Establishment of adult mouse Sertoli cell lines by using the starvation method

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Abstract

Sertoli cells were isolated from the testes of 6-week-old mice and stable Sertoli cell lines with higher proliferation rates were subcloned after starvation of primary cultured cells. After two rounds of this subcloning, 33 subcloned lines were selected on the basis of their proliferation rates. In addition, these subclones were screened according to their phagocytic activity and the characteristics of mature Sertoli cells, such as the expression of androgen receptors (ARs) and progesterone receptors, by using western blotting and immunocytochemical analysis, in addition to their morphology and proliferation rates. After the third round of subcloning, 12 subclones were selected for the final selection using RT-PCR for identification of genes specifically expressed by various testicular cells. Three clones were selected that expressed Sertoli-cell-specific genes, i.e. stem cell factor, clusterin, AR, α -inhibin, transferrin, Wilms' tumour-1, Müllerian inhibitory substance, sex-determining region Y-box 9, FSH receptor (*Fshr*) and occludin; however, these clones did not express globulin transcription factor 1, steroidogenic factor or androgen-binding protein. These clones also expressed growth and differentiation factors that act on germ cells, such as leukaemia inhibitory factor, transforming growth factor β 1 and basic fibroblast growth factor 2, but did not express c-kit (specific for germ cells), LH receptor and 3 β -hydroxyl-dehydrogenase (specific for Leydig cells). Immunocytochemical data confirmed the expression of clusterin in these clones. Furthermore, the Bromodeoxyuridine incorporation assay confirmed the proliferation activity of these clones through *Fshr* after treatment with FSH. These clones are considered to be valuable tools for the study of Sertoli cell-specific gene expression and function.

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Introduction

The culture of primary Sertoli cells has become an important resource in the study of their function (Buzzard *et al.* 2002); however, their use is limited because the isolated cells are contaminated with other testicular cells, mainly myoid cells. Furthermore, the proliferation of primary Sertoli cells, especially from pubertal and adult mice, is very slow, the cells are short-lived and experiments with Sertoli cells require a continual supply of animals, thereby making it difficult to replicate experimental conditions. Thus, the establishment of appropriate functional Sertoli cell lines is necessary.

Sertoli cells have two functionally separate roles: one in the process of testis formation or sexual differentiation and the other in spermatogenesis (Sharpe *et al.* 2003).

Disorders of testicular function may have their origins in foetal or early life because of the abnormal development or proliferation of Sertoli cells (Archambeault & Yao 2010). Sertoli cells also play an important role in spermatogenesis by providing a unique microenvironment that is essential for the development of germ cells in the testis and the formation of the blood–testis barrier (Cheng & Mruk 2010). Without the physical and metabolic support of Sertoli cells, germ cell differentiation, meiosis and development into spermatozoa would not occur (Sharpe 1994, Cheng *et al.* 2010).

The development and function of Sertoli cells are controlled by their interaction with other testicular cell types – either through direct cell–cell contact or via endocrine or paracrine factors (Plant & Marshall 2001, Cheng *et al.* 2010). Increasing concentrations of FSH and

testosterone at puberty, coincident with the expression of the androgen receptor (AR) in Sertoli cells, may induce their final maturation (Sharpe *et al.* 2003, Rey *et al.* 2009). Foetal Sertoli cells are clearly capable of supporting foetal germ cells (e.g. primordial germ cells and gonocytes), and immature, neonatal or infantile Sertoli cells are able to support spermatogonia. Conversely, in the adult testis, mature Sertoli cells support full spermatogenesis because the failure of Sertoli cells to mature functionally makes them incapable of supporting the survival and development of various germ cells that appear after puberty.

Several cell lines have been established from immature Sertoli cells isolated from the testis of a neonatal or infant mouse (Mather 1980, Hofmann *et al.* 1992, 2003, Peschon *et al.* 1992, Walther *et al.* 1997). However, because cells from these lines did not show the highly differentiated phenotype of adult Sertoli cells (McGuinness *et al.* 1994, Strothmann *et al.* 2004), they have not been used to study the function of mature Sertoli cells. Mature cells often lose their proliferative ability, making it difficult to establish a stable cell line from mature Sertoli cells. The establishment of mature Sertoli cell lines requires the introduction of a gene for inducing cell proliferation. Several cell lines have been established by the immortalisation of primary cultured mature Sertoli cells using the temperature-sensitive SV40 or the polyoma T-antigen transgenic mouse (Rassoulzadegan *et al.* 1993, Tabuchi *et al.* 2002). However, several problems have occurred during the establishment of cell lines after gene engineering; for example, after immortalisation, rat Sertoli cell lines expressed epithelial genes, but also unexpectedly expressed mesenchymal genes (Konrad *et al.* 2005).

To establish the cell lines of Sertoli cells with many characteristics similar to mature Sertoli cells in the adult testis, we adopted a different approach in which transgenesis was not required. After culturing primary Sertoli cells, we subcloned 12 Sertoli cell lines without changing the culture medium. These lines were characterised by light microscopy, western blotting, immunocytochemistry and RT-PCR. The gene expression pattern displayed by

three selected Sertoli cell lines indicated that they could be useful as an *in vitro* model for studying several factors related to the function of mature Sertoli cells.

