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RNA-binding protein TIAR is essential for primordial germ cell development

(gene targeting/partial embryonic lethality/RNA recognition motif/ribonucleoprotein/sterility)

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ABSTRACT **Primordial germ cells (PGCs) give rise to both eggs and sperm via complex maturational processes that require both cell migration and proliferation. However, little is known about the genes controlling gamete formation during the early stages of PGC development. Although several mutations are known to severely reduce the number of PGCs reaching and populating the genital ridges, the molecular identity of only two of these genes is known: the c-*kit* receptor protein tyrosine kinase and the c-*kit* ligand (the steel factor). Herein, we report that mutant mice lacking TIAR, an RNA recognition motif/ribonucleoprotein-type RNA-binding protein highly expressed in PGCs, fail to develop spermatogonia or oogonia. This developmental defect is a consequence of reduced survival of PGCs that migrate to the genital ridge around embryonic day 11.5 (E11.5). The numbers of PGCs populating the genital ridge in TIAR-deficient embryos are severely reduced compared to wild-type embryos by E11.5 and in the mutants PGCs are completely absent at E13.5. Furthermore, TIAR-deficient embryonic stem cells do not proliferate in the absence of exogenous leukemia inhibitory factor in an *in vitro* methylcellulose culture assay, supporting a role for TIAR in regulating cell proliferation. Because the development of PGCs relies on the action of several growth factors, these results are consistent with a role for TIAR in the expression of a survival factor or survival factor receptor that is essential for PGC development. TIAR-deficient mice thus provide a model system to study molecular mechanisms of PGC development and possibly the basis for some forms of idiopathic infertility.**

The development of primordial germ cells (PGCs) into gametes is an essential feature of sexual reproduction. Although the development of PGCs and their differentiation into sperm and oocytes is well understood on the morphological level, relatively little is known about the genes controlling gamete formation, particularly during the early stages of PGC development (1, 2). Murine PGCs originate from a founder population of ≈45 cells formed just after embryonic day 7 (E7.0) in the extraembryonic mesoderm posterior to the primitive streak (3). They then localize to the hindgut endoderm by E8.5 and migrate through the hindgut mesentery to reach the genital ridges around E11 (4, 5). During migration and colonization, PGCs proliferate to reach a total of ≈25,000 PGCs per gonad by E13 (6). This migration and proliferation of PGCs is regulated by multiple growth factors, as well as cell–cell and cell–matrix interactions (2). Although several mutations are known to reduce severely the number of PGCs reaching and populating the genital ridges, the molecular identity of only two of these mutations is known: the c-*kit* receptor protein tyrosine kinase and the c-*kit* ligand (the steel factor) (7–9). Herein, we report that disruption of the gene encoding the RNA binding protein TIAR severely affects mouse PGC development before E13.5. TIAR is thus the third molecularly identified gene product necessary for PGC development. TIAR belongs to the RNA recognition motif (RRM)/ribonucleoprotein family of RNA binding proteins (10, 11) and was implicated as a regulator of apoptosis (12). RRM family members generally function in the splicing, transport, translation, and stability of mRNA (13–15). In *Drosophila*, RRM proteins regulate diverse developmental processes, including sex determination, neuron development, and pattern formation (16–22). Thus, TIAR-deficient mice provide a model system to study molecular mechanisms of PGC development.

MATERIALS AND METHODS

Gene Targeting. The TIAR targeting vector consists of a 6.5-kb *Xba*I–*Sal*I fragment containing exons 3–5 of the *tiar* gene followed by a PGK promoter neo^r cassette, a 2.5-kb *Eco*RV–*Hin*dIII fragment containing part of exon 6 and extending through exon 8 of the *tiar* gene, and a PGK–thymidine kinase cassette inserted in pSP73 (Fig. 1*A*). D3 embryonic stem (ES) cells were electroporated with the *Eco*RI linearized targeting construct and selected in G418 (150 μg/ml) and gancylovir (2 μM) (Calbiochem) and expanded for Southern analysis. Two resulting clones of ≈750 clones analyzed yielded the expected size fragments and were used for injection into C57BL/6 blastocysts. Blastocysts were transferred to the uterus of pseudopregnant Swiss–Webster mice. Germ-line transmission of one of the clones was obtained on further crossing of male chimeras with C57BL/6 females.

