SUMO-1, human male germ cell development, and the androgen receptor in the testis of men with normal and abnormal spermatogenesis

Margarita Vigodner,† Tomomoto Ishikawa,† Peter N. Schlegel,1,2 and Patricia L. Morris1,3

1Center for Biomedical Research, Population Council; 2Department of Urology, Weill Medical College of Cornell University; and 3The Rockefeller University, New York, New York

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Vigodner, Margarita, Tomomoto Ishikawa, Peter N. Schlegel, and Patricia L. Morris. SUMO-1, human male germ cell development, and the androgen receptor in the testis of men with normal and abnormal spermatogenesis. Am J Physiol Endocrinol Metab 290: E1022–E1033, 2006. First published December 13, 2005; doi:10.1152/ajpendo.00527.2005.—Sumoylation affects multiple cellular events, including chromatin inactivation and transcriptional repression. Our data provide the first characterization of small ubiquitin-related modifier-1 (SUMO-1) expression during human spermatogenesis by the use of high-resolution cellular SUMO-1 bioimaging. During human meiotic prophase, SUMO-1 localizes to sex chromosomes and centromeric and pericentromeric chromatin. As human spermatocytes progress toward the end of prophase in meiosis I, SUMO-1 is no longer detected within the sex body and pericentromeric heterochromatin but localizes exclusively to cenotromes. SUMO-1 localization along sex chromosome axes, pseudoautosomal region, and centromeres of both chromosomes supports a role for SUMO-1 sumoylation in epigenetic events occurring over the entire sex body, e.g., meiotic sex chromosome inactivation and chromatin condensation. Centromeric SUMO-1—one meiotic prophase suggests a role in centromeric chromatin condensation and/or other centromere/kinetochore functions. SUMO-1 is likely involved in both facultative and constitutive heterochromatin processes in spermatocytes. Haploid round spermatids show a consistent association of SUMO-1 with centromeric clusters. During spermatid elongation, SUMO-1 localizes in the manchette perinuclear ring. Stereodigenic Leydig cells show some cytoplasmic but strong nuclear and perinuclear SUMO-1. Peritubular myepithelial cell SUMO-1 colocalizes with centromeric heterochromatin. In epithelial Sertoli cells, when associated with centromeric heterochromatin, SUMO-1 is adjacent but not colocalized with the nucleolus. Male germ cells demonstrate no SUMO-1 nucleolar association. Human and rodent Sertoli cells consistently show an inverse correlation between androgen receptor (AR) and SUMO-1 expression and compartmentalization. Sertoli cells from certain infertile patients, however, showed greatly decreased SUMO-1 and AR. Our data suggest that human testicular SUMO-1 has specific functions in heterochromatin organization, meiotic centromere function, and gene expression.

small ubiquitin-related modifier-1; meiosis; chromatin; centromere; spermatids; Sertoli cells; Sertoli cell-only; maturational arrest; infertility

THE SMALL UBQUITIN-RELATED MODIFIER 1 (SUMO-1) is a member of a family of ubiquitin-related proteins. In contrast to the process of ubiquitination, which is largely but not always associated with proteolytic pathways, sumoylation is instead implicated in cellular events such as protein-protein interactions, nuclear-cytoplasmic transport, and the formation of specific nuclear heterochromatin domains. Thus sumoylation participates in the regulation of transcription and DNA replication and repair processes. To date, SUMO-1 has been correlated with chromatin inactivation and transcriptional repression in nontesticular cells (14, 22, 36, 45, 46, 51, 53). To our knowledge, neither a SUMO-1-deficient animal model nor its proteotypic characterization or the presence of SUMO-1 protein expression within the human testis has been published.

Spermatogenesis consists of phases of stem cell and spermatogonial proliferation and meiosis of spermatocytes, and the postmeiotic maturation of spermatids (spermiogenesis) coordinate processes that include developmental cues, autocrine factors, and paracrine influences by the Sertoli cell that, together, result in the production of testicular sperm. The critical progression of meiotic events and the onset of posthaploid differentiation depend on the temporally integrated regulation of male germ cell gene expression. Pathologies showing maturational arrest at various germ cell stages, although not common, offer unique opportunities to study human meiosis and spermiogenesis.

To better understand the role of SUMO-1 during spermatogenesis, we recently localized SUMO-1 to the sex chromosomes of meiotic spermatocytes, the centrosome and manchette of spermatids, and specific domains of the somatic cells of the mouse and rat testes, findings that are consistent with diverse and potentially multiple roles of SUMO-1 during spermatogenesis (52). Yet little is known concerning the distinct roles of sumoylation during spermatogenesis (42, 52).