Results

Characterisation of the cell lines by western blotting, immunocytochemistry, phase contrast microscopy and fluorescent microscopy for the third round of subcloning

We subcloned primary cultured Sertoli cells twice by using the starvation method. Thirty-three subclones were obtained after the second subcloning step. We then selected the cell lines on the basis of their morphology, phagocytotic activity and protein expression levels of the AR and the progesterone receptor (PR). From this third subcloning, we obtained 12 subclones. Six cell lines derived from the subcloning of line A3 were designated as A31B1, A31B4, A31C1, A31C4, A32B4 and A33A4. In addition, six cell lines derived from the subcloning of line C2 were designated as C21C3, C21C4, C22A3, C22A4, C22B4 and C24A4. These new cell lines were cultured for more than 1 year.

These 12 subcloned cell lines were mostly spindle-shaped with some lipid inclusions within the cytoplasm, and these Sertoli cells often showed a linear alignment when cultured at a high density (Fig. 1 and Table 1). Furthermore, these cell lines efficiently took up Fluoresbrite latex beads and fluorescence was detected in the cytoplasm (Fig. 2), indicating that the cells have phagocytic activity. All the 12 subclones showed phagocytic activity (Fig. 2 and Table 1). The A3 and C2 cell lines expressed the AR and PR (Fig. 3), and both proteins were localised to the nucleus (Fig. 4). Furthermore, the expression of the AR was higher in the A3 cell line than in the C2 cell line (Fig. 3).

The 12 subclones showed a doubling time of 13–26 h, whereas the doubling times of the final three selected clones, determined from the expression of Sertoli-cell-specific genes, were 18 h (A31C4), 21 h (C22A4) and 25 h (C24A4) (Table 1). Furthermore, the chromosome number of the 12 subclones was between 80 and 180

Figure 1 Phase contrast micrographs of the cell lines. (A) A31C4, (B) C22A4 and (C) C24A4. The immortalised Sertoli cells often showed a linear alignment (arrows) when cultured at a high density. Scale bar: 100 μ m.

Table 1 Characteristics of the new cell lines established from the adult mouse testis.

	Doubling time (h)	Chromosome number	Phagocytic activity	Linear alignment	AR in the nucleus	PR in the nucleus
A31B1	18	180	+	+	+	+
A31B4	26	160	+	+	+	+
A31C1	23	80	+	+	+	+
A31C4	18	80	+	+	+	+
A32B4	23	80	+	+	+	+
A33A4	18	80	+	+	+	+
C21C3	13	80	+	+	+	+
C21C4	18	80	+	+	+	+
C22A3	21	80	+	+	+	+
C22A4	21	80	+	+	+	+
C22B4	20	80	+	+	+	+
C24A4	25	80	+	+	+	+

chromosomes, whereas it was 80 chromosomes for the final three selected clones (Table 1).

Characterisation of the cell lines by using RT-PCR for the final selection

Total RNA was extracted from the cell lines and the adult testis. Three clones (A31C4, C22A4 and C24A4) were selected from the 12 subclones because the number of Sertoli-cell-specific genes expressed in these cell lines was more than that in the other subclones (Fig. 5). The gene expression patterns in the C24A4 and C22A4 cell lines were found to be similar (data not shown). The housekeeping gene cyclophilin was detected by RT-PCR in all the cell lines and adult testes, indicating the presence of cDNA in every sample (Fig. 5A). All Sertoli-cell-specific cDNAs tested were present in the adult testis. The PCR product *Kit*, a marker of spermatogenic cells, was detected in the adult testis, but not in any of the cell lines (Fig. 5B), indicating that none of the lines were of germ cell origin. cDNAs for the following genes expressed by Sertoli cells in the testis were present in all the cell lines (Fig. 5C): AR (*Ar*), clusterin (*Clu*), α -inhibin

(*Inha*), basic fibroblast growth factor (*Fgf2*), transforming growth factor β 1 (*Tgfb1*), leukaemia inhibitory factor (*Lif*), stem cell factor (*Kitl*), Müllerian inhibiting substance (*Mis/Amh*), Wilms' tumour 1 (*Wt1*), transferrin (*Trf*), sex-determining region Y box 9 (*Sox9*), FSH receptor (*Fshr*) and occludin (*Ocln*). *Ar*, *Tgfb1* and *Fgf2* are known to be expressed in Sertoli cells and myoid cells; however, the possibility that the cell lines were derived from myoid cells can be excluded because myoid cells do not express Sertoli-cell-specific genes that are expressed by these cell lines. Although all the cell lines expressed *Ar*, *Lif*, *Amh* and *Sox9*, which are also expressed by Leydig cells, no specific PCR bands were observed for the LH receptor (*Lhr/Lhcgr*) or 3β -ol-hydroxysteroid-dehydrogenase (*Hsd3b*) genes, which are specifically expressed by Leydig cells in the adult testis, in any of the cell lines (Fig. 5B), indicating that none of the cell lines were of Leydig cell origin. No specific bands for steroidogenic factor 1 (*Sf1*), androgen-binding protein/sex hormone-binding globulin (*Shbg*) or GATA-binding protein 1 (*Gata1*) were observed for the cell lines, although these genes were expressed in the adult testis (Fig. 5C).

Figure 2 Phagocytic activity of the cell lines. (A) Fluorescence micrograph of A31C4, (A') phase contrast micrograph of A, (B) fluorescence micrograph of C22A4 and (B') phase contrast micrograph of B. Fluoresbrite beads were phagocytised by the cells and located in the cytoplasm. n, nucleus. Scale bar: 100 μ m.

