Dual role of the basic helix-loop-helix transcription factor scleraxis in mesoderm formation and chondrogenesis during mouse embryogenesis

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SUMMARY

Scleraxis is a basic helix-loop-helix (bHLH) transcription factor shown previously to be expressed in developing chondrogenic cell lineages during embryogenesis. To investigate its function in embryonic development, we produced scleraxis-null mice by gene targeting. Homozygous mutant embryos developed normally until the early egg cylinder stage (embryonic day 6.0), when they became growth-arrested and failed to gastrulate. Consistent with this early embryonic phenotype, scleraxis was found to be expressed throughout the embryo at the time of gastrulation before becoming restricted to chondrogenic precursor cells at embryonic day 9.5. At the time of developmental arrest, scleraxis-null embryos consisted of ectodermal and primitive endodermal cell layers, but lacked a primitive streak or recognizable mesoderm. Analysis of molecular markers of the three embryonic germ layers confirmed that scleraxis mutant embryos were unable to form mesoderm. By generating chimeric embryos, using lacZ-marked scleraxis-null and wild-type embryonic stem cells, we examined the ability of mutant cells to contribute to regions of the embryo beyond the time of lethality of homozygous mutants. Scleraxis-null cells were specifically excluded from the sclerotomal compartment of somites, which gives rise to the axial skeleton, and from developing ribs, but were able to contribute to most other regions of the embryo, including mesoderm-derived tissues. These results reveal an essential early role for scleraxis in mesoderm formation, as well as a later role in formation of somite-derived chondrogenic lineages, and suggest that scleraxis target genes mediate these processes.

Key words: Mouse, Scleraxis, Transcription, Mesoderm, Chondrogenesis, Basic helix-loop-helix

INTRODUCTION

The morphogenic movements and intercellular signals involved in specification of the three embryonic germ layers during gastrulation have been carefully documented in a variety of organisms, but the underlying transcriptional events that control these processes remain largely unknown. During mouse embryogenesis, gastrulation is marked by formation of the primitive streak in the posterior region of the epiblast at embryonic day (E) 6.5. The primitive streak expands anteriorly by progressive recruitment of epiblast cells that ingress and undergo an epithelial-to-mesenchymal transition, giving rise to mesoderm between the epiblast and primitive endoderm (Wilson and Beddington, 1996; Tam and Behringer, 1997). Different mesodermal tissues are subsequently derived from distinct regions of the primitive streak as a consequence of regional signaling.

Fibroblast growth factors (FGFs) and members of the transforming growth factor type-β (TGF-β) family, which includes the bone morphogenetic proteins (BMPs), nodal and activin, have been shown to play important roles in mesoderm induction (Kimelman and Kirschner, 1987; Hogan, 1996; Conlon et al., 1994; Smith et al., 1990). In Xenopus embryos, FGFs and TGF-β synergize to induce mesoderm and, in gastrulating mouse embryos, these factors are expressed in mesoderm-forming regions (Kimelman et al., 1992; Manova et al., 1992; Crossley and Martin, 1995).

Moreover, null mutations in the genes encoding FGF8, FGF receptor 1 (Fgfr1), BMP receptor 1 (Bmpr1) and Smad proteins, which transduce intracellular signals from TGF-β receptors, result in an arrest in mouse development at gastrulation and a lack of embryonic mesoderm or mesodermal patterning defects (Meyers et al., 1998; Lewandoski et al., 1997; Yamaguchi et al., 1994; Mishina et al., 1995; Nomura and Li, 1998; Sirard et al., 1998; Waldrip et al., 1998; Weinstein et al., 1998; Yang et al., 1998). While the peptide growth factors and signaling pathways involved in mesoderm formation have been studied in considerable detail, relatively little is known of the transcription factors that mediate these signals in the gastrulating embryo. In Drosophila, mesoderm formation is controlled by the basic helix-loop-helix (bHLH) protein Twist
Several Twist-related bHLH proteins have been identified in vertebrates, but their potential functions in mesoderm development are unknown and only two have been eliminated in mice by homologous recombination, m-twist and paraxis (Chen and Behringer, 1995; Burgess et al., 1996). In mice homozygous for both the twist and paraxis-null mutations, mesoderm is formed, suggesting either that other related Twist-like factors have redundant functions in mesoderm induction or that mesoderm formation in the mouse does not depend on Twist-like bHLH proteins.

Scleraxis is a Twist-related bHLH protein (Atchley and Fitch, 1997) first identified in mouse as a heterodimeric partner of the ubiquitous bHLH protein E12 (Cserjesi et al., 1995a). The bHLH region of scleraxis is nearly identical to that of the bHLH protein paraxis, which is expressed in primitive streak mesoderm and developing somites (Burgess et al., 1995). Initial in situ hybridization studies revealed scleraxis expression in the sclerotomal compartment of the somites, which gives rise to ribs and vertebrae. Scleraxis expression also marks mesenchymal precursors of axial, appendicular and craniofacial skeletal elements and cells that form tendons, ligaments and bronchial cartilage, and is downregulated in developing bones at the onset of ossification (Cserjesi et al., 1995a). In the chondrocyte-like cell line TC6, scleraxis is upregulated by TGF-β and BMPs and overexpression of scleraxis in the ROS17/2.8 osteosarcoma cell line increases expression of the cartilage markers aggrecan and type II collagen and suppresses expression of the osteoblast markers, type I collagen and alkaline phosphatase (Kawa-uchi et al., 1998; Liu et al., 1996). Scleraxis is a potent transactivator and binds the E-box consensus sequence (CANNTG) as a heterodimer with E12 (Cserjesi et al., 1995a). The aggrecan promoter contains a high-affinity binding site for scleraxis and can be transactivated by scleraxis in vitro (Liu et al., 1997).

