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Asymmetric Distribution of UCH-L1 in Spermatogonia Is Associated With Maintenance and Differentiation of Spermatogonial Stem Cells

Jinping Luo, Susan Megee, and Ina Dobrinski

Department of Clinical Studies, Center for Animal Transgenesis and Germ Cell Research, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pennsylvania

Abstract

Asymmetric division of germline stem cells in vertebrates was proposed a century ago; however, direct evidence for asymmetric division of mammalian spermatogonial stem cells (SSCs) has been scarce. Here, we report that ubiquitin carboxy-terminal hydrolase 1 (UCH-L1) is expressed in type A (A_s , A_{pr} , and A_{al}) spermatogonia located at the basement membrane (BM) of seminiferous tubules at high and low levels, but not in differentiated germ cells distant from the BM. Asymmetric segregation of UCH-L1 was associated with self-renewal versus differentiation divisions of SSCs as defined by co-localization of UCH-L1^{high} and PLZF, a known determinant of undifferentiated SSCs, versus co-localization of UCH-L1^{low/-} with proteins expressed during SSC differentiation (DAZL, DDX4, c-KIT). In vitro, gonocytes/spermatogonia frequently underwent asymmetric divisions characterized by unequal segregation of UCH-L1 and PLZF. Importantly, we could also demonstrate asymmetric segregation of UCH-L1 and PLZF in situ in seminiferous tubules. Expression level of UCH-L1 in the immature testis where spermatogenesis was not complete was not affected by the location of germ cells relative to the BM, whereas UCH-L1-positive spermatogonia were exclusively located at the BM in the adult testis. Asymmetric division of SSCs appeared to be affected by interaction with supporting somatic cells and extracellular matrix. These findings for the first time provide direct evidence for existence of asymmetric division during SSCs self-renewal and differentiation in mammalian spermatogenesis.

Spermatogenesis is a complex process of cell proliferation and differentiation including spermatogonial stem cell (SSCs) self-renewal and differentiation to ultimately form all stages of male germ cells. This dynamic process originates from SSCs and is maintained in the testis for the entire adult life of the male. Among the many unresolved questions in mammalian spermatogenesis, the mechanisms governing the decision by SSCs to proliferate or differentiate are the least understood. SSCs are a subset of undifferentiated type A spermatogonia, residing in a stem cell niche at the basement membrane (BM) of the seminiferous tubules. Previous studies in the mouse suggested that undifferentiated spermatogonia at specific stages of spermatogenesis are not randomly distributed, but instead position themselves preferentially in a specific region of the tubules opposite the interstitium in proximity to the vasculature (Chiarini-Garcia and Russell, 2001; Yoshida et al., 2007). The factors regulating a balance between maintenance of the SSCs reservoir and production of appropriate numbers of

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Correspondence to: Ina Dobrinski.

Correspondence to: Ina Dobrinski, HRIC 2AC58, Faculty of Veterinary Medicine, Department of Comparative Biology and Experimental Medicine, University of Calgary, 3330 Hospital Drive, Calgary, Alberta, Canada T2N 4N1. idobrins@ucalgary.ca.

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differentiated germ cells are largely unknown. Currently, there are three models for SSCs renewal: the A_s model (Huckins, 1971; de Rooij, 1973), the A0/1 model (Clermont and Bustos-Obregon, 1968; Bartmanska and Clermont, 1983), and the clone fragmentation model (Erickson, 1981; Erickson and Hall, 1983). The A_s model is the predominant model in rodents which suggests that single undifferentiated type A spermatogonia (A_s) are the only population of stem cells. According to this concept, A_s spermatogonia divide into two cells that either migrate apart or remain interconnected as cell pairs, called A_{pr} cells, and divide further to A_{al} cells which are destined to eventually differentiate. This model does not consider the possibility of asymmetric division of SSCs in mammals (Huckins, 1971; de Rooij, 1973). In 1997, it was proposed that asymmetric division of germline stem cells contributes to their self-renewal and differentiation (Lin, 1997). In *Drosophila* testis, germline stem cell divisions normally have asymmetrical outcomes: the daughter cell adjacent to the hub retains stem cell identity and self-renewal capacity, while the daughter cell displaced from the hub becomes a gonialblast and initiates differentiation (Kiger et al., 2000; Chen and McKearin, 2003; Yamashita and Fuller, 2005). Asymmetric division of adult stem cells was also found in other tissues such as neuronal system (Matsuzaki, 2000; Shen et al., 2002; Sun et al., 2005), skin (Koster and Roop, 2005; Lechler and Fuchs, 2005), muscles (Kuang et al., 2007), and blood (Faubert et al., 2004). However, little evidence has been reported to support asymmetric division of mammalian SSCs. We hypothesized that asymmetric division of SSCs is accompanied by asymmetric segregation of proteins acting as SSC determinants that would be up-regulated in SSCs but down-regulated in differentiating/differentiated spermatogonia where in turn expression of the proteins involved in differentiation will spontaneously increase.

