twist is required in head mesenchyme for cranial neural tube morphogenesis

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To understand the role of *twist* during mammalian development, we generated *twist*-null mice. *twist*-null embryos died at embryonic day 11.5. Their most prominent phenotype was a failure of the cranial neural folds to fuse. Mutant embryos also had defects in head mesenchyme, somites, and limb buds. Chimera analysis suggested that head mesenchyme was required for cranial neural tube closure and that *twist* acted in a cell-autonomous manner in this tissue. In addition, in the head mesenchyme region of chimeras, *twist*-null cells were segregated from wild-type cells, and in the forebrain they lacked mesenchymal characteristics. These results suggest that *twist* regulates the cellular phenotype and behavior of head mesenchyme cells that are essential for the subsequent formation of the cranial neural tube.

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twist was originally identified in *Drosophila* as one of the zygotic genes required for dorsoventral patterning during embryogenesis (Simpson 1983; Nüsslein-Volhard et al. 1984; for review, see Anderson 1987). *twist* encodes a protein that contains a basic helix-loop-helix (b-HLH) motif and, therefore, most likely functions as a transcription factor (Thisse et al. 1988; Murre et al. 1989). *twist* is expressed initially at the cellular blastoderm stage, in a band of cells on the ventral midline that is destined to form mesoderm and mesectoderm (Thisse et al. 1987a, 1988). In *twist* mutant embryos, the first morphogenetic movements of gastrulation are disrupted: The ventral furrow fails to form and no mesoderm is generated (Thisse et al. 1987b; Leptin and Grunewald 1990). These studies indicate that *twist* has an important role in mesoderm determination and morphogenesis.

Vertebrate homologs of *twist* have also been isolated (Hopwood et al. 1989; Wolf et al. 1991). In *Xenopus*, *twist* (*Xtwi*) transcripts are detected initially at the early gastrula stage, specifically in mesoderm. Expression occurs later at the neurula and tail bud stages in the notochord and lateral plate mesoderm but not in the myotome of somites. *Xtwi* transcripts detected in cells around the notochord and the ventral regions of the somites indicated expression in the sclerotomal regions of the somites. At the neurula stage, abundant *Xtwi* transcripts were also detected in the neural crest tissues of the cranial region. These findings suggested that *Xtwi* functions after the primary response to mesoderm induction, most likely in the spatial differentiation of meso-

derm and also in the development of cranial neural crest derivatives.

In the mouse, *twist* expression is first detected at embryonic day 7.5 (E7.5) in anterior-lateral mesoderm underlying the head folds (Ang and Rossant 1994). Subsequently, at E8.0 *twist* transcripts are detected in somites and the somatopleura of the lateral plate (Wolf et al. 1991). Expression in somites is initially homogeneous, but as the dermamyotome and sclerotome differentiate, *twist* transcripts become most abundant in the sclerotome. *twist* transcripts are also abundant in limb bud mesenchyme but not in the limb ectoderm. Although no expression is detected in the neural plate and neural tube, *twist* transcripts are expressed at high levels in the mesenchyme cells of the head and branchial arches. *twist* transcripts have also been detected in primary osteoblastic cells derived from newborn mouse calvariae (Murray et al. 1992). Thus, consistent with the findings in *Xenopus*, the expression pattern of *twist* during mouse embryogenesis indicates that in vertebrates *twist* probably regulates genes involved in the specification or differentiation of mesoderm and in the development of mesenchyme tissues in the cranial region.

To understand the role of *twist* during vertebrate development, we deleted the *twist* gene in mouse embryonic stem (ES) cells by homologous recombination. Mice homozygous for the *twist* deletion allele died at E11.5. Surprisingly, *twist*-null embryos exhibited a failure of neural tube closure specifically in the cranial region. The mutants also had defects in head mesenchyme, branchial arches, somites, and limb buds. To better understand the mechanisms involved in the development of this complex phenotype, we also examined *twist* function by analyzing mouse chimeras with a β -galactosidase (β -gal)

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cellular marker. Our findings indicate that *twist* regulates the morphology and behavior of head mesenchyme cells that support the morphogenesis of the cranial neural tube.

