Promotion of Trophoblast Stem Cell Proliferation by FGF4

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d-wave–like spin gap in the underdoped regime being the most successful example. The problem with the mean-field solution of the *t-J* model is that it does not agree with exact numerical calculation results (*24*), and the half-filled state was found by neutron scattering to have long range order (*40*). If these numerical calculations are right then the *d*-wave RVB is not the right solution of the *t-J* model. However, the *d*-wave RVB-like state may still be a reasonable way to think about the experimental data that describes the situation of the spin state near a hole (*41*). It is just that one has to start with a model where the single hole Neél state is destabilized, as in the *t-t′-t″-J* model. We leave this open question as a challenge to theory.

The presence of *d*-wave–like dispersion along the remnant Fermi surface shows that the high-energy pseudo gap is a remnant of the *d*-wave gap seen in the insulator. The details of the evolution of this gap, and its connection to the low-energy pseudo gap (which is likely due to pairing fluctuations) as well as the superconducting gap is unclear at the moment. However, we believe that there has to be a connection between these gaps of the similar $|\cos k_x a - \cos k_y a|$ form, as their presence is correlated with each other (*7*).

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Promotion of Trophoblast Stem Cell Proliferation by FGF4

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The trophoblast cell lineage is essential for the survival of the mammalian embryo in utero. This lineage is specified before implantation into the uterus and is restricted to form the fetal portion of the placenta. A culture of mouse blastocysts or early postimplantation trophoblasts in the presence of fibroblast growth factor 4 (FGF4) permitted the isolation of permanent trophoblast stem cell lines. These cell lines differentiated to other trophoblast subtypes in vitro in the absence of FGF4 and exclusively contributed to the trophoblast lineage in vivo in chimeras.

In mammals, the trophoblast cell lineage is specified before implantation into the uterus. In mice, this lineage appears at the blastocyst stage as the trophectoderm, a sphere of epithelial cells surrounding the inner cell mass (ICM) and the blastocoel. After implantation, the ICM forms the embryo proper and also some extraembryonic membranes. However, the trophectoderm is exclusively restricted to form the fetal portion of the placenta and the trophoblast giant cells. The polar trophectoderm (the subset of the trophectoderm that is in direct contact with the ICM) maintains a proliferative capacity and forms the extraembryonic ectoderm (ExE), the ectoplacental cone (EPC), and the secondary giant cells of the early conceptus (*1*). The rest of the trophectoderm ceases to proliferate and becomes primary giant cells. Studies in primary culture and chimeric mice have suggested that stem cells exist in the ExE that contribute de-

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scendants to the EPC and the polyploid giant cells (*2*). Further evidence has indicated that the maintenance of these stem cell–like characteristics was dependent on signals from the ICM and, later in development, from the epiblast (*3*, *4*).

Expression and functional analyses indicated that *Fgf4* and *Fgfr2* may be involved in trophoblast proliferation (*5*–*8*). The reciprocal expression domains of *Fgfr2* and *Fgf4* suggested that the trophoblast could be a target tissue for an embryonic fibroblast growth factor (FGF) signal. *Fgfr2* null and *Fgf4* null mice show similar peri-implantation lethal phenotypes (*6, 8*). This may result from defects in the ICM and its endoderm derivatives. However, this similarity is also consistent with the possibility that FGF4 acts on the trophoblast through FGFR2 to maintain a proliferating population of trophoblast cells. Support for both possibilities is provided by a recent study showing that inhibiting FGF signaling blocked cell division in the ICM and the trophectoderm (*9*).