To determine the function of scleraxis in embryogenesis, we generated scleraxis-null mice. Here we show that scleraxis mutant embryos fail to gastrulate and do not form mesodermal cells or express mesodermal markers. Consistent with this early phenotype, we show that scleraxis is expressed throughout the embryo at the time of gastrulation before becoming restricted to chondrogenic cells later in development. By creating chimeric embryos containing wild-type and scleraxis mutant cells, we show that scleraxis mutant cells are largely excluded from sclerotome-derived chondrogenic lineages, but contribute to most other cell types in the embryo, including mesodermal tissues. Our results reveal multiple roles for scleraxis in mesoderm formation and cartilage development and suggest that scleraxis mutant embryos lack a functional signaling network for mesoderm induction during gastrulation.

MATERIALS AND METHODS

Targeting of the mouse scleraxis gene

The scleraxis targeting vector was constructed using a 12 kb genomic fragment containing the two coding exons, obtained from a 129SvEv mouse genomic library by screening with a mouse scleraxis cDNA. A Ncol site was introduced by PCR mutagenesis at the translation initiation codon so that a 1.7 kb EcoRI-Ncol fragment upstream of this codon could be ligated to the initiation codon of lacZ in the β-galactosidase reporter gene, pLacF. This scleraxis-lacZ cassette was then subcloned upstream of a neomycin-resistance gene linked to the phosphoglycerokinase (PGK) promoter. A 3 kb BglII-XbaI genomic fragment that extended from the distal 13 residues of exon 1 past the intron and exon 2 was ligated to a thymidine kinase gene under control of the herpes simplex virus promoter to create the 3' vector arm. This cassette was ligated into the S' scleraxis-lacZ/PGKneo cassette to produce the final targeting vector, which was then linearized by digestion with NotI. 25 µg of linearized vector was electroporated into 10^7 cells of two different ES cell lines, AB1 and AB2.2, which were then plated onto G418-resistant, mitotically inactivated STO fibroblasts and cultured in the presence of G-418 (Geneticin, 180 µg/ml of active concentration, GIBCO/BRL) and 0.2 µM FIAU (1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-S-iodouracil) for positive-negative selection, respectively. 400 individual ES cell colonies were isolated from both electroporations, and homologous recombination, detected by Southern analysis, was observed at a frequency of 1:30 from both targeting procedures. Germline transmission was obtained from chimeras produced by the injection of four independent ES clones representing both cell lines into C57Bl/6 blastocysts which were subsequently implanted into pseudopregnant Swiss foster female mice.

Genotyping

ES cell DNA, as well as tail genomic DNA, were screened for the scleraxis targeted mutation by BamHI digestion and Southern blot hybridization with a unique 5' 600 bp BamHI-EcoRI probe external to the region of vector homology. Insertion of pLacF introduced a BamHI site that could be used to distinguish the wild-type and targeted alleles which produced 11 kb and 2.3 kb fragments, respectively. Correctly targeted ES clones were then verified by EcoRV digestion and Southern blot analysis with a unique 3' 500 bp SacI-XbaI probe external to the region of vector homology. In this case, an EcoRV site was introduced by the pLacF replacement and wild-type and targeted alleles produced fragments of approximately 35 kb and 20 kb, respectively. Embryos at E8.5 and earlier were genotyped by PCR amplification of yolk sac DNA or DNAs isolated from embryos from which maternal tissues and Reichert’s membranes were carefully removed. A three-primer PCR strategy was developed where a scleraxis-specific forward primer to a region of the scleraxis gene that was deleted in the homologous recombinant allele and a neomycin cassette-specific forward primer which was present only in the recombinant allele were made. A scleraxis sequence downstream of this region was used to produce a reverse primer that would allow for a product from either allele. Sequences of these primers were as follows:

scleraxis forward primer: 5'–GAGACCGGCGGCGAGAACACC-3'
neomycin forward primer: 5'–GAGGGGAAAGAACCAGCTGG-3'
scleraxis reverse primer: 5'–AATGGGATCTGTGTCTCCAGG-3'

The PCR products for the wild-type and mutant scleraxis loci were 240 bp and 280 bp, respectively.

Histology, in situ hybridization and β-gal staining

Embryos were processed for histological analysis as previously described (Lin et al., 1998). Whole-mount in situ hybridizations of wild-type mouse embryos were performed with digoxigenin-labeled scleraxis RNA antisense probes (Cserjesi et al., 1995a), as described by Hogan et al. (1994). In situ hybridizations to embryo sections were performed with 35S-UTP-labeled probes (Maxiscript, Ambion) as previously described (Edmondson et al., 1994). The following antisense probes were used: Fgfb (Crossey and Martin, 1995), gsc (Blum et al., 1992), brachyury (Wilkinson et al., 1990), HNF3 (Sasaki and Hogan, 1993), ApoE (Basheeruddin et al., 1987), crypt (Dono et al., 1993), Bmp4 (Anson et al., 1995), nodal (Conlon et al., 1994) and H19 (Poirier et al., 1991).

E6.0-7.5 embryos were processed and embedded in paraffin in
littermate groupings and sectioned in 5-7 μm increments. Sections subjected to in situ hybridization with 35S-UTP-labeled probes were counterstained with hematoxylin. Sections stained for lacZ expression were counterstained with nuclear fast red. All others were stained with hematoxylin and eosin or left unstained and just coverslipped with Cytoseal or Vectashield.

lacZ transgene expression was detected in the chimeric embryos by procedures previously described by Cheng et al. (1993). Briefly, whole embryos were fixed for 1 hour in 2% parafomaldehyde-0.2% glutaraldehyde, rinsed in PBS and incubated in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 μM MgCl2 and 1 mg/ml X-gal overnight at room temperature. Embryos were then rinsed in PBS and fixed further in 4% paraformaldehyde.

