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Role of the Basic Helix-Loop-Helix Transcription Factor, Scleraxis, in the Regulation of Sertoli Cell Function and Differentiation

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Sertoli cells are a postmitotic terminally differentiated cell population in the adult testis that form the seminiferous tubules and provide the microenvironment and structural support for developing germ cells. The transcription factors that regulate Sertoli cell differentiation remain to be elucidated. The basic helix-loop-helix transcription factors are involved in the differentiation of a variety of cell lineages during development and are expressed in pubertal Sertoli cells. A yeast-two-hybrid procedure was used to screen a Sertoli cell library from 20-d-old pubertal rats to identify dimerization partners with the ubiquitous E47 basic helix-loop-helix transcription factor. Scleraxis was identified as one of the interacting partners. Among the cell types of the testis, scleraxis expression was found to be specific to Sertoli cells. Analysis of the expression pattern of scleraxis mRNA in developing Sertoli cells revealed an increase in scleraxis message at the onset of puberty. Sertoli cells respond to FSH to promote expression of differentiated gene products such as transferrin that aid in proper development of the germ cells. Analysis of the hormonal regulation of scleraxis expression revealed a 4-fold increase in scleraxis mRNA in response to the presence of FSH or dibutryl cAMP in cultured Sertoli cells. An antisense oligonucleotide procedure and overexpression analysis were used to determine whether scleraxis regulates the expression of Sertoli cell differentiated gene products. An antisense oligonucleotide to scleraxis down-regulated transferrin promoter activity in Sertoli cells. A transient overexpression of scleraxis in Sertoli cells stimulated transferrin and androgen binding protein promoter activities and the expression of a number of differentiated genes. Observations suggest scleraxis functions in a number of adult tissues and is involved in the regulation and maintenance of Sertoli cell function and differentiation. This is one of the first adult and nontendon/chondrocyte-associated functions described for scleraxis. (*Molecular Endocrinology* 19: 2164–2174, 2005)

SERTOLI CELLS are epithelial cells that help form the seminiferous tubules and support the developing germ cells. Formation of tight junctions between adjacent Sertoli cells contributes to the blood testis barrier and creates a serum-free microenvironment within the tubule (1). Due to the blood testis barrier, the developing germ cells are dependent on Sertoli cells for nutrients (2). Sertoli cells produce a variety of transport proteins, such as the iron binding protein transferrin, that deliver nutrients to the developing germ cells (3, 4). Sertoli cell functions are sensitive to the gonadotropin FSH (5). FSH stimulates Sertoli cells via receptor-mediated activation of the cAMP/protein kinase A pathway that can influence a number of early response transcription factors such as c-*myc*, c-*fos*, and GATA-1 (5–10). At the onset of pubertal development (*i.e.* postnatal d 10 in rat) Sertoli cells cease to proliferate and become a postmitotic terminally differentiated somatic cell population. The expression of specific genes such as transferrin (3), androgen binding protein (ABP) (11), and inhibin (12) increase in expression as the Sertoli cells differentiate to maintain normal testicular function. Abnormal testicular function in the adult is seen when problems arise with the expression of these Sertoli cell differentiated gene products. Knockout mice for the FSH receptor show normal Sertoli cell development during embryogenesis but have a delay in postnatal differentiation and are subfertile (13). Transgenic mice overexpressing ABP show abnormal sperm morphology and localization within the seminiferous epithelium (14). Previous studies suggest that proper testicular function depends on the appropriate and timely expression of Sertoli cell differentiated gene products. The current study investigates a transcription factor that appears to regulate and maintain pubertal and adult Sertoli cell differentiation.

The basic-helix-loop-helix (bHLH) family of transcription factors has been shown to play a critical role

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Abbreviations: ABP, Androgen binding protein; bHLH, basic-helix-loop-helix; E, embryonic day; Id, inhibitors of differentiation; MMLV, Moloney mouse leukemia virus; mTf, mouse transferrin; P, postnatal day; RT, reverse transcription.

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in cell fate determination and in progression of cellular differentiation in a variety of tissues (15–17). Examples of these proteins include the myogenic bHLH factors MyoD and myogenin in muscle development (15, 16, 18, 19), neuronal factors such as neurogenin and Mash1 (20, 21), and TAL gene involved in hematopoiesis (22). Typically, these class B bHLH proteins dimerize with the ubiquitously expressed class A bHLH proteins, also known as the E family of proteins (23). The class A bHLH proteins are those encoded by the differentially spliced transcripts of the E2A gene products E12 and E47 (23) and by ITF2 (24–26). bHLH proteins contain a common structural motif that consists of two amphipathic helices separated by a loop. The helix-loop-helix domain mediates homo- and heterodimerization between other bHLH proteins (23, 27). The presence of an adjacent DNA-binding region rich in basic amino acids mediates binding of the dimer to a common DNA sequence known as an E-box (CANNTG) (28). bHLH proteins form an interacting network of factors that regulate transcription of a variety of genes. The activities of bHLH proteins often are negatively regulated by another class of HLH proteins that lack the basic domain and are known as inhibitors of differentiation (Id) (29). Because the Id family of proteins lack the basic region, which is essential for DNA binding, the heterodimer is not able to bind to DNA and transcription is inhibited (29, 30).

A number of bHLH transcription factors have been found to be expressed in Sertoli cells. These factors include the class A family of bHLH proteins E47 (31), REBα (32), and ITF2 (Muir, T., and M. K. Skinner, unpublished observation), as well as the Id family of proteins, Id1–4 (33). Expression of ITF2 and the Id family of proteins in Sertoli cells are hormone responsive and are able to regulate transferrin promoter activity (33). Conserved E-box sequences are present in the promoters of Sertoli cell differentiated genes such as c-*fos* (34), transferrin (35), SF-1 (36), and FSH receptor (37). The importance of the E-box in Sertoli cell differentiated genes was demonstrated through mutational analysis of the transferrin and ABP promoters. Mutations in the E-box of the transferrin or ABP promoters cause a dramatic decrease in promoter activity (11, 38). These observations suggest that bHLH proteins play a role in regulating the expression of Sertoli cell differentiated gene products. The expression of class A bHLH proteins and the presence of functional E-boxes within the promoters of Sertoli cell differentiated genes suggest the existence of cell-specific bHLH proteins or known bHLH that have yet to be characterized in Sertoli cells.

