GLI1 Localization in the Germinal Epithelial Cells Alternates Between Cytoplasm and Nucleus: Upregulation in Transgenic Mice Blocks Spermatogenesis in Pachytene

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ABSTRACT

The zinc finger transcription factor GLI1 is the mediator of signaling by members of the Hedgehog (Hh) family. Male mice in which Desert hedgehog (Dhh), an Hh homologue expressed in Sertoli cells of the testis, was knocked out are sterile, suggesting that the Dhh/GLI1 pathway plays a role in spermatogenesis. Using an antiserum raised against human GLI1, we found that during the first round of spermatogenesis, GLI1 expression is initially cytoplasmic, then shifts to the nuclei of Sertoli and germ cells, and finally shifts back to the cytoplasm. In the adult mouse testis, GLI1 expression localized to the nuclei of germ cells, beginning with pachytene cells and persisting through round spermatids. Localization of GLI1 in elongating spermatids shifted from the nucleus to the cytoplasm and became associated with microtubules. We also examined a line of transgenic mice that overexpressed human GLI1. Male mice in this line were sterile. Spermatogenesis was blocked at the pachytene stage, and a subset of the morphologically indistinguishable pachytene cells underwent apoptosis. Patched-2, which is a Dhh receptor, and Fused, another component of the signal transduction pathway, are expressed in Leydig cells and in primary and secondary spermatocytes. Expression of GLI1 in the same cell types as Patched-2 and Fused and the disruption of spermatogenesis by GLI1 overexpression suggest that GLI1 is the mediator of the Dhh signal in the testis, and that it may be a regulator of spermatogenesis.

INTRODUCTION

Compared to other developmental pathways, relatively little is known regarding the signaling events that control spermatogenesis, the complex process by which spermatogonia differentiate into spermatocytes, undergo meiosis, and then undergo morphogenesis to become spermatids, and then undergo morphogenesis to become spermatids and then undergo morphogenesis to become spermatids [1, 2]. Recent evidence suggests that the Desert hedgehog (Dhh) signaling pathway is involved in the regulation of spermatogenesis. Male mice with a Dhh-null mutation are sterile—spermatogenesis is blocked at the pachytene primary spermatocyte stage [3]. When bred into a mixed genetic background [4], the phenotype becomes more severe, with many males displaying a pseudohermaphrodite phenotype. Patched-2 (Ptc-2), a membrane-associated receptor for Dhh, is found on the cell surface of Leydig cells [3] and both primary and secondary spermatocytes [5]. Fused (Fu), a kinase and part of a complex associated with the cytoskeleton that transduces the Dhh signal from the cell surface, is also found in primary and secondary spermatocytes. These findings suggest a signal transduction pathway by which Sertoli cells communicate with Leydig cells and developing germ cells through Dhh.

Like all members of the Hedgehog (Hh) family, Dhh signals through members of the GLI family of five-zinc-finger transcription factors [6]. GLI family members are involved in numerous developmental pathways, and both overexpression and mutations lead to disease states in both human and mouse (reviewed in [7–9]). In the current model of Hh signaling (reviewed in [7, 9–11]), Dhh binds to the Ptc-2 receptor on the cell surface. This binding relieves Ptc-2 repression of Smoothened (Smo), a G-protein-like transmembrane protein that transduces the Dhh signal from the cell surface to the cytoplasm. The final result of Dhh signaling is the activation of the GLIs, which modulate the transcription of Dhh target genes. The presence of other members of the Dhh signal transduction pathway in the testis suggests that GLI1 may also be present and involved in the control of spermatogenesis. The mRNA encoding GLI1 and GLI3 has been detected in spermatogonia [12], but the cell types in which the corresponding proteins are expressed remain unknown.

To localize expression of GLI1 during mouse spermatogenesis, serum raised against human GLI1 was used to probe mouse testis sections. We wanted to determine the expression pattern of GLI1 during the first wave of spermatogenesis following birth and in adult mice, with an emphasis on whether expression is cytoplasmic or nuclear. To investigate further the role that GLI1 plays in spermatogenesis, a line of transgenic mice, in which human GLI1 overexpression results in male sterility, was analyzed as well.

MATERIALS AND METHODS

Paraffin Embedding and Sectioning

Testes were placed in Bouin fixative or 4% (w/v) paraformaldehyde in PBS overnight. Fixed tissue was embedded in paraffin according to standard methods and cut at a thickness of 6 μm. Hematoxylin-and-eosin staining was performed according to standard procedures.