RT-PCR analysis
Poly(A)+ RNA was isolated (Micro-FastTrack, Invitrogen) from separately pooled mutant and wild-type embryos at various stages and RT-PCR analysis was performed as previously described (Lin et al., 1998). Nested primer sets representing coding regions that spanned the scleraxis intron were produced. The first primer sets used were as follows.
scleraxis forward primer in exon 1:

\[ 5'-CGAGAACACCCAGCCTAAACAGAT-3' \]
scleraxis reverse primer in exon 2:

\[ 5'-ATCACCGCTGTCACTCT-3' \]

These primers yielded a PCR product of 282 bp. The nested primer set used was as follows. scleraxis nested forward primer in exon 1:

\[ 5'-CCTTCTGCCTCAGAACAGC-3' \]
scleraxis nested reverse primer in exon 2:

\[ 5'-GGTCCAAAGTGGGGCTTCGACT-3' \]

These primers yielded a PCR product of 149 bp. Primer sets representing coding regions that spanned introns were also produced for analysis of marker genes expressed in scleraxis mutant and wild-type embryos. L7 was used as a control for RNA integrity (Hollenberg et al., 1993). (Sequences will be provided upon request.) Reverse transcriptase (Superscript II, Gibco-BRL) and random hexamer primers (Roche) at 200 ng/20 μl reaction volume were used to produce cDNA from poly(A)+ RNA. Due to the low quantities of RNA produced from scleraxis mutants, quantitative analysis could not be performed. PCR amplification and the resultant products from 30 cycles were analyzed by agarose gel electrophoresis and nested PCR amplification was performed when necessary.

Generation of lacZ-expressing scleraxis+/− ES cells and chimera analysis
Scleraxis heterozygous mice were mated with heterozygous scleraxis mice carrying one copy of the lacZ transgene ROSA 26 (Friedrich and Soriano, 1991). Blastocysts were collected and ES cells were derived as described by Robertson (1987). ES cells were genotyped by Southern blot analysis of EcoRI-digested genomic DNA, using the lacZ gene as probe. Both the targeted scleraxis allele and the ROSA 26 transgene insertion contain the lacZ gene so that the probe hybridized to a 2.4 kb scleraxis lacZ-containing fragment and to a 2.8 kb ROSA 26 lacZ-containing fragment (data not shown). This ROSA-26+/−/Scleraxis+/− cell line was in a Swiss/129/SV × C57Bl/6/129/Sv genetic background. These cells were used to generate control chimeric embryos. A gene targeting strategy was used to produce a Rosa-26+/−/Scleraxis−/− ES cell line. This targeting vector was constructed with a PGK-hygromycin cassette to replace exon 1 at the scleraxis locus and to be used for positive selection. The 5′ homologous arm consisted of a 1 kb EcoRI-BglII genomic fragment and the 3′ arm and thymidine kinase expression cassette were identical to that of the first targeting vector described above. Positive and negative selection were used to screen for the second targeting event with 120 μg/ml hygromycin C (Roche) and 0.2 μM FIAU. Targeted ES cells were selected and isolated and Southern blot analysis confirmed that both scleraxis loci were mutant. A HindIII site was introduced by the hygromycin replacement whereby after genomic ES cell DNA was digested with HindIII, the resultant fragments would identify the scleraxis wild-type locus to be 11 kb and the lacZ/neomycin and hygromycin-targeted loci to be 6 kb and 3.4 kb, respectively, when probed with the 5′ BamHI-EcoRI scleraxis genomic probe used previously. Targeting was verified by BamHI digestion and Southern blot analysis using the 3′ SacI-XbaI probe used previously. In this case, the scleraxis wild-type locus produced a 12 kb fragment while the lacZ/neomycin and hygromycin targeted loci produced 11 kb and 14 kb fragments, respectively. Scleraxis+/− and scleraxis−/− ES cells were injected into Swiss or C57Bl/6 wild-type blastocysts.

BrdU labeling of embryos and TUNEL assay
BrdU labeling of cells was performed as previously described (Hayashi et al., 1988). Pregnant females at E6.0-6.5 were injected intraperitoneally with BrdU at 500 mg/kg body weight and embryos were collected 1 hour later. Embryos were prepared for histology in litter groupings as described above. A combined TUNEL/BrdU immunohistochemical procedure was then performed. Briefly, sections were deparaffinized and rehydrated and then incubated in Proteinase K (20 mg/ml in PBS) for 15 minutes at 37°C. After washing with PBS, slides were incubated with 0.3% Triton/PBS for 10 minutes at room temperature and then covered with DAB buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 5 minutes at room temperature. The TUNEL reaction was performed using 0.3 units terminal deoxynucleotransferase (Gibco-BRL)/μl DAB buffer and a 1:50 biotin-dCTP (Gibco-BRL) dilution for 60-90 minutes at 37°C. The reaction was terminated with stop buffer (300 mM NaCl, 30 mM sodium citrate) and then the specimen were incubated for 30 minutes in FITC/Streptavidin (Roche) in a 1:90 dilution. After four rinses in PBS, sections were incubated with anti-BrdU antibody (Roche) at a 6 μg/ml dilution using standard immunohistochemical staining procedures. The secondary antibody used for fluorescent detection of BrdU incorporation was anti-mouse Rhodamine Red or anti-mouse Texas Red (Vector Laboratories) and after several washes, each specimen was coverslipped with Vectashield.

