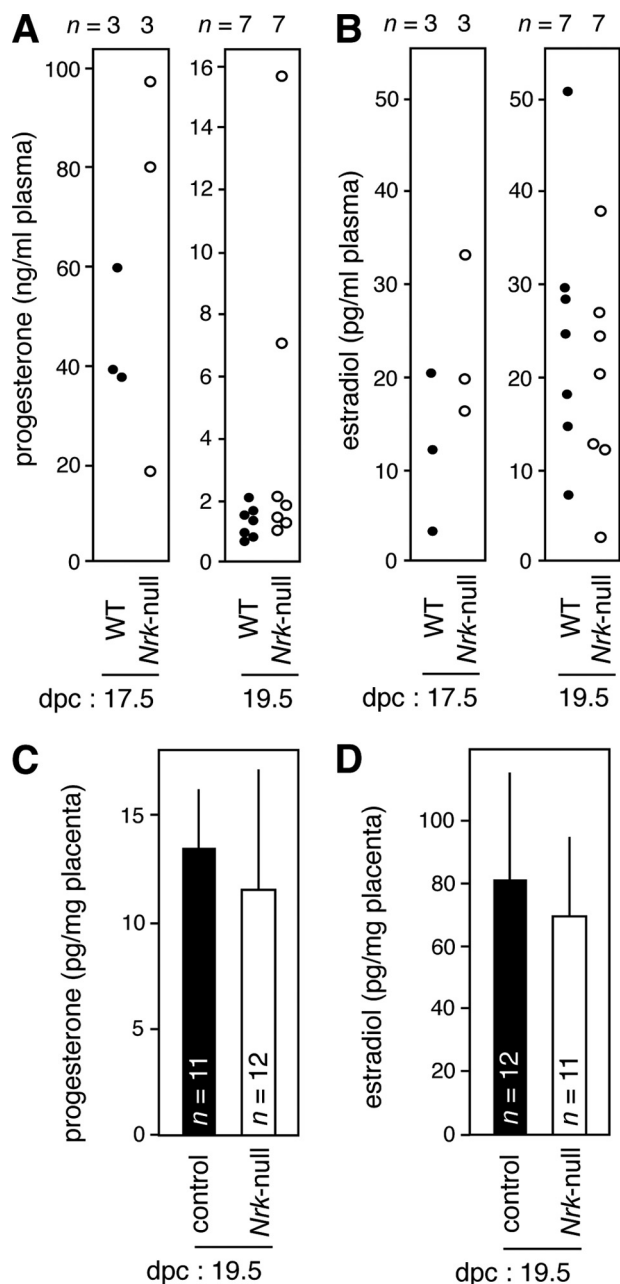


FIGURE 8. *Nrk* does not regulate the levels of progesterone and estrogen. A and B, the levels of progesterone (A) and estradiol (B) in the plasma of dams were measured at 17.5 and 19.5 dpc using LC-MS/MS. WT, WT dams with WT fetuses; *Nrk*-null, *Nrk*-null dams with *Nrk*-null fetuses. Each dot represents a single plasma sample. Numbers (n) of samples examined are indicated (top). $p = 0.48$ (A, 17.5 dpc), 0.16 (A, 19.5 dpc), 0.19 (B, 17.5 dpc), 0.47 (B, 19.5 dpc). C and D, the levels of progesterone (C) and estradiol (D) in control (XY or XX^{*Nrk*}) and *Nrk*-null (X^{*Nrk*}Y or X^{*Nrk*}X^{*Nrk*}) placentas were measured at 19.5 dpc using ELISA. The mean \pm S.D. values are shown. Numbers (n) of placentas examined are indicated. $p = 0.33$ (C) and 0.37 (D).

Nrk is required for labor of the dam, providing the first genetic evidence for fetoplacental induction of labor. Collectively, we suggest a novel, X-linked and protein kinase-mediated signaling for labor induction, which is possibly derived from the placenta.

Role of *Nrk* in Placental Development—In the fetus, *Nrk* is shown to be expressed predominantly in the skeletal muscle (11). In contrast to the drastic morphological defect in *Nrk*-

null placentas, however, embryonic development appeared mostly normal in *Nrk*-null mice (Fig. 2). Although *Nrk*-null fetuses were slightly larger than WT fetuses (Fig. 2) and a population of *Nrk*-null mice died neonatally (supplemental Table S1) for unknown reasons, those that survived the neonatal stage were apparently normal and fertile. In addition, *Nrk* is not expressed in any adult tissues tested in mice (11, 12) (Fig. 7). Thus, the predominant *Nrk*-null phenotype in the placenta and *Nrk* expression pattern suggest that *Nrk* plays a major role in the placenta. Because *Nrk* was restrictively expressed in the spongiotrophoblast layer (Figs. 4 and 5), which was specifically overgrown in *Nrk*-null placentas (Figs. 2 and 3), *Nrk* negatively regulates the proliferation of spongiotrophoblasts in a cell-autonomous fashion. Currently, it is unknown whether the large fetal size and neonatal death of *Nrk*-null mice is secondary to the placental defect.