Ubiquitin-dependent proteolysis has been implicated in the control of mammalian gametogenesis (Sutovsky, 2003; Kwon et al., 2004). Ubiquitin carboxy-terminal hydrolase 1 (UCH-L1; also known as protein gene product 9.5, PGP 9.5) is a deubiquinating enzyme that regenerates monoubiquitin from ubiquitin-protein complexes. In the testis, UCH-L1 is exclusively expressed in spermatogonia among male germ cells across species (Tokunaga et al., 1999; Kwon et al., 2003; Luo et al., 2006). In mice, UCH-L1 was found to play specific roles in apoptosis, meiosis, and mitotic proliferation of germ cells. Loss of UCH-L1 decreased the rate of apoptosis in the first round of spermatogenesis and increased the numbers of premeiotic germ cells in immature mice (Kwon et al., 2005), but spermatogenesis was partially impaired with significant reduction of spermatogonia and primary spermatocytes which mainly go to apoptosis in adult mice (Kwon et al., 2003). Overexpression of UCH-L1 was accompanied by reduced PCNA expression and abolishment of apoptosis in spermatogonia; spermatogenesis was blocked at pachytene stage of meiosis, and primary spermatocytes underwent apoptosis (Wang et al., 2006). In pigs, gonocytes and spermatogonia expressing UCH-L1 include the population expressing PLZF (Luo et al., 2006), the first protein shown to be required for SSCs self-renewal in mammals (Buaas et al., 2004; Costoya et al., 2004; Filippini et al., 2007). We previously observed varying expression levels of UCH-L1 (high, low, or none) in freshly isolated spermatogonia (unpublished), and we hypothesized that precise regulation of UCH-L1 expression levels in spermatogonia is associated with SSCs self-renewal and differentiation as well as cell proliferation. UCH-L1 and PLZF are likely candidates for asymmetric distribution during SSC self-renewal and differentiation.

Materials and Methods

Preparation of tissue samples for histology

Testes obtained at castration from 1-week-old, 10-week-old, and adult Yorkshire boars were maintained in ice-cold PBS without calcium during transfer to the lab. Small pieces of testis tissue were fixed in 4% paraformaldehyde overnight at 4°C, washed three times in PBS, and processed for paraffin embedding and sectioning.

Immunohistochemistry and immunofluorescence imaging

After deparafinization and rehydration, tissue sections were boiled in 10 mM citrate buffer (pH 6.0) for 30 min and cooled down to room temperature. Sections were washed three times with PBS for 5 min each followed by 0.5% Triton X-100 in PBS for 10 min at room temperature or ice-cold methanol for 5 min (only for PLZF immunohistochemistry). Samples were washed and blocked with 3% BSA for 1 h at room temperature. For double labeling, two primary antibodies raised in different species (Table 1) were combined in 3% BSA and incubated with tissues at 4°C overnight or 3 h at room temperature. Incubation without primary antibodies served as a negative control. Sections were washed five times with PBS for 5 min each and incubated with Alexa Fluor 555 (488) donkey anti-mouse IgG (H + L) and Alexa Fluor 488 (555) donkey anti-rabbit IgG (H + L) (Invitrogen Corporation, Carlsbad, CA; 4 µg/ml each in 3% BSA) for 1 h at room temperature. Slides were washed and mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescent images were captured on a Leica CTR5000 microscope (Leica Microsystems, Inc., Bannockburn, IL) with a CoolSNAPfx digital camera and Image-pro plus (Media Cybernetics Inc, Bethesda, MD) under a 40× objective. Images were combined and processed in Adobe Photoshop 7.0.