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Immunohistochemistry

A modification of the method described by Morales and Hecht [13] was employed. Paraffin-embedded sections were deparaffinized by incubating twice for 5 min each time in xylene and then rehydrated by incubating twice for, each time, 5 min in 100% (v/v) ethanol, 3 min in 95% ethanol, 20 min in 70% ethanol containing 1% (w/v) H₂O₂ (to inactivate endogenous peroxidase activity), 20 min in 70% ethanol saturated with Li₂CO₃ (to neutralize picric acid from the fixative), 3 min in 50% ethanol, 10 min in ddH₂O, 10 min in PBS (pH 7.2), and 5 min in 300 mM glycine in PBS. The remainder of the protocol was carried out using reagents from the Zymed Histostain-Plus kit (Zymed Laboratories, Inc., South San Francisco, CA). All operations were performed at room temperature unless otherwise stated. Tissue sections were circled with a PAP Pen (Research Products International Corp., Mount Prospect, IL) to provide a well for reagents. Sections were blocked for 10 min with blocking solution and then incubated overnight in a humidity chamber at 4°C.

Sections were incubated for 30 min with secondary antiserum and then washed twice for 5 min each time and once for 10 min in PBS. Staining was visualized by incubation with 10% (v/v) normal goat serum in PBS for 45 min. Sections were incubated overnight in a humidity chamber with α-GLI1 serum diluted in PBS containing 3% BSA and 0.3% Triton X-100 at 4°C and then washed twice for 5 min each time and once for 10 min with PBS. Tissue was incubated for 45 min at room temperature with a Texas Red-conjugated goat α-rabbit secondary antiserum (Jackson Immunoresearch Laboratories, West Grove, PA) diluted in PBS containing 1.5% BSA and 0.3% Triton X-100 and washed twice for 5 min each time and once for 10 min with PBS. Sections were mounted using Aquamount (VWR Scientific). To ensure the specificity of the GLI1 antiserum, the serum was adsorbed for 1 h at room temperature with an excess of the polypeptide used to raise the antiserum. Adsorbed antiserum was used in the protocol above as a negative control. Images were obtained using a Zeiss deconvolution microscope (Carl Zeiss, Thornwood, NY).

GLI1 Antiserum Production

An affinity-purified α-GLI1 rabbit polyclonal serum was generated from a C-terminal GLI1-glutathione-S-transferase (GST) fusion protein shown to have activity in vivo [14]. The fusion construct consists of the C-terminal 227 amino acids of GLI1 (amino acids 879–1106) expressed in the pGEX 2T GST-expression construct (Amersham Pharmacia Biotech, Piscataway, NJ). Fusion protein (600 μg) was injected into New Zealand White rabbits, followed by two booster injections of 300 μg each. The antiserum was affinity-purified against the GLI1-GST construct bound to nitrocellulose filters, washed extensively, and eluted.

Immunofluorescence

Fresh adult testes were fixed overnight in 4% (w/v) paraformaldehyde in PBS (pH 7.2), washed with PBS, frozen on dry ice, sectioned to a thickness of 10 μm, thaw-mounted onto slides, and allowed to air-dry for 15 min at room temperature. Sections were rinsed with PBS and blocked by incubation with 10% (v/v) normal goat serum in PBS for 45 min. Sections were incubated overnight in a humidity chamber with α-GLI1 serum diluted in PBS containing 3% BSA and 0.3% Triton X-100 at 4°C and then washed twice for 5 min each time and once for 10 min with PBS. Tissue was incubated for 45 min at room temperature with a Texas Red-conjugated goat α-rabbit secondary antiserum (Jackson Immunoresearch Laboratories, West Grove, PA) diluted in PBS containing 1.5% BSA and 0.3% Triton X-100 and washed twice for 5 min each time and once for 10 min with PBS. Sections were mounted using Aquamount (VWR Scientific). To ensure the specificity of the GLI1 antiserum, the serum was adsorbed for 1 h at room temperature with an excess of the polypeptide used to raise the antiserum. Adsorbed antiserum was used in the protocol above as a negative control. Images were obtained using a Zeiss deconvolution microscope (Carl Zeiss, Thornwood, NY).

D259MG Tissue Culture and Immunofluorescence

The D259MG cells, a human glioma-derived cell line [15] in which GLI1 is overexpressed [16], were grown on LabTek II culture slides (Nalge Nunc, Rochester, NY) in Dulbecco modified Eagle medium supplemented with 10% (v/v) fetal calf serum and 1% (w/v) l-glutamine. Cells were fixed at room temperature for 2 h using freshly prepared, 4% (w/v) paraformaldehyde in PBS. Cells were washed three times for 10 min each time in PBS and then blocked for 1 h at room temperature in blocking solution (1% BSA and 0.1% Triton X-100 in PBS). Cells were incubated overnight at 4°C in a 1:4 dilution (in blocking solution) of affinity-purified α-GLI1 serum with the peptide used to generate it eliminated GLI1 signal (D). Magnification in all panels ×200.