To initially characterize SUMO-1 expression and potential functions in the human testis, in the present study high-resolution cell imaging and immunodetection analyses were used to determine cell-specific SUMO-1 during normal spermatogenesis. SUMO-1 was localized to specific chromatin and other cellular domains in germ and somatic cells of the human testes, but not in a manner identical to the pattern that we determined in mouse and rat germ cells, findings that reveal novel insights into the potential roles of SUMO-1 in human male germ cell function. In addition to studies performed on testes from men with normal spermatogenesis, SUMO-1 expression was also evaluated in somatic and germ cells isolated...
from testicular biopsy samples that were obtained from infertile patients with variable defects in spermatogenesis, which ranged from maturation arrest to “Sertoli cell-only” pathologies.

**MATERIALS AND METHODS**

**Human Testicular Cells**

Otherwise healthy men with normal spermatogenesis but confirmed azoospermia secondary to epididymal obstruction underwent testicular biopsy for testicular sperm extraction (44). Tissue was obtained for study under ongoing Institutional Review Board approval at Weill Cornell Medical Center (New York, NY). The obstructed patients whose germ cells are reported in this paper and their respective partners successfully achieved clinical pregnancy with assisted reproduction (in vitro fertilization with intracytoplasmic sperm injection), using the sperm removed during these biopsies. Each clinical pregnancy resulted in the delivery of a healthy child. Matched histopathology sections revealed quantitatively normal testicular histology and spermatogenesis, with 39–52 spermatids/tubule (normal >15–20). All obstructed patients evaluated for this study had normal serum FSH, LH, and testosterone levels. Spermatogenesis was quantitatively evaluated by formal histology with >15–20 spermatids/tubule and considered well within the range for men with normal sperm production. Patients were evaluated with karyotype examination of peripheral leukocytes. In addition, DNA was extracted and amplified from peripheral leukocytes by the use of a polymerase chain reaction-based assay to evaluate for microscopic deletions of the Y chromosome (15, 18, 41). Each man was negative for Y chromosome microdeletions and karyotypic abnormalities. For comparison, testicular cells were freshly isolated from biopsy samples obtained from men with severely defective spermatogenesis, including men with severe hypospermatogenesis, maturation arrest, and Sertoli cell-only patterns on biopsy.

**Antibodies and Reagents**

An affinity-purified rabbit polyclonal antibody raised against full-length SUMO-1 protein (SUMO-1\(^{ABR}\) antibody) was purchased from Affinity BioReagents (Golden, CO). A mouse monoclonal antibody against full-length recombinant SUMO-1 protein (SUMO-1\(^{Zymed}\) antibody) was obtained from Zymed Laboratories (San Francisco, CA). A monoclonal antibody against phosphorylated histone H2A.X (Ser\(^{139}\), γH2A.X) was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). A human autoimmune antiserum directed against the dense fibrillar component (DFC) of the nucleolus was purchased from Abcam (Cambridge, MA). The monoclonal anti-β-tubulin antibody (manchette marker) was purchased from Sigma (St. Louis, MO), as were all other general reagents unless otherwise specified.

**Isolation and Purification of Male Germ Cells, Sperm, Somatic Cells, and Seminiferous Tubules from Rodent Testes**

Germ cell suspensions enriched for specific cell types were isolated from the adult testes of mice or rats by elutriator purification as previously reported (21, 25, 30). In certain experiments, suspensions of mixed germ cells were obtained directly after isolation of tubules and used before elutriator purification procedures. Leydig cells were isolated from Sprague-Dawley (SD) rats (55–65 days old) and purified by Percoll gradient and centrifugal elutriation as previously reported (purity ≥98% 3β-hydroxysteroid dehydrogenase positive, a well-characterized marker of the adult Leydig cell) (24). Sertoli cells were isolated from 18-day-old SD rats to ≥95% purity as previously described (21). Isolated cells were then processed for bioimaging as indicated above for human cells. For isolation of the seminiferous tubules, each testis was detunicated in a small amount of phosphate-buffered saline (PBS), and tubules were gently manually dissociated and unraveled using fine forces. Portions of separated seminiferous tubules were fixed in 1% paraformaldehyde (10 ml of fixative was added to ~1 ml of tubules overnight, 4°C). Fixed tubules were rinsed three times in PBS using unit gravity and then stored in 0.1% (wt/vol) Na-azide in PBS (4°C) until use. When required, propidium iodide (PI) was used to label DNA. Confocal microscopy was performed as we previously reported (52).

**Accreditation of Laboratory Animal Care**

Procedures involving the use of animals strictly followed the Guidelines for Care and Use of Laboratory Animals set forth by NIH, and protocols received Institutional Animal Care and Use Committee approval.

**Bioimaging Analysis**

**Slide preparation.** Testicular cells were freshly isolated aseptically from human biopsy tissue after their manual dissection and gentle dispersion by repetitive pipetting to cell suspensions and small aggregates. Isolated cells were then attached to glass microscope slides with the use of cytocentrifugation (Cytofuge; StatSpin, Norwood, MA), fixed in 1% paraformaldehyde [15 min, room temperature (RT)], washed three times in PBS, and stored at −80°C until staining and subsequent analysis as indicated.