Protein Analysis. $tiar^{-/-}$ ES cell lines were derived from $tiar^{+/-}$ ES cells grown in 0.3 mg/ml G418 (23). For protein analysis, $tiar^{-/-}$ ES cells were lysed in a buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris·HCl (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Samples were sonicated, and insoluble material was removed by centrifugation (10 min at 10,000 × *g*). Proteins were resolved by 14% SDS/PAGE under reducing conditions, transferred to Immobilon P membrane (Millipore), and blotted with anti-TIAR 6E3 mAb (1 μg/ml). Antibody was detected with protein A/G–horseradish peroxidase (Pierce) and the chemiluminescence reagent luminol (DuPont/NEN).

Southern Blot Analysis. Tail biopsies, kidneys, or yolk sacs were subjected to proteinase K digestion for 10–16 h at 55°C in a buffer containing 50 mM Tris·HCl (pH 8.5), 20 mM EDTA, 10 mM NaCl, 0.5% SDS, and 0.5 mg/ml proteinase K.

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Abbreviations: E, embryonic day; ES, embryonic stem; LIF, leukemia inhibitory factor; PGC, primordial germ cell; RRM, RNA recognition motif.

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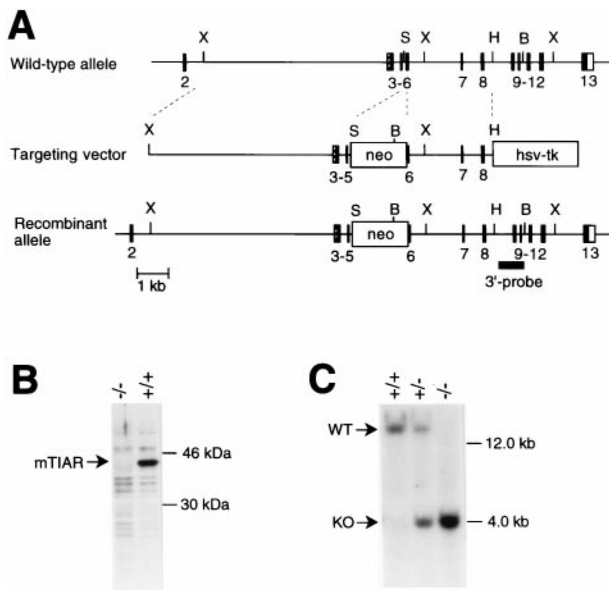

FIG. 1. Gene targeting at the *tiar* locus. (*A*) Schematic representation of the *tiar* gene exon–intron structure with restriction enzyme sites (11), the structure of the targeting vector, and the structure of the *tiar* locus after integration of the targeting vector. Correct gene targeting results in replacement of portions of intron 5 and exon 6 by the marker gene for positive selection (PGKneo). The hsv-TK (thymidine kinase) expression cassette was used for negative selection. The 3′ probe used for Southern blot analysis is shown as a stippled box. B, *Bam*HI; H, *Hin*dIII; S, *Sal*I; X, *Xba*I. (*B*) Protein immunoblot of total lysates of $tiar^{-/-}$ and $tiar^{+/+}$ ES cells by using the anti-TIAR mAb 6E3 (11) confirming absence of TIAR protein in $tiar^{-/-}$ cells relative to wild-type cells. Use of an anti-TIAR mAb reactive with a different TIAR epitope (anti-3E6) (11) gave the same result (data not shown). (*C*) Southern blot analysis of DNA from offspring derived from heterozygous matings. Genomic DNA was digested with *Bam*HI and analyzed by using the 3′ probe, yielding the >12-kb and 4.0-kb fragments expected for the wild-type allele and mutant allele, respectively. Southern blot analysis of ES cell DNA using a 5′ DNA probe and a neo DNA probe showed proper targeting and single insertion of the transfected vector (data not shown).

Genomic DNA was recovered by phenol extraction and precipitation, and ≈20 μg of genomic DNA digested with *Eco*RI was used for Southern blot analysis.