At 6.5 days postcoitum (dpc), ExE cells were isolated from conceptuses (*4*), disaggregated by trypsin, and cultured on a feeder layer of primary mouse embryonic fibroblast (EMFI) cells in the presence of FGF4 (25 ng/ml) and heparin (1 μg/ml) in trophoblast stem (TS) cell medium (*10*). This allowed the passage of colonies with a tight epithelial morphology (Fig. 1A). The removal of FGF4, heparin, or the EMFI cells resulted in a rapid decline in proliferation, with a subsequent differentiation into cells with a giant cell-like phenotype (Fig. 1B). Even under optimal conditions, some giant cells consistently appeared at the edges of colonies after each passage, which suggests that a small percentage of the cells underwent differentiation (Fig. 1A). Because the giant cells were relatively trypsin-resistant, they were left behind after each passage and therefore remained at a relatively constant amount.

The cell colonies were similar to epithelial-like colonies that occasionally appear during the isolation of embryonic stem (ES) cells (*11*) and to a trophectoderm cell line, TE1, established from porcine blastocysts (*12*). Therefore, we attempted to isolate cell lines directly from mouse blastocysts. Using the culture conditions required for isolating cell lines from ExE, we derived lines from blastocysts at 3.5 days dpc that exhibited a morphology and a behavior indistinguishable from those of ExE-derived cell lines (*13*). The blastocyst- and ExE-derived lines are referred to here as $TS_{3.5}$ and $TS_{6.5}$ cell lines, respectively. The generation of $TS_{3.5}$ and $TS_{6.5}$ cell lines was efficient and reproducible. Fifty-eight clonal $TS_{3.5}$ cell lines have been obtained from 91 blastocysts (64%), and 17 $TS_{6.5}$ cell lines have been obtained from 39 ExEs of 6.5-dpc embryos (44%); these lines have been derived from different strain backgrounds (129/Sv and ICR) and from both sexes. Some of these TS cell lines have been stably maintained for more than 50 passages over a period of more than 6 months with no apparent change in their morphology or viability.

To address the possibility that FGF4 stimulated the proliferation of TS cells indirectly by inducing the secretion of mitotic factors from the feeder cells, we prepared EMFI-conditioned medium (EMFI-CM) in the absence of FGF4. TS cells were maintained in an undifferentiated state on gelatin-coated

