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Neuregulins are essential for spermatogonial proliferation and meiotic initiation in neonatal mouse testis

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SUMMARY

The transition from mitosis to meiosis is unique to germ cells. In murine embryonic ovaries and juvenile testes, retinoic acid (RA) induces meiosis via the stimulated by retinoic acid gene 8 (*Stra8*), but its molecular pathway requires elucidation. We present genetic evidence in vivo and in vitro that neuregulins (NRGs) are essential for the proliferation of spermatogonia and the initiation of meiosis. Tamoxifen (TAM) was injected into 14-day post-partum (dpp) Sertoli cell-specific conditional *Nrg1*$^{Ser-/-}$ mutant mice. TAM induced testis degeneration, suppressed BrdU incorporation into spermatogonia and pre-leptotene primary spermatocytes, and decreased and increased the number of STRA8-positive and TUNEL-positive cells, respectively. In testicular organ cultures from 5-6 dpp wild-type mice and cultures of their re-aggregated spermatogonia and Sertoli cells, FSH, RA [all-trans-retinoic acid (ATRA), AM580, 9-cis-RA] and NRG1 promoted spermatogonial proliferation and meiotic initiation. However, TAM treatment of testicular organ cultures from the *Nrg1*$^{Ser-/-}$ mutants suppressed spermatogonial proliferation and meiotic initiation that was promoted by FSH or AM580. In re-aggregated cultures of purified spermatogonia, NRG1, NRG3, ATRA and 9-cis-RA promoted their proliferation and meiotic initiation, but neither AM580 nor FSH did. In addition, FSH, RAs and NRG1 promoted *Nrg1* and *Nrg3* mRNA expression in Sertoli cells. These results indicate that in juvenile testes RA and FSH induced meiosis indirectly through Sertoli cells when NRG1 and NRG3 were upregulated, as NRG1 amplified itself and NRG3. The amplified NRG1 and NRG3 directly induced meiosis in spermatogonia. In addition, ATRA and 9-cis-RA activated spermatogonia directly and promoted their proliferation and eventually meiotic initiation.

KEY WORDS: Neuregulins, Retinoic acid, Meiotic initiation, Neonatal mouse testis

INTRODUCTION

Meiosis is a process that produces gametes with extensive genetic diversity. In testis and ovary spermatogonia and oogonia, respectively, undergo species-specific rounds of mitotic divisions followed by the initiation of meiosis. In the mammalian ovary, germ cells enter meiosis during embryogenesis, whereas those in the testis do not until puberty. Initiation of meiosis in developing ovaries, but not in testes, may involve environment cues (McLaren, 1984; McLaren et al., 1995).

Vitamin A is required for normal spermatogenesis in the form of retinoic acid (RA), which is an active metabolite of vitamin A and is essential for mammalian spermatogonial differentiation and meiotic initiation (Hogarth and Griswold, 2010). In vitamin A-deficient (VAD) animals, germ cells are depleted from the seminiferous tubules and only undifferentiated type A spermatogonia remain. Replacement of vitamin A reinitiates spermatogenesis (Van Pelt and de Rooij, 1990). RA regulates the initiation of meiosis in mouse embryonic ovaries and juvenile testes via the *Stra8* gene (stimulated by retinoic acid gene 8) (Koubova et al., 2006; Bowles et al., 2006; Baltus et al., 2006; Anderson et al., 2008). CYP26B1, a P450 enzyme that catabolizes all-trans RA (ATRA) into inactive metabolites, is expressed more in male than in female gonads between 12.5 and 14.5 dpc, the crucial time when female, but not male, germ cells enter meiosis (Menke and Page, 2002). As germ cells enter meiosis precociously in the testes of *Cyp26b1*-knockout (KO) mouse embryos (Bowles et al., 2006), CYP26B1 might protect male gonads from the action of RA in embryos by reducing retinoid levels during fetal gonadal development (Bowles and Koopman, 2007). Fibroblast growth factor 9 (FGF9) produced in the fetal testis acts directly on germ cells and inhibits meiosis (Bowles et al., 2010). However, the molecular pathway in which RA induces meiosis remains to be clarified.

We have previously identified neuregulin (NRG) 1 in newt testes as a FSH-upregulated clone that is homologous to mouse NRG1 (Oral et al., 2008). *Nrg1* is one of the Nrg genes (*Nrg1*-*Nrg4*) that belong to the epidermal growth factor (EGF) family, and play essential roles in the nervous system, heart and breast (Falls, 2003). The family of EGF receptors includes four closely related transmembrane tyrosine kinases: ERBB1 (EGFR – Mouse Genome Informatics), ERBB2, ERBB3 and ERBB4. NRG1 and NRG2 both bind ERBB3 and ERBB4, whereas NRG3 and NRG4 bind ERBB4, but not ERBB3 (Hynes et al., 2001). ERBB2 has no direct ligand, but works as a co-receptor. NRGs induce not only homodimers of ERBB3 and ERBB4, but also heterodimers of ERBB2/ERBB3 and ERBB2/ERBB4. As *Nrg1* and *Nrg3* mRNAs are expressed in mouse neonatal testes, in this report we analyzed the functions of NRG1 and NRG3 in the proliferation of spermatogonia and their meiotic initiation in relation to retinoic acid and FSH in vivo and in vitro. Because *Nrg1* is expressed in Sertoli cells only, and pan-*Nrg1* KO mice are known to be embryonic lethal (Falls, 2003), we generated conditional Sertoli cell-specific *Nrg1*$^{Ser-/-}$ mutant mice. RA acts through the nuclear

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RA receptors (RARs) (α, β, γ) and retinoid X receptors (RXRs) (α, β, γ). RARs and RXRs may function as homodimers and heterodimers (RAR/RXR) (Mark and Chambon, 2003). As the expressed receptor isotypes of Sertoli cells and spermatogonia are different, we examined the effect of three kinds of retinoids on the proliferation of spermatogonia and their meiotic initiation in vitro: AM580, a stable retinoic acid receptor α (RARα)-specific agonist; ATRA, which can bind RARs; and 9-cis-RA, which can act on both RARs and RXRs (Mark and Chambon, 2003).

MATERIALS AND METHODS

Mice

C57BL/6J mice were purchased from Japan CLEA (Tokyo, Japan) and maintained on a 12-hour-day/12-hour-night schedule at constant temperature and humidity in the Center for Animal Resources and Development of Kumamoto University according to protocols for animal experiments approved by the Institutional Animal Care and Use Committee.

Generation of conditional *Nrg1* mutant mice

Conditional *Nrg1* mutant mice were generated as shown in Fig. S1 in the supplementary material.

Injection of TAM into mutant and control mice

TAM was injected daily into $Nrg1^{Ser-/-}$ mutant and control mice (0.3 mg/10 g weight) for 5 days, and 1 month later the testes were fixed in Bouin's solution.

Organ cultures of testicular fragments from wild-type and mutant mice

One testis from a 6 dpp wild-type mouse was cut into 16 fragments (about 1×1 mm). Eight fragments were placed on a float of nuclepore filter (Track-Etch Membrane, 25 mm, 0.2 μm pore size, Whatman) in a 35 mm plastic dish (Falcon; #1008) and cultured in Dulbecco's Modified Eagle's Medium (DMEM, 2 ml) containing 0.1% bovine serum albumin (BSA, Sigma) for 72 hours at 32°C with 5% CO_2 aeration. Porcine FSH [National Hormone & Peptide Program (NHPP)], ATRA (1 μM; Sigma), AM580 (1 nM; Sigma), 9-cis-RA (1 μM; Biomol), or recombinant EGF-domain of NRG1 or NRG3 was added to the culture dishes. Various inhibitors [AG879 (Calbiochem), an ERBB2 inhibitor; AG1478 (Wortmannin), an EGFR (ERBB1) inhibitor; and PD153035 (Biaffin), an ERBB1/ERBB4 inhibitor] were added to the medium 3 hours prior to the addition of FSH, NRGs or RA. Two to six mice were used for one experiment, depending on the number of different reagents added to the cultures.