Results

Generation of a *twist*-null allele in the mouse germ line

The mouse *twist* gene is encoded by two exons, and the entire protein-coding region is located within exon 1 (Wolf et al. 1991). To mutate the *twist* gene in mouse ES cells, we generated a vector that deletes the *twist* protein-coding sequences by replacing them with a neomycin-resistance expression cassette (Fig. 1A). When the vector is recombined homologously with the mouse genome, novel *Xba*I and *Hind*III sites are introduced (Fig. 1A). Correctly targeted clones can therefore be detected by the presence of an additional 6.0-kb mutant band when digested with *Xba*I and hybridized with a 5' probe external to the region of vector homology or by the presence of a 2.3-kb mutant band when digested with *Hind*III and hybridized with a 3' probe external to the region of

vector homology (Fig. 1B). Correct targeting results in the deletion of the *twist* protein-coding region, thereby creating a null allele. Two correctly targeted ES clones successfully contributed to the germ line of chimeric mice generated by blastocyst injection (Fig. 1B). The phenotype of *twist*-null mice from these two independently derived ES clones were identical.

Embryonic lethality and cranial neural tube closure defects in *twist*-null mice

Mice heterozygous for the *twist* deletion allele appeared normal and were fertile. No homozygous mutants were identified among the progeny born from matings between heterozygotes, indicating that *twist*-null mice died during embryogenesis.

Embryos from timed matings between heterozygotes were analyzed at different gestation stages (Fig. 2). No gross abnormalities were observed in E8.0 embryos. At E8.5, the neural folds of wild-type embryos initiate fusion within the cranial region at multiple sites, including the forebrain-midbrain boundary and the anterior extremity of the forebrain (Geelen and Langman 1977;

Figure 1. (A) Strategy for targeted deletion of *twist*. Boxes above the line, *twist* exons: (Solid box) Translated region; (shaded boxes) *twist* homologous regions used in the targeting vector; (*neo*) PGK-*neo* expression cassette that introduces novel *Hind*III and *Xba*I restriction sites; (*tk*) MC1-*tk* expression cassette for negative selection. The unique Southern probes that lie 5' and 3' of the region of vector homology are shown. (X) *Xba*I; (B) *Bam*HI; (H) *Hind*III; (S) *Sac*I; (N) *Nco*I. (B) Southern analysis of genomic DNA isolated from E9.5 embryos and ES cell lines derived from heterozygote matings. (+/+) Wild type; (+/-) heterozygote (-/-) mutant. (Top left) *Xba*I-digested embryo DNA, hybridized with the 5' probe. The 8.0-kb wild type (WT) and 6.0-kb mutant bands are shown. (Top right) *Hind*III-digested embryo DNA hybridized with the 3' probe. The 6.0-kb wild type and 2.3-kb mutant bands are shown. (Bottom) *Xba*I-digested DNA from ES cell lines (C1-C6) hybridized with the 5' probe. The 8.0-kb wild type (WT) and 6.0-kb mutant bands are shown.

Figure 2. Comparison of *twist*-null embryos with wild-type littermates examined by light microscopy (a–c) and scanning electron microscopy (d–k). (a) Frontal view of E9.5 wild-type (left) and *twist* mutant (right) embryos. (b,c) Lateral view of E10.5 wild-type (b) and mutant (c) embryos. The mutant is actually about half the size of a wild-type embryo but is shown at higher magnification for detail. Multiple sites of blood accumulation (arrows) are observed in the cranial region of the mutant. (d,e) Frontal view of the forebrain region of E9.5 wild-type (d) and mutant (e) embryos. In the mutant, surface ectoderm fails to cover the neuroepithelium except at the anterior extremity of the cranial region (arrow). (f,g) Lateral view of cranial region of E9.5 wild-type (f) and mutant (g) embryos. The asterisk (*) indicates the everted neuroepithelium of the presumptive midbrain region. Note that the mandibular arch in the mutant (curved arrow) no longer exhibits the typical C-shaped morphology as seen in the control (f, arrow). (h,i) Lateral view of E10.5 wild-type (h) and mutant (i, higher magnification) embryos. In the mutant, the size of the forelimb bud (arrowhead) is smaller than that of the hindlimb bud (arrow), whereas in the control the opposite is true. (j,k) Dorsal view of the hindbrain region of E9.5 (j) and E10.5 (k) mutant embryos. The surface ectoderm covers the region of the neural tube just rostral to the otic vesicles (arrowheads).