Enrichment of gonocytes and spermatogonia

For enrichment of gonocytes, seminiferous tubules were isolated from testes of 1- to 2-week-old boars by enzymatic digestion (Honaramooz et al., 2002) without trypsin/EDTA. After removing the majority of connective tissue and Leydig cells by washing with Dulbecco's modified eagle's medium (DMEM) three times, tubules were suspended in DMEM supplemented with 30 mg/L sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 2 mM glutamine (Invitrogen), 1 × MEM vitamin solution (Invitrogen), 1 × MEM nonessential amino acids (Invitrogen), 55 µM 2-mercaptoethanol (Invitrogen), and 5% fetal bovine serum (HyCLone, Logan, UT) and plated in T75 flasks. Tubules were cultured overnight to 1 day. To collect gonocytes from cultured tubules, medium was removed and cells were rinsed in PBS without calcium followed by 2 ml of a 1:20 dilution of 0.25% trypsin/1 mM EDTA in PBS at room temperature with horizontal shaking of the flasks. Collected gonocytes were cultured in DMEM with supplements for 2–4 h to allow remaining somatic cells to attach to the culture dish. Enriched gonocytes were then harvested from the supernatant.

Spermatogonia were enriched from 10-week-old boar testicular cells by performing STAPUT isolation followed by differential plating (Luo et al., 2006).

Culture of enriched gonocytes or spermatogonia on extracellular matrix (ECM)

Four-well tissue culture chambers (Thermo Fisher Scientific, Rochester, NY) were coated with 0.01% poly-L-lysine (Sigma; 0.3 ml/well) and dried for 1 h, followed by coating with laminin (Sigma; 3 µg/cm) or collagen IV (Collaborative Biomedical Products Inc., Bedford, MA; 3 µg/cm²) at room temperature or with Matrigel (BD Biosciences, San Jose, CA; 0.3 ml of 1:10 or 1:1 in DMEM/well) at 4°C for 1 h before use. Culture wells coated only with poly-L-lysine served as control. Before loading cells, all wells were washed once with DMEM. Enriched gonocytes or spermatogonia were suspended in DMEM with supplements and plated in chambers at 4.5×10^4 /cm². After overnight culture at 37°C in 5% CO₂ in air, cells were fixed in 2% PFA at 4°C for 30 min and processed for immunocytochemistry and immunofluorescence imaging as above. Dividing gonocytes or spermatogonia connected with cytoplasmic bridges were identified under a 40× objective to record distribution of UCH-L1 and PLZF. The percentage of cells displaying asymmetric protein distribution in each group was calculated (n = 3) for statistical comparison.

Fluorescence intensity measurement

MetaMorph Offline Version 7.1.6.0 (Molecular Devices, Sunnyvale, CA) was applied for the analysis of immunofluorescence intensity in stained cells to compare the expression level of a protein. To measure fluorescence intensity of a target protein in immunostained cells on paraffin sections, the whole cell was circled as a measurement region by drawing a line at the edge of a cell. Average intensity of each region was transformed to digital data for comparison. In some cases, the relative fluorescence intensity to the lowest observed level was used for comparison.

Statistical analysis

Dual fluorescence staining was executed for UCH-L1 and DAZL, c-KIT, DDX4, or PLZF on paraffin sections prepared from 1-week-old, 10-week-old, and adult boar testes. To analyze protein expression levels, four representative regions of stained samples were used for image capture under a 40× objective as described above and average staining intensity was measured using MetaMorph software. The location of gonocytes/spermatogonia relative to the BM was identified by DAPI staining. Gonocytes where nuclei were located at the same distance from the BM as Sertoli cell nuclei were classified “close to BM,” otherwise they were judged to be “distant from BM.” Similarly, germ cells in samples from 10-week-old boars were classified as either “contact with BM” or “distant from BM.” Differences between groups (“close to BM” vs. “distant from BM,” “contact with BM” vs. “distant from BM”) were compared by *t*-test.

To analyze an effect of ECM on asymmetric allocation of UCH-L1 and PLZF, three independent experiments were performed to compare percentages of cells exhibiting asymmetric protein distribution when cultured on poly-L-lysine, laminin, collagen IV, matrigel diluted 1:10, and matrigel diluted 1:1 by one-way RM ANOVA followed by Holm–Sidak analysis. SigmaStat for Windows 3.0 Software (SPSS Inc., Chicago, IL) was used in the statistical analysis. Significance was assumed at $P < 0.05$.