Histology and Immunohistochemistry. For histology, tissues or embryos were fixed in 10% buffered formalin (Sigma), embedded in paraffin, sectioned, and stained with hematoxylin and eosin according to standard procedures. For immunohistochemical analysis, sections were deparaffinized, rehydrated, and treated with antigen unmasking solution (Vector Laboratories). Sections were incubated for 1 h with 10% horse serum and 5% BSA in PBS and then for 30 min with 5 μg/ml anti-TIAR mAb 3E6 with 10% horse serum and 5% BSA in PBS. Sections were washed in PBS adjusted to 0.5 M NaCl, and primary mAb was detected with the Vecta Stain Elite ABC Peroxidase Kit (Vector Laboratories) and counterstained with Gill's hematoxylin. To detect GCNA1 antigen, sections were treated essentially the same way, except that the sections were incubated for 20 min in PBS with 1.5% horse serum and then for 30 min with undiluted hybridoma supernatant conditioned with mAb against GCNA1 and washed in PBS. For alkaline phosphatase staining (4), embryos were obtained at E11.5 or E13.5 (day 0.5 = day of finding a copulation plug) from crosses of $tiar^{+/-} \times tiar^{+/-}$ mice. The yolk sac was used for genotyping, and embryos were fixed in absolute ethanol-to-glacial acetic acid (7:1) at 4°C for 1 h followed by two changes of absolute ethanol at 4°C for 24 h each. After embedding in paraffin and sectioning (7 μm), sections were dewaxed in xylenes, rehydrated through a graded ethanol series, and stained for alkaline phosphatase activity during 20 min at room temperature in a buffer containing 25 mM sodium tetraborate, 3.5 mM $MgCl_2$, 0.1 mg/ml sodium α-naphtylphosphate, and 0.1 mg/ml Fast Red TR salt (Sigma).

***In Vitro* Methylcellulose Assay for ES Cell Proliferation.** For the *in vitro* methylcellulose proliferation assay, ES cells were dissociated by trypsinization followed by 6 passages through a 20-guage needle. ES cells were then suspended in ES cell media [DMEM/15% fetal calf serum/1X penicillin–streptomycin solution (GIBCO/BRL)/2 mM L-glutamine/0.1 mM MEM nonessential amino acids solution (GIBCO/BRL)/0.1 mM 2-mercaptoethanol] containing 1% (wt/vol) methylcellulose (Fluka), with or without the addition of 1,000 units of leukemia inhibitory factor (LIF; GIBCO/BRL) per milliliter. Cells were plated into nontreated, 96-well plates at ≈1,000 cells/0.15 ml/well. To measure cell proliferation at different days after cell plating, 1 μCi (1 Ci = 37 GBq) [^{3}H]thymidine/25 μl of ES cell media was added per well, and after 9 h of incubation, the cells were harvested, and [^{3}H]thymidine incorporation was determined. Assays were done in triplicate with two independent $tiar^{-/-}$ and two independent control cell lines. The two $tiar^{-/-}$ ES cell clones were derived from independent $tiar^{+/-}$ ES clones by selection in 0.3 mg/ml G418 (23). As control, two ES cell lines were used, one of which had undergone selection in 0.3 mg/ml G418 (23).

RESULTS

Generation and Characterization of TIAR-Deficient Mice. TIAR-deficient mice ($tiar^{-/-}$) were generated by using ES

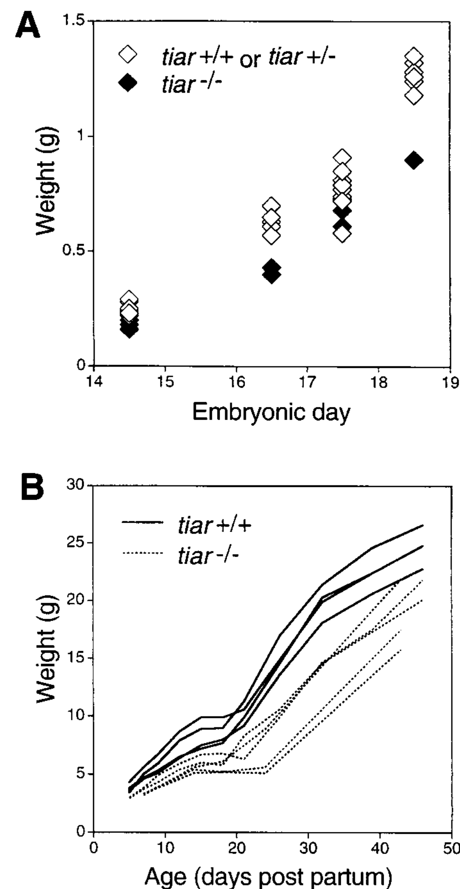

FIG. 2. Reduced weight of $tiar^{-/-}$ mice during development. (*A*) Weights of $tiar^{+/+}$ and $tiar^{+/-}$ embryos (open diamonds) or $tiar^{-/-}$ (solid diamonds) embryos are shown at different days of embryonic development. No significant weight difference was found between $tiar^{+/+}$ and $tiar^{+/-}$ embryos. (*B*) Weights of $tiar^{+/+}$ mice (solid line) and $tiar^{-/-}$ mice (dotted line) between 5 and 45 days postpartum. Each line represents the weight of an individual mouse.

cells containing a targeted mutation of the *tiar* gene. Proper integration of the TIAR targeting construct (Fig. 1*A*) into the *tiar* gene was confirmed by Southern blot analysis of mutant ES cells (data not shown), and immunoblot analysis using antibodies against TIAR verified the absence of TIAR protein in $tiar^{-/-}$ ES cells (Fig. 1*B*). Transmission of the $tiar^{-}$ allele through the germ line was demonstrated by Southern blot analysis of DNA from offspring of mice derived from the $tiar^{+/-}$ ES cells (Fig. 1*C*).