Kaufman 1979; Sakai 1989). In *twist*-null embryos, the cranial neural folds elevated and approached each other medially but never initiated fusion. Focal hemorrhages were occasionally detected in the cranial region of mutants but not in wild-type or heterozygous littermates (data not shown). At E9.0, the cranial neural folds are fused in wild-type embryos, whereas the neural tube in the cranial region of mutant embryos was completely open. Many of the mutant embryos had developed cranial neural fold hemorrhages. At E9.5, the cranial neu-

roepithelium in mutant embryos was everted and exposed (exencephaly) (Fig. 2a,d–g). The neural tube was open from the anterior extremity of the cranial region caudally to the approximate level of rhombomere 4 in the hindbrain (Fig. 2a,e,j,k). In contrast, the neural folds caudal to rhombomere 4 in the hindbrain region and trunk had closed to form the neural tube.

The external morphology of the branchial arches was also altered in the mutants (Fig. 2f,g). The mandibular arches in the *twist*-null embryos were straight, whereas

the arches from controls were curved. The *twist*-null embryos were comparable in size to their wild-type siblings until E10.5, when mutant embryo growth was retarded, although the neuroepithelium continued to proliferate and often formed cystic outpockets (Fig. 2b,c,i,k). Frequently, large pools of blood were found at multiple sites within the cranial region (Fig. 2c). All E10.5 mutant embryos had beating hearts. At E11.5, the mutant embryos were dead and were beginning to be resorbed. All phenotypically abnormal embryos E9.5 or older were homozygous for the *twist* mutation (Table 1). Therefore, the neural tube defect in the cranial region and the embryonic lethality correlated exactly with the mutant genotype, and the defects were completely penetrant.

Cranial neuroepithelium and mesenchyme abnormalities in *twist*-null embryos

Histological analysis revealed cellular disorganization of the cranial neuroepithelium of E9.0 *twist*-null embryos (Fig. 3a,b). At E9.5, some of the neuroepithelial cells had begun to degenerate. However, these abnormalities could have been secondary to primary defects in other tissues. To determine whether the identity of the cranial neuroepithelium was altered, we examined E9.0 *twist*-null embryos for the expression of engrailed protein, a region-specific neuroepithelial marker (Fig. 4a). At this stage in wild-type embryos, engrailed protein is localized to the developing midbrain/hindbrain boundary (Davis et al. 1991). No differences in the domain of engrailed protein expression were observed in *twist*-null embryos in comparison to controls, suggesting that the identity of the neuroepithelium in that region of the cranial neural tube of the mutants was unchanged and that the neuroepithelial abnormalities were secondary lesions.

We next examined *twist*-null embryos earlier at E8.5. At this stage, the cranial neuroepithelium was morphologically normal. However, in the mesenchymal region where *twist* is highly expressed, abnormalities were observed. In the forebrain region of wild-type embryos, the mesenchyme cells were well organized between the neuroepithelium and the surface ectoderm. These mesenchyme cells exhibited their typical morphology: They were stellate-shaped cells that were interconnected to

form a mesh-like structure. In contrast, in *twist*-null embryos, these cells did not exhibit their typical mesenchymal morphology and were rounded. In general, intercellular contacts were reduced and the extracellular space was expanded (Fig. 3c-f). By E9.0, the presumptive forebrain region contained fewer mesenchyme cells, and some of the mesenchyme cells in the presumptive midbrain region also exhibited a poor mesenchymal phenotype (i.e., they were rounded with fewer intercellular contacts; data not shown). The mesenchymal cells in the hindbrain region appeared to be less affected. Thus, the defects observed in the cranial neuroepithelium were preceded by the mesenchyme abnormalities.

In the cranial region, blood vessels were dilated and sometimes contained large pools of blood cells (Fig. 3d). Blood cells were also found frequently outside vessels. The disorganization of surrounding mesenchyme cells in the cranial region may have compromised the integrity of the blood vessels, leading to improper blood circulation and most probably to embryo death. In addition, the defects in the cranial vasculature could have been caused directly by the *twist* mutation.

As early as E9.0, disorganization and expansion of the intercellular space in mesenchyme in the branchial arches was also observed, reminiscent of the abnormal mesenchymal phenotype observed in the forebrain of *twist*-null embryos (Fig. 3c,d). Also, from E9.5 on, significant numbers of pyknotic nuclei were found in the trigeminal (V), facioacoustic (VII–VIII), and glossopharyngeal–vagal (IX–X) cranial ganglia (data not shown).