Organ cultures of 5 dpp testes from $Nrg1^{Ser-/-}$ mutant mice were produced as follows. One testis was cut into 12 fragments (about 1×1 mm) and only six dishes (four fragments/dish) could be cultured from one mutant mouse. TAM (100 ng/ml) was added to the medium on day 0, followed 1 day later by the addition of FSH, ATRA, AM580, 9-cis-RA, NRG1 or NRG3, and cultured for 6 days.

Cell fractionation and re-aggregated cultures

Re-aggregated cultures of spermatogonia and Sertoli cells were produced as follows. After the tunica albuginea was removed from 6 dpp testes, whole testes were treated with 0.1% collagenase (type N-2, Nitta Gelatin, Japan) for 30 minutes at 25°C. The supernatant was used as a Leydig cell-rich fraction. The tubules were then dissociated by 0.1% collagenase and 1.5 kU/ml DNase I (type IV, Sigma) for 1 hour at 25°C, followed by pipetting (~100 times). Then the tubules were dissociated completely by 0.05% trypsin containing 0.1 mM EDTA for 10 minutes at 37°C. The cell suspension was filtered through nylon gauze (50 μm) and washed in DMEM, and 5 ml of the cell suspension in DMEM was placed on a 15% Nycodentz (Sigma) solution in Krebs ringer (5 ml) and centrifuged at 110 ***g*** for 3 minutes. Dead cells sedimented at the bottom of the tube. The layer formed between DMEM and 15% Nycodentz was collected as live germ and Sertoli cells, and washed with DMEM three times. The dissociated cells were re-aggregated by rotation culture (70 rotations/minute, R-30, TAITEC, Koshigaya, Saitama, Japan) for 1.5 hour at 25°C, followed by centrifugation in a 0.5 ml siliconized tube (Assist, Japan) (~10^5 cells/tube) at 230 ***g*** for 5 minutes. The cell pellet formed was detached from the tube by a needle in 50 μl medium, to which DMEM (200 μl) containing 0.24 mg/ml collagen (Cellmatrix type I-P, Nitta Gelatin) was added. The solution containing a re-aggregate was then placed on a nuclepore filter for 1 hour until the collagen hardened and the filter was floated on the medium as an organ culture. The medium was changed after 3 days of culture.

The fractionation of Sertoli cells and spermatogonia was performed as follows. After the testes were dissociated by collagenase and the cell suspension was filtered through nylon gauze, the supernatant (5 ml) was placed on 5% Nycodentz in Krebs ringer (5 ml) that was underlayered by 15% Nycodentz (5 ml) and centrifuged at 110 ***g*** for 3 minutes. The cells recovered from the layer formed between 5% Nycodentz and DMEM were used as a Sertoli cell-rich fraction, and the cells between 5% and 15% Nycodentz were used as a germ cell-rich fraction. Each fraction was further purified by seeding on dishes coated with collagen (type I; Nitta Gelatin) and incubated at 32°C. For the Sertoli cell-rich fraction, after the floating germ cells were removed daily for 3 days, the attached Sertoli cells were used directly as purified Sertoli cells (purity 94.3±2.6%). For the germ cell-rich fraction, the floating germ cells were collected after 6 hours incubation in collagen-coated dishes and, after pipetting, inoculated again into another collagen-coated dish. Then the supernatant spermatogonia were recovered after 6 hours incubation as purified spermatogonia (purity 90.3±2.7%). Re-aggregated cultures containing only spermatogonia were produced in the same way as that described above for spermatogonia and Sertoli cells.

Histology, and analysis of spermatogonial proliferation and differentiation

Cultured testicular fragments, re-aggregates and whole testes were fixed in Bouin's solution and dehydrated in a graded ethanol series. All testicular fragments cultured in one dish were embedded together in paraffin wax. Sections were made serially at 5 μm thickness, treated with xylene, dehydrated in an ethanol series and stained with Delafield's Hematoxylin followed by Eosin.

To assay for spermatogonial proliferation, 5-bromo-2-deoxy-uridine (BrdU) was injected intraperitoneally (0.3 mg/10 g weight) or incubated in the cultures, for 3 hours prior to fixation and processed for immunohistochemistry with a kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, Buckinghamshire, England). The number of positive cells was counted in three photographed areas (157,500 μm^2/area), taken at random in a section (in vivo) containing three or four fragments (in organ culture) × three sections, each derived from every five sections. Sertoli cells and germ cells were identified by cell size, nuclear morphology and localization within the seminiferous tubules (in the case of in vivo and organ culture), according to the descriptions by Huckins and Oakberg (Huckins and Oakberg, 1978) and Russell et al. (Russell et al., 1990).

The proliferative activity and extent of differentiation were expressed as the number (mean±s.e.m.) of BrdU-positive cells and SYCP3 (synaptonemal complex protein 3)-positive cells/tubule, respectively, in cross-section of testes (at least 100 tubules/testis were counted) and organ cultures (at least 45 tubules were counted for one experiment) from three independent experiments. For re-aggregated cultures the number (mean±s.e.m.) of BrdU-positive cells and SYCP3-positive cells/100,000 μm^2/section × three sections from three independent experiments indicated their proliferative activity and extent of differentiation.

Immunofluorescence

Testes, cultured testicular fragments or re-aggregates were fixed in Bouin's and embedded in paraffin wax. Sections (5 μm) were boiled in 0.01 M citrate (pH 6.0), washed three times in 0.1% Tween20/PBS, transferred to blocking solution containing 3% BSA and 10% goat serum (without serum in case of goat anti-GATA4) in 0.1% Triton/PBS for 1 hour, and incubated with primary antibody at 4°C overnight. After washing, the secondary antibody was added and the sections were incubated for 2 hours at room temperature. Combinations of the first antibody and the second antibody were as follows: rabbit anti-SYCP3 polyclonal antibody (Abcam, 1:200) or rabbit anti-STRA8 (Abcam, 1:200) and Alexa Fluor 488 goat anti-rabbit

IgG (Invitrogen, 1:200); mouse anti-CRE (Covance, 1:100) or mouse anti-TRA98 (a gift from Dr Nishimune, Osaka University, Japan) and Alexa Fluor 488 chicken anti-mouse IgG (Invitrogen, 1:200); mouse anti-γ-H2AX (Millipore, 1:200) and Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, 1:200); goat anti-GATA4 (Santa Cruz, 1:200) and Alexa Fluor 594 donkey anti-goat IgG (Invitrogen, 1:200). Microscopic images were obtained using a CCD camera (DP72, Olympus, Tokyo) mounted on a fluorescence microscope (BX60, Olympus).

Chromosome extension

Preparation of nuclear spreads was performed according to the method described by Peters et al. (Peters et al., 1997). Some cultured testicular fragments were placed in 0.1 M sucrose for hypotonic treatment, then in 1% paraformaldehyde and 0.15% Triton-X 100 for the spreading and fixation. Spreading was blocked with 3% BSA and 10% normal goat serum in PBS for 1 hour. The anti-SYCP3 antibody (a gift from Dr Chuma; 1:500 in 0.1% BSA in PBS) was incubated at 4°C overnight. FITC-conjugated swine anti-rabbit IgG (1:1000) (DAKO) was used for the second antibody.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining

The sections from paraffin-embedded testes were subjected to TUNEL staining using the In situ Apoptosis Detection Kit (Takara Biomedical, Japan) according to the manufacturer's instructions. The number of positive cells was counted in three photographed areas (157,500 μm^2/area) taken at random in a section × 3 sections, each derived from every five sections. The number of dead cells was counted as the number of cells (mean±s.e.m.)/tubule (for organ culture and in vivo) or /100,000 μm^2 (for re-aggregated culture) from three independent experiments.