Results and Discussion

Expression level of UCH-L1 (high or low) is associated with SSC self-renewal and differentiation

UCH-L1 is exclusively expressed in A_s , A_{pr} , and A_{al} spermatogonia which are characterized as undifferentiated spermatogonia (Frankenhuis et al., 1982) as well as distributed in cytoplasmic bridges of A_{pr} and A_{al} spermatogonia in adult boar testis (Fig. S1); therefore, the UCH-L1-positive population contains SSCs. Quantification of UCH-L1 expression level in single spermatogonia through measurement of immunofluorescence intensity in freshly isolated spermatogonia disclosed that the expression level of UCH-L1 is variable in porcine spermatogonia at 10 weeks of age when spermatogonia are beginning to differentiate and asymmetric distribution was observed in spermatogonia connected by cytoplasmic bridges (Fig. S2). Based on these findings, we determined whether there is a robust association of distinct expression levels of UCH-L1 to particular sub-populations of spermatogonia. To this end, we first analyzed the expression of UCH-L1 in germ cells at 1 week, 10 weeks, and adult ages when germ cells are gonocytes, spermatogonia to spermatocytes, and a complete population of spermatogenic cells, respectively. To distinguish the expression status of UCH-L1 in the undifferentiated spermatogonia and the germ cells entering differentiation, we performed double immunofluorescence staining of UCH-L1 and a SSC marker PLZF, or any one of DAZL (Schrans-Stassen et al., 2001; Saunders et al., 2003), c-KIT (Tajima et al., 1994; Albanesi et al., 1996; Vincent et al., 1998; Ohta et al., 2000), or DDX4 (Castrillon et al., 2000; Tanaka et al., 2000; Lee et al., 2005) which are expressed in germ cells beginning differentiation.

At 1 week of age, there was no obvious relationship between expression levels of UCH-L1 and DAZL, c-KIT, DDX4, or PLZF (Fig. 1). At this stage, the majority of gonocytes are located in the lumen of the seminiferous tubules in the boar testis (Hughes and Varley, 1980) and few have migrated to the BM. UCH-L1 is uniformly expressed in all gonocytes; PLZF can only be detected in a subpopulation of gonocytes. DAZL and c-KIT are weakly expressed in the majority of gonocytes; DDX4 expression was variable in gonocytes; at this stage of testis development, interaction between germ cells and their environment is restricted to contact with Sertoli cells since gonocytes have not migrated to the putative stem cell niche on the BM. This observation is consistent with our hypothesis because differential expression levels of UCH-L1 would not be expected before the onset of germ cell differentiation.

At 10 weeks of age, differential protein expression in germ cells became evident (Fig. 1). The expression levels of UCH-L1 and PLZF versus DAZL, c-KIT, and DDX4 became inversely correlated. For example, as illustrated in Figure 1, DDX4 is highly expressed in some germ cells in which expression of UCH-L1 is low or absent (UCH-L1^{low/-}), whereas expression of DDX4 is low in spermatogonia with high expression of UCH-L1 (UCH-L1^{high}). At this developmental stage, spermatogonia are the primary germ cells in seminiferous tubules (Frankenhuis et al., 1982). The majority of spermatogonia expressing both PLZF and UCH-L1 reside on the BM; however, UCH-L1^{high} or UCH-L1^{low/-} is not tightly correlated with expression of PLZF in these spermatogonia.

In the adult testis, UCH-L1 (high or low) was expressed only in a single layer of spermatogonia at the BM (Fig. 1). It was highly expressed in SSCs (PLZF-positive) and weakly in PLZF-negative spermatogonia at the BM, but absent in differentiated spermatogonia (DAZL, c-KIT, and DDX4 highly expressed) at a distance from BM. This pattern of UCH-L1 (UCH-L1^{high}, low, or absent) expression in germ cells suggests that down-regulation of UCH-L1 in SSCs might be a prelude for differentiation.

In mice, transgenic over-expression of UCH-L1 in spermatogonia causes a block during spermatogenesis at an early stage (pachytene) of meiosis and increased apoptosis of primary spermatocytes (Wang et al., 2006). In contrast, absence of UCH-L1 in spermatogonia did not affect meiosis but decreased the spermatogonia population (Kwon et al., 2003). Together with the current studies comparing expression of UCH-L1 to expression of DAZL, c-KIT, and DDX4 that are responsible for spermatogonia differentiation (RFS proteins) and PLZF, an essential factor for SSCs maintenance, the findings consistently suggest that a high level of UCH-L1 is associated with maintenance of SSCs, whereas reduction of UCH-L1 in spermatogonia is associated with differentiation toward meiosis.

Contact with the basement membrane affects protein expression level in spermatogonia

To compare the protein expression level in germ cells at different locations in the seminiferous tubules and analyze their correlation according to expression levels, measurement of immunofluorescence intensity of UCH-L1, DAZL, DDX4, or PLZF was performed on paraffin sections.

