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Transmembrane Versus Soluble Stem Cell Factor Expression in Human Testis

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ABSTRACT: The interaction between stem cell factor (SCF), a ligand produced by Sertoli cells, and its *c-kit* receptor on germ cells is necessary for successful spermatogenesis in animal models. SCF can be alternatively spliced into soluble and transmembrane forms, and it is the transmembrane form that is required for spermatogenesis in rodents. *c-Kit* receptors are also present on Leydig cells, and soluble SCF has been implicated in the regulation of testosterone production. This study had two goals: To test the hypothesis that the extent of germ cell production in human males is correlated with the expression of transmembrane SCF, and to examine the relationship between testosterone production and the expression of soluble SCF in humans. Reverse transcriptase polymerase chain reaction was used to determine the ratio of transmembrane-to-soluble SCF in testicular tissue. Clinical analysis, hormonal measurements, and histological methods were used to evaluate the causes of infertility and to seek correlations with the pattern of SCF expression. SCF was preferentially expressed as the transmembrane type in all testicular samples, regardless of the state of germ cell production. Furthermore, the percent of transmembrane SCF expression was independent of clinical and histopathological diagnosis ($r_s = -0.111$, $n = 28$) and unrelated to the extent of spermatogenesis. This contrasts with rat models of testicular injury that exhibit a decreased proportion of transmembrane SCF with atrophy. A significant correlation ($r_s = 0.665$, $P < .02$, $n = 16$) was found between testosterone levels and percent soluble SCF, which suggests that, in humans, there may be a regulatory interaction between soluble SCF and testosterone.

Key words: Spermatogenesis, c-kit, testosterone, infertility.

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Stem cell factor (SCF) is required for successful spermatogenesis in animal models. SCF is produced in the testis by Sertoli cells, the supportive cells of the seminiferous epithelium (Manova et al, 1993; Rossi et al, 1991). SCF interacts with the *c-kit* receptor, a transmembrane tyrosine kinase (Sandlow et al, 1996; Qiu et al, 1988) expressed by spermatogonia and Leydig cells (Loveland and Schlatt, 1997). The *c-kit* receptor has been immunolocalized to the acrosomal granules of spermatocytes and the acrosomes of spermatozoa (Sandlow et al, 1999).

The Steel (*Sl*) mouse and Dominant White Spotting (*W*) mouse contain mutations in their SCF and *c-kit* genes, respectively, and those *W* and *Sl* mice with severe homozygous mutations have deficient melanogenesis, erythropoiesis, and germ cell development (Morrison-Graham and Takahashi, 1993). Heterozygous *W* and *Sl* mice and those that are homozygous with less severe mutations demonstrate white spots, only slightly deficient hematopoesis, and variable fertility (Majumdar et al, 1996). The disrupted spermatogenesis seen in *W* and *Sl* mice can be mimicked in normal mice by blocking the *c-kit*-SCF interaction with an anti-*c-kit* antibody (Yoshinaga et al, 1991). Thus, in animal models, the interaction between SCF and *c-kit* is necessary to produce mature germ cells from spermatogonia.

SCF is alternatively spliced. Messenger ribonucleic acid (mRNA) that contains exon 6, which codes an amino acid sequence that can be cleaved by proteases on the surface of the cell, forms soluble SCF, whereas mRNA that lacks exon 6 codes for a protein that remains surface-bound and, thus, forms transmembrane SCF (Anderson et al, 1990; Flanagan et al, 1991). In rodents, expression of soluble SCF alone cannot support spermatogenesis, but expression of transmembrane SCF is associated with successful spermatogenesis. The Steel-Dickie (*Sld*) mouse, a spontaneous mutant that expresses only soluble SCF caused by a 4-kb intragenic deletion in the SCF genomic sequence, is infertile in both sexes, severely anemic, and lacks any coat pigmentation (Tajima et al, 1991; Brannan et al, 1992). *Sl/Sld* mice are anemic, infertile, and the color of their coats is white (Majumdar et al, 1996). *Sld/Sld* mice exhibit reduced germ cell binding to Sertoli cells in culture (Marziali et al, 1993), but in vitro, when a