RT-PCR

Total RNA was extracted from testes from various stages, from cultured 6 dpp testes, and from fractionated cells by homogenization in ISOGEN (Nippon Gene) using a Dounce homogenizer. cDNA was reverse transcribed with random hexamers by a reverse transcriptase Superscript III (Invitrogen). PCR was performed using ExTaq polymerase (Takara) or Go Taq polymerase (TOYOBO) with a sense and antisense primer specific for each cDNA clone isolated from the samples. PCR was performed as shown in Table 1.

Expression and purification of the EGF-like domain of recombinant NRGs

Expression and purification of the EGF-like domain of recombinant NRG1 and NRG3 was performed as previously reported (Eto et al., 2010). The sense and antisense primers were 5′-CAT ATG ACC AGC CAT CTC ATA AAG TGT GC-3′ (*Nrg1*) and 5′-CAT ATG TCT GAG CAC TTC AAA CCC TGT C-3′ (*Nrg3*), and 5′-CTC GAG TTA CTC CGC TTC CAT AAA TTC-3′ (*Nrg1*) and 5′-ATT CCT CGA GCT AGT GGT CTG TTG GA-3′ (*Nrg3*).

Statistics

Cell proliferation and differentiation activities were analyzed using a Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

RESULTS

Expression of mRNA for Nrg and Erbb genes in neonatal testes

mRNAs of *Nrg1*, *Nrg3*, *Erbb1*, *Erbb2*, *Erbb2/2* and *Erbb4* were expressed in the neonatal testes during the first 8 weeks (see Fig. S2A in the supplementary material), but mRNAs for *Nrg2*, *Nrg4* and *Erbb3* were barely detected. RT-PCR analysis of mRNAs from fractionated Sertoli cells, Leydig cells and spermatogonia from 6 dpp testes, where anti-Mullerian hormone (*Amh*), luteinizing hormone receptor (*Lhr*; *Lhcgr* – Mouse Genome Informatics) and A-kinase anchor protein 12 (*Akap 12*) were used as markers for the respective cell types, showed that *Nrg1* and *Erbb2* were expressed only in Sertoli cells, while *Nrg3*, *Erbb1*, *Erbb2/2* and *Erbb4* were expressed in both somatic cells (Sertoli cells and Leydig cells) and spermatogonia (see Fig. S2B in the supplementary material).

Tamoxifen injected into Sertoli cell-specific *Nrg1$^{Ser-/-}$* mutant mice induces testis degeneration

To examine the functional role of NRG1 in spermatogenesis, we generated Sertoli cell-specific *Nrg1$^{Ser-/-}$* mutants (Accession Number CDB0743K: http://www.cdb.riken.jp/arg/mutant%20mice%20list.html), in which both alleles of *Nrg1* exon 6 were excised

Table 1. Sequences of primers, number of cycles and temperature by which PCR was performed for each gene

Gene	Forward pimer	Reverse primer	Cycles	Temperature (°C)	Reference or Accession Number
Nrg1	GCATGTCTGAGCGCAAAGAAGG	CGTTACTTGCACAAGTATC	40	54	NM_178591.2
Nrg3	GGGCTAGCCCGGATGGGAAC	GTTCCCATCCGGGCTAGCCC	40	58	NM_008734.2
Erbb1 (*Egfr*)	GTGACAGATCATGGCTCATG	CATGTTGCTTTGTTCTGC	40	58	NM_007912.4
Erbb2	GAGTACCTGGTACCCCAG	CTTGGGTCTTTTCTAGAGTAG	40	54	NW_001030424.1
Erbb2/2	CAATCGTTCCCGGGCCTGTC	GAGTGCAGGATCCCACTTCC	40	54	NM_001003817.1
Erbb3	GTATGTATTACCTCGAGGAAC	GTTACCACGTCTAGACCC	40	54	NM_010153.1
Erbb4	GACCAGTGCCTGTCATGTC	CATGATCACCAGGATGAAG	40	54	NM_010154.1
β-Tubulin	TTCTGGGAGGTCATAAGCGAT	GGGAAAGGGAGGCAGGTG	25	58	NM_011655.4
Akap 12	GTCAATGGTGTAGCTGAACA	CGTAGACTGCCCACTTCTAG	40	58	NM_031185.3
Amh	TGACAGTGAGAGGAGAGG	TATCACTTCAGCCAGATGTAGG	40	56	Guyot et al., 2004
Fshr	CGGCAAACCTCTGAACTTCATAACATT	GTTTGTATTGGCTTGTGGTCAGGA	35	56	NM_013523.2
Rara	AGAACTGCATCATCAACAAGG	TCGTTGTTCTGAGCTGTTGTT	28		
Rarb	TGCTCAATCCATCGAGACAC	CTCTTTGGACATGCCCACTT	28	55	Volle et al., 2007
Rarg	TCATCTGTGGAGACCGAATG	GCCTGGAATCTCCATCTTCA	28		
Rxra	CTGCACTCTCCTATCAGCACC	ACTCCACCTCGTTCTCATTCC	40	58	
Rxrb	GTGGGGGTGAGAAAAGAGATG	CATAGTGCTTGCCTGAGCTTC	40	60	Bek et al., 2003
Rxrg	CCAGCTACACAGACACCCCAG	CTCACTCTCTGCTCGCTCTCG	40	60	
Spo11	AATAGTCGAGAAGGATGCAACA	TAGATGCACATTATCTCGATGC	35	57	Keeney et al., 1999
Stra8	GCCTGGAGACCTTTGACGA	GGCTTTTGGAAGCAGCCTTT	35	57	NM_009292.1
Cyclophilin	GGCTTCCACAGTGTTCATGC	GGCACTGGCGGCAGGTCCAT	25	57	NM_8907.1
Nrg1-loxp	GTCTGGCTTCTCATCCTGATTGG	CGGAGACAGTGTGATACCGCTG	35	60	
Mis cre ER	ACGCTGGTTAGCACCGCAGG	TACATTGGTCCAGCCACCAGC	35	68	
Lhr (*Lhcgr*)	AATCCCATCACAAGCTTTCAG	TGCCTGTGTTACAGATGC	40	56	Hofmann et al., 2003

in Sertoli cells when tamoxifen (TAM) was added, by crossing mice carrying *loxP*-flanked alleles of *Nrg1* exon 6 with mice bearing the *MIS-CreER* transgene (see Fig. S1 in the supplementary material). Mutant mice (14 dpp) containing primary spermatocytes were injected with TAM daily for 5 days and 1 month later the testes were fixed. The testicular size and weight in mutant mice were remarkably reduced compared with that in heterozygous mice (Fig. 1A,B). Histological sections showed that vacant areas appeared in the central or peripheral regions of seminiferous tubules (44.3±3.0%) of mutant testes (Fig. 1D,D′), whereas no such areas were observed in those of the heterozygous mice (Fig. 1C). In some cases, only Sertoli cells and undifferentiated spermatogonia remained (Fig. 1D), whereas in other cases Sertoli cells, primary spermatocytes and spermatids were present, but not spermatogonia (Fig. 1D′). Some moribund and dead cells were observed; they were probably spermatogonia (Fig. 1F,H) or primary spermatocytes (Fig. 1J,L), because such live cell types were nearby and also those were the cell types in seminiferous tubules of heterozygous mice (Fig. 1E,G,I,K). This interpretation is consistent with the fact that most of the TUNEL-positive cells were located in the central and peripheral regions of the tubules (Fig. 1N,O). The numbers of TUNEL-positive cells/tubule and TUNEL-positive tubules/section in mutant testes increased to more than twice that in heterozygous mouse testes (Fig. 1P,Q), indicating that NRG1 is required for the survival of spermatogonia and/or primary spermatocytes. The numbers of BrdU-labeled cells/tubule and of BrdU-positive tubules/section in mutant testes were less than half of those in heterozygous mouse testes (Fig. 1R-U), indicating that NRG1 is indispensable for the proliferative activity of spermatogonia and/or DNA synthesis of pre-leptotene spermatocytes. As the majority of STRA8-immunopositive cells in adult testes are pre-leptotene/early leptotene spermatocytes (Zhou et al., 2008b), we compared the number of STRA8-positive cells in the mutant testes with that in heterozygous testes. The numbers of STRA8-positive cells/tubule and of STRA8-positive tubules/section in mutant testes were less than half of those in heterozygous testes (Fig. 1V-Y). In addition, the percentage of BrdU-positive cells in the STRA8-positive cells in mutant testes was significantly lower than that in heterozygous mouse testes (Fig. 1Z). These results indicate that NRG1 plays an important role in the differentiation of spermatogonia into pre-leptotene spermatocytes and their DNA synthesis.

