The winged helix transcription factor MFH1 is required for proliferation and patterning of paraxial mesoderm in the mouse embryo

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The gene mfh1, encoding a winged helix/forkhead domain transcription factor, is expressed in a dynamic pattern in paraxial and presomitic mesoderm and developing somites during mouse embryogenesis. Expression later becomes restricted to condensing mesenchyme of the vertebrae, head, limbs, and kidney. A targeted disruption of the gene was generated by homologous recombination in embryonic stem cells. Most homozygous mfh1 null embryos die prenatally but some survive to birth, with multiple craniofacial and vertebral column defects. Using molecular markers, we show that the initial formation and patterning of somites occurs normally in mutants. Differentiation of sclerotome-derived cells also appears unaffected, although a reduction of the level of some markers [e.g., mtwist, mfl, scleraxis, and α1(II) collagen] is seen in the anterior of homozygous mutants. The most significant difference, however, is a marked reduction in the proliferation of sclerotome-derived cells, as judged by BrdU incorporation. This proliferation defect was also seen in micromass cultures of somite-derived cells treated with transforming growth factor β1 and fibroblast growth factors. Our findings establish a requirement for a winged helix/forkhead domain transcription factor in the development of the paraxial mesoderm. A model is proposed for the role of mfh1 in regulating the proliferation and differentiation of cell lineages giving rise to the axial skeleton and skull.

Key Words: Mouse embryogenesis; winged helix, transcription factor; proliferation; patterning; paraxial mesoderm

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A major challenge in vertebrate embryology is to elucidate the mechanisms by which mesodermal cells acquire and achieve their wide-ranging developmental fates. Mesoderm specification is tightly linked with the process of gastrulation, in which epiblast cells enter the primitive streak and become allocated to different mesoderm populations. Midline cells give rise to the prechordal plate and notochord, paraxial mesoderm cells generate the unsegmented mesoderm of the head and paired somites on either side of the neural tube, and the lateral mesoderm forms the splanchnopleure and somatopleure. The paraxial mesoderm contributes extensively to many adult tissues, including most of the axial skeleton, the muscles of the trunk, and the dermis of the skin, as well as to specific skull bones and muscles of the head and neck. Mutations that affect paraxial mesoderm development are therefore likely to have considerable effects on body form (for review, see Tam and Trainor 1994).

The formation of somites begins at the rostral end of the presomitic mesoderm, a population of multipotent cells generated from either the primitive streak or the tail bud mesenchyme. Fate mapping and orthotopic transplantation studies in the mouse have shown that cells in the primitive streak of 7.5 and 9.5 days post coitum (dpc) embryos tend to colonize the more anterior somites (up to about somite 21), whereas tail bud mesenchyme normally contributes to posterior somites (Bedington 1981, 1982; Tam and Trainor 1994; for review, see Smith et al. 1994). In either case, newly formed somites are generated by the compaction and epithelialization of loosely associated mesenchymal units known as somitomeres, and become patterned along their anteroposterior, dorsoventral, and mediolateral axes. The ventromedial region of the somite forms the sclerotome, whereas the dorsolateral domain gives rise to the epaxial dermomyotome. Recent in vitro studies have provided evidence that dorsovenricular and mediolateral patterning are under the influence of local signaling molecules from the notochord, surface ectoderm, neural tube, and lateral mesoderm (Fan and Tessier-Lavigne 1994; Fan et al. 1995; Pourquie et al. 1995, 1996).

Following induction by signals from the notochord...
and ventral neural tube, sclerotome cells proliferate and migrate toward the notochord, giving rise to a medioventral domain that will form the vertebral body and intervertebral discs, a ventrolateral domain that forms the ribs, and a ventromedial domain that gives rise to the neural arches and the pedicles (Verbout 1985; Johnson 1986; Christ and Ordahl 1995; Pourquie et al. 1996). Lineage tracing and orthotopical transplantation experiments in chick embryos have also shown that within each developing somite, sclerotomal cells segregate into rostral and caudal domains, expressing different genes and with looser and more compact cell densities, respectively (Sterne and Keynes 1987; Norris et al. 1989; Ranscht and Bronner-Fraser 1991). Moreover, cell labeling studies have shown that each vertebra is derived from the caudal half of one somite, and the rostral half of the posteriorly adjacent somite (Bagnall 1992), a finding consistent with the theory of sclerotomal resegmentation proposed by Remak in 1855 (Verbout 1985; Bagnall 1992).

Intense genetic and molecular analysis has led to the identification of many genes expressed in subpopulations of the dermomyotome of the somite and required for the development of specific muscle groups derived from them (Bober et al. 1994; Buffinger and Stackdale 1994; Olson and Klein 1994; Christ and Ordahl 1995; Pourquie et al. 1995, 1996). However, much less is known about the genes regulating the patterning of the sclerotome, and the proliferation and the differentiation of the various cell types derived from it. The large number of mouse mutants with vertebral abnormalities promises to be an invaluable resource for investigating this problem (Johnson 1986; Theiler 1989; Dietrich et al. 1993). One of the first mutants to be studied at the molecular level was undulated (Un), caused by a point mutation in the pax 1 gene, encoding a paired domain/homeodomain transcription factor. The mutant phenotype is characterized by malformation of vertebral bodies and intervertebral discs and the proximal ribs (Koseki et al. 1993; Wallin et al. 1994; Dietrich and Gruss 1995). This phenotype, and in vitro studies in with embryonic tissue, have provided strong evidence that pax 1 is required for ventral sclerotome differentiation during mouse development (Fan and Tessier-Lavigne 1994; Fan et al. 1995). The functional analysis of other genes expressed in the sclerotome, including scleraxis and paraxis, which encode basic helix-loop-helix (bHLH) transcription factors (Burgess et al. 1995; Cserjesi et al. 1995), will add greatly to our understanding of vertebral patterning.