Production and analysis of embryoid bodies derived from wild-type and scleraxis+/− ES cells
Embryoid bodies were produced as previously described (Doetschman et al., 1985; Robertson, 1987) and were collected after 7, 11 and 14 days of culture. Poly(A)+ RNA was isolated from each collection (Micro-FastTrack, Invitrogen), and RT-PCR analysis was performed as previously described (Lin et al., 1998). Reverse transcriptase (Superscript II, Gibco-BRL) and random hexamer primers (Roche) at 200 ng/20 μl reaction volume were used to produce cDNA from 500 ng embryoid body poly(A)+ RNA. 2 μl of each cDNA reaction was used for PCR amplification and the resultant products from 30 cycles were analyzed by agarose gel electrophoresis.

RESULTS
Targeting the scleraxis gene
To define the function of scleraxis during mouse embryogenesis, we inactivated the scleraxis gene by homologous recombination in ES cells. A targeting vector was constructed to replace the first exon, encoding the bHLH
region, with lacZ and a neomycin-resistance cassette (Fig. 1).

Two different ES cell lines were electroporated with the targeting vector and subjected to positive-negative selection; both demonstrated a targeting frequency of 1:30 by Southern blot analysis (Fig. 1B). Multiple targeted clones from both cell lines were injected into C57BL/6 blastocysts to produce chimeras, four of which transmitted the mutation through the germline. Wild-type and mutant alleles were identified by Southern hybridization to unique BamHI or EcoRV fragments or by PCR, using primers that spanned the deleted region of the gene (Fig. 1C).

Germline mutant mice were produced in two mixed genetic backgrounds, C57BL/6 × 129/SvEv and Swiss × 129/SvEv. RT-PCR and whole-mount in situ hybridization strategies confirmed the lack of scleraxis transcripts in mutant embryos (Fig. 3 and data not shown, respectively).

Loss of scleraxis results in recessive lethality at the time of gastrulation

Mice heterozygous for the scleraxis mutant allele showed no phenotypic abnormalities and were bred to obtain homozygous null offspring. Bone and cartilage staining with alizarin red and alcian blue, respectively, confirmed that skeletal development was normal in heterozygotes (data not shown). We failed to detect null neonates by Southern blot analysis from greater than 300 offspring of heterozygous intercross matings, indicating that the homozygous null mutation resulted in embryonic lethality (Table 1). We therefore examined the morphologies and determined the genotypes of embryos from staged litters beginning at E6.0 (Fig. 2). Litters from heterozygous intercrosses up to E8.5 contained the mutant gene at the predicted Mendelian ratios, as determined by PCR of genomic DNA. There were no observable phenotypic differences between mutants produced from the two different ES cell lines or from the two mouse strains used to produce germline heterozygotes, leading us to believe the null phenotype was not a result of a second site mutation.

Scleraxis mutant and wild-type embryos collected at the early egg cylinder stage (E6.0) were indistinguishable in size and morphology (Fig. 2A). However, mutant embryos did not develop beyond this stage and were readily identifiable by growth-retardation at E6.5 and E7.5 (Fig. 2B,C). By E8.5, homozygous mutants appeared to be resorbing or degenerating and lacked distinct features (data not shown). No homozygous mutants were detected after E8.5.

By histological analysis, mutant embryos appeared to develop normally up to E6.0 and contained embryonic and extraembryonic structures and proamnionic cavities (data not shown). However, by E6.5, the simple egg cylinder of the
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Mutants failed to elongate and the extraembryonic regions appeared to be underdeveloped when compared to wild-type littermates (Fig. 2D-G). In addition, mutant embryos contained abnormal pyknotic cells (Fig. 2F,G) and did not form a primitive streak or histologically recognizable mesoderm, which normally forms between the embryonic ectoderm and visceral endoderm. The absence of mesoderm did not simply reflect a developmental delay because the few mutant embryos that survived to E7.5 still had not formed mesoderm. A cavitated extraembryonic region also failed to form in the majority of observed mutants. The ectoplacental cones of the mutants were proportionately correct in size, but appeared to degenerate after E6.5 (data not shown).

During normal development, the visceral endoderm surrounds the embryo and at E5.5 appears columnar or cuboidal. As the embryo elongates and forms definitive embryonic and extraembryonic regions, endodermal cells in the distal portion surrounding the embryonic ectoderm become squamous in shape, while the extraembryonic endodermal cells remain cuboidal. The visceral and parietal endoderm layers were present in mutant embryos at E6.5 and E7.5, but appeared developmentally delayed and failed to form the flattened endodermal cell phenotype in the embryonic region (Fig. 2F,G).

We conclude that scleraxis mutant embryos develop normally until E6.0-6.5 and form embryonic and extraembryonic structures characteristic of the early egg cylinder stage. However, by the early primitive streak stage, when gastrulation normally occurs, mutant embryos become arrested and mesodermal cells fail to form.

Scleraxis is expressed throughout mouse embryogenesis

In previous in situ hybridization studies of sectioned mouse embryos, scleraxis expression was first detected between E9.5 and E10.5 in the sclerotomes of the somites and subsequently in developing chondrogenic cell lineages throughout the embryo (Cserjesi et al., 1995a). Because scleraxis mutant embryos died prior to these stages, we further analyzed scleraxis expression at various preimplantation and postimplantation embryonic stages.