Finally, examination of Hematoxylin and Eosin-stained sections revealed several differences in cell types between mutant and wild-type mice (Fig. 1M): (1) the numbers of Sertoli cells per seminiferous tubule in mutant and heterozygous testes were almost

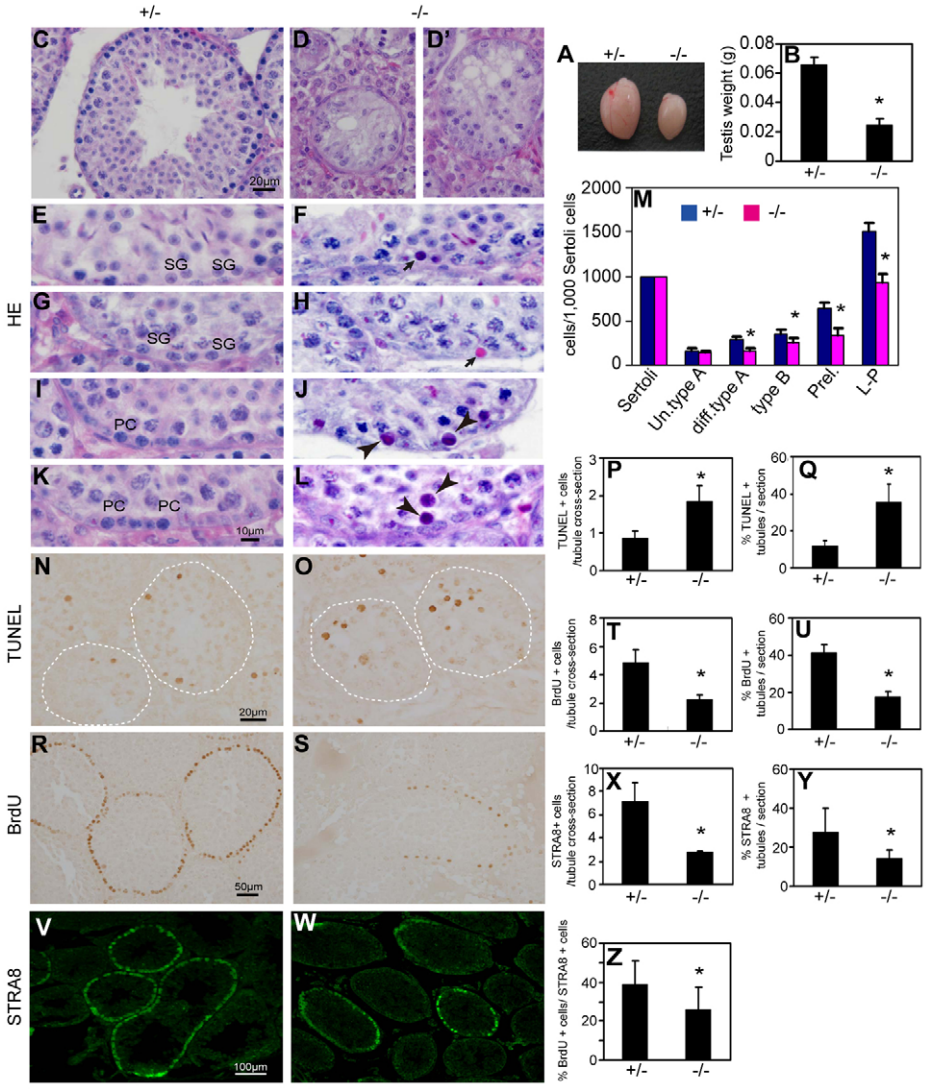

Fig. 1. Injection of TAM into *Nrg1*$^{Ser-/-}$ mutants induces testis degeneration. (**A**,**B**) Intraperitoneal injection of TAM into *Nrg1*$^{Ser-/-}$ mutants (14 dpp) caused reduction of testicular size and testicular weight (three testes for each), compared with heterozygous mice. (**C-L**) Hematoxylin and Eosin staining of the sections of heterozygous (C,E,G,I,K) and mutant (D,D′,F,H,J,L) mouse testis. D and D′ show examples of vacant seminiferous tubules. Arrows (F,H) and arrowheads (J,L) show dead or moribund cells. Scale bars: 20 μm in C-D′; 10 μm in E-L. (**M**) Number of cells in each cell type per 1000 Sertoli cells in heterozygous and mutant testes (stages beyond diplotene spermatocytes were neglected). (**N**,**O**) TUNEL staining. (**R**,**S**) BrdU staining. (**V**,**W**) STRA8 staining. (**P**,**T**,**X**) Number of TUNEL-positive cells, BrdU-positive cells and STRA8-positive cells/tubule cross-section in heterozygous and mutant testes. (**Q**,**U**,**Y**) Percentages of TUNEL-positive tubules, BrdU-positive tubules and STRA8-positive tubules/section in heterozygous and mutant testes. (**Z**) Percentages of BrdU-positive cells/STRA8-positive cells in heterozygous and mutant testes. Data are mean±s.e.m.;*P<0.01.

the same; and (2) the number of undifferentiated spermatogonia per 1000 Sertoli cells was almost the same in mutant and heterozygous testes; but (3) the numbers of differentiating type A and type B spermatogonia and pre-leptotene and leptotene-zygotene primary spermatocytes per 1000 Sertoli cells in mutant testes were remarkably lower than those in heterozygous testes; (4) the ratio of differentiating type A spermatogonia to undifferentiated type A spermatogonia in mutant testes (1.15) was remarkably reduced compared with that in heterozygous testes (1.75); (5) the ratio of pre-leptotene primary spermatocytes to type B spermatogonia in mutant testes (1.34) was remarkably lower compared with that in heterozygous testes (1.78); however, (6) the ratio of type B spermatogonia to differentiating type A spermatogonia and that of leptotene-zygotene primary spermtocytes to pre-leptotene primary spermatocytes were similar in mutant testes (1.31 and 2.78, respectively) and heterozygous testes (1.25 and 2.35, respectively). These results indicate that development from the undifferentiated type A spermatogonia to the differentiating type A spermatogonia was blocked and/or that the proliferative activity of the differentiating type A spermatogonia was inhibited in the mutant testes. In addition, we conclude that the process from type B spermatogonia to pre-leptotene primary spermatocytes was largely suppressed in the mutant testes.