Figure 3 Expression of the AR and the PR in cell lines. (A) Western blot analysis of the AR (upper panel) with Coomassie Brilliant Blue R (CBB) staining (middle panel) and the expression levels of the AR (lower graph). The AR band was at 110 kDa in each lane (arrow in the upper panel). The loading control was set at a clear band around 50 kDa in each lane (arrow in the middle panel). (B) Western blot analysis of the PR (upper panel) with CBB staining (middle panel) and the expression levels of the PR (lower graph). The PR band was at 81 kDa in each lane (arrow in the upper panel). The loading control was set at a clear band around 50 kDa in each lane (arrow in the middle panel). The position of the molecular weight markers is indicated on the left.

Clusterin protein expression in the selected cell lines

The immunocytochemical study revealed that clusterin was expressed in these cell lines. However, the localisation of clusterin expression differed among the cell lines. In the A31C4 cell line, clusterin was mainly expressed in the cytoplasm (Fig. 6A); however, in the C24A4 (Fig. 6B) and C22A4 cell lines (data not shown), it was expressed in both the nucleus and the cytoplasm.

Effects of FSH on Sertoli cell proliferation

To examine the functionality of *Fshr* in the three cloned Sertoli cell lines, the cells were first stimulated with FSH and then the BrdU incorporation assay was performed. BrdU incorporation rates were significantly higher in the FSH-treated Sertoli cell lines than in the controls ($P < 0.001$; Fig. 7). No significant difference was observed in the rate of BrdU incorporation among the three clones treated with FSH, although the C22A4 cell line showed slightly higher BrdU incorporation rates than did the other cell lines that did not receive any stimulation ($P < 0.05$).

Discussion

We isolated Sertoli cells from adult mouse testes and established stable Sertoli cell lines after the starvation of primary cultured cells. These immortalised Sertoli cell lines, which express several genes specific for mature Sertoli cells and show proliferation and phagocytic activity, may provide a useful *in vitro* model for studying

several factors related to Sertoli cell function and spermatogenesis.

An appropriate regulation of androgen activity through AR-mediated signalling is necessary for both the initiation and maintenance of spermatogenesis (Sharpe *et al.* 2003). Poor germ cell differentiation has been observed in the testes of Sertoli-cell-specific AR knockout mice (De Gendt *et al.* 2004, Wang *et al.* 2009). Therefore, the expression of AR is important for the selection of Sertoli cell lines. Only Sertoli cell lines prepared from neonatal mice maintain AR expression (Mather 1980). AR expression is lost in immortalised rat Sertoli cell lines established through SV40-induced transformation (Konrad *et al.* 2005) and in most Sertoli cell lines established from the cells of adult mouse testes via gene transfer. Therefore, transgenic techniques may not be adequate for maintaining AR expression in immortalised Sertoli cells. In the present study, we did not use transgenic techniques to establish our Sertoli cell lines and, instead, opted for the specific selection of clones on the basis of AR expression. This approach may result in the successful establishment of Sertoli cell lines expressing AR from adult testes.

PR expression in Sertoli cells changes during the maturation process (Weber *et al.* 2002), and the expression is altered in the testes of infertile men (Shah *et al.* 2005). Furthermore, the binding affinity of testosterone to AR may be modulated through testosterone binding to PR (Tindall *et al.* 1984). However, no available reports have described the expression of PR in mature Sertoli cell lines derived from adult testes,

Figure 4 Immunolocalisation of AR and PR in the cell lines. (A) AR nuclear localisation in the C22A4 cell line, (A') negative control of A, (B) PR localisation in the nucleus of the A31C4 cell line and (B') negative control of B. Scale bars: 50 µm.

suggesting that it is not expressed or has not been investigated. Therefore, we thought it was important to study the expression of PR in our Sertoli cell lines to investigate the involvement of PR in spermatogenesis.

The Sertoli cell is the only cell type among the testicular cells, which possesses phagocytic activity to eliminate apoptotic germ cells (Grandjean *et al.* 1997, Nakanishi & Shiratsuchi 2004). This process is thought to be essential for the production of normal spermatozoa because half of the produced germ cells disappear during spermatogenesis (Wang *et al.* 2006, Chen *et al.* 2009, Elliott *et al.* 2010). Our established Sertoli cell

lines show active phagocytosis and may be useful for the analysis of this Sertoli cell function in detail.

Transferrin has a role in the transport of iron to developing germ cells and is produced by Sertoli cells (Skinner & Griswold 1980). Transferrin synthesis increases during puberty and is a differentiation marker for Sertoli cells before and during puberty (Anthony *et al.* 1991). Hence, the maintenance of *Trf* gene expression in our established cell lines may indicate that Sertoli cells are fully differentiated. This characteristic suggests that these cell lines may be useful for the analysis of iron transportation between Sertoli cells and germ cells.

Figure 5 RT-PCR analysis of Sertoli cell lines and mouse testis. (A) Expression of the housekeeping genes. (B) Expression of spermatogenic cell- and Leydig-cell-related molecules. (C) Expression of Sertoli-cell-related molecules.

Figure 6 Immunolocalisation of clusterin in the cell lines. (A) Cytoplasmic localisation of clusterin in the A31C4 cell line, (A') negative control of A, (B) nuclear and cytoplasmic localisation of clusterin in the C24A4 cell line and (B') negative control of B. Scale bar: 50 μ m.