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plasmid that expresses transmembrane SCF is introduced into Sertoli cells that have been isolated from these mice, they regain their ability to bind to germ cells (Marziali et al, 1993). Rats exposed to 2,5-hexanedione (2,5-HD) exhibit germ cell loss, testicular atrophy, and a decrease in the ratio of transmembrane-to-soluble SCF expression (Boekelheide and Hall, 1991). Reversal of 2,5-HD-induced testicular atrophy with leuprolide, a gonadotropin-releasing hormone agonist, increases the ratio of transmembrane-to-soluble SCF expression (Blanchard et al, 1998).

In humans, a mutation in the *c-kit* gene causes piebaldism (Giebal and Spritz, 1991), an autosomal dominant disorder that results in nonpigmented areas of hair and skin. Piebaldism has not been associated with abnormalities in hematopoiesis or spermatogenesis (King et al, 1995); thus, it incompletely parallels the heterozygous mutations in the *W* mouse. The absence of identified humans with homozygous mutations at the *c-kit* locus raises the possibility that, unlike mice, this genetic condition is lethal in human embryos. A decrease in the expression of *c-kit* receptors has been correlated with subfertility in human males (Feng et al, 1999).

This study tested the hypothesis that human male infertility is associated with altered expression of either soluble or transmembrane SCF. We used reverse transcriptase–polymerase chain reaction (RT-PCR) to determine the ratio of transmembrane-to-soluble SCF in testicular tissue from infertile patients with normal (obstructed) and abnormal spermatogenesis. Patients underwent clinical assessment, hormone measurements, and histopathological analysis of testicular tissue to evaluate the cause of infertility and to seek correlations with the pattern of SCF expression.

Materials and Methods

General

With the informed consent of patients and approval by the Rhode Island Hospital's human investigational review board, male infertility patients who were undergoing testicular biopsy were recruited into the study. Small portions (~2 mm^3) of testicular tissue were immediately placed in liquid nitrogen for RNA extraction and in 10% neutral buffered formalin for histopathology.

RNA Extraction

Testis RNA was isolated using the method described by Chomczynski (1993). Testis tissue was weighed, homogenized in TRI Reagent (Molecular Research Center, Inc, Cincinnati, Ohio), and extracted with 1-bromo-3-chloropropane solution. After centrifugation and precipitation with isopropanol, the RNA was washed with ethanol, solubilized in water treated with diethyl pyrocarbonate (DEPC), and stored at −20°C.

Reverse Transcription–Polymerase Chain Reaction

We used 5-μg of testis RNA as a template to synthesize first-strand DNA in a reaction mixture that contained 2.5 μM of random hexamer primer, 0.5 mM of 2′-deoxynucleoside 5′-triphosphate mix (dNTPs), 0.01 M of dithiothreitol, 1× of First Strand Buffer (Gibco/BRL, Gaithersburg, Md), and 200 units of Superscriptase Rnase H reverse transcriptase (Gibco/BRL). Reverse transcription consisted of a 1-hour incubation at 37°C followed by a termination step of 70°C for 10 minutes.

The PCR mix contained 2 μL of complementary DNA (cDNA), 1× of PCR buffer (Gibco/BRL), 150 nM of $MgCl_2$, 200 μM of dNTPs, 150 nM of antisense and sense primers, and 2.5 units of Taq DNA polymerase (Gibco/BRL) per 50-μL reaction. The SCF primers we used were 5′-CCATTGATGCCTTCAAGGAC-3′ and 5′-GGCTGTCTCTTCTTCCAGTA-3′, which were based on human SCF cDNA as described by Martin et al (1990). We amplified SCF under the following conditions: initial denaturation at 94°C for 5 minutes for 1 cycle; denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute for 40 cycles, followed by a final cycle extension at 72°C for 15 minutes.