FSH, RA and NRG1 promote spermatogonial proliferation and meiotic initiation in organ culture of wild-type mouse testes

As the above results indicate that NRG1 is indispensable for the proliferative activity of spermatogonia and/or DNA synthesis of pre-leptotene spermatocytes and/or survival of germ cells, we examined the roles of RA and NRG1 in the proliferation of spermatogonia and their entrance into meiosis in 3-day organ cultures from 6 dpp testes when differentiating A-type spermatogonia were the most advanced stage. Recombinant EGF-like domains of NRG1 and NRG3, sufficient for binding and activating receptors to induce cellular responses (Holmes et al., 1992; Wen et al., 1994), promoted spermatogonial proliferation in a dose-dependent manner (Fig. 2A). Both AM580 and 9-cis-RA

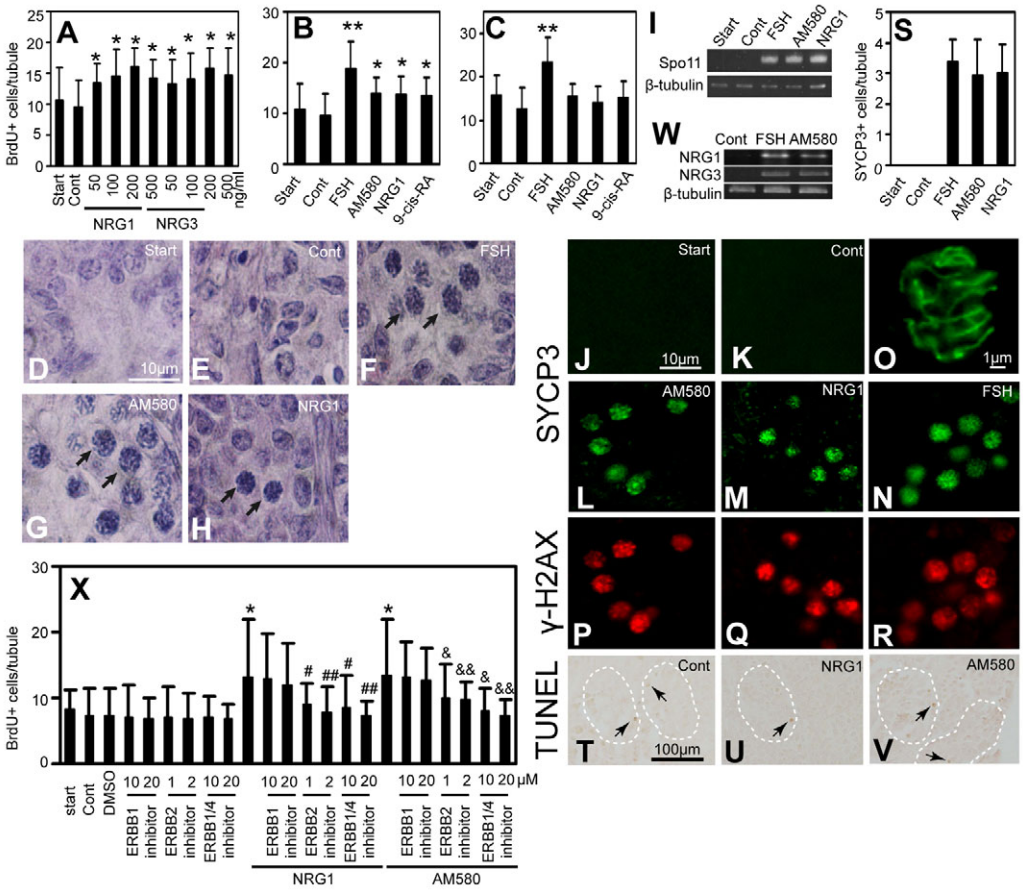

Fig. 2. FSH, NRGs and RAs promote proliferation of spermatogonia and meiotic initiation in organ culture of 6 dpp testes after 3 days. (**A**) Dose-dependent effect of NRG1 and NRG3 on proliferation of spermatogonia. Data are mean±s.e.m.; **P*<0.01. (**B**,**C**) Effect of FSH (100 ng/ml), AM580 (1 nM), 9-cis-RA (1 μM) and NRG1 (50 ng/ml) on proliferation of spermatogonia (B) and of Sertoli cells (C). Data are mean±s.e.m.; ***P*<0.01; **P*<0.05. (**D-H**) Hematoxylin and Eosin staining of the sections show meiotic cells (arrows) in the presence of FSH, AM580 and NRG1. (**I**) RT-PCR shows expression of *Spo11* mRNA in the presence of FSH, AM580 and NRG1. (**J-N**,**P-R**) Immunostaining shows expression of SYCP3 (L-N) and γ-H2AX (P-R) protein in the presence of AM580 (L,P), NRG1 (M,Q) and FSH (N,R). Scale bar: in J, 10 μm for J-N,P-R. (**O**) Representative micrograph showing immunofluorescent SYCP3 on the spread meiotic chromosomes. (**S**) Number of SYCP3-positive cells/tubule. Data are mean±s.e.m. (**T-V**) TUNEL staining shows few dead cells (arrows). Dotted lines encircle some seminiferous tubules. (**W**) RT-PCR shows upregulation of *Nrg1* and *Nrg3* mRNAs by FSH and AM580. (**X**) Effect of ERBB inhibitors on spermatogonial proliferation promoted by NRG1 (200 ng/ml) and AM580 (1 nM) in organ culture of 6 dpp testicular fragments. Data are mean±s.e.m.; **P*<0.01 versus DMSO (0.1%); $^{\#\#}P$<0.01 and $^{\#}P$<0.05 versus NRG1 only; $^{\&\&}P$<0.01 and $^{\&}P$<0.05 versus AM580 only.

stimulated the proliferation of spermatogonia to an extent similar to NRG1, whereas FSH strongly stimulated their proliferation to more than double that of the control number (Fig. 2B). Only FSH, but neither NRGs nor RAs stimulated the proliferation of Sertoli cells (Fig. 1C).

To determine whether NRG1 and RA promote the differentiation of spermatogonia into primary spermatocytes, we examined in organ cultures the nuclear morphology and the expressions of *Spo11* mRNA, which is specifically expressed in meiotic cells (Keeney et al., 1999) and the protein that it encodes is required for double-strand break formation and synapsis (Romanienko and Camerini-Otero, 2000), by RT-PCR. We also examined SYCP3 (Heyting, 1996) and γ-H2AX (phosphorylated histone H2AX) (Richardson et al., 2004), both of which appear during early meiotic prophase, by immunofluorescence. In culture media, containing FSH, AM580 or NRG1 (Fig. 2F-H), some nuclei showed chromosomal condensation that was characteristic of the zygotene or pachytene stages, whereas no such nuclei were observed in the initial (Fig. 2D) or control (Fig. 2E) cultures. *Spo11* mRNA was detected in the presence of FSH, AM580 or NRG1, whereas it was barely detectable in the initial or control cultures (Fig. 2I). In addition, SYCP3 (Fig. 2L-N) and γ-H2AX (Fig. 2P-R) proteins were expressed when FSH, AM580 or NRG1 was added, but not in the initial or control cultures (Fig. 2J,K). Extended chromosomes revealed that the SYCP3 protein was distributed throughout the chromosomes (Fig. 2O). The number of TUNEL-positive cells was few in the control (1.20±0.20 cells/tubule) and in the cultures to which AM580 (1.30±0.25 cells/tubule) and NRG1 (1.28±0.21 cells/tubule) (Fig. 2T-V) were added. Thus, FSH, AM580 and NRG1 stimulated differentiation of spermatogonia into primary spermatocytes in culture (Fig. 2S), indicating that both RA and NRG1 play a pivotal role in the initiation of meiosis.