**Indirect immunofluorescence on isolated cells.** Fixed cells on slides were treated with 0.3% Igepal (10 min; Sigma) and preblocked with Image-IT FX Signal Enhancer (30 min; Molecular Probes, Eugene, OR). Cells were washed and then incubated with either of the anti-SUMO-1 antibodies at dilutions of 1:200–1:300 for SUMO-1\(^{ABR}\) or 1:100–1:200 for SUMO-1\(^{Zymed}\) in PBS containing 1% bovine serum albumin (BSA) for 2 h (RT). After one wash with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG or rabbit anti-mouse (Jackson Immunoresearch Laboratories, Bar Harbor, ME) for SUMO-1\(^{ABR}\) and SUMO-1\(^{Zymed}\), respectively, at a 1:150 dilution in PBS containing 1% BSA and then washed three times. In the experiments where SUMO-1 expression was colocalized with the γH2A.X, cells were first incubated with both SUMO-1\(^{ABR}\) and γH2A.X (1:300 final dilution for γH2A.X) followed by incubation with a mixture of FITC-conjugated goat anti-rabbit IgG and Alexa fluor 633 goat anti-mouse IgG (1:200 dilution; Molecular Probes). For colocalization of SUMO-1 with centromeres, cells were first incubated with both SUMO-1\(^{Zymed}\) and anti-centromere/kinetochore antibody (1:10 final dilution) followed by incubation with a mixture of affinity-purified Texas Red goat anti-human IgG (1:150 final dilution, Vector Laboratories) and FITC-conjugated rabbit anti-mouse IgG. For colocalization of SUMO-1 and β-tubulin (manchette), cells were first incubated with both SUMO-1\(^{Zymed}\) and β-tubulin antibodies (1:300 final dilution for β-tubulin) followed by incubation with a mixture Alexa fluor 633 goat anti-rabbit IgG (1:200 dilution; Molecular Probes) and FITC-conjugated rabbit anti-mouse IgG. For localization of SUMO-1 and the nucleolus, cells were first incubated with both anti-SUMO-1\(^{Zymed}\) and anti-fibrillarin (1:100) antisera followed by incubation with a mixture of FITC-conjugated goat anti-rabbit IgG and Alexa fluor 633 goat anti-mouse IgG. For androgen receptor (AR) localization, a rabbit immunoaffinity-purified polyclonal IgG antiserum (PG-21, Upstate Biotechnology) was used at the recommended dilution (1:40); colocalization studies were performed using the anti-SUMO-1\(^{Zymed}\) antisera (1:100).

Cell nuclei were localized by chromatin staining with 4’,6-diamidino-2-phenylindole (DAPI; 4 μg/ml, 5 min). Slides were mounted using ProLong Antifade kit (Molecular Probes). Images were captured using real-time 2-D-deconvolution Delta Vision Image Restoration Microscopy (Applied Precision, Issaquah, WA). An Olympus IX-70 inverted microscope was used with ×40, ×60, or ×100 oil objectives.
together with DAPI, FITC, and CY-5 filter sets as indicated. Cell types were identified according to their characteristic morphology, centromere content, and localization (23, 43). After side-by-side specific immunostaining and dye labeling of slides prepared from the cells obtained from these patients, digital photomaging for documentation and analysis of ≥100–200 cells each was conducted for the particular human testicular somatic, pachytene spermatocytes (PS), and spermatid cells reported herein. For spermatogonia and meiosis I germ cells, ≥30–50 cells were analyzed. By phase-contrast microscopy, the nuclei of human secondary spermatocytes, characterized by a mixture of fine and coarse chromatin, are smaller than those of primary spermatocytes but larger than those of round spermatids (23). Meiosis II germ cells, found less frequently in normal testes due to their brief existence in the second meiotic division (MeII), are, hence, comparatively rare, but all were documented.

RESULTS

Human Male Germ Cells and SUMO-I Expression

To determine SUMO-I expression during human spermatogenesis, testicular cells freshly isolated from surgical biopsy specimens were subjected to immunofluorescence analyses. Bioimaging, using two different antibodies for immunodetection of human SUMO-I, was evaluated by using high-resolution Delta Vision Restoration microscopy. Human testicular somatic, pachytene spermatocytes (PS), and spermatid cells were processed and compared alongside those obtained from normal mouse and rat testes (52).