The PCR reaction mix to amplify sulfated glycoprotein-1 (SGP-1) was the same as that for SCF amplification. We used 5′-ATTCAGCTCTGCTAGGTGCTTCT-3′ and 5′-CTTGGACTGAAAGAATGCACCAG-3′ as the SGP-1 primers, which are derived from the sequence for human SGP-1. To amplify SGP-1, it underwent an initial denaturation of 94°C for 5 minutes for 1 cycle; denaturation at 94°C for 1 minute, annealing at 53°C for 1 minute, and extension at 72°C for 1 minute for 35 cycles, followed by a final extension at 72°C for 15 minutes for 1 cycle.

RT-PCR products from all reactions were loaded onto Tris-acetate-buffered 1.75% agarose gels and separated by electrophoresis (125 volts). DNA fragments were estimated using a 100-bp ladder. PCR products were confirmed by sequencing as well as by restriction digests using *Spe*I for SCF and *Pst*I for SGP-1.

Confirming the Hybrid Band Resulting from SCF RT-PCR

We analyzed the 3 SCF amplified products using a variety of methods. First, the RT-PCR product was digested with S1 Nuclease (Gibco/BRL). The SCF-PCR product was added to 0.3 M of NaCl, 1× of S1 Nuclease buffer, and 1× of S1 Nuclease enzyme. We incubated this reaction for 30 minutes at 37°C and terminated the reaction by adding 20 mM of EDTA and then electrophoresing it on a 1.75% agarose gel. Second, we used two types of agarose gels to analyze the SCF PCR products (see ''Reverse Transcription–Polymerase Chain Reaction'' section above for the first type). RT-PCR SCF products were also loaded onto denaturing 1.75% agarose gels to obtain single-stranded DNA. These gels were run at 100 volts in alkaline running buffer, and then neutralized, stained, and destained.

Quantitation of the SCF RT-PCR Products

We quantified the amplified DNA products using the Gel Doc 2000 system with Quantity One software (BIO-RAD, Hercules, Calif). The software estimates the density of each band per sample as a percentage of total SCF expression. Each band density was adjusted for differences in PCR product length to account

for the difference in ultraviolet absorbance that results from proportionate dye binding. The percent of transmembrane SCF was calculated as the transmembrane density divided by the total SCF density measured.

Cloning

We isolated and purified individual bands of DNA from a 1.75% agarose gel using the QIAquick Gel Extraction Kit (Qiagen) and used the TOPO TA Cloning (Topoisomerase A-T overhang) Kit (Invitrogen, Carlsbad, Calif) to clone the PCR products. Adenosine overhangs were added by reacting the DNA with 0.5 units of Taq DNA polymerase at 72°C for 10 minutes in a reaction mixture containing 1× of PCR buffer, 1.5 mM $MgCl_2$, and 0.3 mM dNTPs. The resulting mixture was then inserted into the PCR TOPO. The plasmid was transformed into DH5α-competent cells by heat shock for 45 seconds at 42°C. S.O.C. medium (Gibco/BRL) was added to the cells, which were then shaken for an hour at 37°C. Colonies grew overnight at 37°C on LB/amp agar plates containing *x-gal*. We selected white colonies and isolated DNA using a QIAprep spin Miniprep kit. DNA was further analyzed by an *Eco*R1 restriction digest and then finally sequenced. Sequencing was performed at the Brown University Sequencing Facility using the ABI PRISM Model 377 Version 2.1.1 Instrument 1500. Sequences were confirmed by comparing them to the SCF sequence published by Martin et al (1990).

Histopathology

Testicular tissues were embedded in glycol methacrylate (Leica Historesin, Deerfield, Ill), sectioned into 2-μM sections, and stained with periodic acid, Schiff's Reagent, and hematoxylin (Chapin et al, 1984). We assessed spermatogenesis by determining the presence or absence of specific types of germ cells using the Johnsen numerical scoring system (Johnsen, 1970). We counted every available seminiferous tubule cross section (the average was 46 seminiferous tubules per biopsy; range, 19–111; n = 9) for biopsies other than those that were severely depleted of germ cells (Johnsen's score of 2 to <3; n = 18) or normal (Johnsen's score of 10; n = 6).