We initially attempted to examine scleraxis expression by...
staining \textit{scleraxis}+/- embryos for \textit{lacZ} activity, but there was no detectable expression of the \textit{lacZ} gene that had been inserted in-frame with the initiation codon of the targeted \textit{scleraxis} allele. If the original heterozygous ES cell clones were allowed to differentiate into embryoid bodies in vitro, \textit{lacZ} was expressed, indicating that the \textit{lacZ} allele was potentially functional (data not shown). Conceivably, the deletion mutation introduced into the \textit{scleraxis} gene disrupted \textit{cis}-regulatory elements essential for expression in vivo.

We also examined \textit{scleraxis} expression by RT-PCR using nested primer sets to detect \textit{scleraxis} cDNAs produced by reverse transcriptase and random primers in assays of RNAs isolated from early stage wild-type mouse embryos. Transcripts were detected in blastocysts at E3.5, as well as in E6.5 (Fig. 3), E7.5, E9.5 and E11.5 embryos (data not shown). \textit{Scleraxis} mutant embryos failed to show expression of transcripts that would have been produced from sequences left unaltered in the genome of the targeted allele.

To analyze the spatial expression pattern of \textit{scleraxis} in early embryos, whole-mount in situ hybridizations were performed with several probes representing different regions of the \textit{scleraxis} transcript. At E6.0-6.5, \textit{scleraxis} appeared to be expressed throughout the egg cylinder, with highest expression in the epiblast (Fig. 4A,B). By E7.5, \textit{scleraxis} transcripts became restricted to embryonic and extraembryonic mesoderm, particularly in the posterior/proximal/lateral regions (Fig. 4C) and was detected at an earlier stage in the mesodermal wings forming from the primitive streak (data not shown). Between E8.5 and E9.0, \textit{scleraxis} transcripts were weakly detected in the branchial arches, the frontonasal region and along the paraxial mesoderm and somitic regions (Fig. 4E,G). Whole-mount in situ hybridization also confirmed the restricted expression of \textit{scleraxis} to chondrogenic precursors of the axial and appendicular skeletons and connective tissues such as tendons and ligaments from E11.5-14.5 (Fig. 4D,F,H). None of the \textit{scleraxis}-specific probes detected \textit{scleraxis} transcripts in mutant embryos (not shown).

Together, these observations demonstrated that \textit{scleraxis} was expressed at a time consistent with the early embryonic lethality observed in mutant embryos. Although \textit{scleraxis} is expressed as early as E3.5, it is apparently dispensible for embryonic development prior to E6.0. The lack of \textit{scleraxis} transcripts in mutant embryos also confirmed that the targeted \textit{scleraxis} allele was unable to produce functional \textit{scleraxis} transcripts.

\textbf{Scleraxis mutant embryos fail to form mesoderm}

Given the apparent absence of mesodermal cells in \textit{scleraxis} mutant embryos, we used in situ hybridization and RT-PCR to examine expression of mesodermal markers in the mutants. \textit{Fgf8} is one of the earliest markers of primitive streak formation in the mouse (Crossley and Martin, 1995); it is detected initially in the epiblast prior to streak formation.
and subsequently throughout the primitive streak as the embryo proceeds through gastrulation. Fgf8 expression was detected by RT-PCR (Fig. 5) and in situ hybridization in wild-type embryos at E6.5 (Fig. 6A) and E7.5 (data not shown), whereas transcripts were not detected by either technique in mutants at these stages (Figs 5, 6A). Transcripts for three other primitive streak and mesodermal markers, brachyury (T), goosecoid (gsc), and cripto (Herrmann, 1991; Blum et al., 1992; Dono et al., 1993), were also undetectable in scleraxis-null embryos (Figs 5, 6A). Cripto gene expression is very similar to that of Fgf8, where early expression is seen in the epiblast at the region of primitive streak formation and subsequent expression is detected in the primitive streak and newly forming embryonic mesoderm (Dono et al., 1993). The lack of cripto expression in scleraxis null embryos confirms the observation that scleraxis mutants are unable to show even the earliest signs that gastrulation could be initiated. The same results were obtained by whole-mount in situ hybridization (not shown), but because of fragility of mutant embryos, it was difficult to maintain their morphologic integrity through the procedure. These results demonstrate that scleraxis is essential for induction of cellular and molecular markers of mesoderm.

Expression of primitive endodermal and embryonic ectodermal markers in mutant embryos

To determine whether scleraxis was required specifically for mesoderm formation, we examined expression of several primitive endoderm and embryonic ectodermal markers in mutant embryos. HNF3β, ApoE and HNF4, which are specific for endoderm (Filosa et al., 1997; Basheeruddin et al., 1987; Duncan et al., 1994), were expressed in appropriate regions and at normal levels in mutant embryos at E6.5 (Figs 5, 6B). Similarly, Bmp-4 was expressed in wild-type and mutant embryos in a region of the extraembryonic ectoderm positioned above the presumptive origin of the primitive streak (Fig. 6C). Bmp-4 expression was also observed in the most posterior mesodermal cells produced from the streak in wild-type embryos, but not in mutants, supporting the conclusion that mesodermal cells are selectively missing in the mutant (Figs 5, 6C). In addition, nodal expression was detected in the embryonic ectoderm and overlying visceral endoderm in mutant and wild-type embryos (Figs 5, 6C). It is interesting to note that, even though nodal signaling in the ectoderm is believed to be required for primitive streak formation (Varlet et al., 1997) and BMP-4 for epiblast cell proliferation and mesodermal differentiation (Mishina et al., 1995; Winnier et al., 1995), the expression of these two genes in scleraxis mutants is not sufficient to support these processes. Previous studies have demonstrated that the cripto transcript is detected in the embryonic ectoderm in E5.5-6.5 embryos (Dono et al., 1993). However, we did not detect cripto expression in this region in either the wild-type or scleraxis mutant embryos. This may be due to low levels of expression. Cripto expression is known to be reduced in ectodermal cells at gastrulation and then upregulated in the newly formed embryonic mesoderm (Dono et al., 1993). Transcripts from the H19 gene (Fig. 6C), which mark extraembryonic cell types such as the ectoplacental cone, extraembryonic ectoderm and extraembryonic endoderm (Poirier et al., 1991), were expressed correctly in mutant embryos, confirming that these cell types were not significantly affected. The eHAND gene, which encodes a bHLH protein expressed in extraembryonic membranes and ectoplacental cone (Cserjesi et al., 1995b), was also expressed normally in scleraxis mutants (data not shown). The ubiquitously expressed transcript L7 was used as a control for RNA integrity (Fig. 5).