Clusterin is thought to control the expression of many other Sertoli cell products; however, its role remains unclear. Two types of clusterin, a conventional secreted isoform and a cytoplasmic and nuclear isoform, are thought to be involved in survival or cell death (Leskov *et al.* 2011). Although clusterin expression in the nuclei is thought to be associated with apoptosis, our cell lines survive, despite clusterin expression in the nuclei. The prostate tumour cell line PC-3 has been reported to survive when clusterin is overexpressed in both the cytoplasm and the nucleus because of the acquisition of apoptotic inhibitory properties during cloning of the cell line (Scaltriti *et al.* 2004). During selection, our cell lines also may have acquired anti-apoptosis properties similar to those in PC-3 cell lines. Functionally immature Sertoli cells of the adult testis containing only Sertoli cells or Sertoli cells with carcinoma *in situ* do not express clusterin, suggesting that clusterin expression is a marker of functionally mature Sertoli cells and may play a role in normal spermatogenesis (Sharpe *et al.* 2003). Our established Sertoli cell lines may be useful for studying the control function of *Clu* on the expression of many other Sertoli cell products required for spermatogenesis.

Our cell lines also expressed the transcription factor *Wt1*, which is expressed in Sertoli cells (Pelletier *et al.* 1991). WT1 plays an essential role in gonadal development, sexual differentiation and spermatogenesis (Kreidberg *et al.* 1993, Scharnhorst *et al.* 2001, Myers *et al.* 2005). Furthermore, WT1 is thought to be a stable marker of Sertoli cells compared with other protein markers because it is continuously expressed at high levels in Sertoli cells throughout life (Mackay 2000). The 15P1 Sertoli cell line expresses *Wt1*, which induces transmeiotic differentiation of premeiotic germ cells under

co-culture conditions (Rassoulzadegan *et al.* 1993). Therefore, the expression of *Wt1* in our Sertoli cell lines may be expected to support spermatogenesis.

FSHR expression is initiated during foetal development and is maintained throughout development. FSH acts through FSHR, induces Sertoli cell proliferation (Riera *et al.* 2012), establishes the final Sertoli cell number (Singh & Handelsman 1996) and stimulates transcription and metabolic activity in Sertoli cells,

Figure 7 Effects of proliferation activity on the cell lines after exposure to FSH. After the incubation of cell line monolayers for 24 h with (+) or without (-) FSH (100 ng/ml), the cells were further incubated with BrdU (200 μ M) for 24 h. BrdU incorporation was evaluated by immunocytochemical analysis. Each bar represents the percentage of BrdU-positive cells. Results represent mean $\pm$ s.e.m. of more than three independent experiments with triplicate incubations. Asterisks indicate the significant difference in the percentage of BrdU-positive cells between groups with or without (-) FSH treatment in the cell lines. (Student's *t*-test, $P < 0.001$).

which contributes to germ cell survival and development (George *et al.* 2011). Our present results confirm the functionality of FSHR in our cell lines through the evaluation of proliferation activity with FSH supplementation. GATA-1 is thought to be one of the requirements for Sertoli cell-specific activity through the *Fshr* promoter (Kim & Griswold 2001). Although our established cell lines do not show *Gata1* expression, other elements may be used for the activation of the *Fshr* promoter to induce the proliferation activity of the cell lines (Hermann & Heckert 2007). In our current routine culture conditions, we did not add FSH to the media; however, culture media containing bovine serum had a small amount of FSH and may induce the proliferation of Sertoli cells.

Some immature Sertoli cell markers were also expressed in our Sertoli cell lines. *Amh* expression is switched on when the undifferentiated gonad begins to differentiate into the testis (Mackay 2000, Josso *et al.* 2001), and the production of *Amh* continues at high levels before around puberty (Tran *et al.* 1987). The down-regulation of *Amh* expression correlates with the onset of meiosis in germ cells (Rey *et al.* 1996) and the presence of meiotic cells may be required to switch off *Amh* expression in our cell lines.

The transcription factor *Gata1* is initially expressed in murine Sertoli cells as they are maturing and show stage-specific expression after the first wave of spermatogenesis (Yomogida *et al.* 1994). Three of our Sertoli cell lines did not express *Gata1*. Thus, we can assume that our Sertoli cell lines expressed *Amh*, but not *Gata1*, because the expression of *Gata1* and *Amh* are inversely related during testis development (Beau *et al.* 2000), i.e. *Gata1* may be switched off by *Amh* expression in our cell lines.

Several paracrine growth factors produced by Sertoli cells, e.g. *Kitl*, *Lif*, *Tgfb1* and *Fgf2*, alone or in combination, are expected to play a crucial role in the proliferation and/or differentiation of spermatogonia (Mullaney & Skinner 1993, Dym *et al.* 1995, Huleihel & Lunenfeld 2004, Loveland *et al.* 2005, Mirzapour *et al.* 2012). Our established three cell lines, all of which express these molecules, will be valuable for investigating the effects of these factors on spermatogenesis.

In the mammalian testis, Sertoli cells make the blood–testis barrier using tight-junction-associated proteins such as occludin and claudin (Mruk & Cheng 2010). These tight-junction-associated proteins show restructuring during spermatogenesis and participate in coordinated spermiation (Cheng *et al.* 2011). Although many other tight-junction-associated molecules need to be investigated, our cell lines may be used for establishing a tight junction barrier that mimics the blood–testis barrier *in vitro* because these established cell lines express *Ocln*.