In experiments using cultured mammalian cells in which *Nrk* was ectopically expressed, *Nrk* has been reported to activate the JNK pathway (12), promote apoptosis in a kinase-independent manner (15), and regulate the actin cytoskeleton by phosphorylating an actin-depolymerizing protein, cofilin (16). However, we failed to find changes in the levels of JNK phosphorylation, TUNEL staining, and phalloidin staining in *Nrk*-null placentas (Figs. 3 and 4, and data not shown). Further study is necessary to understand the molecular mechanism underlying the regulation of spongiotrophoblasts by *Nrk*. However, the same phenotype (*i.e.* placentomegaly caused by the protrusion of overproliferated spongiotrophoblasts into the labyrinth layer) was previously reported for mice lacking the X-linked homeobox gene *Esx1* (35) and mice cloned from somatic cells (36). Therefore, *Nrk* and *Esx1* may participate in the same signaling pathway that regulates placental development. In addition, *Nrk* expression may be reduced in the placenta of mice generated by somatic cell cloning, a possibility consistent with specific down-regulation of a number of X-linked genes in the cloned mouse embryo (37).

Inactivation of Paternally Derived *Nrk* in Placenta—In eutherian mammals, including mice, the paternally derived X chromosome is preferentially inactivated in the female placenta (29, 30). Therefore, female placentas heterozygous for mutations in X-linked genes such as *Esx1* (35) and *Cited1* (38) exhibit the same phenotype as placentas null for the genes when WT alleles are paternally derived. Consistently, X^{*Nrk*}X placentas also exhibited the phenotype observed in *Nrk*-null tissues (Fig. 5 and supplemental Fig. S1). Interestingly, however, the phenotype was milder than that in *Nrk*-null tissues. Concomitantly, a slight amount of *Nrk* mRNA was detected in X^{*Nrk*}X placentas. These results indicate that the phenotype is ameliorated in X^{*Nrk*}X placentas by a partial escape of the paternally derived *Nrk* from X inactivation. No such amelioration was reported in the *Esx1* and *Cited1* heterozygous mutant mice (35, 38). Our results thus present the first case in which a partial escape of an X-linked gene from paternal X inactivation generates a functional gene product in the placenta.

Role of *Nrk* in Labor—Pregnant mice exhibited a severe defect in labor when all the fetuses and placentas were *Nrk*-null

in utero (Fig. 6). Together with the finding that *Nrk* is not expressed in any adult tissues examined (11, 12) (Fig. 7), these results suggest that *Nrk* in the fetus or placenta is required for labor of the dam. The drastic placental, but not fetal, phenotype upon *Nrk* disruption suggests a role for placental, rather than fetal, *Nrk* in labor induction. The skeletal muscle-specific *Nrk* expression in the fetus (11) may also argue against a role for fetal *Nrk* in the process. This possibility is further supported by the following observation. When *Nrk*-null females were mated to WT males and carried $X^{Nrk}Y$ and $X^{Nrk}X$ fetuses/placentas *in utero*, they completed labor normally in eleven cases and failed to do so in six cases (Fig. 6). This partial defect can be explained if placental *Nrk*, which is weakly expressed in $X^{Nrk}X$ placentas, is responsible for labor induction. If maternal *Nrk* is responsible, *Nrk*-null dams in this experiment would also exhibit a labor defect in most cases as when crossed to *Nrk*-null males. If fetal *Nrk* is responsible, normal labor would be expected in most cases, because $X^{Nrk}X$ fetuses express *Nrk* (paternal and maternal X chromosomes are randomly inactivated in the fetus). Finally, if placental *Nrk* is responsible, *Nrk*-null dams would or would not exhibit a defect depending on the intrauterine number of $X^{Nrk}X$ placentas expressing a low level of *Nrk*: when the number of $X^{Nrk}X$ placentas is high, the total placental level of *Nrk* expression may exceed the threshold required for labor induction, whereas when the number is low, the total *Nrk* level may be below the threshold. In addition, because the levels of overgrowth and *Nrk* mRNA expression varied considerably in different $X^{Nrk}X$ placentas (Fig. 5), the total placental *Nrk* level would also be affected by the degree of escape of WT *Nrk* from X inactivation in individual $X^{Nrk}X$ placentas. Collectively, we suggest that *Nrk* in the placental spongiotrophoblast layer plays a critical role in labor induction.

A possible mechanism for *Nrk*-mediated labor induction is that *Nrk* promotes the production of an unidentified soluble factor that is secreted from the placenta and acts on the dam. Because our knowledge of the molecular function of *Nrk* is limited (12, 15, 16), further study is necessary to understand the role of *Nrk* in labor induction. *Nrk*-null mice will provide a powerful tool for elucidating the precise mechanism of this feto-maternal interaction. The mechanisms of labor induction differ considerably between species, including humans, sheep, and mice (4, 9). Therefore, it is also important to know whether *Nrk* participates in the labor induction of other eutherian mammals. We, however, found the levels of progesterone and estrogen to be unaffected in *Nrk*-null dams carrying *Nrk*-null fetuses (Fig. 8). These results suggest that *Nrk* does not act upstream of the actions of these hormones in labor. In contrast to many mammalian species, including mice, humans maintain a high maternal level of circulating progesterone throughout pregnancy (2, 3). The observation that *Nrk* does not participate in progesterone withdrawal, which does not occur in humans, together with the expression of *Nrk* mRNA in the human placenta (Fig. 4), suggest that *Nrk*-mediated signaling may also operate in human labor. *Nrk* therefore serves as a potential target to therapeutically control human labor.

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