Crosses between $tiar^{+/-}$ mice yielded $tiar^{-/-}$ offspring that exhibited at least three phenotypes: partial embryonic lethality, reduced weight, and male and female sterility. $tiar^{+/-} \times tiar^{+/-}$ matings demonstrated a marked lethality among $tiar^{-/-}$ embryos and offspring. Only 14% of the expected number of $tiar^{-/-}$ mice were alive at 2–3 weeks of age ($tiar^{-/-}$:$tiar^{+/-}$:$tiar^{+/+}$ = 11:157:74). Mortality was not progressive beyond this point, however, and surviving $tiar^{-/-}$ mice lived beyond 12 months of age. Genotype analysis of E10.5–E12.5 embryos and E13.5–E18.5 embryos from $tiar^{+/-}$ matings identified 35% ($tiar^{-/-}$:$tiar^{+/-}$:$tiar^{+/+}$ = 19:105:57) and 38% ($tiar^{-/-}$:$tiar^{+/-}$:$tiar^{+/+}$ = 15:77:40) of the expected number of $tiar^{-/-}$ embryos, respectively, indicating that >60% of the embryos died *in utero*. Slightly less than half of the remaining embryos survived to adulthood. Weight measurements of embryos demonstrated that $tiar^{-/-}$ embryos were smaller than their $tiar^{+/-}$ or $tiar^{+/+}$ littermates at all embryonic stages examined (Fig. 2*A*). This size discrepancy persisted until they reached maturity at ≈6 weeks of age (Fig. 2*B*). The basis for the partial lethality and reduced weight of $tiar^{-/-}$ embryos and mice is unknown. Generation of inbred strains of $tiar^{-/-}$ mice onto several different backgrounds will be necessary to further characterize these phenotypes because the analyses were done using mice of mixed genetic backgrounds.

TIAR-Deficient Mice Are Sterile Because They Lack Spermatogonia or Oogonia. Breedings of either male or female $tiar^{-/-}$ mice with wild-type mice were unsuccessful, indicating that both sexes were sterile. Histologic analysis revealed that the $tiar^{-/-}$ testes lacked spermatogonia, spermatids, and ma-