In the prepubertal testis, gonocytes (1 week of age) and germ cells (spermatogonia and spermatocytes, 10 weeks of age) can be divided into two groups according to their location in relationship to the BM identified by DAPI staining of nuclei. We compared UCH-L1, DAZL, DDX4, and PLZF expression levels between gonocytes (1 week of age) close to and distant from BM (Fig. 2a–c); and their expression levels between spermatogonia/spermatocytes (10 weeks of age) in contact with or distant from the BM (Fig. 2d–f). At 1 week of age, about one-third of gonocytes are close to BM, and two-thirds are distant from BM (Fig. 2b). Expression level of PLZF was significantly increased in the gonocytes close to BM compared to those distant from BM; however, expression of UCH-L1, DAZL, or DDX4 was not affected by the

distance of the gonocytes to the BM (Fig. 2a). At 10 weeks of age, over two-thirds of germ cells (spermatogonia) are in contact with BM, while other germ cells (mainly spermatocytes) are distant from BM (Fig. 2e). Expression of PLZF was significantly increased and expression of DDX4 was significantly decreased in the spermatogonia in contact with BM compared to those distant from BM (Fig. 2d). There was no significant difference in expression of UCH-L1 and DAZL in germ cells in contact with BM and distant from BM at 10 weeks of age (Fig. 2d). The expression level of UCH-L1 was not tightly associated with the position of the germ cells relative to the BM before puberty, but differential expression of UCH-L1 became prominent at 10 weeks (Fig. 1 and Fig. S2). In the adult testis, only spermatogonia expressing PLZF (PLZF positive) and/or UCH-L1 (high and low) reside on the BM (Fig. 1), and a high proportion of spermatogonia expressing high levels of UCH-L1 are also PLZF positive (data not shown). These results show that expression of proteins associated with germ cell self-renewal or differentiation in spermatogonia is influenced by the position of the cells relative to the BM; proteins important for self-renewal are highly expressed in cells close to the BM whereas RFSD proteins are expressed predominantly in cells at a distance from the BM.

Asymmetric segregation of UCH-L1 and PLZF in spermatogonia occurs in vivo and in vitro

Based on the inverse correlation in the expression level of UCH-L1 and PLZF versus RFSD proteins in spermatogonia during spermatogenesis, we hypothesized that to balance differentiation and stemness, an SSC performs asymmetric division where decrease of UCH-L1 and PLZF expression accompany onset of expression of RFSD proteins in one daughter cell, whereas high levels of UCH-L1 and PLZF remain in the other. Through measurement of UCH-L1 immunofluorescence intensity in spermatogonia in the adult testis, we observed both symmetric and asymmetric segregation of UCH-L1 in A_{pr} spermatogonia (Fig. 3). Further, we found asymmetric segregation of UCH-L1 in spermatogonia is associated with asynchrony of the cell cycle in their daughter cells (Fig. 4I,m–p). At 10 weeks and adult ages, concurrent asymmetric allocation of UCH-L1 and PLZF (Fig. 4II,a–f) as well as DDX4 and PLZF (Fig. 4III,a–i) was apparent in situ. During an asymmetric division, expression of UCH-L1 and PLZF remains strong in spermatogonia located at the BM, but expression is reduced or absent in daughter cells more distant from the BM (Fig. 4II,a–c). Asymmetric segregation of UCH-L1 was often found in A_{pr} spermatogonia. It was not possible to determine whether asymmetric distribution of UCH-L1 exists in A_{al} spermatogonia (4-cell, 8-cell, and so on) by measuring fluorescence intensity due to an uneven focal plane (Fig. 1 and S1). These results indicate that asymmetric segregation of UCH-L1 and PLZF is tightly associated with the fate of SSCs in self-renewal or differentiation. The positioning of SSCs relative to the BM appears to determine asymmetric division through affecting expression of certain proteins. This observation of asymmetry implies that daughter cells are not equivalent with one cell (close to the BM) remaining undifferentiated and the other (more distant from the BM) committed to differentiation. This is in contrast to the A_s theory that postulates that daughter cells resulting from division of an A_s cell either both remain undifferentiated or both undergo differentiation (Huckins, 1971; de Rooij, 1973). Therefore, our findings suggest asymmetric division of SSCs is another choice to regulate mammalian SSCs self-renewal and differentiation.