FIG. 1. Tubules from wild-type mice were double-labeled with antisera that recognize human GLI1 (red) and β-tubulin (green). GLI1 expression was detected in pachytene primary spermatocytes (A, arrow P), round spermatids (A and B, arrow R), and elongating spermatids (C, arrow E), where localization of GLI1 was cytoplasmic. GLI1 also appeared in the nuclei and cytoplasm of Sertoli cells (arrow S, A and C, respectively). Preadsorption of the α-GLI1 serum with the peptide used to generate it eliminated GLI1 signal (D). Magnification in all panels ×200.
GLI1 SIGNALING IN MOUSE SPERMATOGENESIS

RESULTS

GLI1 Protein Localizes to the Nuclei of Germ Cells

We examined the distribution of GLI1 protein in wild-type testis sections using a rabbit polyclonal antiserum raised against amino acids 879–1106 of human GLI1 expressed as a GST fusion protein. This region of human GLI1 is highly homologous to mouse GLI1, and a good reaction across the species was expected. Frozen sections of wild-type mouse testis were double-labeled with GLI1 antiserum (Fig. 1, red) and with a mouse monoclonal antiserum that recognizes β-tubulin (Fig. 1, green) to mark the cytoskeleton. The GLI1 protein appeared in the nuclei of a subset of tubules from each cross-section examined. The GLI1 appeared in the nuclei and cytoplasm of Sertoli cells (Fig. 1, A and C, respectively, arrow S), the nuclei of pachytene cells (Fig. 1A, arrow P), and round spermatids (Fig. 1, A and B, arrow R) and persisted through elongating spermatids, where protein localization shifted to the cytoplasm (Fig. 1C, arrow E). Sections incubated with serum that had been adsorbed with the immunizing peptide showed no signal (Fig. 1D).

GLI1 Associates with Microtubules During Mitotic Cell Division

Alternate localization of GLI1 between the cytoplasm and nucleus raises the question of whether GLI1 can associate with microtubules as a possible transit mechanism. To answer this question, dividing D259MG cells, which constitutively overexpress GLI1, were labeled for GLI1 (red) and β-tubulin (green). Overlap of GLI1 and β-tubulin was observed on the spindles of the mitotically dividing cells (Fig. 2).

GLI1 Expression Pattern Changes from Cytoplasmic to Nuclear During the First Round of Spermatogenesis

We examined localization of GLI1 expression during the first round of spermatogenesis after birth by labeling paraformaldehyde-fixed, paraffin-embedded sections of testes taken from mice aged 9.5–30.5 days at 3-day intervals. Both testes from each mouse were examined. The GLI1 localization was cytoplasmic on Days 9.5–15.5 (Fig. 3A, from a 15.5-day-old animal) but was weakly detectable in the nuclei of pachytene cells (data not shown, but similar to Fig. 3C, arrow P), the nuclei and cytoplasm of Sertoli cells, and the nuclei of round spermatids by Day 18.5 (Fig. 3B, arrows S and R, respectively). On Day 21.5, weak signal was observed in the nuclei of pachytene spermatocytes and round spermatids (Fig. 3C, arrows P and R, respectively), and signal in Sertoli cytoplasm and nuclei was more prominent and widespread (Fig. 3C, arrow S). On Day 24.5, more intense staining was seen in the nuclei of round spermatids in addition to Sertoli and pachytene cells (Fig. 3D, arrows R, S, and P, respectively). Staining on Day 27.5 (Fig. 3E) was the same as on Day 24.5, although less intense in all cell types. By Day 30.5 (Fig. 3F), staining was again seen only in the cytoplasm. Interstitial Leydig cells were found to autofluoresce even in the absence of antisera.