We conclude from these marker analyses that scleraxis is specifically required for mesoderm, but not for endoderm or ectoderm formation.

Scleraxis mutants show altered growth properties at gastrulation

The inability of scleraxis−/− egg cylinders to elongate suggested that cell proliferation was decreased or halted in mutant embryos. To examine cellular growth properties of postimplantation mutant embryos in vivo, we analyzed incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA at the onset of gastrulation (E6.5). Pregnant females from scleraxis+/− intercrosses were injected with BrdU and, after 1 hour, embryos were collected and analyzed histologically. BrdU was incorporated into the majority (>80%) of nuclei in wild-type embryos (Fig. 7B,F). In contrast, little or no BrdU labeling was detected in mutant embryos at this stage (Fig. 7D).

To determine whether enhanced apoptosis contributed to growth retardation of scleraxis mutant embryos, we end-labeled fragmented DNA with biotinylated dCTP by the TUNEL assay. As shown in Fig. 7, there was a significant increase in apoptosis in mutant compared to wild-type embryos at E6.5. These results suggest that a rapid decline of proliferative activity and an increase in cell death in the epiblast at the time of egg cylinder elongation prevents scleraxis mutant embryos from achieving the cell numbers required for initiation of gastrulation. Thus, scleraxis appears to be essential for proliferation and viability of embryonic cells beginning at E6.0, whereas prior to that time proliferation occurs normally in mutant embryos. However, when blastocysts produced from scleraxis heterozygous crosses were cultured in vitro, the mutant colonies were indistinguishable from the wild-type in these culture conditions (data not shown).

Generation of mouse chimeras composed of scleraxis mutant and wild-type cells

The early embryonic lethality of scleraxis mutant embryos precluded an analysis of the potential role of scleraxis during later embryonic stages. In particular, it was of interest to determine whether scleraxis was important for cartilage development, given its restricted expression to chondrogenic precursors in the sclerotomes of the somites and axial, appendicular, and craniofacial skeletal elements after E9.5. To address these issues, we analyzed chimeric embryos generated from scleraxis-null and wild-type cells. For these studies, we first crossed scleraxis+/− mice with the Rosa 26 gene trap insertion line, which harbors a lacZ marker gene expressed ubiquitously during embryogenesis (Friedrich and Soriano, 1991). Scleraxis+/− ES cells hemizygous for the lacZ marker were then established from blastocysts. Homozygous scleraxis mutant cells were generated by a second round of targeting to inactivate the wild-type scleraxis allele in scleraxis+/− ES cells (see Materials and Methods). ES cells from wild-type and scleraxis−/− lines were used to generate chimeric embryos, which were isolated at E8.5, E9.5 and E16.5, stained for lacZ expression, and completely serially sectioned for analysis.

Injection of lacZ-marked scleraxis−/− ES cells into wild-type
blastocysts resulted in chimeric embryos with extensive $\text{lacZ}$ expression throughout ectoderm, endoderm and mesoderm-derived tissues (Fig. 8A,B). However, there was a marked deficiency in the ability of mutant cells to contribute to the sclerotomal compartment of the somites at E8.5 and E9.5 (Fig. 8C-F) and ribs at E14.5 (not shown) and E16.5 (Fig. 8G), while wild-type cells contributed readily to these structures (Fig. 8I). In contrast, mutant cells readily contributed to the dermomyotome and myotome of the somites, as well as to all other cell types examined, including developing cartilage and bone in the limbs at E16.5 (Fig. 8C-F,H,J).

As shown in Fig. 8C,E,F, $\text{lacZ}$-positive cells contributed to the epithelial layer of the dermomyotome, as well as the myotome, of the somite, while the sclerotomal region was essentially devoid of $\text{lacZ}$-positive cells. As the somite matures, cells from the rostral half migrate ventrally, giving rise to a triangular pattern at the somitic junction (Christ and Ordahl, 1995). The caudal half of the somite at this stage is composed of sclerotomal cells. The sagittal section shown in Fig. 8D demonstrates the exclusion of $\text{lacZ}$-marked $\text{scleraxis}^{-/-}$ cells from the caudal sclerotomal region of the somite, whereas the rostral region populated by muscle cells is extensively labeled. Wild-type cells contributed to all major embryonic regions examined, including sclerotomes (data not shown) and developing ribs (Fig. 8I), and showed extensive mixing with cells derived from the recipient blastocysts.

In chimeric embryos containing approximately 50% mutant cells, embryonic development appeared to proceed normally until at least E16.5, indicating that the normal cells rescued viability. In two chimeric embryos at E9.5, in which greater than 95% of cells were mutant, the heart developed to the early looping stage and the neural tube was formed. However, there was a complete absence of discernable somites and the somitic region contained a deficiency of cells. As a result, the dorsal aortae were dilated to fill the space (data not shown). Since no homozygous null embryos developed to this stage, this suggests that the few wild-type cells in this embryo were able to partially rescue the developmental block of the mutant or that the host blastocyst, which supplies trophoblasts, chorion and ectoplacental cone, was able to sustain the embryo to this stage of development.