In conclusion, the three established cell lines are potentially valuable tools for studying Sertoli-cell-specific gene expression. Furthermore, the starvation method used to establish the cell lines from adult Sertoli

cells might be effective in isolating the cell lines with acquired proliferative activity and conserved native characteristics.

Materials and Methods

Primary cell culture preparation

Cells for primary culture were obtained from the testes of 6-week-old male C57BL/6J mice by a modified two-step enzymatic digestion protocol (Brinster & Avarbock 1994, Bellve *et al.* 1997). In this procedure, the tunica albuginea was removed from the testes manually, exposing the seminiferous tubules. The testes were then incubated in 1 ml PBS without calcium and magnesium (PBS (–); Wako, Tokyo, Japan) containing 1 mg/ml collagenase (Type IV; Sigma) at 37 °C with gentle agitation (80 cycles/min) for 1 h. After sedimentation by centrifugation (300 *g* for 5 min at room temperature), the supernatant was removed and the seminiferous tubules were incubated in PBS (–) containing 1 mg/ml hyaluronidase (Type-1S; Sigma) at 37 °C with gentle agitation (80 cycles/min) for 1 h. The cells were washed three times with PBS (–) and centrifuged (300 *g* for 5 min at room temperature). Following digestion, cell suspension was filtered through a nylon mesh (Cell Strainer 100 µm; BD Falcon, Tokyo, Japan) to remove cell clumps and undigested materials. The filtrate was centrifuged (300 *g* for 5 min at room temperature) and the supernatant was removed from the pellet. The cells in the pellet were then resuspended in complete culture medium, which consisted of Eagle's medium (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) supplemented with 0.292 g/l L-glutamine (Nissui Pharmaceutical Co. Ltd), 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and 10% foetal bovine serum (FBS; Gibco). Yields were in the range of 5.0×10^8 cells per testis. The cells were plated in culture dishes or in 24-well culture plates (Falcon; Fisher Scientific, Yokohama, Kanagawa, Japan) containing complete culture medium supplemented with L-glutamine, 10% FBS and antibiotics, as described above, and incubated at 32 °C and 5% CO₂ in a humidified incubator. After culture for 1 day, the culture medium was changed to remove non-adherent cells from the dish or well. This study was approved by the Ethical Committee/Institutional Review Board of St Marianna University School of Medicine.

Isolation of immortalised cell lines and subcloning

After changing the medium once a week and passaging the cells for 1 month, to starve the cells, we altered the medium changes from once a week to once every 2 weeks for two cycles and then to once every 3 weeks for two cycles. After starvation of the cells, the original medium change routine was used, and the cells showing high proliferation rates were selected. Using the cloning ring (Iwaki, Tokyo, Japan) for the collection of the cells from a single colony (McFarland 2000), we performed the first subcloning of the cells and isolated 88 cell groups showing six different morphological types. The first subcloned cell groups were stored in culture medium containing 0.75% dimethyl sulphoxide (DMSO; Sigma) in liquid nitrogen. We confirmed the proliferation of these cell groups over four

passages. Then, we continued with the second subcloning of the cells using the same methods and isolated 33 cell line groups. The second subcloned cell groups were also stored in culture medium containing 0.75% DMSO (Sigma) in liquid nitrogen. The proliferation activity of these cell groups was confirmed over six passages.

Selection markers of the cell lines for the third round of subcloning

The 12 subclones were selected as cell line candidates on the basis of their morphological resemblance to Sertoli cells (i.e. the presence of cytoplasmic projections, lipid inclusions and indented nuclei), phagocytic activity and the characteristics of mature Sertoli cells, such as both the expression and localisation of the AR and PR, by using phase contrast and bright field microscopy.

Analysis of the phagocytic activity of the cell lines

We used a modified version of a previously reported phagocytosis assay (Tokuda *et al.* 1992). Fluoresbrite plain Calibration Grade 6.0 micron YG Microspheres (2.6% Solids-latex; Polysciences, Inc., Warrington, PA, USA) were added to 70% confluent cell lines, and further incubated at 32 °C and 5% CO₂ overnight. Internalisation was assessed using fluorescent microscopy and phase contrast microscopy after washing the cells three times with PBS (–) to remove the latex beads on the outside of the cells.

Immunocytochemistry for AR, PR and clusterin

Cells were grown on Lab-Tek chamber slides (Nalge Nunc International KK., Tokyo, Japan) to 80% confluence and fixed with 4% paraformaldehyde for 1 h or ice-cold methanol (MeOH) for 30 min. After washing with PBS (–), the slides were incubated with 3% H₂O₂–MeOH for 10 min to block endogenous peroxidase activity. After pre-incubating the slides with 10% goat serum in PBS (–) for 1 h, primary antibodies against AR (N-20, rabbit polyclonal, 1:200; Santa Cruz Biotechnology), PR (C-19, rabbit polyclonal, 1:200; Santa Cruz Biotechnology) or clusterin (H-330, rabbit polyclonal, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted in 10% BSA in PBS (–), added to the slides and incubated for 2 h or overnight (clusterin) at room temperature in a humidified chamber. Pre-absorbed antibodies with blocking peptides (AR [N-20]P and PR [C-19]P; Santa Cruz Biotechnology) or rabbit IgG (Dako, Tokyo, Japan), which were adjusted to the working concentration of each primary antibody, were used for the negative control, respectively. After washing several times with PBS (–), the slides were incubated with anti-rabbit IgG (Fab') labelled with an amino acid polymer–peroxidase complex (Histofine Simple Stain MAX PO (R); Nichirei Co., Tokyo, Japan) for 1 h at room temperature. The slides were then washed and the peroxidase complex was detected by incubation with aminoethyl carbazole (AEC, Histofine Simple Stain AEC Solution; Nichirei Co.) according to the manufacturer's instructions. The slides were counterstained with haematoxylin, mounted using an aqueous

permanent mounting solution (Nichirei Co.) with a cover glass and observed under a microscope.