Statistics

When grouped, values were reported as mean ± standard error of the mean (SEM). Statistical testing was performed using the Spearman rank correlation test (r_s) with $P < .05$ considered significant. For those patients in whom 2 testes were biopsied, we conducted statistical comparisons between patient-related parameters (serum testosterone, luteinizing hormone [LH], and follicle stimulating hormone [FSH]) and the average of the two testis-related parameters (Johnsen score, testis volume, and percent transmembrane SCF). For statistical comparisons of testis-related parameters alone, we used the testis as the basis for comparison.

Results

Histopathological Analysis and Clinical Correlation

A total of 33 testicular biopsies were obtained from 20 infertile men. Each biopsy was evaluated histopathologically to determine the extent of ongoing spermatogenesis. Six biopsies from 5 patients showed normal spermatogenesis (Figure 1a, Johnsen score of 10). Nine biopsies from 6 men showed varying degrees of maturation arrest (Figure 1b, Johnsen score of 3 to <10). Eighteen biopsies from 9 men showed severe germ cell depletion (Figure 1c, Johnsen score of 2 to <3). Johnsen score correlated with clinical diagnosis and with the presence or absence of elongated spermatids. Increased germ cell content (ie, a higher Johnsen score) was positively correlated with testis volume ($r_s = 0.827$, $P < .01$, n = 24), negatively correlated with serum FSH ($r_s = -0.525$, $P < .05$, n = 17), and unrelated to serum testosterone and serum LH (Table 1).

Transmembrane Versus Soluble SCF Expression in Human Testis

Judged by the successful production of a control gene fragment (SGP-1), adequate RNA for RT-PCR was obtained from 28 of the 33 testicular biopsies evaluated histopathologically. SCF RT-PCR was performed using primers that spanned exon 6. Two amplified SCF cDNA fragments were expected of 202 base pairs for transmembrane SCF and 286 base pairs for soluble SCF because of the differential splicing of exon 6. However, 3 bands were observed after electrophoresis of the SCF RT-PCR products in a nondenaturing buffer (Figure 2, lanes 1–3). S1 Nuclease digestion, which cleaves single-stranded DNA, caused the largest band to disappear (Figure 2, lane 4), demonstrating that the largest gel fragment was a hybrid product that contained a single stranded area in its secondary structure, as previously reported for other amplified alternatively spliced products (Bernal et al, 1993). Moreover, denaturing alkaline gels contained only 2 bands, which correspond to the single strands of soluble and transmembrane SCF (data not shown). The PCR products were cloned and sequenced to verify their identity (data not shown).

SCF was preferentially expressed as the transmembrane type in all samples, regardless of the presence or absence of germ cells (Table 1). The mean ± SEM of the percent transmembrane SCF expressed in all tissue samples was 72.2% ± 1.9%, with a range of 58.4% to 91.2%.

Johnsen Score and Percent Transmembrane SCF

We investigated the relationship between the state of spermatogenesis, as determined histopathologically, and percent of transmembrane SCF. We found no overall correlation between Johnsen score and percent transmembrane SCF (Figure 3, $r_s = -0.111$, n = 28). Further, no trends were apparent when the percent transmembrane SCF was determined for different states of spermatogenesis, as reflected by Johnsen score (Table 1).