Overexpression of GLI1 in Transgenic Mice Results in a Block of Spermatogenesis

Transgenic mice were made that express the human GLI1 gene under control of the zinc-inducible mouse metallothionein-1 (MT-1) promoter [17]. Males from one line were found to be infertile, regardless of whether they were fed zinc. Testes from these mice were fixed with Bouin fixative, embedded in paraffin, and sectioned. Examination of hematoxylin and eosin-stained testis sections revealed that spermatogenesis was halted at the pachytene primary spermatocyte stage (Fig. 4A). Seminiferous tubules were disorganized compared to tubules of wild-type mice (Fig. 4C), and there also appeared to be a hyperplasia of interstitial Leydig cells.
FIG. 3. Testis sections from wild-type mice aged 15.5 days (A), 18.5 days (B), 21.5 days (C), 24.5 days (D), 27.5 days (E), and 30.5 days (F) were labeled with α-GLI1 immunoserum and detected using a Texas Red-conjugated secondary antiserum. Arrows indicate Sertoli cells (S), pachytene spermatocytes (P), and round spermatids (R). Magnification in all panels ×200.

Only Some Pachytene Primary Spermatocytes Express the Marker Lactate Dehydrogenase C₄

Because the pachytene-like cells in the transgenic tubules were indistinguishable morphologically, we employed an immunohistochemical approach to determine whether the cells were equivalent biochemically. Testis sections were stained with an antiserum raised against lactate dehydrogenase C₄ (LDH-C₄), a testis-specific protein that is normally found in the cytoplasm of germinal epithelial cells from pachytene spermatocytes to elongated spermatids [18] (Fig. 4C). Only a subset of the pachytene-like cells stained with the LDH-C₄ antiserum (Fig. 4B). To determine at which stage of the seminiferous epithelium the pachytene-like cells ceased to express LDH-C₄, we examined cells at the periphery of tubules 1 (LDH-C₄ expressed) and 2 (LDH-C₄ not present) depicted in Figure 4, A and B, at a higher magnification (Fig. 5). The system of Russell et al. [2] was used as a guide for staging the tubules. Intermediate/type B spermatogonia were present in tubule 1 (Fig. 5A, I/B) and also in tubules 5 and 6 (data not shown), identifying these tubules as stage IV or V. Preleptotene/leptotene spermatocytes were found at the periphery of tubule 2 (Fig. 5B, P/L) and tubules 3 and 4 (data not shown), identifying these tubules as stage VIII. Our results indicate that cessation of LDH-C₄ expression in the GLI1-overexpressing transgenics occurs between stages V and VIII. Precise staging requires examination of acrosome development and spermatid nucleus morphology [2]. Because we were only able to examine cells at the periphery
Overexpression of GLI1 Leads to Apoptosis of Germ Cells

Testis sections were double-labeled for LDH-C₄ expression and for apoptosis using the antiserum described above to localize LDH-C₄ expression and the terminal deoxynucleotidyl transferase technique for visualizing fragmented nuclear DNA characteristic of apoptotic cells. Of the subset of tubules in which signal was observed, nearly all tubules were positive for either apoptosis (Fig. 6A) or LDH-C₄ (Fig. 6B), but not for both. Only a few tubules could be found that contained LDH-C₄-positive and apoptotic cells (Fig. 6C), but in no case was LDH-C₄ signal and apoptosis detected in the same cell. Apoptosis detection and LDH-C₄ localization were evident in the nuclei and cytoplasm, respectively, of wild-type tubules that were treated with nuclease as a positive control for apoptosis detection (Fig. 6D), but only LDH-C₄ was detected in untreated wild-type tubules (Fig. 6E).
FIG. 5. High magnification of tubules 1 (A) and 2 (B) from Figure 4, A and B. 
A) Tubule 1, in which LDH-C₄ is expressed (Fig. 4B), has intermediate/type B (I/B) spermatogonia at its periphery, identifying it as being at stage IV or V.  
B) Tubule 2, in which LDH-C₄ is not expressed, has pre-leptotene/leptotene (P/L) spermatocytes at its periphery, indicating it is at stage VIII. Magnification ×600.

DISCUSSION

During the first wave of spermatogenesis from Days 9.5–15.5 postpartum, GLI1 can be detected in the cytoplasm of germinal epithelial cells. By Day 18.5, GLI1 appears in nuclei of pachytene cells, round spermatids, and Sertoli cells. By Day 21.5, GLI1 is detectable in the cytoplasm of Sertoli cells as well. A high level of GLI1 signal is found in the nuclei of round spermatids as well as in the nuclei and cytoplasm of Sertoli cells, along with a low level of expression in pachytene cells, at Day 24.5. The signal in all cell types weakens at Day 27.5, and by Day 30.5, GLI1 becomes localized to the cytoplasm. This is reminiscent of the Drosophila GLI homologue cubitus interruptus (Ci), which is found predominately in the cytoplasm [19]. Its import into the nucleus is tightly regulated [20, 21]. The coordinated movement of GLI1 from the cytoplasm to the nucleus of Sertoli and germ cells in a stage-specific manner strongly suggests that signaling through GLI1 plays a role in the regulation of spermatogenesis, possibly involving cell-cycle control. The GLI genes have been shown to function in cell-cycle control in other systems [22, 23]. The absence of nuclear GLI1 signal at Day 30.5, when nearly all germ cell types should be present, suggests that localization of GLI1 to the nucleus may be a transient event. Lack of nuclear GLI1 signal at this time may indicate that no germ cells were at the precise stage of development during which GLI1 is required in the nucleus.