To identify Sertoli cell-specific bHLH proteins, a yeast-two-hybrid system (39) was used to screen a 20-d-old rat Sertoli cell cDNA library whose products could dimerize with E47. One of the clones that formed a functional dimer with E47 was scleraxis. Scleraxis is a class B bHLH protein involved in mesoderm formation and in the formation of somite derived chondrogenic lineages early in embryogenesis (40, 41). Scleraxis also has an important role in somitic tendon progenitors development (42–44). The current study investigates the regulation of Sertoli cell differentiated functions and hormone responsiveness by the bHLH protein scleraxis in the adult.

RESULTS

Identification of E47-Interacting Proteins

A yeast-two-hybrid system was used to screen a 20-d-old rat Sertoli cell cDNA library in an effort to identify Sertoli cell bHLH proteins that form functional dimers with E47. In this screen, the bHLH domain of E47 was fused to the DNA binding domain of yeast Gal4, whereas the cDNA library was fused to the activation domain of yeast Gal4. From a screen of approximately 6.25×10^6 independent transformants, 16 clones were isolated. Eleven of the isolated clones were a known class B bHLH transcription factor scleraxis. Five of the isolated clones were Id2. Comparison with the known sequence of mouse scleraxis with this novel rat homolog shows a 98% similarity with a difference of two amino acids (PPPLP/LAR) being unique within the rat carboxyl terminus sequence.

Scleraxis mRNA Expression

RT-PCR was used to analyze the expression of scleraxis in various pubertal animal tissues. Tissues isolated from 20-d-old postnatal rats (P20) revealed that scleraxis was expressed in tissues arising from the brain (ectoderm), lung (endoderm), heart, kidney, muscle, and spleen (mesoderm) (Fig. 1). However, scleraxis mRNA was not detected in the liver (endoderm), prostate, or ovary (mesoderm) (Fig. 1). Cyclophilin was used as a constitutively expressed control gene. Although shown to be expressed in primarily mesoderm (*e.g.* tendon) tissue in the embryo, scleraxis is expressed in a variety of adult tissues arising from the ectoderm, endoderm, and mesoderm.

To identify the testicular cell types that express scleraxis mRNA, a Northern blot analysis was per-

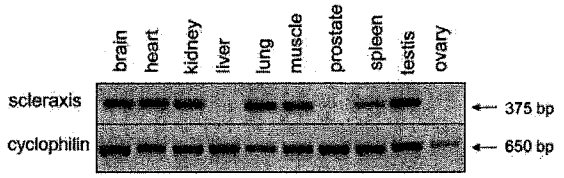

Fig. 1. RT-PCR Analysis of Scleraxis mRNA Expression in Total RNA (2 μg) Isolated from Brain, Heart, Kidney, Liver, Lung, Muscle, Prostate, Spleen, Testis, and Ovary

Cyclophilin (1B15) gene was used as a positive control constitutively expressed gene. The scleraxis PCR product is 375 bp and cyclophilin 650 bp. The data are a representative of three separate analyses on three independent RNA samples.

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formed on total RNA from germ cells, interstitial cells that includes the Leydig cells, peritubular myoid cells, and Sertoli cells isolated from 20-d-old rat testis. Northern analysis revealed a single transcript of approximately 1.2 kb for scleraxis in Sertoli cells (Fig. 2) but none of the other testis cell populations. The constitutively expressed rat ribosomal (S2) gene was used to confirm RNA integrity and normalize the data (Fig. 1). RT-PCR was used to confirm the Northern blot analysis, and scleraxis was only found to be expressed by Sertoli cells (data not shown). No scleraxis antibody currently exists so protein expression could not be confirmed.

Because scleraxis is only expressed by Sertoli cells and not other testis cells, whole testis were used to determine the developmental expression pattern of scleraxis. Scleraxis message was examined during embryonic and postnatal development. RT-PCR analysis determined the presence of scleraxis message in rat testis from embryonic d 13 (E13) and d 16 (E16), prepubertal d 0 (P0) and d 10 (P10), pubertal d 20 (P20), and adult d 65 (P65) ages (Fig. 3A). Cyclophilin gene expression was used as a constitutively expressed control gene. Scleraxis expression was detected in the testis at all the embryonic and postnatal ages examined. To further investigate the pattern of scleraxis expression RNA from 0-, 4-, 8-, 10-, 20-, and 65-d-old rat testis were isolated and analyzed using quantitative real-time PCR. No change in scleraxis RNA levels is observed in postnatal 4- and 8-d-old rat testis. At the onset of puberty, postnatal d 10, there is an increase in scleraxis expression that declines in mid-pubertal d 20 testis (Fig. 3B). The decline in scleraxis expression at P20 may be due to changes in scleraxis expression in Sertoli cells or that scleraxis expression is being masked by the dramatic increase in germ cell number in the testis. To address this total RNA was isolated from prepubertal P10, mid-pubertal P20, and adult P65 rat purified Sertoli cells and analyzed using real-time PCR. Scleraxis expression appears to remain constant in the Sertoli cells at all these

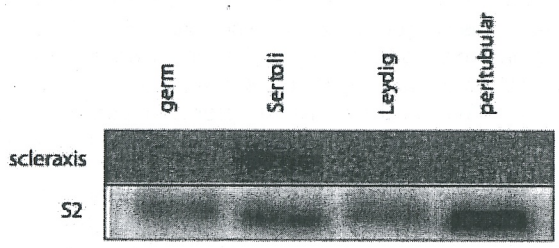

Fig. 2. Northern Blot Analysis of Scleraxis mRNA Expression from Germ Cells, Sertoli Cells, Leydig Cells (*i.e.* Interstitial Cells), and Peritubular Cells Isolated from 20-d-Old Rat Testis

Scleraxis transcript of approximately 1.2 kb were assessed with 10 μg of total RNA. The constitutively expressed rat ribosomal (S2) gene was used to determine loading variability and RNA integrity. The data are representative of three separate experiments on different RNA preparations.