Of the 28 biopsies from 19 men that could be analyzed

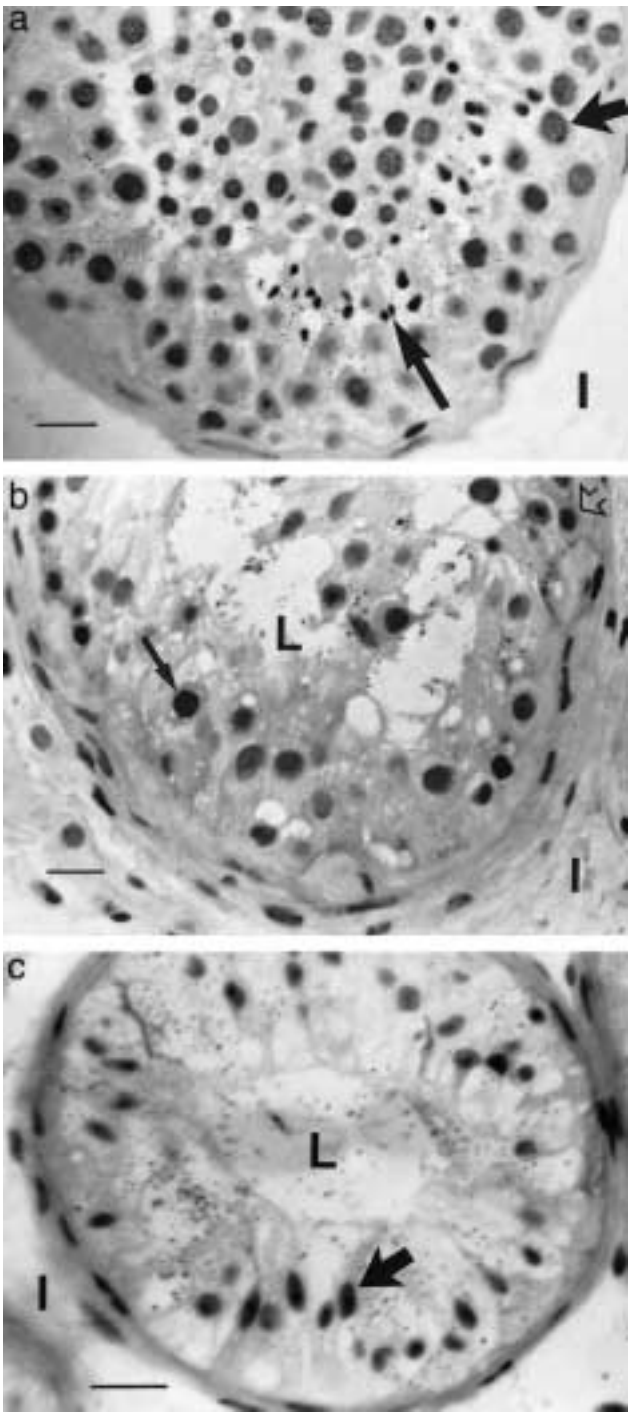

Figure 1. Histopathology of testicular biopsies. To compare among groups of patients, samples were divided into 3 categories based on Johnsen score (Table). **(a)** Normal spermatogenesis in an obstructed patient; spermatocytes (short arrow), spermatids (long arrow). **(b)** In an arrested state of spermatogenesis, some spermatogonia (open arrow) and a few spermatocytes (closed arrow) are seen. **(c)** Severe germ cell depletion shows tubules occupied by Sertoli cells only (arrow). L indicates seminiferous tubule lumen; I, interstitum. Scale bar = 20 μm.

*Johnsen score, serum hormone levels, testis volume, and percentage transmembrane SCF in 20 infertile men**

Johnsen score	Histopathologic category	n	Testosterone, ng/dL	LH, mIU/mL	FSH, mIU/mL	Testis volume, mL	Percentage transmembrane SCF
2 to <3	Severe germ cell depletion	9	390.6 ± 35.3 (n = 8)	10.01 ± 1.87 (n = 7)	23.76 ± 5.19 (n = 9)	20.93 ± 1.36 (n = 7)	74.8 ± 3.3 (n = 9)
3 to <10	Maturation arrest	6	473.3 ± 79.3 (n = 6)	6.13 ± 1.34 (n = 4)	11.82 ± 4.78 (n = 6)	27.80 ± 1.91 (n = 5)	73.9 ± 4.2 (n = 6)
10	Normal spermatogenesis	5	548.0 ± 138.0 (n = 2)	NA	3.45 ± 1.25 (n = 2)	34.25 ± 1.03 (n = 4)	71.0 ± 3.9 (n = 4)

* Some men had both testes biopsied, and in these cases, the left and right testis-related parameters for those men were averaged. For each measurement, the mean ± standard error of the mean is reported for each histopathologic category. NA indicates no sample data were available.