From these analyses, we conclude that although $\text{scleraxis}^{-/-}$ embryos cannot progress through gastrulation or form mesoderm, in the presence of wild-type cells, $\text{scleraxis}^{-/-}$ cells can contribute to mesoderm-derived tissues with the exception of sclerotomal derivatives. Thus, the effect of the $\text{scleraxis}$ mutation on mesoderm formation is non-cell autonomous, whereas its effect in the sclerotome is cell autonomous.

**$\text{Scleraxis}^{-/-}$ ES cells behave normally in culture**

The chimera analyses suggested that $\text{scleraxis}$ mutant cells had the potential to form mesoderm in the appropriate environment and therefore did not contain
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an intrinsic defect in mesoderm specification. To further investigate the potential of scleraxis<sup>-/-</sup> cells to form mesoderm, ES cells homozygous for the scleraxis null mutation and hemizygous for the Rosa 26 gene trap allele were allowed to form embryoid bodies in vitro and expression of mesodermal markers was examined. As shown in Fig. 9, the mesodermal markers, brachyury, cripto and fgf8 (data not shown), which were not expressed in scleraxis mutant embryos in vivo, were expressed in mutant embryoid bodies in vitro at levels comparable to wild-type embryoid bodies.

Similar to what was observed in our analysis of cultured blastocysts, scleraxis mutant and wild-type ES cells formed embryoid bodies that were indistinguishable in size and histological characteristics (data not shown). These results support the conclusion that scleraxis mutant cells are not intrinsically defective in the ability to proliferate or form mesoderm and indicate that the environment of scleraxis mutant embryos at the time of gastrulation is incompatible with these cellular functions.

**DISCUSSION**

The results of this study provide the following insights into the functions of scleraxis during embryogenesis. (1) Scleraxis is required for gastrulation and mesoderm formation, but not for endoderm or ectoderm formation. (2) In chimeric embryos, scleraxis mutant cells are largely excluded from sclerotome-derived chondrogenic cell lineages, indicating an essential cell autonomous role for scleraxis in this chondrogenic developmental pathway. (3) Scleraxis mutant cells can contribute to most mesoderm-derived tissues in the presence of wild-type cells in vivo and can form mesoderm in embryoid bodies in vitro, revealing a non-cell autonomous role of scleraxis in mesoderm formation during gastrulation.

**Scleraxis is essential for mesoderm formation**

Gastrulation begins at E6.5 in the mouse as the primitive streak forms from embryonic ectoderm in the proximal-posterior region of the epiblast (Tam and Behringer, 1997). The primitive streak then extends anteriorly to the distal tip of the egg cylinder and mesoderm forms by ingression of epiblast cells through the streak. Specification of mesodermal cells during gastrulation occurs in response to signaling by FGFs and TGF-β or their related molecules (BMP, activin, etc) (Kimelman and Kirschner, 1987).

The phenotype of scleraxis mutant embryos reveals an essential role for scleraxis in mesoderm formation during gastrulation. Scleraxis mutants first began to show defects after implantation, just prior to egg cylinder elongation (E6.5). At this stage, cell proliferation abruptly ceased and apoptosis, measured by TUNEL activity, strikingly increased in mutant embryos. Epiblast cell numbers increase rapidly from approximately 250 cells in E6.0 prestreak mouse embryos to 3300 cells in E7.0 midstreak embryos (Snow, 1977). Without an accumulation of 1400-1500 cells, gastrulation will not commence (Power and Tam, 1993). Our results demonstrate that scleraxis is not required for cell proliferation up to the egg cylinder stage (E6), but thereafter, cells of scleraxis mutants ceased to divide and began to die between E6.0 and E6.5 before these critical numbers could be achieved. We conclude that scleraxis is required for embryonic...
growth during egg cylinder elongation such that homozygous mutant embryos die before they can progress through gastrulation.

How might the obligatory role of scleraxis in gastrulation and mesoderm formation be explained? At the time of gastrulation, scleraxis is expressed throughout the endodermal and ectodermal layers of the embryo. It is possible that scleraxis acts at this stage to regulate expression of a signaling molecule required for mesoderm induction. In this regard, FGF8, which is expressed in the primitive streak and is...
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Fig. 9. Detection by RT-PCR of mesoderm transcripts in cultured embryoid bodies. Transcripts for the indicated genes were detected by RT-PCR of RNA isolated from wild-type and scleraxis mutant embryoid bodies following culture for 7 or 14 days, as indicated. The mesodermal markers cripto and brachyury were expressed comparably in wild-type and mutant embryoid bodies. The upper bands in the mutant brachyury lanes were produced from the initial primer set used in the nested RT-PCR strategy. L7 transcripts were detected as an internal control. RT-PCR reactions performed in the absence of reverse transcriptase (–RT) failed to detect either transcript. PCR primers spanned introns and yielded the products of the predicted size for the corresponding transcripts. The image is a negative of an ethidium-bromide-stained gel. Markers are to the right.

essential for mesoderm formation (Meyers et al., 1998), was not expressed in scleraxis mutants. It will be of interest to determine whether fgf8 is a target gene for scleraxis, once the regulatory elements that control fgf8 expression in the gastrulating mouse embryo are identified. Scleraxis might also function downstream in a signal transduction pathway for mesoderm induction. Alternatively, scleraxis could be required indirectly for expression of genes essential for migration of epiblast cells through the primitive streak during gastrulation. While scleraxis is required for embryo growth at the egg cylinder stage, it is not required for cell proliferation per se, because scleraxis mutant cells proliferated normally prior to E6.0 and embryoid bodies derived from scleraxis mutant cells grew normally in vitro as did cultured scleraxis-null blastocysts. Nevertheless, we cannot rule out the possibility that scleraxis is essential for proliferation in vivo or for other events necessary for embryonic progression, such as cell-cell interactions or adhesion.