Protein extraction and western blot analysis

Cells were washed twice with cold PBS (–), solubilised with cold RIPA lysis buffer (10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 2% 3-((3-cholamidopropyl)dimethylammonio) propanesulphonate, 1 mM dithiothreitol) containing one protease inhibitor cocktail tablet (Roche Biochemicals) on ice for 10 min, and collected by using a cell scraper and by pipetting on ice for 10 min. The lysate was then centrifuged to remove cellular debris (15 min, 15 000 *g*, 4 °C). Protein concentration was measured by the bicinchoninic acid assay (Smith *et al.* 1985) using BSA as a standard. We loaded 10 µg protein from the testis or 10 µg protein from the isolated cell fractions into Laemmli buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue, 0.0625 M Tris–HCl), separated the proteins on a 7.5% acrylamide gel and semi-dry electroblotted the samples onto a polyvinylidene difluoride membrane (Millipore, Tokyo, Japan). The membranes were blocked for 1 h at 37 °C with a solution of 5% (w/v) non-fat dried milk in Tris-buffered saline (20 mM, pH 7.8) supplemented with Tween-20 (0.05%; TBS-T). The membranes were incubated with the anti-AR antibody (1:2000) or PR antibody (1:1000) in TBS-T containing 5% (w/v) non-fat dried milk for 30 min at 37 °C. After three washes in TBS-T for 5 min, the membranes were incubated with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:3000; Bio-Rad) in TBS-T for 30 min at 37 °C. Control incubations were performed using an antibody pre-absorbed with an excess antigen-blocking peptide (AR[N-20]P or PR[C-19]P). Specific immune complexes were detected using an enhanced chemiluminescence kit (ECL; Amersham-Pharmacia) by following the manufacturer's instructions.

Screening of PR and AR expression in the cell lines by western blot analysis

Double sets of the samples were run on both sides of a gel. After the proteins were transferred to a membrane, half of the membrane was stained with Coomassie Brilliant Blue R (CBB), for which detection was considered as a good quantitative loading control for the excision of bands of the AR or PR. Because the band the size of the AR or PR band in CBB was difficult to detect, the clear band at around 50 kDa was used for the loading control. The other half of the membrane was used for western blotting as described previously. The wet gels were stained with CBB to confirm the efficiency of blotting. The western blot membranes were scanned and volumetric determination of the detected bands was performed using ImageMaster software (Amersham Pharmacia Biotech). To adjust for the running differences between the gels, we used the same protein amount from the same testis sample and compared the results of the expression of the AR or PR in each clone using the following formula:

$$(\text{ECL value of (clone X)/CBB value of (clone X)})/$$

$$(\text{ECL value of (testis)/CBB value of (testis)}).$$

Detection of Sertoli cell transcripts by RT-PCR for final cloning

After the selection of the 12 subclones (A31B1, A31B4, A31C1, A31C4, A32B4, A33A4, C21C3, C21C4, C22A3, C22A4, C22B4 and C24A4), RT-PCR was performed for the final selection of the cell lines using primers sets for genes specifically expressed by various cell types within the testis. Total RNA was extracted from the cell lines or testicular tissue using ISOGEN (Nippon Genes, Tokyo, Japan) according to the manufacturer's protocol. Total RNA was digested by RNase-free DNase I (Gibco) to degrade any genomic DNA present and reverse transcribed into cDNA with the Gene Amp PCR Core Kit (Perkin-Elmer, Yokohama, Kanagawa, Japan) by using oligo d(T) primers in the RT reaction. Aliquots of 1 µl of cell line cDNA or 1 µl of testis-derived cDNA served as PCR templates

(Ex Taq polymerase; Takara Co. Ltd, Tokyo, Japan). We amplified 1 µl of cDNA for 35 cycles (denaturing at 94 °C for 30 s, annealing at specific temperatures for 45 s (Table 2) and elongation at 72 °C for 45 s) by using primers specific for various genes expressed by different testicular cell types: *Kit* expressed by spermatogonia; *Kitl*, *Inha*, *Gata1*, *Clu*, *Fshr*, *Wt1*, *Trf*, *Shbg* and *Ocln* expressed by Sertoli cells; *Hsd3b* expressed by Leydig cells; *Ar* expressed by Sertoli cells, Leydig cells and peritubular myoid cells; *Lhcgr* expressed by Leydig cells; *Sf1* expressed by Sertoli cells and Leydig cells; *Lif* produced by Sertoli cells, germ cells and Leydig cells; *Tgfb1* and *Fgf2* produced by Sertoli cells and peritubular myoid cells; *Amh* expressed by Sertoli cells and Leydig cells until around puberty; and *Sox9* expressed by embryonic Sertoli cells and Leydig cells. As a positive control, PCR primers for cyclophilin, a housekeeping gene, were also used. The primers were

Table 2 Primer sequences of RT-PCR.