Relationship between the NRGs/ERBB and the RA and FSH pathways

To determine the relationship between the NRGs/ERBB and the RA and FSH pathways, we first examined whether *Nrg* mRNA expression is activated by AM580 and FSH. *Nrg1* and *Nrg3* mRNA expression was stimulated by AM580 and FSH (Fig. 2W), indicating that NRG1 and NRG3 act downstream of RA and FSH. Next, we examined whether ERBB inhibitors suppress the spermatogonial proliferation that was stimulated by AM580, because, if RA operates upstream of the NRG pathway, ERBB inhibitors should suppress the effect of AM580. Although NRG1 can potentially bind to ERBB3 and ERBB4 (Holmes et al., 1992), no *Erbb3* mRNA expression was detected in mouse testes (1-8 weeks) (see Fig. S2A in the supplementary material). Therefore, NRG1 may signal through homodimers of ERBB4 and/or heterodimers of ERBB2/ERBB4 and/or ERBB1/ERBB4. The effects of various inhibitors for ERBB1, ERBB2 and ERBB1/ERBB4 on spermatogonial proliferation in the presence of NRG1 revealed that both ERBB2 and ERBB1/ERBB4 inhibitors suppressed spermatogonial proliferation, whereas there was no significant inhibition without NRG1 compared with the control (Fig. 2X). These results indicate that NRG1 binds to ERBB4 and signals via ERBB2/ERBB4 and/or ERBB4/ERBB4. We then examined the effects of the inhibitors on spermatogonial proliferation in the presence of AM580 (Fig. 2X). Both ERBB2 and ERBB1/ERBB4 inhibitors significantly suppressed the proliferative activity that was promoted by AM580. These results indicate that NRG1 operates downstream of the RA signaling system.

Neither FSH nor AM580 promote meiotic initiation in testes from Sertoli cell-specific *Nrg1*$^{Ser-/-}$ mutants in the presence of TAM in vitro

To examine the functional role of NRG1 in spermatogonial proliferation and meiotic initiation, TAM or its vehicle was added to organ cultures of 5 dpp mutant testes. Then, 1 day later AM580, ATRA, 9-cis-RA, FSH, NRG1 or NRG3 was added and the testes were cultured for 6 days. Analysis of histological sections revealed only a few areas with pyknotic cells (data not shown). TUNEL staining also showed very few dead cells in the presence of the vehicle ±TAM (1.32±0.18 cells/tubule, 1.35±0.24 cells/tubule, respectively) and NRG1 ±TAM (1.38±0.22 cells/tubule, 1.42±0.20 cells/tubule, respectively). Thus, cell viability was very good. The proliferative activity of spermatogonia and their differentiation into primary spermatocytes were promoted by FSH, ATRA, AM580, 9-cis-RA, NRG1 and NRG3 in the absence of TAM (Fig. 3A-F). However, the proliferative activity of spermatogonia and their differentiation stimulated by FSH and AM580 were significantly suppressed by TAM (Fig. 3A,B,D,E). However, the addition of NRG1 or NRG3 promoted spermatogonial proliferation and their

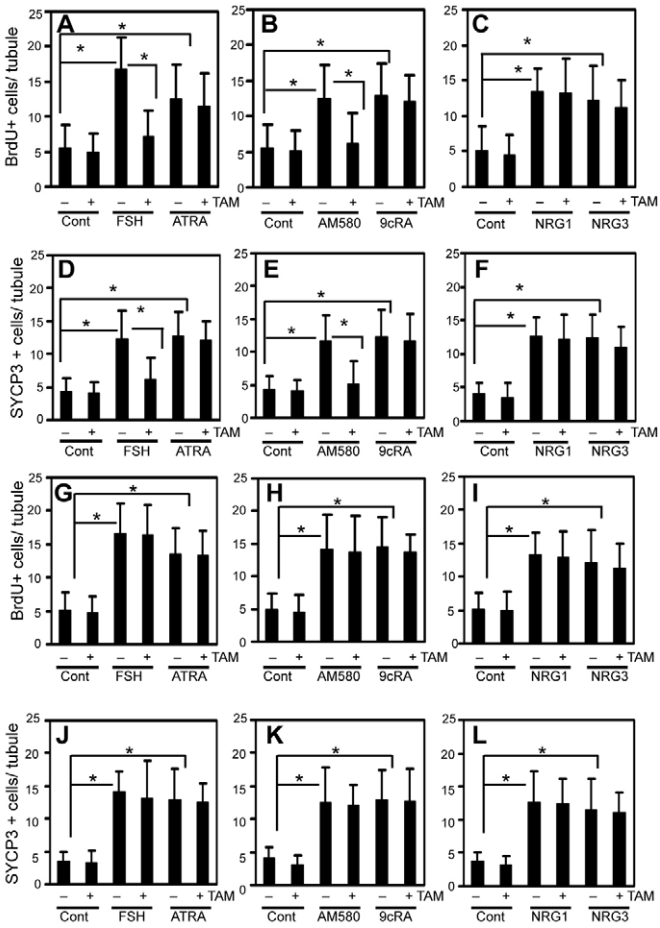

Fig. 3. Proliferative activity of spermatogonia and their differentiation to primary spermatocytes in organ cultures of mutant and heterozygous testes. In organ culture of 5 dpp testes from *Nrg1*$^{Ser-/-}$ mutants (**A-F**) and heterozygous mice (**G-L**), TAM or vehicle was added to culture medium, followed 1 day later by addition of various factors, and cultured for further 6 days. (A-C,G-I) BrdU-positive cells/tubule. (D-F,J-L) SYCP3-positive cells/tubule. Data are mean±s.e.m.; **P*<0.01.

differentiation even in the presence of TAM to almost the same level as when TAM was absent (Fig. 3C,F), indicating that NRG1 and NRG3 rescued the mutant phenotype. On the other hand, ATRA and 9-cis-RA also promoted the proliferative activity and the differentiation even in the presence of TAM (Fig. 3A,B,D,E). TAM had no effect on heterozygous mouse testes in littermates (Fig. 3G-L). These results indicate that NRG1 acts as a downstream factor of FSH and AM580 in promoting spermatogonial proliferation and the initiation of meiosis.

NRGs promote the initiation of meiosis in re-aggregated cultures of purified spermatogonia

To determine whether NRGs and/or RA stimulate spermatogonial proliferation and their differentiation into primary spermatocytes via Sertoli cells or directly, we established cultures (1) consisting of spermatogonia and Sertoli cells that were re-aggregated, and cultures (2) consisting of only re-aggregated spermatogonia, within a collagen matrix. In the re-aggregated cultures of spermatogonia and Sertoli cells, AM580 and NRG1, as well as FSH, promoted spermatogonial proliferation (see Fig. S3A in the supplementary material), whereas only FSH stimulated the proliferation of Sertoli cells (see Fig. S3B in the supplementary material). In 7-day cultures, primary spermatocytes in the zygotene-pachytene stage appeared in the media supplemented either with FSH, AM580 or NRG1 (see Fig. S3D-F in the supplementary material), but not in the control medium (see Fig. S3C in the supplementary material). Differentiation into primary spermatocytes was confirmed by the expression of SYCP3 (see Fig. S3G-J,O in the supplementary material) and γ-H2AX (see Fig. S3K-N in the supplementary material).

To determine whether NRGs and RA act on spermatogonia directly, NRG1, NRG3, AM580, ATRA and 9-cis-RA were added separately in re-aggregated cultures of spermatogonia and cultured for 1 week. NRG1, NRG3, ATRA and 9-cis-RA promoted spermatogonial proliferation, whereas AM580 did not (Fig. 4A). Zygotene-pachytene spermatocytes were identified by Hematoxylin and Eosin staining (Fig. 4B-G), and by immunofluorescence for SYCP3 (Fig. 4H-M) and γ-H2AX (Fig. 4N-P) in the presence of NRG1, NRG3 and ATRA, but were not seen in the presence of AM580 or FSH. NRG1 and NRG3, as well as ATRA, promoted the expression of mRNA for *Spo11* and *Stra8*, while NRG1 and ATRA also upregulated *Nrg3* mRNA, but NRG3 did not. Conversely, AM580 did not activate the three kinds of mRNA (Fig. 4R). Analysis of histological sections and TUNEL staining showed a few dead cells in the control and in the cultures in which NRG1 was added (see Fig. S4A-D in the supplementary material). That FSH did not promote spermatogonial proliferation (Fig. 4A) was consistent with the fact that the percentage of contaminated Sertoli cells was quite low (see Fig. S4E-G in the supplementary material). These results strongly suggest that NRG1, NRG3, ATRA and 9-cis-RA directly stimulated spermatogonial proliferation and the initiation of meiosis.