We further investigated spermatogonial division in vitro. Seminiferous tubules from 2 weeks, 8 weeks, or adult boar testes were isolated and cultured for 1–2 days. No asymmetric allocation of UCH-L1 or PLZF was observed in gonocytes in seminiferous tubules from 2-week-old pigs, and a very low frequency of spermatogonial divisions with asymmetric distribution of UCH-L1 and PLZF was evident in cultured seminiferous tubules from 8-week-old and adult boars (Fig. S3a). When seminiferous tubule fragments are isolated for culture, the majority of Leydig cells are removed but all other cell types comprising the testis microenvironment are present; however, the three-dimensional structure of the tubules is not maintained over time (Fig. S3a–d). While an inverse correlation of expression of SSC determinants and RFSD proteins was

maintained in germ cells in tubule culture, asymmetric allocation of these proteins in spermatogonia connected with cytoplasmic bridges was only very rarely observed (Fig. S3b–d). This supports our hypothesis that interaction of germ cells with the extracellular matrix in a precise three-dimensional niche governs germ cell self-renewal and differentiation. Loss of the three-dimensional niche microenvironment could disturb communication of germ cells with somatic cells and the extracellular matrix and change protein expression and allocation during SSC division.

To further investigate spermatogonial divisions in vitro, enriched gonocytes (over 70% purity) from 1- to 2-week-old pigs and spermatogonia (up to 70% purity) from 8- to 10-week-old pig testicular cells were cultured overnight before analysis. Clear asymmetric segregation of UCH-L1 and PLZF was evident in dividing gonocytes cultured overnight, while β -tubulin, RET, and DAZL were distributed evenly between cells (Fig. 5a–d). As observed in situ, expression of UCH-L1 and DAZL or DDX4 appeared inversely correlated in cultured spermatogonia (Fig. 5e). These results suggest that dividing SSCs isolated from supporting somatic cells maintain stem cell activity and tend to undergo asymmetric divisions in short-term culture.

Based on our observation that proximity of cells to the BM affects protein expression level, we hypothesized that presence of ECM might affect cell fate decision in vitro. The percentage of asymmetric distribution of UCH-L1 and PLZF in cultured gonocytes was not significantly different between cells cultured on poly-L-lysine (as a negative control), collagen IV, laminin, or matrigel at a high dilution (1:10) ($P = 0.082$; Fig. 6). However, when cells were cultured on matrigel at a very low dilution (1:1), a significantly higher percentage of cells showed asymmetric segregation of UCH-L1 instead of PLZF compared to those cultured on laminin ($P = 0.032$; Fig. 6). Laminin and collagen IV are the major compositions of BM of seminiferous tubules in testis (Hadley et al., 1990; Richardson et al., 1995; Glattauer et al., 2007). The ability of SSCs to selectively bind to laminin is broadly used to enrich SSCs (Hamra et al., 2008). The association of laminin with β 1-integrin was attributed to homing of SSC to BM (Kanatsu-Shinohara et al., 2008). Matrigel is a commercial product composed of 56% laminin, 31% collagen IV, and 8% entactin including traces of EGF, bFGF, NGF, PDGF, and other growth factors. Matrigel diluted at 1:10 in culture medium promoted binding of rat type A spermatogonia (van Pelt et al., 2002), and diluted matrigel induced differentiation of embryonic stem cells (Levenberg et al., 2003; Philp et al., 2005). Asymmetric distribution of UCH-L1 and PLZF in cultured spermatogonia most likely reflects an increase in differentiation in the absence of SSC maintenance factors or even induction by soluble factors present in matrigel.

Recently, it has become possible to maintain mouse SSCs in culture with addition of cytokines, such as LIF and glial-cell-derived neurotrophic factor (GDNF) to maintain self-renewal and inhibit differentiation in vitro (Kubota et al., 2004a,b; Kanatsu-Shinohara et al., 2005, 2006, 2007). Therefore, it may be possible to prevent asymmetric allocation of SSC determinants and differentiation by addition of certain cytokines. However, so far only mouse SSCs can be propagated in vitro for extended time periods while maintaining stem cell activity. It remains to be determined which cytokines or growth factors are required to maintain stem cell activity of nonrodent SSCs during extended periods in vitro.

Controlled degradation of cellular proteins by the ubiquitin–proteasome system has been identified as a key regulatory mechanism in many eukaryotic cells. Accumulating evidence reveals that the ubiquitin–proteasome system is involved in the regulation of fundamental processes in mammalian stem and progenitor cells of embryonic, neural, hematopoietic, and mesenchymal origin (Naujokat and Saric, 2007). Our results are consistent with studies on the function of UCH-L1 in murine spermatogenesis (Kwon et al., 2003, 2004; Wang et al., 2006) and first establish a potential role for UCH-L1 in SSCs. The findings presented here might suggest that UCH-L1 maintains SSCs through precise degradation of the RFS to prevent

differentiation. Since UCH-L1^{high} spermatogonia represent a very small proportion of the whole UCH-L1-positive population and the majority of these UCH-L1^{high} spermatogonia express PLZF, these UCH-L1^{high} spermatogonia might represent a population of “resting SSCs.” In addition, several functions of UCH-L1, other than as a ubiquitin hydrolase, have been proposed; these include acting as a ubiquitin ligase and stabilizing monoubiquitin (Setsue and Wada, 2007). Therefore, the function of high expression levels of UCH-L1 in SSCs remains to be defined.