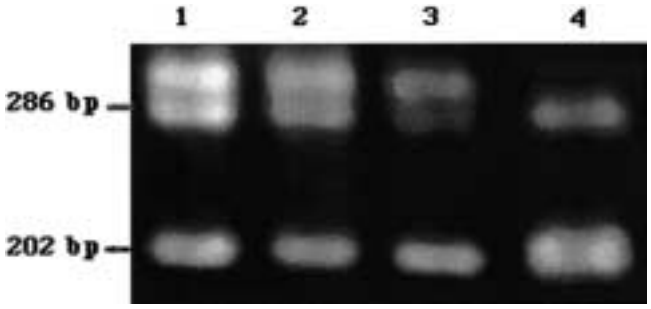

Figure 2. Reverse transcription–polymerase chain reaction (RT-PCR) and S1 Nuclease digestion identifies the alternatively spliced SCF products. Using SCF primers that span exon 6, RT-PCR was followed by agarose gel electrophoresis and ethidium bromide staining to visualize the alternatively spliced mRNA species. Lane 1: SCF expression in a testis with normal spermatogenesis (transmembrane SCF, 72.7%). Lane 2: SCF expression in a testis with maturation arrest (transmembrane SCF, 66.9%). Lane 3: SCF expression in a germ cell–depleted testis (transmembrane SCF, 84.6%). Lane 4: S1 Nuclease digestion of the SCF RT-PCR products eliminates the upper, hybrid cDNA band in this sample from a cryptorchid testis (transmembrane SCF, 61.2%).

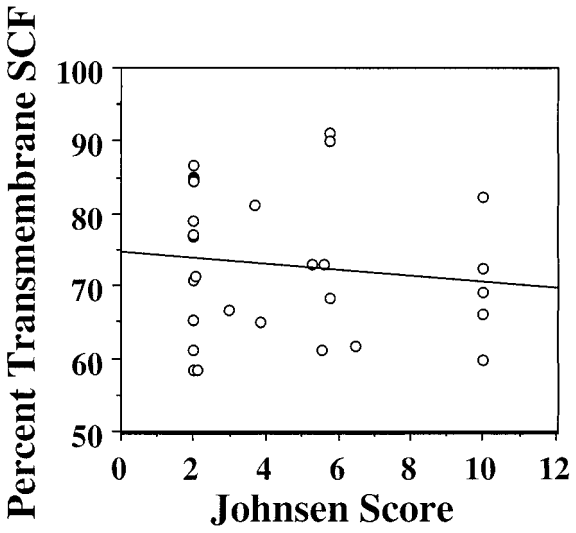

Figure 3. Johnsen score is not related to the percent of SCF mRNA expressed as the transmembrane splice variant. The linear regression is presented for illustrative purposes.

both by histopathology and by SCF RT-PCR, the percent transmembrane SCF by patient and category showed the following: severe germ cell depletion, 74.8% ± 3.3% transmembrane SCF (n = 9, Johnsen score 2 to <3); maturation arrest, 73.9% ± 4.2% (n = 6, Johnsen score of 3 to <10); and normal spermatogenesis, 71.0% ± 3.9% (n = 4, Johnsen score of 10).

Serum Testosterone and Percent Transmembrane SCF

Animal studies have suggested that testosterone may be regulated, at least in part, by soluble SCF (Yoshinaga et al, 1991); therefore, we investigated the relationship between testosterone level and percent of transmembrane SCF. Serum testosterone levels, measured clinically in 16 patients, and percent transmembrane SCF in testis tissue were strongly negatively correlated (Figure 4, $r_s = -0.665$, $P < .02$). No correlation was apparent between serum LH levels and the percent of transmembrane SCF ($r_s = -0.509$, n = 11) or between serum FSH levels and the percent of transmembrane SCF ($r_s = -0.032$, n = 17).

Discussion

A previous, limited study of human testicular SCF reported an invariant and equal transmembrane-to-soluble ratio in all human testis samples examined (Fujisawa et al, 1998). In striking contrast, we observed a wide range of ratios of transmembrane-to-soluble SCF expression that varied from 1.4-fold to 10.4-fold greater transmembrane transcript relative to soluble transcript. It is interesting that we found no apparent correlation between the extent of germ cell production and the ratio of expressed transmembrane-to-soluble SCF. These results in humans are markedly different from observations of SCF expression in rodents, in which a decreased transmembrane-to-soluble ratio of SCF expression has been strongly associated with decreased germ cell production in the testis (Blanchard et al, 1998; Boekelheide and Hall, 1991; Tajima et al, 1991).