In human PS, several prominent SUMO-I nuclear foci are observed (Fig. 1A), findings that are different from those in both mouse and rat PS, where SUMO-I localizes almost exclusively to the sex body (Fig. 1A, inset). Double immunolabeling using anti-SUMO-I and anti-γH2A.X (used here as a marker of the sex body) antibodies confirms the presence of SUMO-I in other nuclear regions of the spermatocyte in addition to that seen within the sex body (Fig. 1B). In mouse and rat PS, the two proteins are completely colocalized over the whole sex body (Fig. 1B, top inset). Interestingly, in human spermatocytes, γH2A.X covers the sex body region but SUMO-I is detected largely along the XY chromosomal axes (Fig. 1B, arrows and bottom insets).

SUMO-I Associates with Centromeric Chromatin in Human Male Germ Cells

Previous studies in PS showed that, besides the sex body, heterochromatin regions adjacent to clustered centromeres (i.e., pericentromeric heterochromatin) are also enriched in proteins and histone modifications implicated in chromatin silencing (8, 10, 27, 32, 38, 50). To determine potential association of SUMO-I with the centromeric regions of repressed chromatin in germ cell nuclei, double labeling with anti-SUMO-I and anti-centromere/kinetochore antisera was performed (Fig. 1, C and D). Double labeling revealed localization of SUMO-I with centromeric chromatin and with regions adjacent to clustered centromeres. On the XY chromosomes, SUMO-I is detected in the pseudo-autosomal region (PAR) along the XY axes and on the X and Y centromeres (Fig. 1, C and D, arrows and insets). Interestingly, when anti-SUMO-IABR is used for such double immunolabeling, SUMO-I is similarly located in the pericentromeric heterochromatin (Fig. 1E) but is not detectable in the centromeres themselves (Fig. 1E, inset and arrow).

The centromeric marker enabled us to distinguish germ cells in premeiotic-early meiotic stages (Fig. 2, A and B), because centromeres cluster at the nuclear periphery during this developmental stage (43). These human cells show low to nondetectable levels of SUMO-1, findings similar to murine premeiotic germ cells (Fig. 2A, inset).

As human spermatocytes progress toward the end of prophase of the first meiotic division (MeI), SUMO-1 is no longer detected within the sex body and pericentromeric heterochromatin but instead localizes exclusively to the centromeres (Fig. 2, C and D). Similar patterns were also observed in human germ cells, completing MeII (Fig. 2, E, left inset, and F). Similarly, SUMO-1 is not detected in the sex chromosomes of the mouse from the ddiplotene stage during MeI (Fig. 2C, inset). No SUMO-1-positive nuclear regions are observed during MeII in the mouse (Fig. 2E, right inset).

In oocytes and various somatic cells, certain heterochromatin regions are associated with nucleoli, which, in turn, associate with centromeres (1, 28, 48). To evaluate whether SUMO-I is present in the spermatocyte nucleolus, SUMO-I was localized simultaneously with the nucleolar DFC fibrillarin (29, 39). Fibrillarin marks, in part, the DFC (26).

In human spermatocytes, SUMO-1 does not localize to the nucleolus (Fig. 2G). Nucleoli were randomly distributed relative to the centromere in PS (Fig. 2H). Some distinct SUMO-I association with centromeres is observed in specific early meiotic cells (Fig. 2H, arrow).

Human Spermiogenesis and SUMO-I Expression

During human spermiogenesis, round spermatids show that prominent nuclear punctate dots of SUMO-I clearly associated with the clustered centromeres (Fig. 3, A and B), consistent with findings when either of the anti-SUMO-1 antibodies was used (Fig. 3, C and D). Strikingly, in certain murine round spermatids, we observe a single SUMO-I-positive spot in the chromocenter region, which consists of centromeric heterochromatin (Fig. 3A, inset).

Consistently, in a subset of elongating spermatids (ES), a linear pattern of SUMO-I in the postacrosomal region is observed (Fig. 3E). Similarly, in murine ES, SUMO-I is in the perinuclear ring, the structure that gives rise to the microtubules of the manchette during spermatid elongation (Fig. 3E, inset). To determine whether such a pattern exists for human ES, we next performed immunostaining of SUMO-I with β-tubulin to mark the manchette (Fig. 3F). Human SUMO-I is present at the manchette initiation site.

Human Testicular Somatic Cell SUMO-I

In somatic cells freshly isolated from human testes that display normal spermatogenesis, Leydig cells (Fig. 4A) show strong nuclear and perinuclear SUMO-I signals, with some clearly cytoplasmic as well, a pattern similar to those in rat (Fig. 4A, inset) and mouse Leydig cells (Fig. 4D, red). In Leydig cells, SUMO-I is observed throughout the nucleus as well as within the nucleolus (Fig. 4D, arrow). Approximately one-half of the Sertoli cells isolated from samples obtained from four patients with normal spermatogenesis were SUMO-I