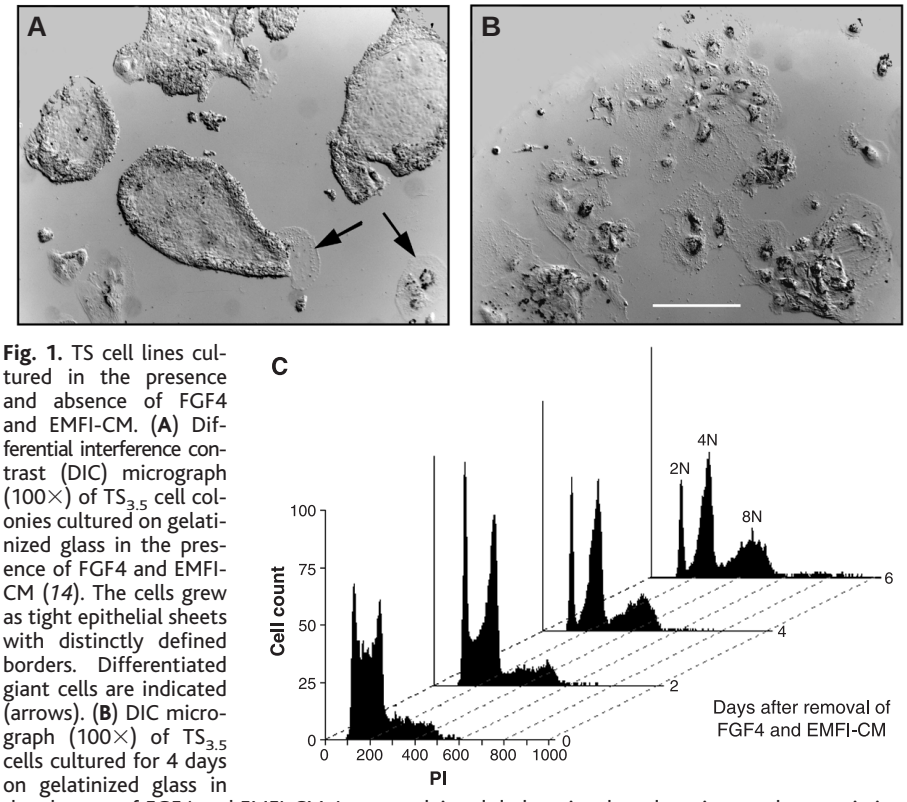

Fig. 1. TS cell lines cultured in the presence and absence of FGF4 and EMFI-CM. (**A**) Differential interference contrast (DIC) micrograph (100×) of $TS_{3.5}$ cell colonies cultured on gelatinized glass in the presence of FGF4 and EMFI-CM (*14*). The cells grew as tight epithelial sheets with distinctly defined borders. Differentiated giant cells are indicated (arrows). (**B**) DIC micrograph (100×) of $TS_{3.5}$ cells cultured for 4 days on gelatinized glass in the absence of FGF4 and EMFI-CM. Large nuclei and dark perinuclear deposits are characteristic of giant cells. Scale bar, 5 μm. (**C**) DNA content was analyzed by flow cytometric studies of cells stained with PI (*16*). TS cells were analyzed at 0, 2, 4, and 6 days after the removal of FGF4 and EMFI-CM. Diploid (2N), tetraploid (4N), and octaploid (8N) DNA contents are indicated.

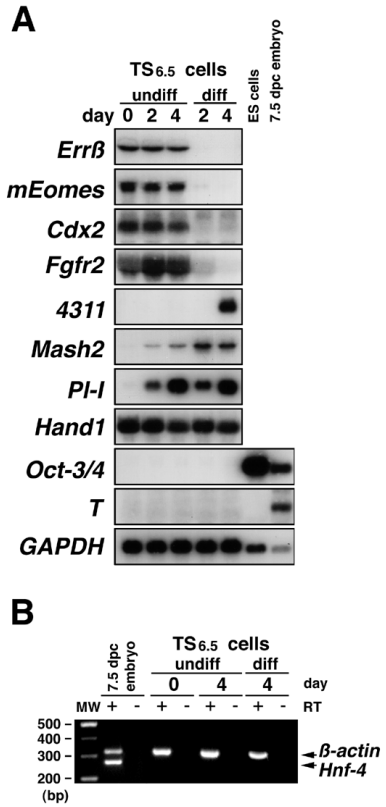

Fig. 2. RNA analysis of a $TS_{6.5}$ cell line. (**A**) TS cells were grown in 70% EMFI-CM and 30% TS medium supplemented with FGF4 and heparin for 2 days (*14*). The undifferentiated samples (undiff) were allowed to proliferate further in the same conditions for 0, 2, and 4 days. The differentiated samples (diff) had FGF4, heparin, and EMFI-CM removed for 2 and 4 days. The total RNA (10 μg) from TS cells, undifferentiated ES cells, and 7.5-dpc embryos was fractionated on a 1% denaturing agarose gel and blotted onto a nylon membrane. Three blots were made and sequentially probed and reprobed with antisense RNA probes as indicated (*18*). *T*, *brachyury*. (**B**) A reverse transcriptase (RT)–PCR analysis of *Hnf4* expression in the TS cells. From 0.5 μg of total RNA, first-strand cDNA was synthesized with (+) or without (–) RT. Primers specific for β-*actin* and *Hnf4* were added in a single reaction tube to amplify both β-*actin*– and *Hnf4*-specific fragments simultaneously (*18*). The predicted sizes of the β-*actin* and *Hnf4* bands are 321 and 270 base pairs, respectively. Similar results were obtained from a $TS_{3.5}$ cell line (*15*). MW, molecular weight.

plates in medium supplemented with 70% EMFI-CM, FGF4, and heparin (*14*); lower concentrations of EMFI-CM were not effective. The leukemia inhibitory factor, which is the EMFI factor that maintains ES cells in an undifferentiated state, could not substitute for EMFI-CM. These results suggest that (i) EMFI cells secrete an unidentified factor or factors that act with FGF4 to maintain the TS cells in a proliferative and undifferentiated state, (ii) the secretion of this factor or factors is not a result of the addition of FGF4 to the medium, and (iii) FGF4 acts directly on the TS cells.