AM580, ATRA and FSH promote the expression of *Nrg1* and *Nrg3* mRNAs, whereas NRG1 amplifies itself and *Nrg3* mRNAs in Sertoli cells

To determine the relationship between RA signaling and the NRGs/ERBB pathway in Sertoli cells, we examined the effects of FSH, AM580, ATRA, NRG1 and NRG3 on mRNA expression of *Nrg1* and *Nrg3* in Sertoli cells cultured for 3 days (Fig. 4S). FSH, AM580 and ATRA promoted the expression of *Nrg1* and *Nrg3* mRNAs. Interestingly, NRG1 also stimulated the expression of *Nrg1* and *Nrg3* mRNA, but NRG3 did not. These results indicate that the NRGs/ERBB system operates downstream of RA and FSH, and that NRG1 exerts an autocrine effect on the production of NRG1 and NRG3 in Sertoli cells (Fig. 5).

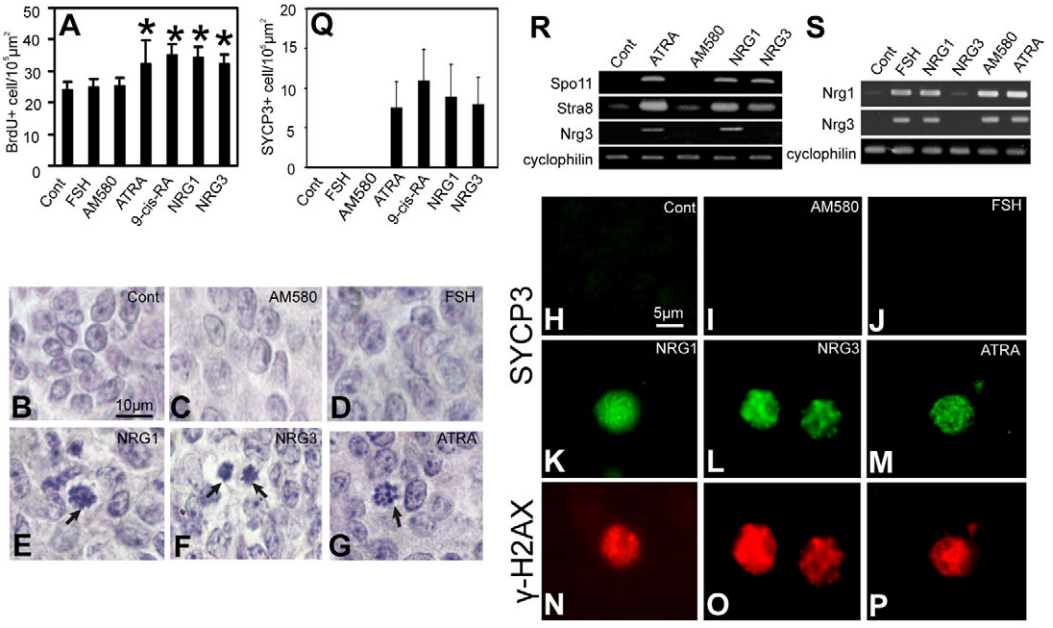

Fig. 4. Proliferative activity of spermatogonia and meiotic initiation in re-aggregated cultures of purified spermatogonia after 7 days of culture. (**A**) Proliferative activity of spermatogonia by various factors. Data are mean±s.e.m.; *P<0.01. (**B-G**) Hematoxylin and Eosin staining of the sections show meiotic cells (arrows). (**H-P**) SYCP3 (H-M) and γ-H2AX (N-P) expressed in the presence of NRG1 (K,N), NRG3 (L,O) and ATRA (M,P). (**Q**) Number of SYCP3-positive cells/10^5 μm^2 that differentiated in the presence of various factors. Data are mean±s.e.m. (**R**) RT-PCR shows expressions of mRNAs for *Spo11, Stra8* and *Nrg3* by various factors in purified spermatogonia. Cyclophilin was used as an internal control. (**S**) RT-PCR shows expressions of mRNAs for *Nrg1* and *Nrg3* by various factors in purified Sertoli cells cultured for 3 days.

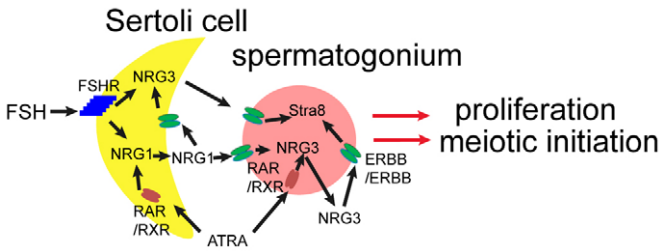

Fig. 5. Possible mechanism of meiotic initiation by RA and FSH through activation of NRG1 and NRG3 in Sertoli cells. ATRA and FSH work on Sertoli cells to promote expression of NRG1 and NRG3; the NRG1 produced acts to amplify itself and NRG3 in an autocrine manner in Sertoli cells. The amplified NRG1 and NRG3 directly act on spermatogonia to promote their proliferation and meiotic initiation. ATRA can also act directly on spermatogonia to produce NRG3 that upregulates Stra8. Thus, NRG3 may finally induce meiotic initiation in spermatogonia.

DISCUSSION

NRGs are essential in spermatogonial proliferation and meiotic initiation

In the above study, we presented novel findings that FSH and RA activate Sertoli cells to produce NRG1 and NRG3, which act directly on spermatogonia, promoting their proliferation and initiation of meiosis (Fig. 5). Our findings are based on the analyses of *Nrg1*$^{Ser-/-}$ mutant mice and of re-aggregated cultures consisting of spermatogonia and Sertoli cells, and of spermatogonia only. Thus, NRG1 and NRG3 play a pivotal role, probably through ERBB4 expressed on spermatogonia, in promoting spermatogonial proliferation and their entrance into meiosis. Our results in re-aggregated cultures containing only spermatogonia (Fig. 4) indicate that ATRA and 9-cis-RA also act directly on spermatogonia.

Nrg1 is known to play essential roles in the nervous system, heart and breast (Falls, 2003), but little is known about its role in spermatogenesis except for a few reports. For example, recombinant NRG1 in combination with GDNF effectively stimulated the formation of aligned rat spermatogonia up to the 32-cell stage (Hamra et al., 2007). Our analysis of *Nrg1*$^{Ser-/-}$ mutants showed that ablation of NRG1 in Sertoli cells caused cell death of spermatogonia and/or primary spermatocytes, and also the reduction of spermatogonial proliferation and the number of STRA8-positive cells (Fig. 1). These results indicate that two processes were mainly affected in mutant testes: (1) the transition from undifferentiated type A spermatogonia to differentiating type A spermatogonia and (2) that from type B spermatogonia to pre-leptotene primary spermatocytes. The first transition is known to involve the action of retinoic acid and stem cell factor (SCF)/c-kit system (de Rooij, 2001). RA was shown to induce directly the transition of undifferentiated spermatogonia to differentiating spermatogonia by stimulating *Stra8* and *Kit* gene expression (Zhou et al., 2008a). It is possible that NRG works as a downstream factor of RA in this process. It is also likely that NRG1 stimulates the proliferative activity of differentiating type A spermatogonia. It has been suggested that the SCF/c-kit interaction is required for the proliferation and/or survival of type A spermatogonia (Sette et al., 2000). As NRG1 stimulates the expression of *SCF* mRNA and vice versa in organ culture of testes (our unpublished results), it is possible that both SCF and NRG1 interact with each other and promote the proliferation of type A spermatogonia. Future experiments should examine the relationship between the SCF/c-kit and NRG/ERBB systems. The second process, the transition from type B spermatogonia to pre-leptotene primary spermatocytes, also seems to be largely dependent on the NRG1/ERBB system. RA was shown to regulate the initiation of meiosis in mouse embryonic ovaries and juvenile testes via *Stra8* (Koubova et al., 2006; Bowles et al., 2006; Baltus et al., 2006; Anderson et al., 2008). As the number of STRA8-positive cells in mutant testes was remarkably reduced compared with those in heterozygous testes (Fig. 1V-Z), we suggest that NRG1 plays a crucial role in regulating the initiation of meiosis. We further support this suggestion with our in vitro results to be discussed below.