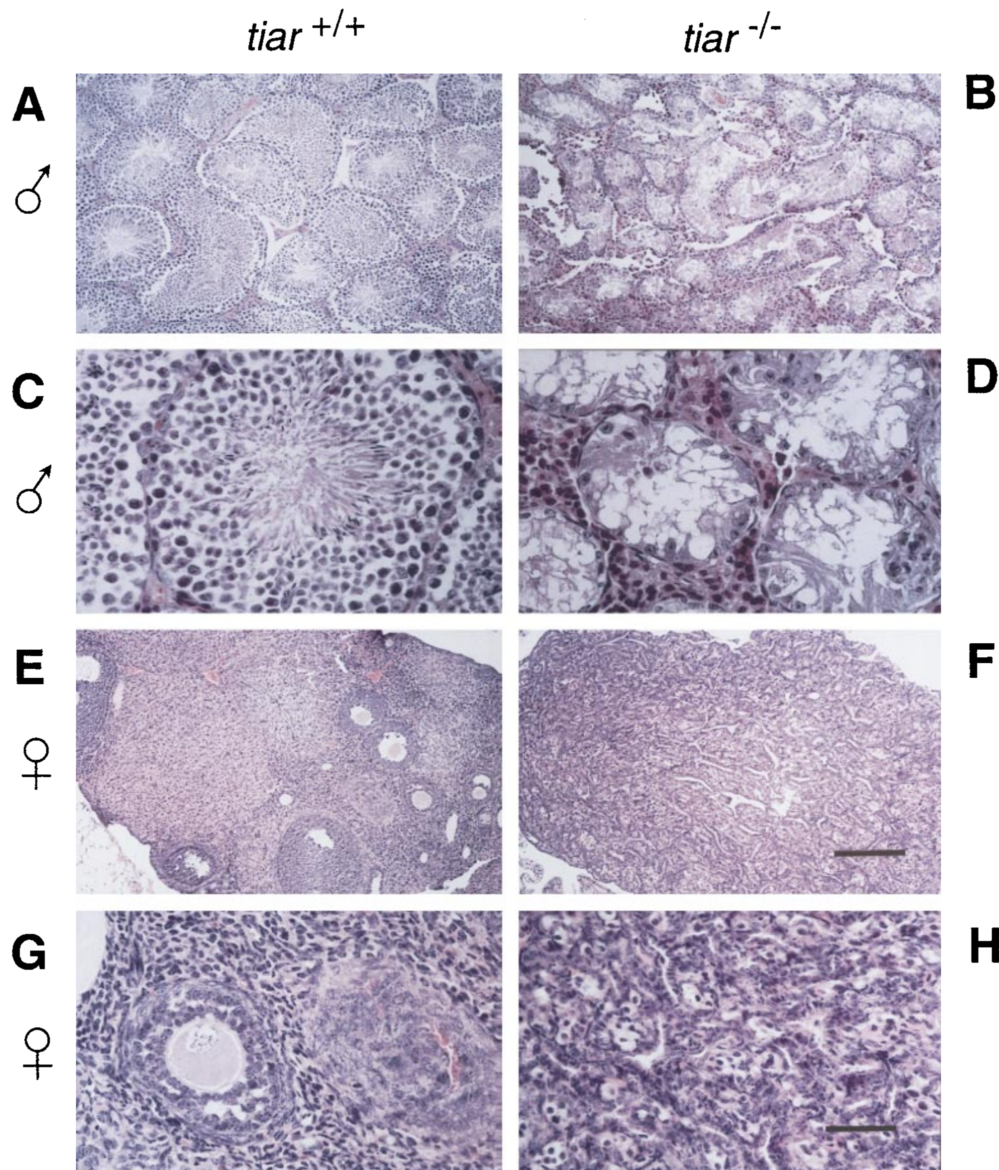

FIG. 3. Lack of germ cells in TIAR-deficient testes and ovaries. Hematoxylin and eosin-stained histological sections of $tiar^{+/+}$ (*A*) and $tiar^{-/-}$ (*B*) adult testes at low magnification (60-fold), $tiar^{+/+}$ (*C*) and $tiar^{-/-}$ (*D*) adult testes at high magnification (240-fold), $tiar^{+/+}$ (*E*) and $tiar^{-/-}$ (*F*) adult ovaries at low magnification (60-fold), and $tiar^{+/+}$ (*G*) and $tiar^{-/-}$ (*H*) adult ovaries at high magnification (240-fold). No significant difference was observed between $tiar^{+/+}$ and $tiar^{+/-}$ adult gonads (data not shown). (Bar = 200 μm for low magnification and 50 μm for high magnification.)