Chromosome spreads from two TS cell lines that were passaged over 20 times revealed an apparently normal euploid karyotype (*15*). The ploidy of the stem cells and differentiated giant cells was determined by fluorescence-activated cell sorting (FACS) analysis (*16*). The profile for cells maintained in EMFI-CM supplemented with FGF4 and heparin revealed prominent peaks at 2N (diploid) and 4N (tetraploid), indicative of the first gap phase (G_1) and the second gap phase (G_2) or mitotic (M) DNA content of a diploid cell line (Fig. 1C). A small shoulder of higher ploidy cells (>4N) was also observed and was likely due to the presence of differentiating giant cells in the culture. Upon removal of FGF4 and EMFI-CM, an 8N peak appeared within 4 days. The 2N peak was reduced, and the 4N peak, which would include diploid G_2 or M cells and tetraploid G_1 cells, increased in size. By 6 days, higher ploidy cells (>8N) were starting to appear. The time required for TS cells to double their ploidy agreed with other giant cell analyses (*17*). These observations are consistent with the morphological differentiation of TS cells to giant cells.

Several genetic markers were analyzed during stem cell and differentiative culture conditions to confirm the trophoblast identity of these cell lines and to characterize their differentiation in the absence of FGF4 (*18*). Markers of the diploid ExE were highly expressed in TS cells. *Err*β (*19*), *Cdx2* (*20*), *Fgfr2* (*6*), and the mouse homologue of *eomesodermin* (*mEomes*) (*21*) were highly expressed in TS cells grown in the presence of FGF4 and 70% EMFI-CM but were downregulated when differentiation was induced (Fig. 2A). In contrast to the ExE-specific genes, *4311* [an EPC-specific gene (*22*)] was not detected in the undifferentiated cells but was induced 4 days after the removal of FGF4 and EMFI-CM. *Mash2*, which encodes a basic helix-loop-helix (bHLH) transcription factor expressed in the EPC (*23*), was upregulated in differentiating TS cells (Fig. 2A). *Placental lactogen 1* (*Pl-1*), a specific marker for giant cells (*24*), was induced in cultures after the removal of FGF4 (Fig. 2A). *Mash2* and *Pl-1* transcripts were also progressively induced in TS cells that were cultured in stem cell conditions. *Hand1*, another bHLH transcription factor that functions in the development of giant cells but is not