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positive (range 40–60%). Of these Sertoli cells, prominent SUMO-1-positive dots were detected within the nucleolus, a pattern quite characteristically observed with samples from four different patients with normal spermatogenesis but azoospermia secondary to obstruction by using either anti-SUMO-1 antisera (FITC; green) specified below; chromatin was counterstained using 4’,6-diamidino-2-phenylindole (DAPI; blue). Scale bar, 5 μm. For comparison, images from rodent cells are provided as insets. Colored text denotes specific immunolabeling in each panel. A: human pachytene spermatocytes (PS) show several prominent SUMO-1 nuclear foci (anti-SUMO-1\textsubscript{Zymed} antibody, green). Mouse spermatocytes (\textit{inset}) show SUMO-1 localized exclusively to the sex body. B: human PS are shown after double labeling with anti-SUMO-1\textsubscript{ABR} (green) and anti-phosphorylated H2A.X (\(\gamma\text{H}2\text{AX};\) red) antibodies. As \(\gamma\text{H}2\text{AX}\) covers the sex body region, SUMO-1 is detected largely along the XY chromosomal axes. [See bottom insets of the marked spermatocyte (arrows). \textit{Red frame:} DAPI image showing the sex body region; \textit{yellow frame:} pattern of green and red fluorescence in the sex body region.] Mouse PS (top \textit{inset}) showing complete colocalization of SUMO-1 and \(\gamma\text{H}2\text{AX}\) over the whole sex body. C and D: human PS are shown after double labeling with anti-SUMO-1\textsubscript{Zymed} (green) and anti-human centromere protein (red) antibodies. D is the identical field to C, where the green and red fluorescence patterns alone are shown. SUMO-1 is colocalized with centromeric chromatin and is associated with the chromatin regions adjacent to clustered centromeres. In the sex body region (arrows), SUMO-1 is detected in the pseudo-autosomal region (PAR) along the XY axes and on the X and Y centromeres (C, \textit{inset}: DAPI image showing the sex body region; D, \textit{inset}: patterns of green and red fluorescence in the sex body region). E: human PS are shown double labeled with anti-SUMO-1\textsubscript{ABR} (green) and anti-centromere (red) antibodies (\textit{inset}: green and red fluorescence alone are shown for the particular cell indicated by arrow). SUMO-1 is located in the pericentromeric heterochromatin but not in the centromeres (aligned with red arrows). F: mouse PS are shown after double labeling with anti-SUMO-1\textsubscript{Zymed} (green) and anti-human centromere (red) antibodies. No SUMO-1 is detected in the centromeric regions.

SUMO-1 (red) was specifically localized in the regions of the nucleolus adjacent to, but not overlapping with, fibrillarin (green). No correlation was found in the intensity of fibrillarin staining between SUMO-1-positive and -negative Sertoli cells. In mouse Sertoli cells (Fig. 4C, \textit{bottom left}), fibrillarin is detected solely in the central fibrillar area of the nucleolus (green) and not in the adjacent regions of heterochromatin that is positive for SUMO-1 (red; also Fig. 4B, \textit{inset}). This nuclear pattern of Sertoli cell SUMO-1 and fibrillarin is different from that observed in murine Leydig

cells, where SUMO-1 is seen throughout the entire nucleus (Fig. 4D). Consistent with nucleolar SUMO-1 in Sertoli cells, the nucleolus of Leydig cells contains SUMO-1. Thus the findings with human Leydig and Sertoli cells are comparable to those in rodents. Human peritubular myoid epithelial (My) cells display prominent SUMO-1 foci along their elongated nuclei (Fig. 4E).

Both nuclear and cytoplasmic SUMO-1 are observed in murine myoepithelial cells with the use of confocal microscopy [Fig. 4C, inset, and PI staining (red)]. Interestingly, similar to what was demonstrated in human germ cells, SUMO-1-positive regions in human peritubular myoepithelial cells are associated with centromeric heterochromatin (Fig. 4F and inset).
SUMO-1 and AR Expression in Somatic Cells of Patients with Normal Spermatogenesis or Infertility Associated with Sertoli Cell-Only or Maturation Arrest

Recent studies (7, 40) showed that several steroid receptors can be sumoylated. Given the critical dependence of spermatogenesis on androgens, it is interesting that two SUMO sites are present in the amino-terminal domain of the AR and that sumoylation can modify its transcriptional activity. Therefore, the AR, a functional marker of androgen-responsive human Sertoli and Leydig cells, was used for double labeling with SUMO-1 to evaluate colocalization. For Sertoli cells isolated from human testes with normal spermatogenesis (Fig. 5A), a cell-specific correlation between apparent nuclear-limited punctate AR [Fig. 5A2 (green)] and nucleolar SUMO-1 [Fig. 5A2 (red), red arrows] was seen. SUMO-1 is located specifically in the periphery of the nucleoli, where it is adjacent to but not colocalized with fibrillarin, findings that are suggestive of the association of SUMO-1 with centromeric DNA rather than the rDNA-containing dense fibrillar and fibrillar center regions of the nucleolus (Fig. 5A1).
Sertoli cells with nuclear-restricted AR foci concomitant with barely detectable cytoplasmic AR patterns, SUMO-1 was strikingly limited to the nucleolus, with some signal overlap with the AR within (Fig. 5A2, red arrows). Human Sertoli cells with both abundant nuclear and cytoplasmic AR demonstrated isolated punctate SUMO-1-positive cytoplasmic localization (Fig. 5, A2 and A3, red arrowheads). The majority of SUMO-negative cells demonstrated intense AR staining through the entire nucleus and cytoplasm. Similar to these findings with human Sertoli cells, an AR-to-SUMO-1 inverse correlation was representative of many mouse Sertoli cells that were isolated in testicular suspensions (Fig. 5A2, inset, red arrowhead).