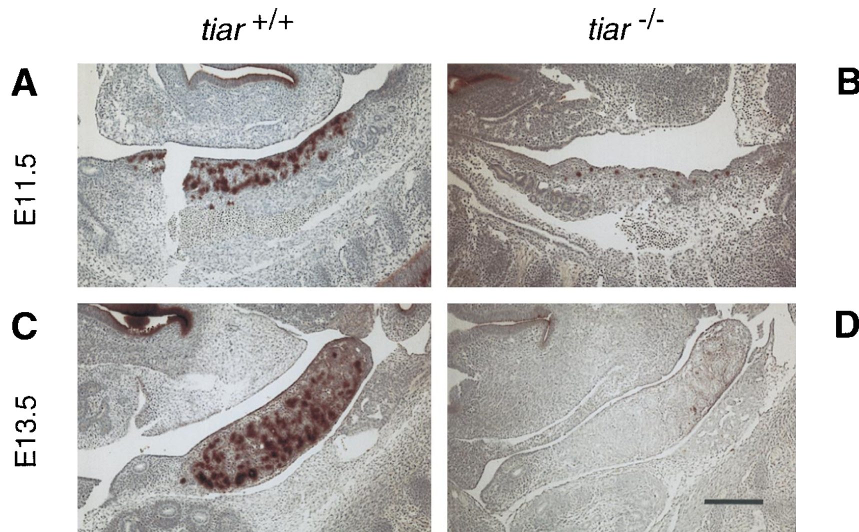

FIG. 4. TIAR expression is required for development of primordial germ cells. Staining for alkaline phosphatase activity (brown) of E11.5 $tiar^{+/+}$ (*A*) and $tiar^{-/-}$ (*B*) gonads and E13.5 $tiar^{+/+}$ (*C*) and $tiar^{-/-}$ (*D*) gonads. (Bar = 200 μm for paramedial sagittal sections.) The mucosal lining of the embryonic stomach lumen in the upper left corner of each panel also shows alkaline phosphatase activity. The number of alkaline phosphatase staining cells seen is representative of two $tiar^{+/+}$ and nine $tiar^{-/-}$ E11.5 embryos. Sectioning of four entire $tiar^{-/-}$ E11.5 embryos and staining for alkaline phosphatase activity did not reveal any ectopic alkaline phosphatase staining cells (data not shown). The number of alkaline phosphatase staining cells in 3 $tiar^{+/-}$ E11.5 embryos was reduced slightly compared with $tiar^{+/+}$ embryos.

ture spermatozoa (Fig. 3 *A–D*). At 3 months of age, testes of $tiar^{-/-}$ mice were approximately one-third the normal size. The architecture of the seminiferous tubules was largely normal, and interstitial Leydig cells and Sertoli cells appeared in their expected locations (Fig. 3 *B* and *D*). The $tiar^{-/-}$ ovaries also were slightly smaller and lacked developing follicles and oocytes, accounting for the female sterility (Fig. 3 *E–H*). However, the residual ovary showed hyperplasia of sex cord derivatives and underlying stroma in a trabecular and tubular pattern. Continued growth of these elements caused $tiar^{-/-}$ ovaries to grow to approximately five times the size of wild-type ovaries by 8 months (data not shown).

Primordial Germ Cell Development Defect in TIAR-Deficient Mice. The absence of both sperm and eggs in $tiar^{-/-}$ mice suggested a developmental defect at the level of PGCs. Histology of wild-type and $tiar^{-/-}$ embryos revealed marked differences in the number of PGCs populating the genital ridge at E11.5 and E13.5 (Fig. 4). PGCs were present at the genital ridge of both wild-type (Fig. 4*A*) and $tiar^{-/-}$ embryos at E11.5 (Fig. 4*B*), although the number of PGCs was reduced drastically in $tiar^{-/-}$ embryos. Whereas PGCs proliferated to fully populate the genital ridge of wild-type embryos by E13.5 (Fig. 4C), PGCs were absent from E13.5 $tiar^{-/-}$ embryos (Fig. 4*D*). It is unlikely that the migration of $tiar^{-/-}$ PGCs simply was delayed or aberrant because sectioning of entire embryos did not reveal any unusually located PGCs (data not shown). The decreased number of PGCs at the genital ridge in $tiar^{-/-}$ embryos could have resulted from the inability of $tiar^{-/-}$ PGCs to respond to a survival signal during migration or on arrival at the genital ridge or could have resulted from the inability of the surrounding tissues to provide a survival signal or both. Although it is likely that PGCs arriving at the genital ridge in $tiar^{-/-}$ mice died by apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) analysis did not detect large numbers of apoptotic cells in these sections (data not shown). This was probably a consequence of the small numbers of PGCs present at the genital ridge and the rapidity with which apoptotic cells were phagocytosed by neighboring cells.

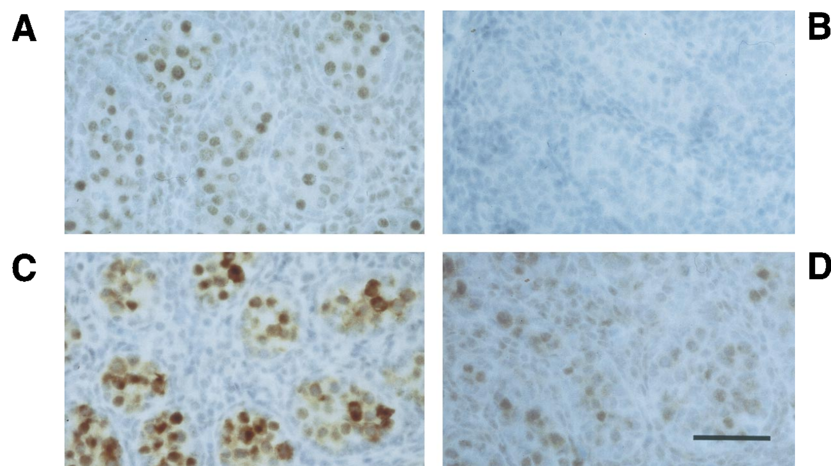

FIG. 5. Primordial germ cells express high levels of TIAR protein. Immunostaining of sections of E14.5 $tiar^{+/+}$ gonads (*A*) and $tiar^{-/-}$ gonads (*B*) using the anti-TIAR 3E6 mAb (11). A neighboring section of the $tiar^{+/+}$ gonad stained for GCNA1 (*C*), a PGC-specific antigen (24). (*D*) A section of a E12.5 $tiar^{+/+}$ gonad stained with mAb 3E6. (Bar = 50 μm.) Staining with an Ig-type matched control mAb further confirmed the specificity of mAb 3E6 staining (data not shown).