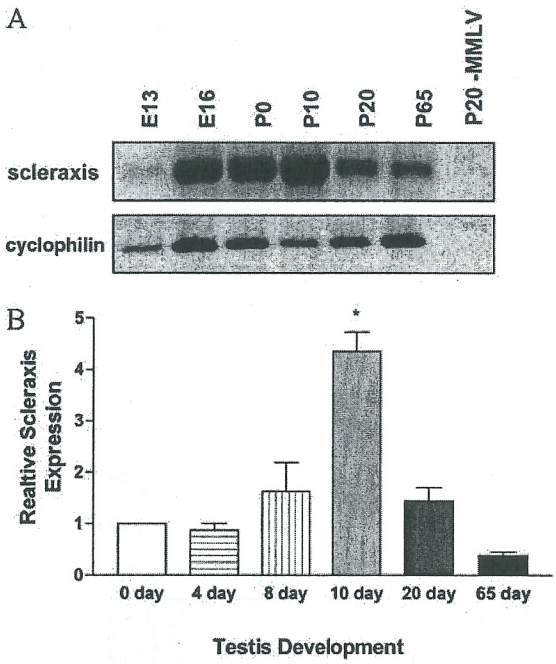

Fig. 3. RT-PCR Analysis of Scleraxis mRNA Expression from Testes from Rats of E13, E16, P0, P10, P20, and P65

Cyclophilin gene was used as a constitutively expressed gene and to monitor the efficiency of the PCR. RT reaction lacking RT enzyme was used as a negative control (*i.e.* P20-MMLV). The data are a representative of three separate analyses on three independent RNA samples (B). Quantitative real-time PCR of scleraxis mRNA of freshly isolated P0, P4, P8, P10, P20, and P65 rat testis. Data are mean ± SEM from three separate experiments with three replicates per experiment. *Asterisk* indicates a significant difference between the 0-d-old testis and the other time points at $P < 0.01$ by Student's *t* test.

developmental stages (Fig. 4A). Therefore, the decline in scleraxis expression in Fig. 3B is due to a dilution effect by germ cell proliferation. Transferrin is a Sertoli cell differentiated gene product that increases in expression during puberty and on into the adult. A quantitative real-time PCR analysis of transferrin mRNA levels demonstrates a low level of expression in 10-d-old rat Sertoli cells (onset of puberty) (Fig. 4B). In mid-pubertal 20-d-old rat Sertoli cells transferrin mRNA levels increase dramatically (*i.e.* greater than 100-fold) and continued to increase (*i.e.* 500-fold) in adult 65-d-old rat Sertoli cells (Fig. 3B). Therefore, transferrin is a useful marker of Sertoli cell differentiation. The observations indicate that scleraxis is expressed during embryonic development at the time of testis determination (*i.e.* E13) and then during postnatal testis development. Expression increases in Sertoli cells at the onset of pubertal development (*i.e.* P10) and then remains constant throughout pubertal development and in the adult when maintenance of Sertoli cell differentiation is required. The rise in scleraxis mRNA levels at P10 precedes the rise in transferrin expression during pubertal development.

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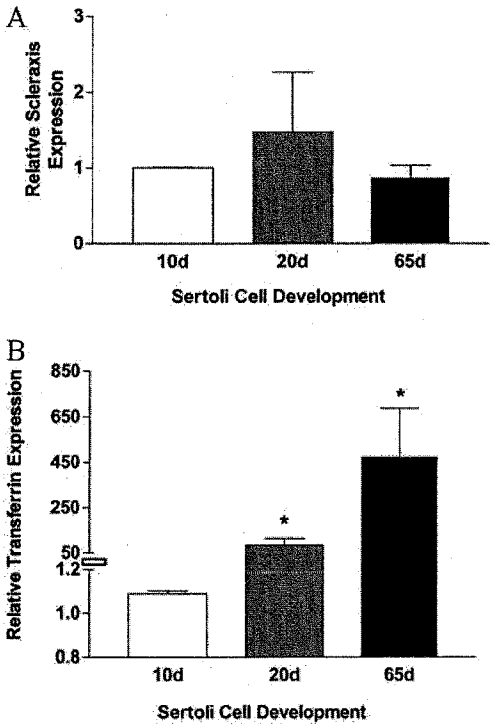

Fig. 4. Quantitative Real-Time PCR Analysis of (A) Scleraxis and (B) Transferrin mRNA Expression from Purified Sertoli Cells from P10, P20, and P65 Rats

Ribosomal (S2) gene was used as a constitutively expressed control gene to normalize data. Data are mean ± SEM from three separate experiments with three replicates per experiment. *Asterisk* indicates a significant difference between 10-d-old Sertoli cells and the other time points at $P < 0.01$ by Student's *t* test.

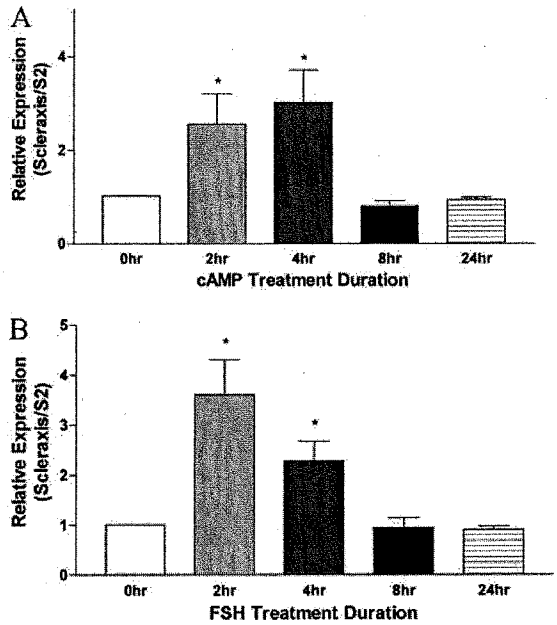

Fig. 5. Quantitative Real-Time PCR of Scleraxis mRNA in Cultured Sertoli Cells after Dibutryl cAMP (A) and FSH (B) Treatment for 0, 2, 4, 8, and 24 h

Scleraxis and S2 levels were analyzed using quantitative real-time PCR. All data were normalized to the 0-h time point for each treatment group. Data are mean ± SEM from three separate experiments with three replicates per experiment. *Asterisk* indicates a significant difference between the 0-h treatment of cultured Sertoli cells and the other treatment time points at $P < 0.01$ by Student's *t* test.