As indicated above, in samples obtained from human testicular biopsy material from four patients with obstructive azoospermia but quantitatively normal spermatogenesis, a typical range of SUMO-1-positive Sertoli cells was correlated with defined patterns of AR and fibrillarin (Fig. 5A). In comparison, an almost total absence of SUMO-1 in Sertoli cells was consistently correlated with abnormally low to nondetectable...
able AR levels such as those illustrated from two patients with a histological confirmation of the phenotypes of an arrest in spermiogenesis (Fig. 5, B and B1) or Sertoli cell-only (Fig. 5, C and C1). In this first study of infertility phenotypes, although a single cell may show either a relatively abundant or very low diffuse cytoplasmic AR (Fig. 5B1, bottom inset, and C1, green arrow), SUMO-1 levels were barely detectable against negative-control background. Whereas very low AR and SUMO-1 levels were observed in Sertoli cells obtained from a patient with germ cell arrest during spermiogenesis compared with
those with apparently normal spermiogenesis, fibrillarin was the same [Fig. 5B1, top inset (green)]. In Sertoli cells obtained from the SUMO-1-negative Sertoli cell-only patient illustrated, no nucleolar AR was observed, although particular Sertoli cells showed diffuse cytoplasmic staining (Fig. 5C1, green arrow). Such findings suggest that the presence of particular germ cells influences human Sertoli SUMO-1 and AR patterns. Additionally, in human Sertoli cells, the relationship of SUMO-1 relative to the AR may exert paracrine effect(s) on the progression of spermatogenesis.

To further evaluate the relationship of SUMO-1 and AR, larger numbers of Sertoli cells were purified after their isolation from rat testes. Interestingly, a strict cell-to-cell inverse correlation between intracellular AR and SUMO-1 could clearly be seen in this highly enriched rat Sertoli cell population (≥95%; Fig. 6, A and B). In contrast, highly purified rat Leydig cells (≥98%) show extensive and diffuse cytoplasmic AR with abundant nuclear AR within the same cells. Although SUMO-1 is present in both cellular compartments, no significant areas of overlap with AR were demonstrated (Fig. 6, C and D). SUMO-1 was clearly present in all cells with concomitant abundant nuclear and cytoplasmic AR. Interestingly, in this same field a Leydig cell with nuclear-limited AR is shown without a detectable SUMO-1 signal (Fig. 6, white arrows). Therefore, data from both rat and mouse Sertoli cells support the findings for human Sertoli cells obtained from testes with normal spermatogenesis. Further quantitative studies will be required to resolve the functional significance of these findings.

**DISCUSSION**

**SUMO-1 and Human Sex Chromosomes**

During meiotic prophase in human male germ cells, these data indicate that SUMO-1 localizes to the sex chromosomes and peri- and centromeric chromatin. The association of SUMO-1 or its sumoylated substrates with human sex chromosomes is consistent with our recent findings for mouse and rat spermatocytes (52). The presence of human SUMO-1 along the sex chromosome axes, PAR, and centromeres of both chromosomes supports a role in the events occurring over the whole sex body, such as meiotic sex chromosome inactivation and chromatin condensation similar to that proposed in animals, and yet species-specific differences are demonstrated. SUMO-1 appears more restricted and comparatively abundant in the mouse sex body than that of the human. In human PS, SUMO-1 and γH2A.X show discrete patterns, whereas in rodent PS the two proteins colocalize and overlap the entire sex body. In rodents, SUMO-1 is restricted to the sex chromosomes during prophase of meiosis. In human spermatocytes, our data indicate that SUMO-1 likely plays a role in the organization of other heterochromatin regions as well.