Consistent with a role for TIAR in PGC development was the high level of TIAR expression in PGC nuclei as determined by immunohistochemistry. Staining of embryo sections with a TIAR-specific mAb revealed that TIAR was expressed highly in selected cells of the developing gonad (Fig. 5), brain, neural tube, and ganglia, as well as in certain epithelial cells (data not shown). Cells in many other tissues exhibited a relatively low level of TIAR expression (data not shown). Staining of a E14.5 $tiar^{+/+}$ testis showed that TIAR was highly expressed in the nuclei of PGCs contained within seminiferous tubules (Fig. 5*A*). In contrast, precursor Sertoli cells lining the seminiferous tubules expressed little or no TIAR (Fig. 5*A*). The precursor Leydig cells between the seminiferous tubules expressed relatively low levels of TIAR similar to those found in many tissues throughout the embryo. Staining of a E14.5 $tiar^{-/-}$ testis confirmed the absence of TIAR protein and the specificity of the staining reaction (Fig. 5*B*). Wild-type E14.5 testes expressed the PGC-specific antigen GCNA1 (24), confirming (by location and morphology) that cells expressing high levels of TIAR were PGCs (Fig. 5*C*). Analysis of a wild-type E12.5 gonad (Fig. 5*D*) indicated that TIAR expression in PGCs was not stage-specific and presumably occured throughout development. $tiar^{-/-}$ embryos derived from crosses between $tiar^{+/-}$ mice backcrossed onto BALB/c mice for four generations also lacked PGCs. Taken together, these results demonstrate that TIAR is essential for PGC development.

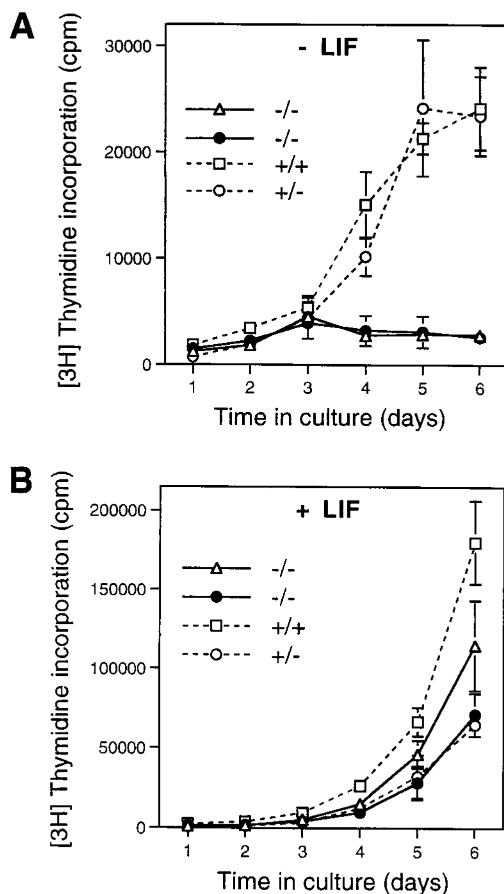

FIG. 6. $tiar^{-/-}$ ES cell proliferation defect. $tiar^{-/-}$ ES (solid lines) and control $tiar^{+/+}$ or $tiar^{+/-}$ ES (dashed lines) cell lines were grown in media containing methylcellulose in the absence (*A*) or presence (*B*) of exogenous LIF. Cell growth was determined by using [^{3}H]thymidine incorporation at the indicated times. Values represent mean values of triplicate samples with vertical bars indicating the SDs. Data are shown for two independent $tiar^{-/-}$ ES cell lines and control $tiar^{+/+}$ and $tiar^{+/-}$ cell lines. Similar results were obtained in four additional experiments.