Previous studies from this laboratory and others have identified a number of mouse genes in the winged helix/forkhead domain superfamily of transcription factors. In particular, the expression patterns of mfh 1, mfh 1(fkh 1), mfh 2, and mfh 3(fkh 5) suggest that they are involved in paraxial mesoderm and somite patterning and differentiation during mouse development (Kaestner et al. 1993, 1996a,b; Miura et al. 1993; Sasaki and Hogan 1993; Kaufman and Knochel 1996). Here we focus on mfh 1, which is initially expressed in a dynamic pattern in the presomatic mesoderm, somites, and cephalic mesoderm. As development proceeds, mfh 1 transcripts are localized to condensing mesenchymal lineages of the vertebral column, nasal process, limbs, and the developing kidney (Miura et al. 1993; Kaestner et al. 1996a).

To investigate the role of mfh 1, we have generated a null allele by homologous recombination in embryonic stem (ES) cells. Most mfh 1 homozygous null embryos die prenatally, beginning -13.5 dpc. However, mutants that survive to later stages exhibit multiple craniofacial and vertebral defects that result from the absence or malformation of skeletal elements primarily derived from cephalic and somitic mesoderm. Analysis of early mfh 1 mutant embryos reveals a reduction in the proliferation and possibly also the differentiation of sclerotome-derived cell lineages. These findings suggest that mfh 1 encodes a transcription factor that is required for the proliferation of a subset of paraxial mesoderm precursor cells involved in the formation of the axial skeleton and skull.

Results

Localization of mfh 1 RNA during mouse embryogenesis

mfh 1 expression is first detected by whole-mount in situ hybridization at 7.0 dpc in non-notochordal mesoderm surrounding the node and notochord (data not shown). Figure 1A shows expression of mfh 1 in these locations at 7.5 dpc. By 8.5 dpc, mfh 1 RNA is detected in the anterior presomitic mesoderm adjacent to the youngest somites, in the somites, and in the cephalic mesoderm (Fig. 1B, data not shown). Later, at 9.5–10.5 dpc, mfh 1 is still expressed in the presomitic mesoderm and epithelial somites. However, as the somite differentiates, overall mfh 1 expression levels decrease, so that transcripts are always highest in the most caudal or youngest somites and the anterior presomitic mesoderm (Fig. 1C,D). Moreover, the localization of transcripts is very dynamic; initially, expression is detected throughout the epithelial somites, but later becomes progressively restricted, first to the dermomyotome, then to the dorsomedial and dorso-lateral myotomal precursors and sclerotome, and finally to the sclerotome of differentiated somites (Fig. 1G–I, summarized in Fig. 9).

By 10.5 dpc, mfh 1 expression is detected in the branchial arches and mesenchymal cells surrounding the eye (Fig. 1D,E). At 11.5 dpc, transcripts are present in the nasal processes, the eye region, branchial arches, blood vessels, endocardium, and mesenchymal condensations that will form the future appendicular and axial skeleton (Fig. 1E,F, data not shown). In later embryos, sites of expression include the developing otic capsule, joints of the digits, and the mesenchyme of the kidney (Miura et al. 1993; Kaestner et al. 1996a, data not shown).

Targeted disruption of the mouse mfh 1 gene

To investigate mfh 1 function, a null allele was generated by homologous recombination in ES cells. Mapping of

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the mfh1 locus reveals a single exon that encodes a 2.7-kb transcript including the forkhead domain (Miura et al. 1993; Kaestner et al. 1996a) (Fig. 2). The targeting strategy (Fig. 2) was designed to delete the entire protein coding region. Two independently targeted ES clones were injected into C57BL/6 blastocysts, chimeras were obtained, and heterozygotes for the mfh1 mutant allele were generated. These appeared normal and were fertile. However, following intercrossing of mfh1 heterozygotes, only six mfh1 (-/-) pups were identified (Table 1), and these all died within hours of birth. Genotyping of embryos before birth demonstrated that the majority of homozygous mutants did not survive beyond 13.5 dpc (Table 1), showing that the mfh1 disruption is a recessive embryonic lethal mutation.

In confirmation of the deletion of the mfh1 allele, whole-mount in situ hybridization with the full-length mfh1 cDNA showed that no mfh1 transcripts were detected in the homozygous mutant embryos (data not shown). To investigate whether deletion of mfh1 had detrimental effects on the downstream winged helix gene, fkh6, we examined the expression of this gene by section in situ hybridization (data not shown). We observed the normal level and pattern of expression of fkh6 RNA in the mfh1 mutant embryos, suggesting that the mutant phenotype results from the loss of mfh1.

Embryonic lethality of mfh1 homozygous null embryos

From 8.5 to 12.5 dpc, mfh1 homozygous null embryos are comparable in size to wild-type +/- or +/- littermates. However, 42% of the mutant embryos exhibited a severely crooked neural tube and irregular somites. Frequently, large pools of blood were found at multiple sites surrounding the hindbrain and spinal cord, suggesting discontinuities in the circulatory system. At 11.5 dpc, all mutant embryos had beating hearts, but from 12.5 to 13.5 dpc most homozygous null embryos were dying and being resorbed.

Discontinuities in the cardiovascular system were examined by histological analysis on the heart and major vessels, revealing no abnormal morphology compared with wild-type littermates (data not shown). To assess
**Figure 2.** Targeted disruption of the *mfh1* gene. (A, top line) Genomic organization of *mfh1* showing one exon with protein coding [black box] and untranslated regions [white boxes]. The winged helix domain is represented by a hatched box. (A, middle line) The targeting vector consists of the PGKneo' cassette (stippled box) flanked by the 1.2-kb 5' homology [Xhol-Xhol fragment] and 2.9-kb 3' homology arms [EcoRI-EcoRI fragment]. The 3' PGK-thymidine kinase cassette (solid box) is located at the 3' end. (A, bottom line) Structure of the targeted locus. The entire protein coding region and the majority of the untranslated sequences of the *mfh1* locus were replaced by the the neo' cassette. (B) Homologous recombinants were detected using a 5' probe (1.3-kb HindIII-Xhol fragment) on Southern blots digested with HindIII. (wt) Wild-type locus; (m) targeted locus. (C) Male and female F1 mice were produced by mating Black Swiss females with chimeric males. Homozygous embryos were produced from an intercross between F1 females and males. Genotyping of embryo was performed by digestion of genomic DNA with Spel and hybridization with the 5' probe. The wild-type and targeted locus generate 12.5-kb and 4.5-kb hybridizing fragments, respectively. (E) EcoRI; (H) HindIII; (N) Ncol; (Nt) NotI; (P) PstI; (S) Sall; (Sp) Spel; (X) Xhol.