In our study, because of the small size of the testis samples obtained, we did not measure the total amount of expressed SCF mRNA or protein. A previous study demonstrated that the amount of immunohistochemically detectable SCF protein expressed varied depending upon

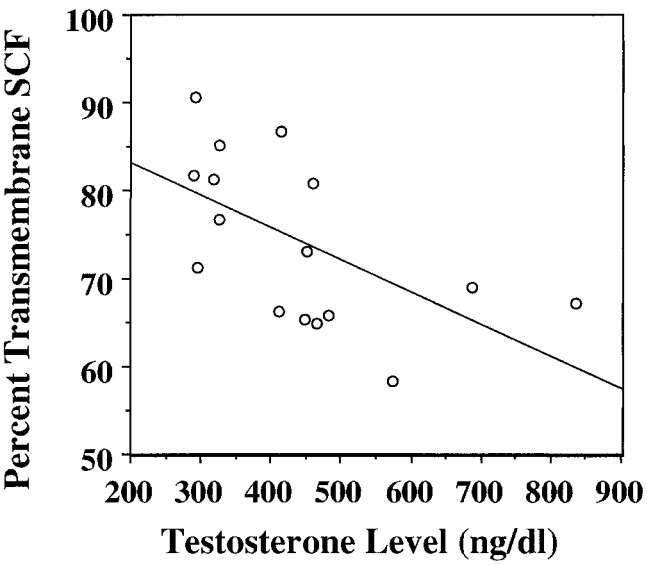

Figure 4. Serum testosterone is significantly negatively correlated with the percent of SCF mRNA expressed as the transmembrane splice variant. The linear regression is presented for illustrative purposes.

the state of spermatogenesis; SCF was most highly expressed in Sertoli cell–only patients with lower expression found in patients with normal spermatogenesis (Sandlow et al, 1996). The significance of this relationship between SCF immunoreactivity and the state of spermatogenesis remains to be determined.

A novel observation in our study was the strong negative correlation between serum testosterone levels and the ratio of transmembrane-to-soluble SCF expression; that is, high serum testosterone was associated with high percent soluble SCF mRNA expression. SCF not only causes the proliferation, adhesion, and survival of spermatogonia but also may regulate testosterone synthesis through its interactions with the *c-kit* receptor on Leydig cells (Qiu et al, 1988). In mice, blocking SCF stimulation of *c-kit* receptors with an anti–*c-kit* antibody produced a transient, large increase in serum testosterone followed by a decreased testosterone level, but one that was higher than that found in controls (Yoshinaga et al, 1991). Thus, at least in this animal model, the soluble SCF–*c-kit* interaction appears to down-regulate testosterone secretion by Leydig cells.

The strong correlation in our study between serum testosterone levels and the percent of testicular SCF mRNA expressed in the soluble form raises the possibility of an intratesticular paracrine negative feedback loop, which influences testosterone secretion, in addition to the classical hypothalamic-pituitary-gonadal hormonal pathway (Marziali et al, 1993). In this model, soluble SCF expressed by Sertoli cells would bind to *c-kit* receptors on Leydig cells to down-regulate testosterone production. In turn, testosterone interaction with the Sertoli cell would up-regulate soluble SCF expression. Although this model is speculative, it is consistent with the observed correlation in this study between testosterone levels and soluble SCF expression and with the results of the antibody blocking experiment in mice (Yoshinaga et al, 1991). Additional experimentation is required to fully understand the significance of the relationship between serum testosterone levels and testicular soluble SCF expression.

We conclude that in humans, unlike rodents, the ratio of transmembrane-to-soluble SCF expression and the state of spermatogenesis are not correlated. This difference between species emphasizes the need to study molecular aspects of spermatogenesis and infertility in humans if the ultimate goal is to have clinical relevance. Indeed, a closer examination of testicular function and fertility status of men with piebaldism may be helpful in determining the role of *c-kit* in the human testis.

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