TIAR-Deficient ES Cells Require Exogenous LIF for Growth in an *in Vitro* Methylcellulose Proliferation Assay. Several growth factors prolong the survival of PGCs *in vitro*, including LIF (25). Because PGCs can be de-differentiated into ES-like cells in the presence of growth factors (26), we assessed whether TIAR regulates ES cell proliferation in the presence or absence of exogenous LIF by using an *in vitro* methylcellulose assay culture system. To this end, two independent $tiar^{-/-}$, and control $tiar^{+/+}$ and $tiar^{+/-}$, ES cell lines were cultured in media containing methylcellulose, which prevents cell–cell interactions, and proliferation was measured by [^{3}H]thymidine incorporation at various times after cell plating (Fig. 6). In the absence of LIF, both the $tiar^{-/-}$ and control ES cells grew about equally well and had a similar morphology for the first 2–3 days, but after 3 days, the $tiar^{-/-}$ ES cells stopped proliferating and appeared dead, whereas the controls continued to grow (Fig. 6*A* and data not shown). In the presence of LIF (1,000 units/ml), both the $tiar^{-/-}$ and control ES cells grew about equally well at all time points measured (Fig. 6*B*). Proliferation of the $tiar^{-/-}$ and control ES cells was also about the same in the absence of LIF when cells were cultured in media lacking methylcellulose (data not shown). Thus, TIAR appears necessary for the clonal outgrowth of ES cells in the absence of exogenous LIF.

DISCUSSION

Although a number of defects in spermatogenesis or oogenesis are known, only a few mutations are ascribed to defects in PGC development. Mice homozygous for the *germ cell-deficient* (*gcd*) mutation, which has not been molecularly defined, have reduced numbers of PGCs and are partially fertile (27). Because the *gcd* germ cell defect is less severe than the TIAR PGC defect and these mice do not have any other apparent abnormalities, the *gcd* mutation is probably not allelic to *tiar* or the mutation is of a different nature (27). The best characterized PGC defects are the *W* (white spotting) and *Sl* (steel) mutations, which are mutations in the c-*kit* tyrosine kinase receptor and its ligand, steel factor, respectively. Sterility in these mice is a consequence of both decreased migration and reduced proliferation of PGCs (7–9). Of interest, *W* and *Sl* females develop hyperplasia of the residual ovarian elements in an identical pattern to the $tiar^{-/-}$ mice (28, 29). In contrast to the *W* and the *Sl* mutations, however, the *tiar* mutation does not appear to affect hematopoiesis or melanocyte development (30). Hematocrits, blood counts, and lymphocyte populations (CD3, CD4, CD8, CD45RA) are grossly normal, and no difference in coat color is observed between $tiar^{+/+}$ and $tiar^{-/-}$ mice (data not shown).

How TIAR promotes the survival of PGCs at the genital ridge is unknown. TIAR belongs to the RRM/ribonucleoprotein family of RNA-binding proteins (14) and binds to short uridylate stretches similar to cis elements regulating RNA metabolism (31). Other RRM/ribonucleoprotein-type RNA-binding proteins such as Elav family members regulate tissue-specific gene expression via selective binding to uridylate-rich sequences within proto-oncogene and growth factor mRNAs (32, 33). Similarly, TIAR may affect the stability of mRNA encoding growth factors such as steel factor, LIF, basic fibroblast growth factor, or interleukin 4 or their respective receptors, all of which promote the survival and proliferation of PGCs (25, 26, 34, 35). Consistent with a role for TIAR in growth factor signaling is our finding that $tiar^{-/-}$ ES cells do not proliferate without addition of exogenous LIF in a methylcellulose proliferation assay. Because $tiar^{-/-}$ ES cell proliferation was similar to that of control cells in the absence of exogenous LIF when cells were allowed to aggregate or when exogenous LIF was added, TIAR appears necessary for cell proliferation only in suboptimal growth conditions. Possibly, during their migration to the genital ridge, PGCs require

TIAR as they encounter suboptimal conditions (e.g., decreased concentrations of growth factors or cell–cell interactions). Although the significance of the difference in growth rates of the *tiar*$^{-/-}$ and control cells in the absence of exogenous LIF requires further evaluation, including the effect of other growth factors on *tiar*$^{-/-}$ ES cell proliferation, analysis of *tiar*$^{-/-}$ cells may provide important insight into the gene products regulated by TIAR. The recent report that murine Dazla, which is also a RRM/ribonucleoprotein-type RNA-binding protein, is essential for development and survival of both male and female germ cells (36) and that human Y-linked, Dazla-like genes are deleted in some infertile males (37, 38) supports the possibility that defects in TIAR function could underlie some forms of idiopathic infertility in humans.

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