Hormonal Regulation of Scleraxis Expression

Quantitative real-time PCR was used to analyze the hormonal regulation of scleraxis expression in Sertoli cells. Sertoli cells from 20-d-old rats were cultured with either dibutryl cAMP or FSH for 0, 2, 4, 8, and 24 h. In the presence of dibutryl cAMP, an increase in scleraxis mRNA message was observed at 2 h after treatment and peaked at 4 h (Fig. 5A). Message levels returned to control levels at 8 and 24 h after treatment. Treatment with FSH had a slightly different effect on scleraxis mRNA levels. After treatment with FSH scleraxis, message peaked at 2 h and returned to the control level after 8 and 24 h (Fig. 5B). Therefore, a transient increase in scleraxis expression is observed in Sertoli cells in response to cAMP and FSH.

Scleraxis Function in Sertoli Cells

The transferrin promoter reporter construct was used to study the actions of scleraxis on Sertoli cell differentiation. An antisense oligonucleotide approach was used to disrupt scleraxis expression to determine effects on expression of the transferrin gene in Sertoli cells. A chimeric reporter construct containing the luciferase gene driven by the proximal 581-bp mouse transferrin promoter was transiently transfected into cultured 20-d-old (pubertal) Sertoli cells. This reporter construct has been shown to be responsive to bHLH proteins (35). After transfection, the Sertoli cells were cultured in the absence (control) or presence of cAMP or serum. For each treatment group, an antisense [Scl (AS)] or sense [Scl (S)] oligonucleotide was added at 1 μM to each well every 12 h for 48 h. The presence of the antisense oligonucleotide to scleraxis inhibited the transferrin promoter activity and suppressed activation of the promoter (Fig. 6). The sense oligonucleotide had no effect on the transferrin promoter construct suggesting that the effect is due to the scleraxis antisense oligonucleotide and not due to nonspecific effects of the oligonucleotide (Fig. 6). Analysis of mRNA levels with a semiquantitative RT-PCR in the Sertoli cells after antisense treatment demonstrated a reduction in scleraxis expression (data not shown). The down-regulation of the transferrin promoter activity in the presence of scleraxis antisense oligonucleotide suggests scleraxis is required to maintain Sertoli cell differentiation and hormone responsiveness.

Scleraxis was overexpressed in cultured Sertoli cells to determine whether scleraxis can induce transferrin promoter activity. In 20-d-old (pubertal) rat Sertoli

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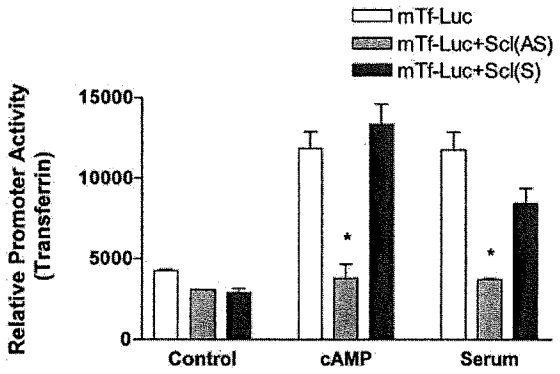

Fig. 6. Antisense Oligonucleotide to Scleraxis Effects on the Transferrin Promoter

Cultured Sertoli cells were transfected with the proximal 600-bp mouse transferrin promoter-luciferase construct (mTf-Luc). Immediately after transfection 2 μM of either the scleraxis sense (S) or antisense (AS) phosphorothionate-modified oligonucleotides were added. The cells were then treated with dibutryl cAMP or serum after the transfection. The oligonucleotides were subsequently added every 12 h until the cells were harvested for luciferase assay (72 h). Data are mean ± SEM from three separate experiments with three replicates per experiment. *Asterisk* indicates a significant difference between scleraxis sense (S) and scleraxis antisense (AS) at $P < 0.01$ by Student's *t* test.

cells, transferrin mRNA levels increase in response to FSH and cAMP levels. Sertoli cells were cotransfected with the transferrin promoter construct and either the control vector (pCMV-myc) or the scleraxis expression construct. Transferrin promoter activity increased by 5-fold in the presence of cAMP as compared with the untreated control cultures (Fig. 7). Interestingly, the overexpression of scleraxis induced a significant increase (1.3-fold for control and 2.5-fold for cAMP) in the transferrin promoter activity in the absence or presence of cAMP treatment (Fig. 7). To confirm that the actions of scleraxis are directly on the promoter and involve an E-box element, ABP reporter constructs containing intake or mutated E-box elements were used (11). ABP is another marker of Sertoli cell differentiation that increases in expression as the cells differentiate. Both wild-type and mutated E-box promoter constructs were transfected separately into Sertoli cells. The overexpression of scleraxis caused an increase in ABP promoter activity (Fig. 8). The presence of the mutated E-box eliminated the ability of scleraxis to stimulate ABP promoter activity (Fig. 8). Therefore, an intact E-box (*i.e.* scleraxis response element) was required to obtain activation by scleraxis (Fig. 8). Observations suggest that scleraxis regulates the transferrin promoter and ABP promoter activity (*i.e.* with an intact E-box element) in Sertoli cells. Analysis of mRNA levels using semiquantitative RT-PCR of Sertoli cells demonstrated an increase in ABP and inhibin-α expression (*i.e.* mRNA levels) after the overexpression of scleraxis (data not shown). Scleraxis was found to stimulate promoter activity of two Sertoli cell differentiated genes and increase mRNA levels of several Sertoli cell differentiated genes.

Scleraxis Binding Partners

To identify possible binding partners for scleraxis in Sertoli cells a yeast-two-hybrid system was used. The Gal4 activation or binding domain and full-length scleraxis were expressed as a fusion protein in yeast cells to verify scleraxis interactions with other bHLH or HLH transcription factors. The interacting partners, E47, ITF2, Id1, Id2, and Id3 were fused to the Gal4 activation or binding domain. Scleraxis fusion vector was cotransformed with each of the potential interact-