In subsequent waves of spermatogenesis, GLI1 protein localizes to the nucleus of the germ cell in a subset of pachytene spermatocytes, round spermatids, and the nuclei and cytoplasm of Sertoli cells. GLI1 also appears in elongating spermatids, where it localizes to the cytoplasm. It is
important to note that many tubules did not display nuclear GLI1 localization in Sertoli or germ cells. This suggests, as mentioned above, that GLI1 nuclear translocation occurs only briefly, at discrete points in the cell cycle. Although Persengiev et al. [12] reported that the mRNA for GLI1 is present only in spermatogonia, whereas we found GLI1 protein only in later germ cell types and Sertoli cells, GLI1 mRNA and protein also do not appear concordantly in the developing mouse gut. Whereas GLI1 protein is found in the epithelial, mesodermal, and mesothelial layers at Embryonic Day 13 (data not shown), GLI1 mRNA is found only in the mesodermal layer [24]. Colocalization of GLI1 with tubulin in D259MG cells suggests that GLI1 may be trafficked or stored along the cytoskeleton, which would explain the appearance of GLI1 in germ cell types distinct from those in which the mRNA is present. An alternative possibility is that GLI1 is present in the cell types in which the mRNA is found but is associated with the cytoskeleton in a conformation that is unrecognizable by our antiserum.

Hedgehog family members signal by binding to the Ptc transmembrane receptor (reviewed in [7, 9]). This, in turn, eliminates repression of Smo, another transmembrane protein, by Ptc. The signal from Smo is thought to be transduced to GLI family transcription factors through the action of a complex consisting of Fu (a kinase), Suppressor of Fu (SuFu), and Costal-2 (Cos-2). Then, GLI1 is translocated to the nucleus, where it binds to the promoters of target genes and modulates transcription [14]. The Dhh-receptor
Double-labeling for DNA fragmentation characteristic of apoptosis and for the presence of LDH-C4 revealed that a population of the pachytene-like cells had entered a cell-death pathway. Furthermore, LDH-C4 was undetectable in the apoptotic cells, whereas none of the cells in which LDH-C4 was detected were apoptotic. Stained tubules generally were positive either for LDH-C4 or for apoptosis. A few rare tubules contained examples of staining for both LDH-C4 and apoptosis, but we never observed apoptosis and LDH-C4 localization in the same cell. This result supports our assertion that cells cease to express LDH-C4 (and, presumably, other normally expressed genes) before entering the apoptotic pathway. Cells that stained for neither LDH-C4 nor apoptosis were presumed to be in between the time points when protein production ceases and fragmented DNA characteristic of apoptotic cells becomes detectable in the nucleus. Based on our staging, germ cell apoptosis in these animals occurs some time during or after stage VIII.

Thus, the phenotype we describe in the GLI1-overexpressing transgenic mice is morphologically similar to one of the phenotypes observed in mice in which the Dhh gene is disrupted [3]. Our data support the suggestion that GLI1 regulates expression of Hh family members through a negative feedback loop. This has been proven for the Dro sophila GLI homologue Ci [25]. We hypothesize that in our transgenic mice, GLI1 overexpression leads to the observed phenotype by causing a functional knockout of Dhh. We argue that the transgene integrates into a chromosomal context where it is constitutively expressed in the testis, and that this ectopic expression is responsible for the observed phenotype. An alternative explanation for these results is that the integration of the transgene disrupted another gene necessary for spermatogenesis. However, because the transgenic mice analyzed were heterozygotes, this possibility seems to be unlikely.

Based on our developmental localization of GLI1 expression, it appears that GLI1 activity is necessary only at discrete points in the spermatogenic cycle. Overexpression of GLI1 would clearly have a devastating effect on spermatogenesis by upsetting the delicate balance of signals required for this complex developmental process.

Our results and other recent reports suggest a role for GLI1 in the regulation of germ cell maturation in the testis. Further studies aimed at elucidating the specific function of GLI1 and the possible involvement of GLI2 and GLI3 will significantly increase our understanding of the signaling events that control spermatogenesis.

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