Table 1. Genotypes of offspring from *mfh1*<sup>+/−</sup> heterozygote matings

| Age (dpc) | Total | +/+ | +/− | −/− | N.D. |
|-----------|-------|-----|-----|-----|------|
| 8.5       | 59    | 19  | 23  | 13  | 4    |
| 9.5       | 173   | 38  | 76  | 45  | 14   |
| 10.5      | 205   | 49  | 90  | 56  | 6(6) |
| 11.5      | 107   | 22  | 51  | 26  | 3(3) |
| 12.5      | 142   | 45  | 66  | 30  | 5    |
| 13.5      | 77    | 23  | 39  | 11  | 4(4) |
| 14.5      | 28    | 3   | 13  | 3(2)| 9(6) |
| 16.5      | 39    | 12  | 17  | 8(6)| 2(2) |
| 17.5      | 26    | 6   | 14  | 4   | 2(2) |
| 19.5      | 28    | 8   | 15  | 1   | 4(4) |
| Total     | 884   | 225 | 404 | 197 | 58   |
| Newborn   | 173   | 73  | 94  | 6   | 4(4) |

*Numbers in parentheses indicate embryos being resorbed.*
Table 2. Summary of skeletal defects observed in mfh1<sup>−/−</sup> newborn mutants

| Structure         | Defect     | Primary origin |
|-------------------|------------|----------------|
| Supraoccipital    | absent     | cephalic       |
| Basisphenoid      | absent     | cephalic       |
| Phesphenoid*      | absent     | cephalic       |
| Gonial*           | malformed  | cephalic       |
| Incus/stapes*     | absent     | cephalic       |
| Exoccipital       | reduced    | somitic        |
| Basiooccipital    | reduced    | somitic        |
| Vertebrae         | malformed  | somitic        |
| Ribs              | fused/absent | somitic    |
| Xiphoid           | malformed  | somitic        |
| Mandibles         | malformed  | neural crest   |
| Interparietal     | reduced    | neural crest   |
| Squamosal         | malformed  | neural crest   |
| Palatine          | absent     | neural crest   |
| Tympanic          | reduced    | neural crest   |

*Some components of these bones derived also from neural crest cells.

served, but the appendicular skeleton, including digit patterning, was normal.

Craniofacial skeletal elements are altered or absent in neonatal mfh1<sup>−/−</sup> mutant mice

In the mfh1<sup>−/−</sup> embryos that were born (and were therefore the least affected phenotypically), no trace of the supraoccipital bone was observed (Fig. 4A,B), and the exoccipital, basioccipital, interparietal, and squamosal bones were misshapen and reduced. At the base of the skull, the pterygoid bone and basisphenoid bone were reduced, and the palatine and presphenoid bones were largely missing (Fig. 4C), resulting in a cleft palate. The mandibles were also slightly shortened. No apparent phenotypic differences were observed in the nasal septum and frontal bones of the skull (Fig. 4D,E).

Besides the craniofacial abnormalities, mfh1<sup>−/−</sup> neonates display several defects in the ossicles of the middle ear, the malleus, incus, and stapes, structures that arise from cephalic mesoderm and neural crest [Novack 1993]. The malleus, formed from Meckel's cartilage, is misshapen in the mfh1<sup>−/−</sup> mutants (data not shown). The incus, which is derived from the proximal end of the palaotoquadrat cartilage (Novack 1993) and forms a separate cartilaginous body attached to the head of the malleus and stapes, was missing. The stapes was absent, whereas the gonial bone was malformed and failed to properly attach to the tympanic ring. The tympanic ring was also reduced [data not shown].

To identify the developmental processes affected in the more advanced homozygous mutants, skeletons from embryos at an earlier stage of development (16.5 dpc) were examined. As shown in Figure 4, the forming occipital bones and otic capsule cartilage were present, but reduced in size (Fig. 4E,F), and the mandible and Meckel’s cartilage were reduced and misshapen in mutants (Fig. 4D-F) compared with wild-type embryos. At the base of the skull, the basisphenoid bone was reduced, and the palatine processes and presphenoid bone were also absent (Fig. 4C).

The abnormalities described above were fully penetrant in the older surviving mutant embryos. Moreover, the craniofacial structures affected were limited to a specific set of skeletal elements, namely, those derived from the cephalic and somitic mesoderm, and only involved to a lesser, and probably secondary, extent structures arising from cranial neural crest populations.

Vertebral defects in mfh1 mutant mice

As summarized in Figure 5, skeletal analysis of those mfh1<sup>−/−</sup> homozygotes surviving to 16.5 dpc and birth revealed consistent abnormalities in both the dorsal and ventral aspects of the vertebrae. These abnormalities were more striking in the more anterior vertebrae, particularly in the cervical (C2-C7) and upper thoracic (T1) region, where the ossification centers of the centrum are absent (Fig. 5, see especially newborn C4). In contrast, in the lower thoracic, lumbar, and sacral vertebrae, these ossification centers form, but fail to fuse medially, giving rise to split centra (Fig. 5, e.g., newborn T13). Defects were also observed in the neural arches, which were misshapen and exhibited a reduction in ossification when compared with wild-type siblings (Fig. 5, e.g., newborn L1 and S1). Examination of the rib cage of mfh1<sup>−/−</sup> mice revealed multiple rib fusions and loss of ribs (Fig. 4I,J). These defects were observed at a high frequency in the

Figure 3. Expression of PECAM-1/CD-31 protein by whole-mount immunohistochemistry in wild-type and mutant embryos. (A,B) Localization in 8.5-dpc wild-type and mutant embryos, showing expression in the heart (h), dorsal aorta (a), and throughout the vascular system. Expression at 10.5 dpc, in wild-type (C) and mutant (D) embryos. [b] Branchial arches; [fib] forelimb bud; [hlb] hindlimb bud; [ivb] intersegmental blood vessel; [op] optic placode; [s] somites.
lower thoracic regions [Fig. 4J]. In addition, the ribs were often fused directly to the neural arches [data not shown]. Examination of 16.5-dpc skeletons revealed abnormalities similar to those observed in the mfh1 -/- neonates.