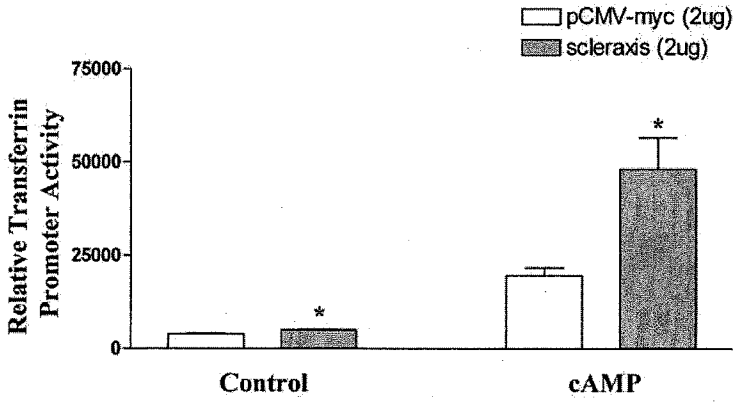

Fig. 7. Constitutive Expression of Scleraxis in Cultured Sertoli Cells

Cultured Sertoli cells were transfected with the proximal 600-bp mouse transferrin promoter-luciferase construct (mTf-Luc). Scleraxis expression indicates the presence of 2 μg of pCMV-scleraxis. Basal expression vector (pCMV-myc) is used as a positive control. The cells were incubated in the absence or presence of dibutryl cAMP after the transfection for 72 h. Data are mean ± SEM from three separate experiments with three replicates per experiment. The control plasmid (pCMV-myc) values are 3911 ± 155 in the absence and 19,576 ± 2074 in the presence of cAMP. *Asterisk* indicates a significant difference between control (pCMV-myc) and scleraxis at $P < 0.01$ by Student's *t* test.

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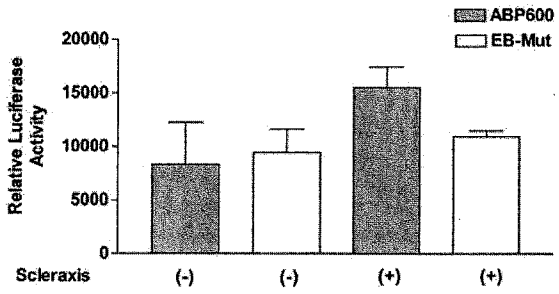

Fig. 8. Scleraxis Effects on the ABP Reporter Construct with and without E-Box Mutated Response Elements

Cultured Sertoli cells were transfected with the ABP luciferase reporter construct (ABP 600) or a construct with a mutated E-box [ABP600 (EB-Mut)] element. Cells were incubated in the presence of cAMP, and in the absence (−) or presence (+) of scleraxis or control plasmid (pCMV-myc) constructs. After 72 h of culture, the relative luciferase activity in the cell was determined. The mean ± SEM from a representative of three experiments is presented.

ing partners. Survival of the double transfected yeast in selection media and the expression of the LacZ reporter gene were used to verify a positive interaction. The pSV40 and p53 constructs were used as the positive control (Table 1). The pBDgal4-E47 and pSV40 constructs were used as the negative control and also excludes E47 ability to autoactivate the reporter genes (Table 1). Similar controls were conducted for all constructs to exclude the possibility of autoactivation (data not shown). A positive binding interaction is observed when scleraxis is cotransformed with either of the class A bHLH transcription factors E47 (45) or ITF2 (Table 1). However, no interaction was observed when scleraxis was cotransformed with Id1, Id2, or Id3 (Table 1). The inability of Id proteins to interact with scleraxis indicates scleraxis may not be inhibited by this normal regulatory pathway. Observations suggest scleraxis can interact with E47 or ITF to promote Sertoli cell differentiated functions.

Table 1. Scleraxis Interactions with Other bHLH and HLH Transcription Factors

	Bait	Prey	-his-ade	β-Galactosidase Activity
1	p53	pSV40	+	+
2	E47	pSV40	−	−
3	E47	Scleraxis	+	++
4	ITF	Scleraxis	+	+++
5	Id1	Scleraxis	−	−
6	Scleraxis	Id2	−	−
7	Scleraxis	Id3	−	−

Under the (-his-ade) column, + represents growth of yeast on -his-ade plates. Under the β-galactosidase activity column, + represents a minimum of 2-fold increase in activity, ++ represents a minimum of 4-fold increase in activity, and +++ represents a minimum of 6-fold increase in activity as compared with the negative control (E47 bait and pSV40 prey). Negative control is set at 1 nmol/min · mg protein.

DISCUSSION

Sertoli cell differentiation at the onset of puberty is in part triggered by the increase in circulatory FSH levels. The increase in FSH induces the expression of Sertoli cell differentiated gene products. The increase in expression of Sertoli cell differentiated genes allows for the maintenance and support of spermatogenesis. Identification of transcription factors involved in promoting the process of differentiation in Sertoli cells is of interest. Scleraxis has been identified as a cell type-specific bHLH protein shown to be expressed in mesenchymal precursors of cartilage and in connective tissue (40). Scleraxis is a bHLH protein that can regulate the expression of cartilage differentiated gene products (46). Scleraxis has been shown to be primarily involved in tendon progenitors and chondrogenesis during embryogenesis (42–44, 47, 48). Interestingly, a yeast-two-hybrid system to detect E47 dimer partners identified scleraxis in a 20-d-old rat Sertoli cell cDNA expression library. This is one of the first adult and nonchondrocyte/tendon tissues shown to express scleraxis. The current study demonstrates the presence of scleraxis in the testis and its role in regulating Sertoli cell function and differentiation.

The majority of information concerning the role of bHLH proteins in the process of differentiation have been investigated during embryogenesis. Few studies have examined the role many of these proteins play later in the adult tissues. Expression of scleraxis was detected in tissues of endodermal (lung), ectodermal (brain), and mesodermal (heart, kidney, muscle, and spleen) origin. However, scleraxis transcript was not detected in the liver, prostate, or ovary. Because scleraxis was previously identified in mesodermal tissue of the embryo, the detection of scleraxis transcripts in tissues arising from the ectoderm and endoderm in the adult suggests that scleraxis is activated later in development in a variety of tissues and may be important for proper adult tissue development. However, connective tissue is present in many organs such that scleraxis function in other cell types requires further investigation. Scleraxis may have a much more diverse role in adult tissue function than the role in cartilage and tendon development in the embryo.

Northern blot and PCR analysis was used to determine whether scleraxis is ubiquitously expressed within the testis or whether it is specific to Sertoli cells. Scleraxis gene expression was only detected within Sertoli cells and not in the germ cells, Leydig cells, or peritubular myoid cells. Scleraxis being specifically expressed in Sertoli cells and not the other cells of the testes suggests scleraxis may be important for Sertoli cell function and differentiation. Sertoli cells undergo a mesenchymal-epithelial cell transition during development and then become postmitotic terminally differentiated cells during pubertal development. Recently, the twist bHLH factor has been shown to be critical for epithelial-mesenchymal transition and morphogenesis

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during development and tumor metastasis (49, 50). Scleraxis is a member of the twist subfamily of bHLH proteins. A potential role for scleraxis in Sertoli cells is in the regulation of the mesenchymal-epithelial transition of the cells.