Maintenance or differentiation of stem cells is generally considered to be dependant on intrinsic and extrinsic factors. The mechanisms of asymmetric stem cell division have been reviewed in different systems (Sutton, 2000; Wong et al., 2005; Lin, 2008). In mammalian SSCs, symmetric and asymmetric segregation of intrinsic determinants appears directly associated with SSC self-renewal and differentiation. For example, PLZF might act to maintain the pool of SSCs through direct repression of KIT expression (Filipponi et al., 2007). In addition, extrinsic factors like GDNF (essential for SSC maintenance) (Braydich-Stolle et al., 2005) and stem cell factor (SCF; inducing SSC differentiation via c-KIT receptor signaling) (Ohta et al., 2000) are produced by Sertoli cells and control SSC fate. Location of the cells in the putative stem cell niche relative to the BM might influence the dominant signaling pathway determining cell fate and result in asymmetric division. The molecular processes resulting in asymmetric allocation of UCH-L1 and PLZF as well as the effects on spermatogonia differentiation/proliferation require further study.

In summary, this study determined that UCH-L1 is exclusively expressed in undifferentiated porcine SSCs. Symmetric and asymmetric segregation of SSC determinants UCH-L1 and PLZF occurs in situ, and the expression level of these proteins was tightly associated with the cells location relative to the BM. In vitro, asymmetric distribution of UCH-L1 and PLZF was more frequent in isolated SSCs compared to those maintained in co-culture with testicular somatic cells. Together, these results for the first time provide direct evidence that self-renewal and differentiation of SSCs is associated with asymmetric division in mammalian spermatogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Dual immunofluorescence detection of UCH-L1 with DAZL, c-KIT, DDX4, or PLZF in testes from 1-week-old, 10-week-old, and adult boars. At 1 week of age, expression of UCH-L1 was present in all gonocytes irrespective of expression of DAZL (positive or negative), c-KIT (positive), DDX4 (strong or weak positive), and PLZF (positive or negative). At 10 weeks of age, different from gonocytes, spermatogonia express various levels of UCH-L1, DAZL, and DDX4 (negative, weak, or strong positive), PLZF (positive or negative), and weak expression of c-KIT. Strong expression of UCH-L1 occurred in spermatogonia that were also positive for PLZF, and was associated with weak expression of DAZL and DDX4, and vice versa. In the adult testis, expression of UCH-L1 was restricted to spermatogonia (A_s , A_p , and A_{al}) located

on the BM. Strong expression of UCH-L1 was associated with expression of PLZF, and weak expression of DAZL, c-KIT, and DDX4. Conversely, in spermatogonia negative for PLZF, but strongly positive for DAZL, DDX4, and expressing c-KIT UCH-L1 was absent or only weakly detected. Spermatogonia with strong (arrow heads) or weak (arrows) expression of UCH-L1 are marked for the 10 weeks and adult samples. PLZF-positive spermatogonia are indicated by arrowheads in the 1-week sample. Basement membranes are highlighted by dotted lines. Bars = 50 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 2.

Protein expression level in gonocytes/spermatogonia associated with their location relative to the basement membrane. Immunofluorescence intensity was measured over the whole cells by MetaMorph software (details in Materials and Methods Section). **a:** At 1 week of age, expression of UCH-L1 ($n = 77$, $P = 0.529$), DAZL ($n = 77$, $P = 0.299$), and DDX4 ($n = 46$, $P = 0.364$) is not affected by the location of the gonocytes relative to Sertoli cells and BM; however, expression of PLZF ($n = 99$, $P = 0.001$) in gonocytes close to BM is significantly higher than those distant from BM. **b:** Percentage of gonocytes close to BM (31.1%) and of those distant from BM (68.9%). **c:** A representative section of 1-week-old seminiferous tubule showing gonocytes close to BM (arrowhead) and distant from BM (arrows) with DAPI staining. **d:** At 10 weeks of age, expression level of UCH-L1 ($n = 66$, $P = 0.201$) and DAZL ($n = 50$, $P = 0.177$) is not influenced by the location of germ cells within the seminiferous tubules, but expression level of DDX4 ($n = 66$, $P = 0.015$) and PLZF ($n = 39$, $P = 0.005$) in spermatogonia in contact with BM is significantly different from that in the germ cells distant from BM. **e:** Percentage of the spermatogonia in contact with BM (73.5%) and of germ cells distant from BM (26.5%). **f:** A representative section of 10-week-old seminiferous tubule showing the spermatogonia in contact with BM (arrowheads) and the germ cells distant from BM (arrows) with DAPI staining. Bars = 50 μm .