**Molecular analysis of mfh1<sup>1<sup>st</sup></sup> null mutants**

The reduction, malformation, or absence of specific skeletal elements observed in the more advanced mfh1 mutants described above most likely involve defects in the growth, differentiation, and/or condensation of cartilage precursor populations earlier in development. To investigate these possibilities, whole-mount and section in situ hybridization were performed on mutant embryos between 8.5 and 12.5 dpc, with markers of mesodermal patterning and differentiation (for review, see Tam and Trainor 1994).

We first examined by whole-mount in situ hybridization the expression of mfh1<sub>1<sub>fkh1</sub></sub>, a closely related winged helix/forkhead gene. mfh1 and mfh1<sub>1<sub>fkh1</sub></sub> are normally expressed in overlapping sites in the presomitic mesoderm, somites, and head mesenchyme and in nonoverlapping sites in the branchial arches (Sasaki...
product. To examine early mesodermal patterning, we partially compensate for the absence of the gene and moxl, mf3(fkh5), paraxis, fgfRl and notchl.

Patterns of the somite, five markers were observed in the cervical (C2, C4) and first thoracic vertebrae in the thoracic vertebrae. No ossification centers of the centrum from wild-type and embryos at 16.5 dpc and at birth.

Figure 5. Morphology of the vertebral column. Cervical vertebrae (C1, C2, C4), thoracic (T1, T13), lumbar (L1), and sacral (S1) from wild-type and mfhl-/- embryos at 16.5 dpc and at birth show defects in the neural arches, centrum, and the foramen transversum (arrows). Arrowheads show the position of the ribs in the thoracic vertebrae. No ossification centers of the centrum are observed in the cervical (C2, C4) and first thoracic vertebrae of the mfhl-/- newborns.

Figure 6. A-D). The winged helix gene mf3(fkh5) is expressed in the presomitic mesoderm, dorsomedial region of the somite, and neural tube (Kaestner et al. 1996a, b; data not shown). The normal pattern of expression of these markers and histological examination of mutants (data not shown) suggests that mfhl is not required for the segmentation of the paraxial mesoderm or for somite epithelialization. To further examine the anterioposterior patterning of the somite, we analyzed the expression of paraxis and moxl. paraxis, which encodes a bHLH protein, is expressed at high levels throughout the newly formed somite. As the somite differentiates, expression in the myotome is down-regulated, whereas expression persists in the dermomyotome and sclerotome (Burgess et al. 1995). moxl is a homeobox gene normally expressed in the presomitic mesoderm, dermomyotome, sclerotome, and lateral plate mesoderm and is differentially expressed in the anterior half of the somite (Candia et al. 1995). No normal expression of paraxis and moxl in the mfhl homozygous null embryos, we conclude that the subdivision into the anterior and posterior halves and the differentiation of the somite occurs normally. These findings provide evidence that the formation of presomitic mesoderm and epithelial somites, and their early patterning (i.e., dorsomedial, dorsoventral, and anterioposterior), is essentially normal in the mfhlmutants.

Because mfhl is transiently expressed in the myotome, myoD and myogenin expression were examined to assess the formation and differentiation of muscle precursors in the somite. At 10.5 dpc, myoD and myogenin are markers of differentiated myoblasts in the limb buds as well as in the axial musculature. As seen in Figure 6, E and F, the somites and myocytes of the mutant embryos at 10.5 dpc express myoD and myogenin normally, even in severely affected embryos, which were identified by the crooked neural tube (data not shown). This demonstrates that myotomal cells form at the appropriate developmental time in the mutant embryos.

To identify the different cell lineages in the sclerotome and to investigate the differentiation of the sclerotome, we analyzed the expression of pax1 and pax9. pax1 and pax9 transcripts are normally restricted to the medioventral region of the sclerotome of differentiating somites between 9.5 dpc and 10.5 dpc (Deutsch et al. 1988; Wallin et al. 1994; Neubuser et al. 1995). In 10.5-dpc mfhl mutant embryos, pax1 expression is not altered in the sclerotome (data not shown). At 12.5 dpc, pax1 is normally expressed in the mesenchyme surrounding the vertebral body primordia (Fig. 6G) and is down-regulated as the mesenchymal condensations differentiate into prechondroblasts (Deutsch et al. 1988; Wallin et al. 1994). At 12.5 dpc, expression of pax1 in the posterior vertebrae of mutant embryos is normal. In contrast, the condensing mesenchymal cells of the anterior vertebrae express pax1 in a dumbbell-like pattern surrounding the notochord (Fig. 6H). The mutant notochord, in turn, is surrounded by irregular condensations.
of pax1-negative cells, presumably prechondroblasts. In contrast, the normal pattern of expression of pax9 in the sclerotome and the anlagen of the intervertebral and vertebral bodies at 12.5 dpc was observed in mfh1 mutants (data not shown). These results suggest that formation and differentiation of sclerotomal cell lineages are essentially normal in the mfh1<sup>mm1</sup> mutants, and that the malformations of the vertebrae and the altered pax1 expression result from either a reduction in the number and density of cells surrounding the notochord and/or from abnormalities in sclerotomal cell migration.

To investigate the ability of mutant sclerotome-derived cells to differentiate into prechondroblasts and chondroblasts, the expression of α1(II) collagen and scleraxis were examined. By 12.5 dpc, α1(II) collagen transcripts mark the chondrogenic mesenchymal cells of the vertebrae and skull, prior to chondrocyte differentiation (Cheah et al. 1991). Examination of the vertebral column of 12.5-dpc mutants reveals a significant reduction in the number of cells expressing α1(II) collagen, particularly in the more anterior vertebrae (Fig. 6L). In transverse sections through cervical and upper thoracic mutant vertebrae, α1(II) collagen transcripts were detected in irregular dumbbell-like condens-
sations of prechondrogenic cells surrounding the notochord (Fig. 6K,L). \(\alpha I(II)\) collagen is also expressed in the cranial mesenchyme destined to form the chondrocranium, the hyoid, and the laryngeal cartilages (Cheah et al. 1991). A marked reduction of \(\alpha I(II)\) collagen hybridization was observed in the region of the basisphenoid, whereas the malformed basioccipital region and Meckel's cartilage expression was normal in 12.5-dpc mutants (Fig. 6J; data not shown). scleraxis, which encodes a bHLH protein, is expressed in the lateral and then ventromedial sclerotome, the mesenchymal cells of the body wall, and the limb buds at 9.5 dpc. Subsequently, scleraxis RNA becomes restricted to the chondroblast condensing mesenchyme prior to terminal chondrogenesis (Cserjes et al. 1995). In 10.5- and 12.5-dpc \(mfh1^{-/-}\) mutants, the level of scleraxis RNA within the limbs was not significantly altered (data not shown). However, scleraxis expression was reduced markedly in the vertebrae, nasal region, and lower jaw region of 12.5-dpc mutant embryos (Fig. 6M–P). These findings suggest that \(mfh1\) is not absolutely required for the differentiation of condensing mesenchyme into chondroblasts but affects the number of more differentiated cells produced, because some scleraxis expression is seen [e.g., Fig. 6M–P].