Scleraxis mRNA was observed to undergo dramatic changes during testis development. Scleraxis is expressed at the time of sex determination and testis determination (E13). The expression of scleraxis remains constant during prepubertal development (P0, P4, and P8). Prepubertally, gonadotropin and testosterone levels are low, and testis function is marked by mitotic division of all cell types. At the onset of puberty (*i.e.* postnatal d 10 in the rat) scleraxis mRNA levels significantly increase compared with prepubertal levels. Early during puberty (*i.e.* d 15 in the rat) is when Sertoli cells become postmitotic and form tight junctional complexes between adjacent cells to create the blood-testis barrier. The increase in scleraxis expression at P10 correlates with the onset of puberty and may therefore be involved in this critical progression of Sertoli cell differentiation. Interestingly, the increased expression of scleraxis at the onset of puberty preceded the increase in transferrin expression during puberty. After the onset of puberty, scleraxis expression remained constant, whereas transferrin expression continued to rise. Many transcription factors that promote differentiation also continue to be expressed to subsequently maintain cellular differentiation (*e.g.* MyoD). The rise in scleraxis expression at the onset of puberty and maintenance of expression in adult Sertoli cells suggests scleraxis also is needed to maintain Sertoli cell differentiation and hormone responsiveness. Observations demonstrate an increase in scleraxis expression correlates with the onset of pubertal testis development, increase in Sertoli cell differentiation, and maintenance of Sertoli cell differentiation in the adult.

Sertoli cell functions are in part regulated by the gonadotropin FSH (9). FSH stimulation is critical in prenatal and newborn rats for Sertoli cell proliferation. FSH is important during pubertal development as a determinant of the spermatogenic capacity of the testis. FSH binds specific cell-surface receptors on Sertoli cells and initiates an intracellular signaling cascade that leads to increased cAMP levels. The cAMP pathway triggers the expression of Sertoli cell differentiated genes. Several genes that are stimulated by FSH and/or cAMP in Sertoli cells include c-*fos*, jun B, inhibin α (6, 51–53). Studies with cultured Sertoli cells revealed that after 2–4 h of treatment with cAMP or FSH scleraxis expression increased and then returned to basal levels after 8–12 h of treatment. Analysis of the mouse and rat scleraxis 1500-bp proximal promoters revealed the presence of consensus cAMP response elements supporting potential regulation by cAMP. The down-regulation of FSH receptor signaling has previously been shown to occur 12–24 h after initial stimulation. The decline in the scleraxis expression may be linked to this FSH receptor down-regulation. Other factors such as an inducible cAMP early repressor (ICER)-type inhibition of the cAMP response at the level of the promoter may also be possible. Observations suggest that scleraxis may help mediate FSH actions in the induction of Sertoli cell differentiation.

Transferrin is a Sertoli cell differentiated gene product that is highly expressed at the onset of puberty. The increase in transferrin expression correlates with the progression of Sertoli cell differentiation. Therefore, transferrin is an important and useful marker of Sertoli cell differentiation. Constitutive overexpression of scleraxis in cultured Sertoli cells stimulated the transferrin promoter suggesting that scleraxis may be involved in regulating the expression of Sertoli cell differentiated gene products. Observations that scleraxis can also stimulate ABP promoter activation when an intact E-box element is present also supports the ability of scleraxis to act on Sertoli cell differentiated genes. This is further supported by the observation that an antisense oligonucleotide to scleraxis inhibited the transferrin promoter activation. Analysis of other Sertoli cell differentiated genes (*i.e.* ABP and inhibin-α) demonstrated that scleraxis also stimulates the expression (*i.e.* mRNA levels) of these genes as well. Therefore, scleraxis appears to play a role in promoting Sertoli cell differentiated functions (*e.g.* transferrin) by activating the promoters of differentiated genes.

To investigate the network of bHLH proteins involved in Sertoli cell differentiation, the binding partners of scleraxis in Sertoli cells were determined with a yeast-two-hybrid procedure. The bHLH and HLH factors known to be expressed in Sertoli cells include E47 (31), ITF2 (Muir, T., and M. K. Skinner, unpublished observation), and the Id family of proteins, Id1–4 (33). As previously shown, scleraxis interacts with E47 (45). The Id family of proteins have been shown to interact with the class A family of bHLH transcription factors, but weakly if not at all with other class B bHLH transcription factors such as MyoD (29, 54, 55). To determine potential interactions with any of the Id proteins scleraxis was coexpressed with Id1, Id2, or Id3. No interaction occurred between any of the Id proteins and scleraxis indicating scleraxis actions may not be directly influenced by Id proteins. Interestingly, scleraxis did interact with another known regulator of cellular differentiation ITF2. ITF2 is a bHLH factor that is ubiquitously expressed and interacts with myogenic and melanogenic bHLH factors (56, 57). Further analysis of the functional importance of ITF2 interactions with scleraxis is required. The bHLH family of transcription factors heterodimerize with a large number of different family members. During testis development, the Sertoli cell expression for different bHLH proteins may promote different heterodimers with scleraxis at different periods of development. This may allow a greater complexity and specificity for the control of cellular differentiation involving scleraxis.

In summary, scleraxis is a bHLH protein that appears to be involved in Sertoli cell differentiation and

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hormone responsiveness. The importance of Scleraxis in Sertoli cell differentiation is suggested from three observations: 1) Scleraxis expression is found only in Sertoli cells within the testis and scleraxis expression increases at the onset of puberty when Sertoli cells become a postmitotic terminally differentiated cell type; 2) FSH and cAMP regulate scleraxis expression and Sertoli cell differentiated functions; and 3) Constitutive expression of scleraxis in cultured Sertoli cells promoted the activation of the transferrin and ABP promoters, whereas the inhibition of scleraxis expression suppressed the activation of the transferrin promoter. The overexpression and antisense oligonucleotide experiments suggest scleraxis may have a role in regulating Sertoli cell differentiation during pubertal testis development and in the adult. The current study suggests scleraxis may have a wide variety of unique functions in adult tissues independent of the embryonic functions in muscle and chondrocyte development. The speculation is made that roles attributed to specific bHLH proteins in the embryo may be distinct and quite different from those required in adult tissue functions.