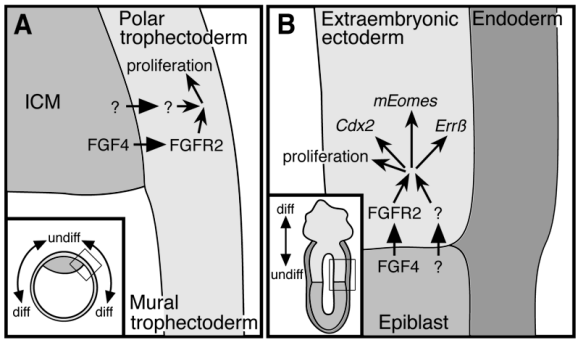

Fig. 4. A model for embryonic-trophoblast interactions and the maintenance of TS cells in vivo. (**A**) A schematic drawing of a 3.5-dpc blastocyst (inset) emphasizing a region where the polar and mural trophectoderms meet with the ICM. FGF4 and at least one other unidentified factor produced in the ICM signal to the overlying polar trophectoderm, maintaining it in a proliferative state. As the trophectoderm cells move away from the ICM to become mural trophectoderm, they cease to receive the ICM-derived signals and, consequently, differentiate. (**B**) A schematic drawing of a 6.5-dpc conceptus (inset) emphasizing the embryonic-extraembryonic boundary. Similar to the blastocyst scenario, FGF4 and an unknown factor or factors from the epiblast signal to the ExE and directly or indirectly mediate the expression of genes such as *Cdx2*, *mEomes*, and *Err*β. These signals maintain a TS cell population in the ExE nearest to the epiblast. As trophoblast cells move away from the embryonic-extraembryonic border, they no longer receive the epiblast signals, and differentiation ensues.