The overall conclusion from these studies is that the reduction in level of expression of several mesodermal and sclerotomal markers in \(mfh1^{1m1}\) mutants (e.g., twist, \(\alpha I(II)\) collagen, and scleraxis) reflects a reduction in the number of sclerotome-derived cells, rather than defects in their lineage and differentiation.

**Reduction of cell proliferation within the \(mfh1^{-/-}\) sclerotome**

No significant difference was observed in the level of programmed cell death in the sclerotome and cephalic mesenchyme of \(mfh1^{1m1}\) mutant or wild-type embryos, as determined by TUNEL labeling (data not shown). We therefore examined the effect of the \(mfh1\) deletion on the proliferation of sclerotome cells, as judged by bromodeoxyuridine (BrdU) incorporation. A striking difference was observed in the BrdU labeling of mutant sclerotome-derived regions from 9.5 dpc to 12.5 dpc, compared with wild-type littermates (Fig. 7). Quantitation of these results is presented in Table 3. These data show a 20% to 58% reduction in the proliferative activity of the sclerotome-derived cells. A similar reduction was not seen in neural tissue.

In conclusion, the reduction of prechondrocyte markers observed in the \(mfh1^{1m1}\) mutants may be attributed, at least in large part, to the reduced proliferation of cells derived from the sclerotome and cephalic mesenchyme, rather than to an increase in programmed cell death.

### Table 3. Cell proliferation in the sclerotome and neural tube/dorsal root ganglia

| Age (dpc) | wild | \(mfh1\) inhibition (%) | wild | \(mfh1\) |
|-----------|------|------------------------|------|--------|
| 9.5       | 65 ± 1 | 52 ± 1 | 20 | 51 ± 1 | 50 ± 1 |
| 10.5      | 29 ± 1 | 15 ± 1 | 48 | 48 ± 1 | 49 ± 1 |
| 11.5      | 32 ± 2 | 14 ± 1 | 58 | 30 ± 2 | 31 ± 1 |
| 12.5      | 31 ± 2 | 13 ± 2 | 56 | 25 ± 1 | 25 ± 2 |

Embryos were labeled in vivo with BrdU for 1.5 hr and sections stained by immunohistochemistry as described in Materials and Methods. As an internal control, nuclei from the neural tube at 9.5 dpc and 10.5 dpc, and dorsal root ganglia at 11.5 dpc and 12.5 dpc were scored. The fraction of BrdU-labeled cells was determined by dividing the number of positive nuclei (brown) by the total number of nuclei. Groups of three to four consecutive sections from the same embryo were used. For each stage examined, wild-type data were pooled from 9 sections from four embryos while the mutant data were pooled from 12 sections from four embryos. Data are expressed as the mean ± s.e.m.

\(mfh1^{-/-}\) somite micromass cultures fail to generate chondrogenic nodules

To test whether the abnormal proliferation of \(mfh1^{-/-}\) sclerotome cells in vivo could be reproduced or rescued in vitro, micromass cultures were prepared from somites of 10.5-dpc \(mfh1\) mutants and wild-type embryos. They were grown for 6 days under controlled conditions [with or without growth factors], and then stained with alcian blue to detect chondrogenic nodules. As shown in Figure 8, A and C, normal and mutant cells attached equally well to the substratum. However, mutant cultures failed to increase in density after 24 hr. By 48 hr, a large num-

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**Figure 7.** Reduced cell proliferation in the sclerotome of \(mfh1\) mutant embryos. Proliferative activity in the sclerotome of 9.5-dpc wild-type and \(mfh1\) mutant embryos (A,B) was detected by BrdU immunohistochemistry. (C,D) The sclerotome of 10.5-dpc wild-type and \(mfh1^{-/-}\) embryos. Note the very high level of BrdU labeling in the lateral sclerotome (ls) of wild type compared with mutant. Bars: [A,B] 25 μm; [C,D] 60 μm. (d) dermomyotome; (drg) dorsal root ganglia; (nt) neural tube; (arrowhead) notochord.
phenotype

proliferation, or increase in cell death. After 6 days, ~25
der of mutant cells began to dissociate from the dish,
blue-stained nodules (n) were observed. (D) No alcian blue-
stained nodules were observed in -/- cultures and most
cells had detached.

After 6 days, cultures were fixed and stained with alcian blue to
detect chondrogenic nodules (n). (B,D)

Figure 8. Somite micromass cultures from wild-type and
-/- cultures after 24 hr
in 2% FBS, showing a uniform layer of fibroblast-like cells. (B,D]
After 6 days, cultures were fixed and stained with alcian blue to
detect chondrogenic nodules [n]. (B) In wild-type cultures, alcian
blue-stained nodules [n] were observed. (D) No alcian blue-
stained nodules were observed in -/- cultures and most
cells had detached.

ber of mutant cells began to dissociate from the dish,
suggesting a loss of cell adhesiveness, a block in cell proliferation, or increase in cell death. After 6 days, ~25
chondrogenic nodules were detected in wild-type and heterozygous cultures [Fig. 8B]. In contrast, no chondro-
genic nodules were observed in the small number of cells
remaining in -/- cultures [Fig. 8D].