RA and FSH promote spermatogonial proliferation and their meiotic initiation by generating NRG1 in Sertoli cells

In 5-6 dpp testes from wild-type mice, we showed that RA (AM580, ATRA, 9-cis-RA) and FSH promoted spermatogonial proliferation and their meiotic initiation in organ culture (Fig. 2) and also in re-aggregated cultures of spermatogonia and Sertoli cells (see Fig. S3 in the supplementary material). All promoted the expression of NRG1 and NRG3 in culture of Sertoli cells (Fig. 4S), indicating that they act on spermatogonia indirectly through Sertoli cells. FSH acts on the specific seven-transmembrane receptor of Sertoli cells (Sprengel et al., 1990). AM580, as well as FSH, act only indirectly through Sertoli cells, because in organ culture of *Nrg1*$^{Ser-/-}$ mutant testes, the stimulating effect of AM580 and FSH on spermatogonial proliferation and their meiotic initiation was abrogated by TAM (Fig. 3). ATRA and 9-cis-RA, however, can act on spermatogonia directly, as well as indirectly, because they promoted spermatogonial proliferation and meiotic initiation in cultures containing only spermatogonia (Fig. 4). The differential effects between AM580 and ATRA (or 9-cis-RA) may be due to different expressions of the isoforms of RAR and RXR in spermatogonia and Sertoli cells. RARα is expressed only in Sertoli cells, whereas RARγ is detected only in spermatogonia from 6 dpp testes (see Fig. S5 in the supplementary material) (Vernet et al., 2006b). Vernet et al. (Vernet et al., 2006a) showed that selective ablation of the RARα gene in mouse Sertoli cells (*Rara*$^{Ser-/-}$ mutation) or in combination with RARβ and RARγ genes (*RARα/β/γ*$^{Ser-/-}$ mutation), delays spermatogenesis in pre-pubertal mice. That finding indicates that a cell-autonomous effect of RA-liganded RARα in immature Sertoli cells is required to promote spermatogonia differentiation during the prepubertal spermatogenic wave. Our current results, together with those of Vernet et al. (Vernet et al., 2006a), suggest that AM580, ATRA and 9-cis-RA act on Sertoli cells by binding to RARα. How RA promotes the expression of NRG1 and NRG3 in Sertoli cells through RARα remains to be elucidated.

NRG1 activates Sertoli cells and amplifies NRG1 and NRG3

FSH and RA stimulated the expression of *Nrg1* and *Nrg3* mRNA, and NRG1 upregulated the expression of *Nrg1* and *Nrg3* mRNA in Sertoli cells (Fig. 4S). This indicates that as long as FSH or RA is present, NRG1 and, accordingly NRG3, are amplified consecutively in Sertoli cells by NRG1 (Fig. 5). A potential mechanism for a positive-feedback loop of NRG1 is implicated in neuroblastoma in which *Nrg1* gene transcription is activated by NF-κB, the transcriptional activity of which is increased by NRG1 (Frensing et al., 2008). It may be intriguing to know whether NF-κB is involved in *Nrg1* gene transcription in Sertoli cells. It is also

interesting that although NRG1 promoted the expression of itself and NRG3, NRG3 did not (Fig. 4S). This may be due to differences of receptor affinity between NRG1 and NRG3; however, the receptors for NRG1 and NRG3 and their downstream signaling pathways in Sertoli cells remain to be identified.

NRG1 and NRG3 activate spermatogonia directly to promote their proliferation and meiotic initiation

NRG1 and NRG3 directly stimulated the proliferation of spermatogonia and their entrance into meiosis in re-aggregated cultures of purified spermatogonia (Fig. 4). These results are based on our successful cultivation of viable (~90%) spermatogonia for as long as 1 week. Although it has been reported that germ cells survived only for 48 to 72 hours under feeder cell-free and serum-free conditions (Zhou et al., 2008a), the reason for our successful culture may be due to the cell adhesion retained in the re-aggregates, and/or the three-dimensional structure of the cells maintained within the collagen matrix, and/or collagen as a substratum on which the cells attached. The reason for our success is now being investigated.

NRG1 is considered to signal through ERBB receptors in spermatogonia. ERBB receptors are indispensable not only because they have essential roles in normal physiological processes occurring during development, but also because of their involvement in various types of human tumors (Holbro et al., 2003). Ligand binding to ERBB receptors induces the formation of homo- and heterodimers leading to the activation of the intrinsic kinase domain, which in turn leads to the activation of intracellular pathways such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI-3K) pathways (Olayioye et al., 2000; Holbro et al., 2003). Amplification of ERBB1 and ERBB2 contributes to processes linked to malignant development and ERBB2 bound with a partner in a heterodimer is responsible for the strong and prolonged activation of downstream signaling pathways leading to cell proliferation (Olayioye et al., 2000; Holbro et al., 2003). However, ERBB4 correlates in general with increased differentiation, such as that produced by NRG3 and ERBB4 signaling when regulating mammary bud specification (Muraoka-Cook et al., 2008). Therefore, distinct receptor combinations formed in response to NRG1 and NRG3 may determine the spermatogonial fates of proliferation or differentiation. The mechanism controlling the usage of the receptor combinations remains to be elucidated.

ATRA and 9-cis-RA act on spermatogonia directly to stimulate their proliferation and meiotic initiation

Our current study showed that ATRA and 9-cis-RA promoted spermatogonial proliferation and the initiation of meiosis in re-aggregated cultures containing only spermatogonia (Fig. 4), indicating that they act directly on spermatogonia. Our results are consistent with those of Zhou et al. (Zhou et al., 2008a) who showed, in cultures of $THY1^+$ spermatogonia, that ATRA directly induced the transition of undifferentiated spermatogonia to differentiating spermatogonia by stimulating *Stra8* and *Kit* gene expression, and those of Pellegrini et al. (Pellegrini et al., 2008) that ATRA increased the meiotic entry of murine spermatogonia in cultures containing only spermatogonia. How, then, does ATRA stimulate spermatogonial proliferation and the initiation of meiosis in spermatogonia? As AM580, a specific agonist of RARα, did not act on spermatogonia directly (Fig. 4), ATRA appears to act by binding RARγ to form a heterodimer with RXR. In addition, our results indicate that ATRA acts on spermatogonia by promoting NRG3 expression within them (Fig. 4R). Thus, NRG3 is considered to act on spermatogonia as an autocrine growth factor. A similar example was reported by Xiao et al. (Xiao et al., 1999) using dominant-negative *Rara* mutants: they suggested that retinoid receptor heterodimers located in differentiated suprabasal cells mediated retinoid induction of heparin-binding EGF-like growth factor, which in turn stimulated basal cell growth via intercellular signaling. In summary, ATRA activates spermatogonia directly to promote proliferation and meiotic initiation, and also indirectly by activating NRG1 and NRG3 expression in Sertoli cells. Though it is currently unknown which pathway is dominant or equally effective, these two ways of retinoid action may provide a 'fail-safe' mechanism for RA to promote meiosis. Future study is required to clarify how ATRA activates NRG3 expression and whether the NRG3/ERBB signaling is essential for ATRA-mediated stimulation of spermatogonial proliferation and meiotic initiation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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