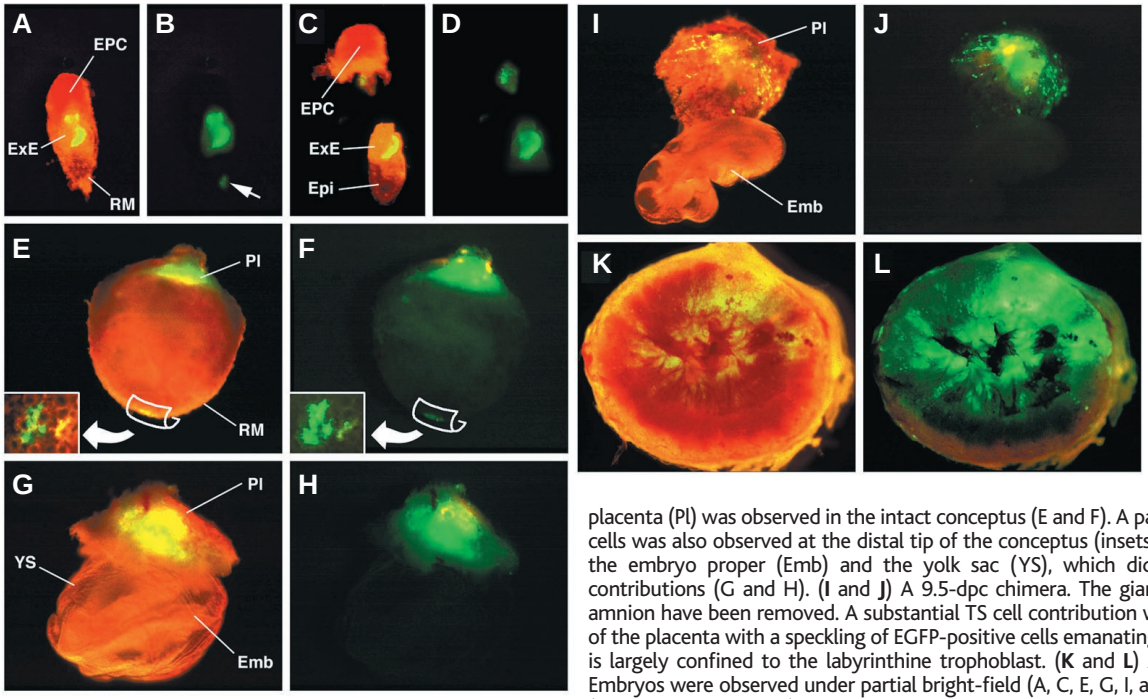

Fig. 3. TS cell chimeras generated by EGFP-$TS_{3.5}$ cell blastocyst injections. (**A** through **D**) A 6.5-dpc chimera. The intact conceptus revealed TS cell contributions to the ExE, a patch in the EPC, and a few giant cells on Reichert's membrane (RM) (arrow) (A and B). Removal of RM and separation of the EPC from the ExE further illustrated the TS cell contributions to extraembryonic regions and not the epiblast (Epi) (C and D). (**E** through **H**) An 8.5-dpc chimera. A large contribution of TS cells to the placenta (Pl) was observed in the intact conceptus (E and F). A patch of EGFP-positive giant cells was also observed at the distal tip of the conceptus (insets). Removal of RM exposed the embryo proper (Emb) and the yolk sac (YS), which did not exhibit any TS cell contributions (G and H). (**I** and **J**) A 9.5-dpc chimera. The giant cell layer, yolk sac, and amnion have been removed. A substantial TS cell contribution was observed at the center of the placenta with a speckling of EGFP-positive cells emanating from it. This contribution is largely confined to the labyrinthine trophoblast. (**K** and **L**) A chimeric term placenta. Embryos were observed under partial bright-field (A, C, E, G, I, and K) and dark-field optics (B, D, F, H, J, and L). Green fluorescence was observed as described (*28*), and all photographs were taken with Kodak P1600 film at 1600 ASA.

expressed in the ExE cells (*25*), was detected throughout the analyzed culture periods regardless of the presence of FGF4 and EMFI-CM (Fig. 2A). *Oct3/4*, *Brachyury*, and *Hnf4* [genes specific for the ICM or epiblast, the mesoderm, and the primitive endoderm, respectively (*26*)] were not detected in TS cells (Fig. 2). Thus, these established cell lines display a gene expression profile that is characteristic of trophoblast cells in the ExE, and they express markers of other trophoblast cell lineages upon differentiation.

To investigate the ability of TS cells to contribute to trophoblast lineages in vivo, we made chimeric embryos by the aggregation method (*27*) and by blastocyst injection. $TS_{3.5}$ and a $TS_{6.5}$ cell lines were derived from B5/EGFP transgenic mice (*28*) that ubiquitously express enhanced green fluorescent protein (EGFP) (Clontech, Palo Alto, California) in all embryonic and extraembryonic tissues. These lines were passaged more than 20 times (over 2 months) before they were used for chimera experiments. Chimeras were obtained from each cell line by both aggregation and blastocyst injection (tables containing these data can be seen at www.sciencemag.org/feature/data/984629.shl). EGFP-positive cells were only observed in tissues of the trophoblast lineage in the 61 chimeric embryos analyzed. TS cells contributed to the ExE, EPC, and giant cells, but they were never observed in the epiblast, primitive endoderm, or other ICM-derived extraembryonic tissues (such as the allantois, yolk sac, and amnion) (Fig. 3). High contributions of TS cells to chimeric placentas at term were also observed, indicating that these cells could functionally support fetal development (Fig. 3, K and L). There was no difference between the EGFP-$TS_{3.5}$ and EGFP-$TS_{6.5}$ cell lines in their ability to contribute to trophoblast subtypes. Thus, TS cells retain the ability to differentiate into all trophoblast cell types in vivo despite being cultured in vitro for extended periods of time. After observing this ability and the results of the Northern (RNA) blot analyses, we conclude that we have established stable pluripotent mouse TS cell lines.

The successful derivation of TS cell lines has led us to propose that FGF4 is a component of the embryonic signal required for the maintenance of the proliferative undifferentiated state of ExE (Fig. 4). Recent studies on the effects of FGF4 on *Oct3/4* mutant embryos have reached the same conclusion (*29*). Because of its expression pattern and null phenotype, FGFR2 is the best candidate to receive the FGF4 signal in the trophoblast. The components downstream of the trophoblast FGF response are not known; however, the T-box gene, *mEomes*, and the caudal-related gene, *Cdx2*, are good candidates because they are expressed in the appropriate cells and because members of these gene families have been shown to be regulated by FGF signaling (*30*, *31*).

The availability of TS cell lines, which can differentiate into trophoblast subtypes in vitro and contribute to normal development in chimeras, opens new possibilities for dissecting the function of genes and signaling pathways that are important to the development of the mammalian trophoblast lineage.