Despite the inability of scleraxis mutant embryos to form mesoderm at gastrulation, scleraxis mutant cells could contribute to mesoderm in chimeric embryos with wild-type cells. Similarly, when scleraxis mutant ES cells were cultured in growth media and allowed to form embryoid bodies, mesodermal markers were expressed normally. These results demonstrate that the block to mesoderm formation in mutant embryos is not cell autonomous and that mutant cells can become mesoderm in the appropriate environment. A possible explanation for these findings is that signals from wild-type cells (FGF8 or BMPs perhaps?) can rescue the ability of mutant cells to enter mesodermal cell lineages.

Similarity of scleraxis mutants and mutants lacking components of mesoderm-inducing pathways

Several mouse mutants lacking components of the TGF-β and FGF signaling pathways have been shown to exhibit defects in gastrulation and mesoderm formation similar to those of scleraxis mutants. The scleraxis mutant phenotype closely resembles that of Bmpr1 mutant embryos, which arrest at E6.5, fail to form mesoderm and show a block in cell proliferation and enhanced apoptosis in the egg cylinder (Mishina et al., 1995). Likewise, Smad2 and Smad4 mutant mice also arrest during the early egg cylinder stage and fail to form embryonic mesoderm (Nomura and Li, 1998; Weinstein et al., 1998; Sirard et al., 1998; Yang et al., 1998), consistent with the role of Smads as transducers of BMP signals. Like scleraxis mutants, Smad4 mutant embryos also show a defect in proliferation of epiblast cells (Sirard et al., 1998). The similarity in phenotypes of scleraxis mutant embryos and embryos lacking components of the TGF-β signaling pathway is also consistent with previous studies demonstrating that scleraxis expression is regulated by TGF-β and BMPs in cells in culture (Liu et al., 1996, 1998; Katagiri et al., 1994; Kawauchi et al., 1998).

Fgf8 mutant embryos and embryos lacking serum response factor, which mediates transcriptional responses to FGF signaling, are also unable to form mesoderm and exhibit a phenotype similar to scleraxis mutants (Meyers et al., 1998; Arsenian et al., 1998; Lewandoski et al., 1997). Whether scleraxis regulates expression of components of the FGF pathway or functions downstream in that pathway remains to be determined.

A cell autonomous role for scleraxis in the developing sclerotome

Somites form as paired epithelial spheres by budding off from the anterior end of the segmental plate mesoderm (Christ and Ordahl, 1995). The sclerotome, which serves as a source of chondrogenic precursors for the axial skeleton, then forms by delamination of the ventral region of epithelial somites in response to signaling from the notochord. The dorsal portion of the somite remains epithelial and gives rise to the dermomyotome, the source of skeletal muscle cells for the body. Scleraxis is expressed throughout the ventral sclerotome at E9.5 and subsequently in developing vertebrae and ribs, which are derived from the sclerotome, as well as in long bones and craniofacial cartilage, which are derived from other mesenchymal precursor cells (Cserjesi et al., 1995).

The early embryonic lethality of scleraxis mutant embryos prevented analysis of the potential role of scleraxis in cartilage formation, which does not begin until about E9.5. However, by assessing the ability of lacZ-marked scleraxis mutant ES cells to contribute to the embryo, we found that cells lacking scleraxis were specifically excluded from the sclerotomal region of the somites, which serves as the precursor pool for the axial skeleton. In contrast, scleraxis+ cells contributed extensively to the dermomyotome and myotome. These results indicate an essential role for scleraxis in development of certain chondrogenic cell lineages. This requirement for scleraxis appears to be specific for sclerotomal derivatives because scleraxis mutant cells contributed to cartilage in the limbs and
craniofacial region. Scleraxis could be required for specification of sclerotomal cell identity, for acquisition of a mesenchymal phenotype or for activation of downstream genes during differentiation of sclerotomal derivatives, as suggested by its ability to activate the aggrecan promoter in vitro (Liu et al., 1997).

**Control of mesoderm formation by bHLH transcription factors**

Several twist-like bHLH proteins are expressed in developing mesoderm during mouse embryogenesis. Paraxis is coexpressed with scleraxis in the primitive streak, before becoming restricted to developing somites (Burgess et al., 1995). **Paraxis** mutant mice show normal primitive mesoderm development, but fail to form epithelial somites (Burgess et al., 1996). Twist and Dermo-1 are coexpressed in anterior-lateral mesoderm subjacent to the head folds, as well as in somites, body wall and limb mesenchyme (Gitelman, 1997; Fuchtbauer, 1995; Li et al., 1995). Mesoderm development also occurs normally in twist mutant mice (Chen and Behringer, 1995). Scleraxis is the only bHLH factor shown thus far to be essential for mesoderm formation in the mouse. The apparent requirement for scleraxis during mesoderm formation is intriguing, in light of the requisite role of Twist in mesoderm formation in *Drosophila*. Whether scleraxis plays a role analogous to Twist in *Drosophila* or whether its actions in mesoderm induction are less direct will require identification of its target genes in the gastrulating embryo.

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