MATERIALS AND METHODS

Yeast Expression Vectors

The GAL4(DBD)-E47 fusion vector was constructed by inserting the region encoding amino acids 501–639 of E47 into the *Eco*RI site of the yeast expression vector pBDGal4 (Stratagene, La Jolla, CA). This generates a chimeric cDNA construct that contains the yeast Gal4 DNA binding domain in frame with the E47 protein coding sequence. The region of E47 expressed contains the bHLH region. The same was done for Id1 (full length), Id2 (full length), Id3 (full length), Id4 (encoding amino acids 27–162), ITF2 (full length) and scleraxis (full length). A Gal4(AD) fusion expression library, from Sertoli cell cDNA was constructed in HybriZapII by Stratagene from poly (A) + RNA isolated from purified 20-d-old rat Sertoli cells.

Yeast-Two-Hybrid Procedure

The Gal4(DBD)-E47 was transformed into the yeast strain AH109 (mat A) (CLONTECH, Palo Alto, CA), whereas the Gal4(AD)-Sertoli cell cDNA library was transformed into the yeast strain Y187 (mat α) (CLONTECH). Following CLONTECH's procedure for performing a mating two-hybrid system 6×10^6 colonies were screened. Colonies able to grow on SD-Trp-Leu-His plates were replica-plated onto SD-Trp-Leu-His-Ade plates and harvested after 5 d. Successful candidates were assayed for β-galactosidase activity. Plasmids from β-galactosidase-positive colonies were isolated and transformed into DH5α cells (Invitrogen, Carlsbad, CA). DH5α cells containing Gal4(AD)-cDNA were selected with ampicillin. Plasmids were isolated and sequenced using Gal4(AD) primers that flanked the cDNA insert in conjunction with dideoxy sequencing using an automated fluorescence-based sequencer (Washington State University/University of Idaho Center for Reproductive Biology, Molecular Biology Core). The sequences were then analyzed against the known sequences in GenBank using FASTA search.

Yeast-Two-Hybrid Interaction Screen

Yeast-two-hybrid interaction screens were performed with the yeast strain AH109 (CLONTECH) harboring *HIS3*, *ADE2*, and *β-gal* as reporter genes. Approximately 2 μg of either the pBDGal4 or pADGal4 constructs were introduced into 50 ml of yeast. Cells were transformed using the lithium acetate method and plated on appropriate drop out medium. To identify the expression of the β-gal reporter a liquid culture assay using 2 nitrophenyl β-D-galacto pyranoside as a substrate was followed according to CLONTECH protocol.

RNA Preparation and Northern Analysis

Cultured Sertoli cells were lysed using TRI reagent (Sigma, St. Louis, MO). The lysate was passed through a pasture pipette to form a homogenous lysate. The whole tissue samples were homogenized in a tissue homogenizer in the presence of TRI reagent. The homogenate was centrifuged at $12{,}000 \times g$ for 10 min at 4 C. Total RNA was then isolated from the cell lysate and whole-tissue homogenate following the manufacturer's protocol for RNA isolation using TRI reagent. The final RNA pellet was dissolved in distilled water. Total RNA from Sertoli cells, germ cells, Leydig cells, and peritubular cells were isolated as described earlier using TRI reagent. Approximately 10 μg of RNA were fractionated on a 1.25% formaldehyde-agarose gel. After fractionation, the RNA was transferred onto Nylon membrane (Hybond n +, Amersham, Little Chalfont, UK) using transfer buffer and UV cross linked. The membrane was prehybridized in Church and Gilbert hybridization buffer for 30 min at 60 C. Hybridization was carried out at 60 C for 1 h with ^{32}P-labeled scleraxis probes. The pADgal4 plasmid containing the scleraxis cDNA obtained in the yeast-two-hybrid screen was used to generate random primed probes for Northern blotting. The membrane was subsequently stripped and rehybridized with the constitutively expressed rat ribosomal (S2) to determine loading variability. All probes were labeled using prime-it II kit from Stratagene.

PCR

Total RNA (2 μg) was treated with deoxyribonuclease I for 30 min at 37 C. RNA was reverse transcribed with oligo deoxythymidine (Invitrogen) using Moloney mouse leukemia virus (MMLV) reverse transcriptase (Invitrogen) To distinguish possible contamination of RNA with DNA, the reverse transcription (RT) reaction was performed without MMLV reverse transcriptase. The absence of any product in the amplification reaction indicated the absence of DNA in the RNA sample. All RT reactions were tested for DNA contamination. Approximately 2 μl of the product were subjected to PCR amplification using the following scleraxis primers: scx 5′-GACCGCACCAACAGCGTGAA and scx 3′-GTGGACCCTCCTCCTTCTAACTTC. Each RT reaction was carried out using three different RNA samples. For an internal control, two primers corresponding to the cychlophilin gene were used as a constitutively expressed gene and to monitor the efficiency of the PCR.

The RT-PCR was carried out at 42 C for 1 h. Each PCR contained 250 pg reverse-transcribed DNA, 1 μM of each 5′ and 3′ oligonucleotide primers, 0.5 U *Taq* polymerase and 0.1 mM of each deoxynucleotide triphosphates. The primer pair sequences used were obtained from published sequences of rat cyclophilin, transferrin, ABP and inhibin-α. The primer sequences are: cyclophilin, 5′ primer (ACA CGC CAT AAT GGC ACT GGT GGC AAG TCC ATC) 3′ primer (ATT TGC CAT GGA CAA GAT GCC AGG ACC TGT ATG); rat transferrin, 5′ primer (ATC TGG GAG ATC CTC AAA GTG GCT C) and 3′ primer (GGC ACT AGT CCA CAC TGG CCT GCT A); ABP, 5′ primer (GAC GGA CCC TGA GAC ACA TT) and 3′ primer

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(GAA CAG TCC AGG TTG CAG GT); and inhibin-α, 5′ primer (TCT GAA CCA GAG GAG GAG GA) and 3′ primer (GGC CTG AGC AAG AAC AGA GT). The possible contamination of RNA with DNA was distinguished by performing the RT reaction without MMLV reverse transcriptase. The absence of any product in the amplification reaction using such a reverse-transcribed preparation indicated the absence of any contaminating DNA in our RNA samples. Each RT reaction was performed using three different samples. The PCR-based amplification reactions were carried out in duplicate on each reverse-transcribed RNA sample. Simultaneous PCRs were also carried out using primers designed to rat cyclophilin to monitor the efficiency of the RT-PCRs. Cyclophilin was faithfully amplified in all the PCRs, indicating consistency in the quality of RT and PCRs. The cycle number used was approximately 25–30, so within the linear range and cyclophilin expression assessed as a constitutive gene for data normalization. The identity of the corresponding PCR products was size and the sequence confirmed by the Center for Reproductive Biology Molecular Biology Laboratory.