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13. $TS_{3.5}$ cell lines were obtained with techniques similar to those used for ES cell line derivation (*11*). Blastocysts (3.5 dpc) were individually plated into four-well plates on EMFI cells and cultured in TS medium with FGF4 and heparin (*10*). The medium was changed after day 2, and the blastocyst outgrowth was trypsinized on day 3. On day 5 or 6, flat colonies [referred to as "epithelial-like cells" in (*11*)] were picked and passaged. Once established, the cell lines were grown without EMFI cells but in the presence of EMFI-CM (*14*). Under these culture conditions, ES cell colonies were not observed.
14. EMFI-CM was prepared by incubating TS medium (*10*) without FGF4 or heparin on confluent plates of mitomycin-treated EMFI cells for 72 hours. The conditioned medium was filtered (0.45 μm) and stored at –20°C. Established TS cell lines were routinely cultured in 70% EMFI-CM, 30% TS medium, FGF4 (25 ng/ml), and heparin (1 μg/ml) on gelatinized plates. The medium was changed every 2 days, and 1 part in 25 of the cells was passaged every 4 days or at 80 to 90% confluency.
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16. TS cells were grown in the absence of EMFI cells (*14*) and were collected by cell scraping at 0, 2, 4, and 6 days after the removal of FGF4, heparin, and EMFI-CM. The cells were fixed and stained with propidium iodide (PI) (Molecular Probes, Eugene, OR) as described [Z. Darzynkiewicz and G. Juan, in *Current Protocols in Cytometry*, J. P. Robinson, Ed. (Wiley, New York, 1997), pp. 7.5.2–7.5.3]. Cell fluorescence was measured by flow cytometry with an argon ion laser (488 nm). The data were analyzed with Coulter EXPO Cytometer software [version 2.0 (1998)] by Applied Cytometry Systems (Sheffield, UK).
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18. Total RNA was prepared from cells and embryos with TRIzol (Gibco BRL) according to the manufacturer's instructions. Northern (RNA) blotting was performed by a standard protocol. Antisense RNA probes for *Err*β (*19*), *eomesodermin* (*21*), *Cdx2* [E. Suh, L. Chen, J. Taylor, P. G. Traber, *Mol. Cell. Biol.* **14**, 7340 (1994)], *Fgfr2* (*7*), *4311* (*22*), *Mash2* (*23*), *Pl-1* [P. Colosi, F. Talamantes, D. I. H. Linzer, *Mol. Endocrinol.* **1**, 767 (1987)], *Hand1* (*25*), *Oct-3/4* (*26*), *Brachyury* (*26*), and *GAPDH* [P. Fort *et al.*, *Nucleic Acids Res.* **13**, 1431 (1985)] were labeled with either [α-^{32}P]uridine 5′-triphosphate (UTP) or digoxigenin (DIG)-11-UTP (Boehringer Mannheim) by using a Strip-EZ RNA kit (Ambion, Austin, TX). Blots were hybridized overnight at 65°C in NorthernMax prehybridization/hybridization buffer (Ambion) and were washed in 0.1× standard saline citrate/0.1% SDS at 65°C. DIG-labeled probes were detected with the DIG luminescent detection kit (Boehringer Mannheim). The removal of hybridized RNA probes was performed with the Strip-EZ RNA kit (Ambion) according to the manufacturer's recommendations. To assess the expression of *Hnf4* in the TS cell lines, we synthesized first-strand cDNA from total RNA (0.5 μg) of TS cells, and 7.5-dpc embryos with random hexamers were subjected to 35 cycles of polymerase chain reaction (PCR) (62°C annealing temperature) with *Hnf4*-specific primers [5′-CACGTCCCCATCTGAAGGTG-3′ and 5′-CTTCCTTCTTCATGCCAGCCC-3′ (0.2 μM each)] and with β-actin–specific primers [5′-GACAACGGCTCCGGCATGTGCAAAG-3′ and 5′-TTCACGGTTGGCCTTAGGGTTCAG-3′ (0.1 μM each)]. The primer sequences were adapted from D. Ioannis *et al.*, *Development* **125**, 1529 (1998).
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