Fig. 6. Rat Sertoli, but not Leydig, cells show an inverse correlation of SUMO-1 with AR. Sertoli and Leydig cells were freshly isolated and purified from rat testes as previously reported. Cells were immediately processed for immunodetection of intracellular AR (green) and SUMO-1 [anti-SUMO-1Zymed (red)]; nuclei were counterstained using DAPI (blue). A strict cell-to-cell inverse correlation between intracellular AR and SUMO-1 was observed in rat Sertoli cells (A and B). In contrast, rat Leydig cells show abundant nuclear and diffuse cytoplasmic SUMO-1 and AR within the same cell (C and D). Scale bar, 10 μm.
SUMO-1 and Pericentromeric Heterochromatin

The centromere is the chromosomal locus adjacent to the regions of constitutive heterochromatin that are responsible for assembly of the kinetochore complex, which then interacts with the spindle microtubules. This process regulates chromosome segregation during mitosis and meiosis (2, 3, 11, 16, 31). The rapidly growing number of proteins identified and localized to the centromere can be divided functionally into two groups. One group is associated with kinetochore function, and the other is comprised of heterochromatin-associated proteins that localize to centromeric and pericentromeric chromatin and other heterochromatin sites (11). In humans, the present study demonstrates SUMO-1 in the centromere region throughout meiotic prophase, data that are consistent with a function during meiosis. Its presence suggests participation by sumoylation or a SUMO-1-based scaffolding in the underlying processes of centromeric chromatin condensation and/or other centromere/kinetochore functions. Condensed chromatin in the pericentromeric region is required for the normal centromere activity (31). Localization of SUMO-1 or sumoylated protein(s) to this region is consistent with transcriptional silencing and formation of heterochromatin domains (22, 51).

Our results indicate that SUMO-1 or sumoylation is likely required for both facultative and constitutive heterochromatin formation in human spermatocytes. Such findings are consistent with the proposed common, but as yet unknown, mechanisms of heterochromatin formation in both sites. Similar to the present findings, other specific protein and histone modifications implicated in chromatin silencing have been localized to both centromeric and sex chromosome heterochromatin, such as heterochromatin protein-1 (HP-1), histone deacetylases, H3K9, SUV39H1, and macroH2A1.2 (8, 10, 12, 19, 20, 27, 35, 38, 50).

Interestingly, several heterochromatin proteins are associated with both centromeres and promyelocytic leukemia (PML) nuclear bodies, nuclear substructures where SUMO-1 or sumoylation may regulate heterochromatin assembly (13, 22). In a similar manner, some components of the PML body were recently detected in the sex body (42). These findings imply functional similarities among all three domains where SUMO-1 could play a common role in remodeling. However, the formation of the sex body in the spermatocytes, which lack PML bodies, indicates that at some level these domains are organized differently (42).

SUMO-1 and Centromere/Kinetochore Function

The persistence of centromeric SUMO-1 to the end of meiotic prophase indicates that SUMO-1 may be involved in other functions of the centromere, such as in sister chromatid cohesion, or, afterwards, in kinetochore formation at metaphase. SUMO-1 has been shown to modify several centromeric proteins during mitosis in Saccharomyces cerevisiae and mammalian cells, where different components of the sumoylation machinery have been demonstrated to be associated with chromatin condensation, chromosome cohesion and separation, and kinetochore assembly (5, 6, 49). One centromeric protein shown to be sumoylated during mitosis is topoisomerase II (TOP2), which regulates a component of chromatin structure that is required for centromeric cohesion (4, 5). During meiotic prophase in mouse spermatocytes, TOP2α is present at the centromeric region and represents a good candidate for sumoylation (9). Interestingly, although both SUMO-1 antibodies recognized SUMO-1 in the pericentromeric regions, only one detects its presence in the centromeres. One explanation could be the differing abilities of the two antisera to detect particular SUMO-1-conjugates within distinct topological areas of centromeric chromatin or particular sumoylated proteins. Given the different nuclear protein composition and heterochromatin properties of centromeric and pericentromeric chromatin, this is reasonable and will need further study (16, 47).

Comparison of SUMO-1 in Mouse and Human Centromeric Heterochromatin

Human and mouse centromeric and pericentromeric heterochromatin are organized differently (34, 43); the different SUMO-1-positive patterns in specific human or mouse male germ cells demonstrated herein could reflect differential heterochromatin arrangement in these two species. Similar to the present human SUMO-1 study, different HP-1 subtypes show different localization patterns between those observed in human and mouse somatic cells (34). A detailed comparison of three different HP-1 subtype localizations has been performed for human spermatocytes but to date has not been reported for the mouse (33).

For the mouse spermatocyte centromere, we cannot entirely rule out the presence of SUMO-1 below detection by the sensitive bioimaging methods we employed. However, comparatively modest but significant signals were clearly detected in the centromeric DNA regions of both mouse round spermatids and Sertoli cells. Protein differences in the composition of the mouse and human centromere could influence the recruitment of SUMO-1. Additionally, a tighter centromeric chromatin structure for the mouse compared with human has been reported (11). This could result in a higher threshold for detection on the basis of adequate antibody accessibility and sufficient amplification.