A two-step real-time PCR was carried out to analyze the expression pattern of scleraxis. RT-PCR was used to generate cDNA from either hormone-treated cultured Sertoli cells, freshly isolated Sertoli cells, or whole testis. Total RNA (2 μg) was reverse-transcribed into cDNA in a reaction primed by oligo deoxythymidine using MMLV reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Reverse and forward oligonucleotide primers, specific to the chosen candidate genes, were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) as described by the manufacturer. The following primers were used: scx 5′-GAC CGC ACC AAC AGC GTG AA and 3′-GTG GAC CCT CCT CCT TCT AAC TTC; ribosomal RNA (S2) 5′-CTG CTC CTG TGC CCA AGA AG and 3′-AAG GTG GCC TTG GCA AAG TT; transferrin 5′-AAA TGG TGC GCA GTG TCA GA and 3′-CTT GCA GAA ATG GCC TTG ATG. Real-time RT-PCR was performed in a 96-well plate using a 7000 ABI prism sequence detection system (Applied Biosystems). The previously synthesized cDNA was used as template. Samples from either cultured Sertoli cells, freshly isolated Sertoli cells, or testes were plated in triplicate PCRs. The PCR contained 5–10 ng of cDNA, 1× SYBR GREEN master mix (Applied Biosystems), and 100 nM of each reverse and forward primers of scleraxis in a final PCR of 50 μl. Amplification parameters were: denaturation at 94 C for 10 min followed by 40 cycles of 94 C for 15 sec; 60 C for 60 sec. Samples were analyzed in triplicate and ribosomal RNA (S2) was used as an endogenous control.

Sertoli Cell Preparation and Culture

Sertoli cells were isolated from 10- and 20-d-old rats by sequential enzymatic digestion procedure described by (58). Decapsulated testis fragments were digested first with trypsin (1.5 mg/ml; Life Technologies, Gaithersburg, MD) to remove the interstitial cells and then with collagenase (1 mg/ml type I; Sigma Chemical Co., St. Louis, MO) and hyaluronidase (1 mg/ml; Sigma). Sertoli cells were plated under serum-free conditions in 24-well Falcon plates (Falcon Plastics, Oxnard, CA) at 1×10^6 cells/well. Cells were maintained in a 5% CO_2 atmosphere in Ham's F-12 medium (Invitrogen) with 0.01% BSA at 32 C. Sertoli cells were left untreated (control) or treated with either FSH (100 ng/ml; ovine FSH-16, National Pituitary Agency), dibutryl cAMP (100 μM), or 10% bovine calf serum. The cells were cultured for up to 72 h. Sertoli cells from 65-d-old rats were isolated by methods previously described (59). All procedures involving animals were approved by the Washington State University Laboratory Animal Research Committee

Transfection Procedure

Sertoli cells cultured for 48 h were transfected with the mammalian reporter and expression vectors by the calcium phosphate method coupled with hyper osmotic shock (10% glycerol) (60). The transferrin luciferase reporter construct contains 581 bp of the mouse transferrin (mTf) promoter that contains the transcriptional initiation site of the transferrin gene, which is 54 bp upstream of the start site of translation (38). The ABP luciferase reporter constructs contain 619 bp of the proximal promoter (11), and both wild-type and E-box response element mutated forms of the reporter construct were also used as previously described (11). The scleraxis expression construct was full-length scleraxis inserted into the *Eco*RI site of pCMV-HA (Promega, Madison, WI). Approximately 1.5 μg of each vector is added to 150 μl of transfection buffer [250 mM NaCl, 50 mM HEPES, and 1.47 mM Na_2HPO_4 (pH 7.05)] and placed in each well of a 24-well plate containing 1×10^6 Sertoli cells. Incubation occurred for 4 h and was followed by a 3-min 10% glycerol shock. Cells were washed twice with Hanks' balanced salt solution (Invitrogen) before adding fresh Ham's F-12. Cells were treated with serum, FSH, or dibutryl cAMP and incubated for 72 h. Transfection efficiency has been shown to be very consistent within a specific experiment by several procedures and indicated in the relatively small error associated with replicates.

Sertoli cells cultured for 48 h were transfected with the luciferase reporter vector as mentioned previously. The transfected Sertoli cells were treated with 2 μM of either the antisense or sense oligonucleotide of scleraxis immediately after transfection. Various treatments were added to the cells after transfection. Continued treatment (1 μM) of either the antisense or sense oligonucleotides was added every 12 h for 72 h. The antisense oligonucleotide to scleraxis was designed to incorporate 20 bases around and including the translational initiation site. The antisense and sense oligonucleotides were synthesized using phosphorothioate modifications at the last three bases from both the 5′ and 3′ ends (MWG, High Point, NC). The sequence for the oligonucleotides were: antisense scleraxis 5′-GGC GAA GGA CAT GGG CCG GGC A-3′ and sense scleraxis 5′-TGC CCG GCC CAT GTC CTT CGC C-3′. Cells were lysed after 72 h with 150 μl of lysis buffer (Promega). Luciferase activity was determined by measuring luminescence of 20 μl of the cell lysate plus 100 μl of luciferase assay reagent for 20 sec in a luminometer (LB96P; Wallac-Berthold, Bad Wildbad, Germany).

Statistical Analysis

The data from quantitative real-time PCR, overexpression analysis, and the antisense procedure were analyzed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). The values were expressed as the mean ± SEM. Statistical analysis was performed using a paired comparison Student's *t* test. Groups were considered significantly different for a two-tailed *P* value if $P \leq 0.05$.

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