Genes	Orientation	Nucleotide sequence	Annealing temperature (°C)	PCR product (bp)	Reference
<i>Cyclophilin</i>	Sense	5'-TGG GCC GCG TCT CCT TCG AG-3'	56	588 bp (exons 1–5)	Hofmann <i>et al.</i> (2003)
	Antisense	5'-GGG GAA TGA GGA AAA TAT GG-3'			
<i>Kit</i>	Sense	5'-TCA ACG ACC TTC CCG AAG GCA CCA-3'	60	389 bp (exons 9–12)	Schrans-Stassen <i>et al.</i> (1999)
	Antisense	5'-CTG GTG GTT CAG AGT TCC ATA GAC-3'			
<i>Lhcgr</i>	Sense	5'-AAT CCC ATC ACA AGC TTT CAG-3'	56	215 bp (exons 2–5)	Walther <i>et al.</i> (1996)
	Antisense	5'-TGC CTG TGT TAC AGA TGC-3'			
<i>Hsd3b</i>	Sense	5'-GTG ACA GGA GCA GGA GG-3'	66	890 bp (exons 2–4)	Walther <i>et al.</i> (1996)
	Antisense	5'-AGG AAG CTC ACA GTT TCC A-3'			
<i>Ar</i>	Sense	5'-GAG GAA CAG CAG CCT TCA CAG CAG C-3'	66	259 bp (exon 1)	Walther <i>et al.</i> (1996)
	Antisense	5'-GCT GCT GCT GAA GAA GTT GCA T-3'			
<i>Inha</i>	Sense	5'-GCA ATG GAT GGG GAA GGT GG-3'	66	238 bp (exons 1–2)	Walther <i>et al.</i> (1996)
	Antisense	5'-GGT GGC TGC GTA TGT GTT GG-3'			
<i>Clu</i>	Sense	5'-TGA ACG GCG ACC GCA TCG AC-3'	60	264 bp (exon 5)	NM_013492.2
	Antisense	5'-TGT GGA AGC TCG GAG GCC CA-3'			
<i>Fgf2</i>	Sense	5'-GTC CGC GAG AAG AGC GAC CC-3'	60	283 bp (exons 1–3)	NM_008006.2
	Antisense	5'-TGG CCT TCT GTC CAG GTC CCG-3'			
<i>Tgfb1</i>	Sense	5'-CGG GGC GAC CAG CCT TCA CAT GAC-3'	68	405 bp (exons 5–7)	Hofmann <i>et al.</i> (2003)
	Antisense	5'-CTG CTC CAC CTT GGG CTT GCG ACC CAC-3'			
<i>Lif</i>	Sense	5'-TCA CCC CTG TAA ATG CCA CC-3'	56	450 bp (exons 4–5)	Hofmann <i>et al.</i> (2003)
	Antisense	5'-CTT CTT TGT CAG AGT GGT CG-3'			
<i>Kitl</i>	Sense	5'-TAA CCC TCA ACT ATG TCG CC-3'	60	344 bp (exons 3–5)	Hofmann <i>et al.</i> (2003)
	Antisense	5'-TGA AGA GAG CAC ACA GTC AC-3'			
<i>Gata1</i>	Sense	5'-TGT GTG AAC TGT GGA GCA ACG GC-3'	60	592 bp (exons 2–6)	NM_008089.1
	Antisense	5'-TAG GTG CAG GAC CGG TGG GG-3'			
<i>Sf1</i>	Sense	5'-GTG AAA TTC CAG AAC AAC CAC AGC-3'	60	242 bp (exon 7)	NM_139051.3
	Antisense	5'-GTC TGC TTG GCC TGC AGC ATC TC-3'			
<i>Fshr</i>	Sense 1	5'-TGA TGA TAT GAC TCA GCC TG-3'	56	478 bp (exon 10)	NM_013523.3
	Antisense 1	5'-TGC CAA TGT GTA GAC TGA CA-3'			
	Sense 2	5'-GTT GAT GTG ACC TGC TCG CC-3'	56	237 bp (exon 10)	NM_013523.3
	Antisense 2	5'-TGA GGC TAT AAG TAG CAA GT-3'			
<i>Amh</i>	Sense	5'-GGC TCT GAT TCC CGC TGT T-3'	50	236 bp (exons 4–5)	Ao <i>et al.</i> (1993)
	Antisense	5'-GCC AGT TGC GTG TTC GAA G-3'			
<i>Sox9</i>	Sense	5'-TGA ATC TCC TGG ACC CCT TCA TG-3'	60	440 bp (exons 1–3)	NM_011448.4
	Antisense	5'-GTT CAG CAG CCT CCA GAG CTT G-3'			
<i>Wt1</i>	Sense	5'-GCA AAC CTG GAA ACC T-3'	56	453 bp (exon 10)	Beverdam <i>et al.</i> (2003)
	Antisense	5'-ACT TGC AGA TTC AAA GAT ACT-3'			
<i>Trf</i>	Sense	5'-ATC TGG GAG ATT CTC AAA GTG-3'	60	579 bp (exons 8–12)	Walther <i>et al.</i> (1996)
	Antisense	5'-AGT GTG GCA GGA CTT CTT GCC-3'			
<i>Shbg</i>	Sense	5'-GTC ATG GTT ATG ACC ATT GA-3'	60	498 bp (exons 2–5)	NM_011367.2
	Antisense	5'-CAG GTC CAC ATC ACA GTT TC-3'			
<i>Ocln</i>	Sense	5'-TGC AGC AGC CCT CAG GTG AC-3'	54	318 bp (exon 9)	NM_008756
	Antisense	5'-TGG TGG GGA ACG TGG CCG AT-3'			