To see whether the -/- mutant cells in vitro can be
rescued by the addition of exogenous transforming
growth factor-β [TGF-β] and fibroblast growth factor
[FGF], two growth factors that have been shown to
stimulate chondrogenesis in somite micromass cultures
[Frenz et al. 1994; Grass et al. 1996], the cultures were
treated with basic FGF [bFGF] alone or with bFGF and
TGFβ1 from the time of plating. Both wild-type and hetero-
yzous cultures generated equal amounts of chondro-
genic nodules, as detected by alcian blue staining. How-
ever, the -/- cultures were not rescued by the ad-
dition of these growth factors. We conclude that the
inability to generate chondrogenic nodules in mutant
somites cultures, even in the presence of several growth
factors, is consistent with defects of sclerotomal cell prolif-
eration observed in vivo.

Discussion

Deletion of the - gene results in a lethal
phenotype

We have shown here that absence of - leads to pre-
or perinatal lethality. The few pups that survive to birth
show loss or malformation of elements of the vertebral
column, ribs, and skull bones. The more severely af-
ected embryos show a specific decrease in the prolifera-
tion of sclerotome-derived cells in vivo and their somite
cells fail to grow and differentiate normally into cartilage
in culture. These results provide the first evidence that a
member of the winged helix/forkhead gene family is re-
quired for the normal growth and patterning of paraxial
mesodermal derivatives during mouse embryogenesis.

Embryos homozygous for the - allele begin to
de -12.5-13.5 dpc, probably because of a failure in the
circulatory system, as judged by the presence of extra-
vascular blood pools. - is normally expressed in en-
dothelial cells of blood vessels, suggesting a role in their
growth and differentiation. Chick-qual fate mapping
studies have revealed that paraxial mesoderm (i.e.,
somites) gives rise to the endothelium of the interseg-
mental blood vessels (Noden 1989; Couly et al. 1995).
Although major abnormalities were not observed in the
heart, defects were observed in the vascular system of the
more severely affected homozygous mutants [Fig. 3].
These observations are consistent with a role for - in
the generation or proliferation of paraxial mesoderm giv-
ing rise to intersegmental vessels. In addition there may
be subtle defects in vessel integrity, permeability, or
other properties that are the cause of death later in em-
byogenesis. Further studies are needed to solve this
problem.

- is not required for the formation of epithelial
somites and their early patterning and differentiation

Our in situ hybridization studies and those of others
[Miura et al. 1993; Kaestner et al. 1996a] show that - is
expressed in the presomitic mesoderm, the epithelial
somites, transiently in the dermomyotome and lateral
myotome [Fig. 1], and then in sclerotome-derived cells.
Our analysis shows that the initial formation of epithe-
lial somites and their dorsoventral, mediolateral, and an-
terioposterior patterning appear to be normal in homo-
zygous mutants, as judged by the expression of a variety
of mesoderm specific markers [Fig. 6]. This result sug-
gests either that - has no function during the early
stages of somitogenesis or, more likely, that the absence
of a functional gene product is compensated for in vivo
by other members of the winged-helix gene family, for
example - (fkh1), mi2, and - (fkh5), which are ex-
pressed in presomitic mesoderm and/or paraxial meso-
derm and somite-derived populations [Sasaki and Hogan
1993; Costa 1994; Kaestner et al. 1996b; Kaufmann and
Knochel 1996; Labosky et al. 1996]. This possibility is
currently being tested by generating embryos homozy-
gous for mutations in some of these genes and by making
double mutants. Compensation by other winged helix/
forkhead family members is also the likely reason why
defects are not seen in the appendicular skeleton of - mutants.

- affects the proliferation of sclerotome-derived
cells in vivo and in vitro

A striking finding from our studies is that - affects the
proliferation of sclerotome-derived cells, as judged by
the reduced incorporation of BrdU into homozygous mu-
tant cells in vivo [Fig. 7; Table 3]. This defect is seen as
early as 9.5 dpc in sclerotome-derived cells, and appears to be specific for these cells that normally express mfhl, because neural tube or dorsal root ganglia proliferation remains unchanged (Table 3). One possible explanation for these results is that mfhl controls the expression of a cell-autonomous receptor for a growth factor that stimulates progression through the cell cycle. Alternatively, or in addition, MFH1 may be part of a downstream signaling pathway from a growth factor receptor (Soriano 1994). A likely candidate would be a receptor for a bone morphogenetic protein (BMP), because these cytokines are known to promote chondrogenesis in vitro, and at least some members of the BMP superfamily, such as BMP5 and GDF5 (Kingsley et al. 1994), are required for the formation of specific cartilage elements in vivo. Moreover, a winged helix/forkhead protein, FAST, recently has been identified in a complex with a xenopus mothers-against-dpp (XMAD) protein that has been linked to activin signaling in Xenopus embryos (Chen et al. 1996). The expression of genes for BMPs or BMP receptors currently is being examined in the sclerotome of mfhl mutants. Another possibility is that mfhl regulates the expression of an autocrine growth factor produced by the sclerotome cells and required for their proliferation. However, preliminary studies failed to show any significant difference between the expression of ihh, bmp2, and bmp7 in wild-type and homozygous mutant embryos (data not shown).

The reduced proliferation capacity of mfhl homozygous mutant cells in vivo also appears to be a characteristic of mfhl mutant cells in micromass cultures in vitro (Fig. 8). Mutant cells attach to the substratum normally for up to 24 hr, suggesting that cell–substrate and cell–cell adhesion are not grossly defective. However, the density of the cultures does not increase, and by 48 hr the cells begin to detach in large numbers, and no cartilage nodules form after 6 days. Recent somite micromass culture experiments have shown that the first 48 hr is the period of maximal cell proliferation (George-Weinstein et al. 1994). The abnormal phenotype of mfhl mutant cells in vitro cannot be rescued by the addition of exogenous TGF-β and FGF, two growth factors that stimulate chondrogenesis in somite micromass cultures (Frenz et al. 1994, Grass et al. 1996). These findings, although consistent with a defect in cell proliferation, do not rule out an independent or related defect in the ability of mutant cells to aggregate, to survive in culture, or to differentiate along the chondrogenic pathway. However, the fact that the mutant phenotype can be revealed in culture opens up possibilities for exploring MFH1 function at the cellular and biochemical level.

Does mfhl affect sclerotomal-derived cell differentiation as well as proliferation?