Fig. 3.

Expression level of UCH-L1 in A_{pr} spermatogonia. a: Double staining of UCH-L1 and vimentin labeling of Sertoli cells in the seminiferous tubule lumen. Three pairs of A_{pr} spermatogonia were enclosed by dotted yellow lines and marked as i, ii, and iii. BM was highlighted by dotted white lines. Bar = 50 μ m. b: Measurement of immunofluorescence intensity of individual cells in A_{pr} spermatogonia using MetaMorph software (see details in Materials and Methods Section). In pair i, UCH-L1^{high} and UCH-L1^{low} were allocated to cell 1 and cell 2 of the pair, respectively; in pairs ii and iii, UCH-L1^{low} (pair ii) or UCH-L1^{high} (pair iii) was equally distributed between both spermatogonia.

Fig. 4.

Symmetric and asymmetric allocation of UCH-L1 and PLZF in spermatogonia in situ. Paraffin sections (I and II) and whole mounts of seminiferous tubules (III) were subjected to dual immunofluorescence staining. I. Dual labeling for UCH-L1 and Ki 67 (marker for proliferating cells) in adult testis: symmetric (arrows, low in part a; high in parts i and q) or asymmetric (arrow heads, parts e, i, and m) allocation of UCH-L1 was found in spermatogonia located at BM. Concurrent symmetric (parts a–d) and asymmetric (parts m–p) expression of both Ki 67 and UCH-L1 is shown in (I). II. Concurrent asymmetric segregation of UCH-L1 and PLZF is evident in 10-week-old (a–c) and adult testes (d–f). III. Asymmetric allocation of PLZF-DDX4 (a–c, stars) and symmetric expression of PLZF (d–f, arrow heads) as well as DDX4 (g–i,

arrows) was found in 8-week-old seminiferous tubules. White dotted lines highlight BM of seminiferous tubules. A_{pr} spermatogonia are outlined by dotted yellow lines. Bar in part (I) = 20 μm ; bars in parts (II) and (III) = 50 μm .

Fig. 5.

Asymmetric segregation of UCH-L1 and PLZF in enriched gonocytes/spermatogonia in vitro, a–c: Distinct asymmetric distribution of UCH-L1 in gonocytes cultured overnight. d: Compared to symmetric expression of β -tubulin, RET, and DAZL, asymmetric segregation of UCH-L1 and PLZF was evident in enriched gonocytes cultured overnight. e: Asymmetric distribution of UCH-L1, DAZL, and DDX4 but not of c-KIT and RET was found in enriched 8-week-old spermatogonia cultured overnight. Bars = 20 μ m.

Fig. 6.

Effect of extracellular matrix (ECM) on asymmetric distribution of UCH-L1 and PLZF in enriched gonocytes cultured overnight. More gonocytes exhibited asymmetric distribution of UCH-L1 when maintained on Matrigel 1:1 than on laminin-coated wells ($n = 3$, $P = 0.032$). Stars indicate significant difference between groups.

TABLE 1

Antibodies used in immunohistochemistry

Protein	M _w (kDa)	Company	Cat. no.	Host species	Dilution
UCH-L1 (PGP 9.5)	27	AbD Serotec, Raleigh, NC	7863-0504	Rabbit	1:300
UCH-L1 (PGP 9.5)	27	Abcam Inc, Cambridge, MA	Ab8189	Mouse	1:30
PLZF	74.3	EMD (Calbiochem), Gibbstown, NJ	OP128	Mouse	1:20
DAZL	33	Custom made	N/A	Rabbit	1:200
c-KIT	120/145	Santa Cruz Biotechnology, Santa Cruz, CA	Sc-168	Rabbit	1:100
DDX4	76	Abcam	Ab13840	Rabbit	1:100
Ki 67	345/395	Dako North America, Inc., Carpinteria, CA	M7240	Mouse	1:100
Ret	150/170	Santa Cruz	Sc-167	Rabbit	1:100
β-tubulin	55	Abcam	Ab15568	Rabbit	1:100
Vimentin	57	Santa Cruz	Sc-7557	Goat	1:100