Ar, androgen receptor; *Clu*, clusterin; *Fgf2*, basic fibroblast growth factor; *Tgfb1*, transforming growth factor β1; *Lif*, leukaemia inhibitory factor; *Kitl*, stem cell factor; *Amh*, Müllerian inhibiting substance; *Wt1*, Wilms' tumour gene; *Sox9*, sex-determining region Y box 9; *Fshr*, FSH receptor; *Sf1*, steroidogenic factor-1; *Shbg*, androgen-binding protein/sex hormone-binding globulin; *Gata1*, globin transcription factor-1; *Kit*, c-kit oncogene; *Lhcgr*, LH receptor; *Hsd3b*, 3β-ol-hydroxysteroid-dehydrogenase; *Ocln*, occludin.

designed using published sequences from mouse or other mammalian species (Walther *et al.* 1996, Schrans-Stassen *et al.* 1999, Beverdam *et al.* 2003, Hofmann *et al.* 2003) or Primer BLAST (NCBI). For the RT-PCR of *Fshr*, after the first-round PCR, the amplified products (50 times diluted) were used for a second-round nested PCR. The PCR products were electrophoresed through 2% agarose gels, and after staining with ethidium bromide, the product bands were visualised under u.v. light. The absence of contaminating genomic DNA was confirmed by running each reaction in parallel with a reaction lacking reverse transcriptase. The RT-PCR products were subcloned into TOPO pCRII (Invitrogen), and DNA was purified for sequencing (Mini-Prep Kit; Qiagen). RT-PCR experiments were performed independently three times.

Growth curves

The final 12 subcloned cell lines were counted and seeded at 2.0×10^3 cells per dish in different 30 mm tissue culture dishes with complete cell culture media containing 10% FBS. For the next 6 days, three of the dishes for each cell line were trypsinised, and the cells were counted using the trypan blue (Gibco) exclusion method. The doubling time of each cell line was calculated from their individual growth curves.

Chromosomal analysis of the cell lines

Cell lines showing 70% confluence were incubated for 2 h at 32 °C in complete culture medium containing 0.1 µg/ml colcemid in order to arrest the cell cycle at metaphase. After colcemid treatment, the cells were washed with PBS (–) and removed from the dish using 0.05% trypsin with EDTA (Invitrogen). The cells were washed three times with PBS (–) and centrifuged (300 g for 5 min at room temperature). After removing the supernatant, the cells were hypotonised with 1% (w/v) sodium citrate for 15 min at room temperature. The cells were fixed in a 2 × volume of Carnoy's fixative for 2–3 min and centrifuged (300 g for 5 min at room temperature). After removing the supernatant, the cells were suspended in a small amount of Carnoy's fixative and placed onto clean glass slides. The slides were air-dried, stained with 10% (V/V) Giemsa solution (Invitrogen) for 10 min, rinsed in distilled water and air-dried again. The chromosome spreads were examined via bright field microscopy to determine the nuclear ploidy of each cell line.

BrdU incorporation assay

After culturing the monolayer Sertoli cell lines on Lab-Tek chamber slides with or without purified native FSH from porcine pituitary glands (100 ng/ml, H031/H; Biogenesis, Dorset, UK) in culture medium for 24 h on day 2 in culture, the cell lines were incubated with BrdU (200 µM; Sigma) in culture medium for 24 h on day 3 in culture. After the incubation, cultured cells were fixed with 4% paraformaldehyde in PBS (–) for 30 min and then permeabilised with 0.1% Triton-100 in PBS (–) for 15 min. After washing with PBS (–), the slides were rinsed with distilled water for 5 min and then incubated with 2 M HCl for 20 min to denature DNA for

allowing the anti-BrdU antibody to interact with incorporated BrdU. To neutralise the acid, the slides were immersed with 0.1 M sodium borate buffer (pH 8.5) for 5 min and then washed with distilled water and PBS (–) several times. After blocking the endogenous peroxidase activity, as described for the immunocytochemical method previously, the cells were incubated with 10% goat serum in PBS (–) for 1 h at room temperature. The immunocytochemical reaction was performed using an anti-BrdU mouse MAB (1:100, NCL-BrdU; Leica Biosystems, Tokyo, Japan) diluted in 10% BSA in PBS (–) as the primary antibody or mouse IgG (Dako) adjusted to the working concentration of the BrdU antibody as the negative control for 2 h at room temperature. After washing the slides with PBS (–), anti-mouse IgG (Fab') labelled with an amino acid polymer-peroxidase complex (Histofine Simple Stain MAX PO (M); Nichirei Co.) was applied on the slides as the secondary antibody and incubated for 1 h at room temperature. Then, AEC was used for the detection of the antigen, as described previously. The slides were counterstained with haematoxylin and observed under a microscope. Data are presented as the mean value of the percentage of proliferating Sertoli cells ((BrdU-positive Sertoli cells/total Sertoli cells) × 100) from more than three independent experiments with triplicate incubations. All values are expressed as mean ± S.E.M. Student's *t*-test was used to assess all differences between the pairs using Windows Excel 2007 (Microsoft Corp.). A *P* value of <0.05 was considered as significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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