We have shown here that mfhl affects the proliferation of sclerotome-derived cells. Studies on other winged helix/forkhead genes have provided evidence for a role in both cell proliferation, differentiation, and cell fate decisions (Miller et al. 1993; Xuan et al. 1995; for review, see Kaufmann and Knochel 1996). For example, disruption of the brain factor 1 (bf1) gene results in a decrease in the rate of proliferation of undifferentiated neur ectodermal cells in the ventral telencephalon (Xuan et al. 1995; Kaufmann and Knochel 1996). In addition, a marker of early postmitotic neurons was expressed prematurely in the dorsal telencephalon of bf1 mutants, suggesting that the precursor cells exiting from the cell cycle were differentiating earlier than normal (Xuan et al. 1995). Another example is the phenotype of embryos with a disruption of brain factor 2 (bf2). This results in reduced differentiation of the condensing mesenchyme of the kidney into tubular epithelium, as well as decreased growth and branching of the ureter and collecting system (Hatini et al. 1996).

Therefore, we have considered the possibility that mfhl mutant cells are defective in their ability to differentiate along the pathway from multipotent sclerotome cells to mature chondroblasts making up different regions of the vertebrae (vertebral body, intervertebral disc, neural arch, rib, etc.). Unfortunately, this analysis is hampered by the absence of molecular markers characterizing specific populations of precursor cells, for example, migrating sclerotome cells, condensing sclerotome cells, prechondroblasts, and chondroblasts. Our experiments to date suggest that mfhl mutant cells are able to differentiate into migrating and condensing sclerotome cells that express pax1 and pax9. They can also give rise to some cells that express a1(II) collagen and scleraxis. However, the overall level of expression of these and other markers (e.g., mf1 and mtwist) is reduced in sclerotome-derived regions of homozygous mutant embryos (Fig. 6), in a manner that suggests that the number and density of expressing cells is reduced. The dumbbell pattern of a1(II) collagen and pax1 expression (Fig. 6) and the formation of two small centers of ossification in the vertebral bodies of the posterior vertebrae is also consistent with a reduced migration of sclerotome-derived cells and/or a delay in endochondral ossification in mutants.

A schematic model summarizing our ideas about the functional role of mfhl is presented in Figure 9. During the normal development of the vertebra, cells of the sclerotome lineage differentiate along a pathway that is still poorly understood but that includes epithelial and early sclerotome, migrating sclerotome, and condensing sclerotome. The condensed sclerotome then differentiates into chondroblasts and chondrocytes, with some relatively undifferentiated chondroblast precursors remaining in the perichondral layer. At each stage, the population of precursor cells needs to undergo clonal expansion to generate a pool of progenitor cells in which the next differentiation program can be initiated. We propose that an important role for mfhl is to regulate or facilitate the clonal expansion that takes place in sclerotome-derived lineages as they migrate ventrally, laterally, and medially, and then condense. We cannot at this time eliminate some role for MFH1 in regulating differentiation steps, or processes such as cell adhesion and migration. However, it is also possible that some abnor-
mhf1 winged helix gene in mouse development

The targeted allele is designated *mhf1<sup>tm1eh2</sup>* according to the guidelines of the International Committee on Standardized Genetic Nomenclature for Mice (The Jackson Laboratory, Bar Harbor, ME).

**Generation of recombinant ES cell clones and mouse chimeras**

Approximately 2–7 x 10<sup>7</sup> ES cells of line TL1 (derived by Patricia A. Labosky, University of Pennsylvania Medical School, Philadelphia; from 129/SmEVTaconic Brosser blastocysts) at passage 15 were electroporated with 100 µg of *Nori*<sup>l</sup>-digested replacement vector DNA in a total of 800 µl of PBS using a single pulse from a gene pulsor [Bio-Rad] at 800 V and 3 µF. The cells were then plated on irradiated neo<sup>-</sup> primary mouse embryo fibroblasts and selected as described by Winnier et al. (1995). Of the 105 double resistant colonies screened, three were found to have the correctly targeted allele, giving an overall frequency of one in 35. Two clones, D1 and 5E, were injected into host (C57BL/6) blastocysts as described (Hogan et al. 1994), and both gave germ-line transmission of the mutant allele. Chimeras were mated with Black Swiss females (Taconic), and agouti offspring analyzed by Southern blot hybridization for the presence of the targeted allele (*mhf1<sup>tm1eh2</sup>*). Intercrossing of heterozygotes yielded no surviving homozygous mutants, and further analyses were performed on a mixed (129 x Black Swiss) background. Both lines exhibited the same phenotype.

**Genomic DNA analysis**

For the extraction of genomic DNA, ES cells, tail biopsies, and yolk sacs were lysed in 100 mM Tris-HCl at pH 7.5, 50 mM EDTA, 0.5% SDS, 0.1 mg/ml of proteinase K (Sigma) and digested overnight at 56°C. DNA was prepared by phenol–chloroform extractions and ethanol precipitation and resuspended in TE (10 mM Tris-HCl at pH 8.0, 0.1 mM EDTA). Southern blots were performed as described (Church and Gilbert 1984). Ten micrograms of genomic DNA was digested with SpeI restriction endonuclease. Southern blots were hybridized with the 5′ 1.1-kb HindIII–XhoI probe. A 12.5-kb fragment corresponding to the wild-type allele and a 4.5-kb fragment corresponding to the targeted allele were identified on Southern blots (Fig. 2C).

**Skeletal preparation**

Skeletal preparations were performed as described by Kimmel and Tramwell (1981). In brief, neonatal mice and embryos were placed in water overnight and eviscerated. Skeletons were fixed in 95% ethanol for 3 days followed by alcian blue staining [15 mg of alcian blue 8G(X) (Sigma), 80 ml of 95% ethanol, 20 ml of glacial acetic acid] for 24 hr. Counterstaining for bone was performed using alizarin red (Sigma, 50 mg/liter of 2% KOH) for 2 hr. Skeletons were cleared in 2% KOH and stored in 100% glycerol.