In human spermatids, SUMO-1 is associated with several centromeric clusters seen throughout the spermatid nuclei. In comparison, in mouse spermatids SUMO-1 was localized within a single chromocenter, which contains centromeric DNA. In human ES, our studies to date do not allow us to determine whether SUMO-1 has a role in microtubular nucleation and nuclear reshaping similar to that of the mouse that we previously reported (52).

Testicular Somatic Cells

Human peritubular myoid cells show prominent SUMO-1 staining along their elongated nuclei, results similar in part to those seen in certain, but not all, mouse peritubular myoid cells. Human Leydig cells show strong nuclear and perinuclear SUMO-1, with some cytoplasmic localization patterns similar to those we reported for the mouse and rat. These findings allow for the possibility that SUMO-1 or SUMO-1 sumoylation mediates regulatory influences on Leydig cell activity and gene expression, thereby affecting androgen-dependent spermatogenesis in both humans and rodents.

In certain human and murine Sertoli cells, SUMO-1 localizes within the nucleolar region but is not colocalized with fibrillarin. In contrast to both human and rodent Sertoli and Leydig cells, in the PS of all the species we investigated, no
apparent association of SUMO-1 was observed within nucleoli. Human Sertoli nucleolar SUMO-1 appears to associate with centromeric heterochromatin, which has been previously shown to be in the nucleolar region (43). In the majority of SUMO-1-positive cells, our consistent finding is an apparent, striking accumulation of the AR in the nucleolus. Here, the two proteins are often detected in close proximity. These findings are suggestive of sumoylation of Sertoli nucleolar AR. Interestingly, although the accumulation of AR in the nucleolus of human Sertoli cells has not been described previously, it may have important functional implications for the regulation of AR-mediated transcriptional activities and paracrine influences on germ cell development (17). By use of cells obtained from four different patients with obstructive azoospermia but histology consistent with normal spermatogenesis, colocalization analyses reveal a correlation between the relative absence of nuclear SUMO-1 whenever extensive AR is present throughout the nucleus. In particular human Sertoli cells, SUMO-1 could participate in AR binding to domains of inactive chromatin in the nucleolus, findings that are consistent with previously suggested nuclear receptor-mediated mechanisms of SUMO-mediated transcriptional repression (46, 51). At this time, we cannot rule out any influence of untoward secondary effects of epididymal obstruction, such as back pressure on the testes, on the expression and compartmentalization of SUMO-1 and AR. However, the findings of quantitatively normal spermatogenesis and normal histology of the tubules in each of the obstructed patient samples suggest otherwise. If such effects are present, their molecular characterization will require further experimental study with the use of models of moderate-to-severe epididymal obstruction. Nonetheless, our observations in human testicular cells are further supported by similar findings where matched cell types obtained from the testes of normal adult rodents are used. In the rat Sertoli cell, SUMO-1 is almost totally absent from AR-positive cells but consistently abundant in those Sertoli cells with low to nondetectable AR levels. It has recently been proposed that sumoylation of the AR could affect cooperativeity of dimer-dependent DNA binding conformation (7, 37). Taken together with our present findings, the molecular ratios of SUMO-1 to AR, as well as their respective compartmentalization, could contribute to the regulation of androgen-dependent transcriptional activities in specific cell types exposed to endogeneous high (normal) or low (dysfunction) intratesticular testosterone.

In patients with nonobstructed infertile phenotypes, our data indicate that, in Sertoli cell-only syndrome and certain forms of germ cell maturation arrest, SUMO-1 levels are abnormally decreased and are at the limits of detection relative to those in normal spermatogenesis. Moreover, in these patients the AR signal was barely or not detectable, findings that suggest a functional connection between the regulation of SUMO-1 and AR expression in normal human Sertoli cells. In contrast, in patients with maturation arrest in spermiogenesis, the levels of fibrillarin, reflecting ribosomal activity of the Sertoli cells, appear not to be correlated with SUMO-1. In the Sertoli cell-only phenotype, Sertoli cells consistently demonstrated abnormally low SUMO-1 and AR levels. To better address the germ cell-Sertoli cell interactions that may underlie such regulation, studies in this laboratory are ongoing with the use of several mutant mouse models with disrupted spermatogenesis. Taken together, abnormal SUMO-1 and AR expression profiles in Sertoli cells from a subset of infertile patients could represent an underlying causative or contributing factor.

Studies to better understand the unique mechanistic role(s) of SUMO-1 and sumoylation in human and rodent pre- and postmeiotic germ cells and differentiating spermatids, as well as in the functionally distinct testicular somatic cells, are ongoing. In summary, this study shows that SUMO-1 localizes to specific domains of distinct human male germ and somatic cells, findings that are suggestive of potentially diverse functions for SUMO-1-mediated sumoylation during spermatogenesis in men.

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