**In situ hybridizations**

Whole-mount in situ hybridization was performed as described [Winnier et al. 1995]. The following murine cDNAs were used as templates for synthesizing antisense or sense strand digoxigenin labeled riboprobes [α<sup>35</sup>S]UTP RNA probes: 3.0-kb *mhf1* cDNA (3BB); 900-bp *mf1* (C52); 850-bp *mf2*, *mf3* (e4.3) (Sasaki and Hogan 1993); 650 bp *mfkb6* (kindly provided by Klaus Kaestner, University of Pennsylvania, Philadelphia); 1.2-kb *bmp2* [Blessing et al. 1993]; 800-bp *bmp7* (kindly provided by
Dr. Karen Lyons, University of California, Los Angeles; 470-bp fgfR1 (kindly provided by Dr. Jill McMahon, Harvard University, Cambridge, MA); 1.8-kb myoD; 1.2-kb myogenin; 400-bp notch1 (kindly provided by Dr. Thomas Gridley, The Jackson Lab, Bar Harbor, ME); 1.0-kb paraxis (kindly provided by Dr. Thomas Quertermous, Vanderbilt University Medical School, Nashville, TN); 1.3-kb twist (kindly provided by Dr. Richard Behringer, MD Anderson Cancer Center, Houston, TX); 900-bp scleraxis (kindly provided by Dr. Eric Olson, UT Southwestern Medical Center, Dallas, TX); 620-bp fgf4 (kindly provided by Dr. Lee Niswander, Sloan Kettering Institute, New York, NY); 313-bp bial (provided by Dr. C.V.E. Wright, Vanderbilt University and 660-bp (3' UTR) (kindly provided by Dr. C.V.E. Wright, Vanderbilt University, Nashville, TN); 1.3-kb twist (kindly provided by Dr. Annette Neusbauer, University of California, San Francisco); 405-bp aII/lI) collagen and 515-bp mox1 (provided by Dr. C.V.E. Wright, Vanderbilt University Medical School, Nashville, TN). In situ hybridization with [α-35S]UTP RNA probes was performed using a protocol described previously by Zhao et al. (1993).

Whole-mount immunostaining

The whole-mount immunostaining protocol described below was kindly provided by Dr. Scott Baldwin (Wistar Institute, Philadelphia, PA). Embryos were dissected in calcium/magnesium free phosphate-buffered saline (PBS) and 0.1% BSA, fixed overnight at 4°C in Dent fixative (80% methanol, 20% DMSO), washed three times in 100% methanol, and then stored at −20°C until use.

Embryos were bleached in 6% hydroquinone in methanol for 1 hr and then rehydrated in a reverse series of methanol dilutions (75%, 50%, 25%) and blocked in antibody dilution buffer (4% BSA, 0.01% NaN₃, 10% goat serum in PBS) twice at 4°C for 1 hr to block nonspecific sites. The embryos were incubated at 4°C overnight in primary antibody (5–20 μg/ml) in dilution buffer, followed by three quick washes with TBST (Tris-buffered saline with 0.1% Tween 20) containing 2 mM levamisole, then 5–7 1-hr washes at 4°C. The embryos were then incubated in 1:200 dilution of goat anti-rat alkaline phosphatase conjugate (Jackson ImmunoResearch Laboratories) in 10% goat serum, 4% BSA in PBS with 0.01% NaN₃ overnight at 4°C. No secondary antibody was added for the negative control. Following the incubation, embryos were given 5–7 1-hr washes with TBST containing 2 mM levamisole, followed by two 15-min washes in NTMT (100 mM NaCl, 100 mM Tris at pH 9.5, 50 mM MgCl₂, 0.1% Tween 20) containing 2 mM levamisole. Coloring reactions were performed in coloring solution (4.5 μl/ml of NBT, 3.5 μl/ml and 2 mM levamisole) in the dark without rocking. Color development was continued for 8–10 min. The reaction was stopped by three washes of PBT, and embryos were stored in the dark at 4°C.

Cell proliferation and apoptosis

BrdU (Sigma) and fluorodeoxyuridine (FUdR) (Sigma) were injected intraperitoneally into pregnant mice, 1.5 hr before sacrifice. Embryos were fixed in 4% paraformaldehyde for 2–4 hr and embedded in paraffin. Sections (8 mm) were dewaxed and stained as described (Bellucci et al. 1996). To determine the rate of cell proliferation in the sclerotome, photomicrographs were taken at 20× magnification and cells surrounding the notochord and lateral regions were counted. Cells in four adjacent sections from the thoracic region, spanning ~160 μm anterio-posteriorly, from four independent wild-type and mfh1 mutants were scored as labeled or unlabeled. All the cells of the neural tube or dorsal root ganglia were scored as labeled or unlabeled as an internal control. The overall percentage of labeled nuclei was determined and analyzed by Student’s t-test using the SAS 6.10 program. Results were determined to be significant if P < 0.05.

Cell death analysis was performed using the ApopTag Plus detection kit (Oncor) essentially following the manufacturer’s instructions with minor modifications. Sections were dewaxed and rehydrated through a series of ethanol (100%, 95%, 75%, 50%, and 25%) and rinsed in PBS. Proteinase K digestion was optimized to 10 min. Sections were incubated with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dUTP for 1 hr before quenching in 3% hydrogen peroxide for 10 min at room temperature. The incorporated digoxigenin-dUTP was detected using an anti-digoxigenin-peroxidase coupled antibody with fast DAB tablets (Sigma). Sections were counterstained with either methylene blue or hematoxylin.

Somite micromass cultures

For micromass cultures, somites (11–36), which were isolated from individual wild-type, heterozygous, and mfh1 null mutant 10.5-dpc embryos, were dissected in ice-cold PBS, and treated with 0.5 mg/ml of dispase for 10 min. Isolated somites were digested with 0.1% collagenase and 0.1% trypsin for 20 min at 37°C with occasional shaking. The somitic cells were dissociated by pipetting and resuspended in DMEM, 2% fetal bovine serum (FBS) in 20 μl drops at 2 x 10⁵ cells/ml in six-well dishes (Nunc). Some cultures were treated with either 10 ng/ml of bFGF alone or with 10 ng/ml of bFGF and 10 ng/ml of TGF-β1. Cultures were grown at 37°C in a humidified incubator for 6 days and fixed for 10 min in 4% buffered formalin. To stain for chondrogenic nodules, cultures were incubated at 37°C overnight in 1 mg/ml of alcin blue in 1 N HCl. The presence of alcin blue staining was determined by visualization using light microscopy.

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