The mesenchyme of the head and the branchial arches originate from two embryonic tissue sources, mesoderm and neural crest (Noden 1988). To examine the cranial neural crest tissues in more detail, we analyzed the expression of the neural crest-specific marker *AP-2* in *twist*-null embryos at E9.5 (Mitchell et al. 1991). At this stage, wild-type embryos showed distinct *AP-2* expression in neural crest cells that were emigrating from rhombomeres 2 and 4 into branchial arches 1 and 2, respectively, as well as crest cells of the forebrain region (Fig. 4b). *twist*-null embryos had essentially the same pattern of *AP-2* expression, although there was weaker expression in the forebrain region. These data suggest that *twist*-null neural crest cells are able to migrate to the branchial arches.

Table 1. Correlation of phenotype with genotype of embryos from *twist* heterozygote matings

Age (days)	Normal embryos (%)	Genotype of normal embryos ^a			Abnormal embryos (%)	Genotype of abnormal embryos ^a			Phenotypically abnormal -/- embryos (%)
		+/+	+/-	-/-		+/+	+/-	-/-	
8.5 ^b	42 (78)	14	22	6	12 (22)	2	2	8	8/12 (67)
9.5	32 (74)	9	23	0	10 (24)	0	0	10	10/10 (100)
10.5	43 (75)	16	27	0	14 (25)	0	0	14	14/14 (100)
11.5	24 (71)	8	16	0	10 (29)	0	0	10	10/10 (100)
15.5	9 (100)	3	6	0	0	0	0	0	0

^a+/+, +/-, and -/- refer to embryos wild type, heterozygous, and homozygous mutant for *twist*.

^bAt E8.5, not all of the *twist* homozygous mutant embryos were distinguishable from the wild-type or heterozygous mutant embryos. Embryos at E8.5 were genotyped by PCR using yolk sac DNA. Later stage embryos were genotyped by Southern analysis.

Figure 3. Histological comparison of wild-type (*a,c,e,g*) and *twist*-null (*b,d,f,h*) embryos. (fb) Forebrain region; (n) notochord; (nt) neural tube; (ot) otic vesicle; (ov) optic vesicle; (s) somite; (v) blood vessel. (*a,b*) Transverse section of E9.0 embryos showing the neural tube at the level of the otic vesicles. In *b*, the apical surface of the neuroepithelium is irregular and loose (arrow), and mitotic figures (curved arrow) are found invading the neural lumen. (*c,d*) Transverse section of E9.0 embryos at the level of the otic vesicles. In *c*, the neural tube in the forebrain region is closed, and there are densely packed mesenchymal cells between the neuroepithelium and the surface ectoderm (arrow) and in the first branchial arch (curved arrow). In *d*, the neural folds of the presumptive forebrain are well elevated and bent medially towards each other but not fused (open arrow). The mesenchymal cells are disorganized in the first branchial arch (curved arrow) and the presumptive forebrain (arrow). (*e,f*) Transverse section of E8.5–9.0 embryos at the level of the forebrain region. There is more space between the head mesenchymal cells (arrows) of the mutant than of the control. (*g,h*) Longitudinal section of E9.5 embryos at the midtrunk region. The somites in *h* are poorly organized, and the somitic subdomains are not distinct. Cells with pyknotic nuclei are found in the mutant (arrow).

Abnormalities in mesoderm derivatives in twist-null embryos

twist is highly expressed in limb bud mesenchyme but not in ectoderm. In *twist*-null embryos, initial forelimb bud formation was observed. During normal development, the growth of the forelimb bud precedes that of the hindlimb bud. However, by E10.5, the relative sizes of the forelimb and hindlimb buds were reversed in the *twist*-null embryos compared with the controls (Fig. 2*h,i*). In addition, although the apical ectodermal ridge (AER) was found in the forelimb buds of controls, no AER was found in the mutants at this stage. Histological analysis revealed no indications of limb bud cell death. These results suggest that *twist* is required in mesoderm cells for limb bud outgrowth.

Whereas somites were readily observed in all of the *twist*-null embryos from stages E8.0 to E10.5 and looked superficially normal (Fig. 2*a,c,i*), histological examination revealed morphological abnormalities (Fig. 3*h*). The

cells of the dermamyotome in E9.5 mutant embryos were not well packed, the sclerotomal cells as a whole were not well segregated, and the individual domains of these somitic tissues were less distinct. Significant numbers of pyknotic nuclei were also observed, especially in the sclerotome, indicating cell death (Fig. 3*h*). The presence of apoptotic cells in the sclerotome of somites was also shown by staining *twist*-null embryos at E9.5 with acridine orange (Fig. 4*c,d*). The sclerotome regions of somites of *twist*-null embryos were stained intensely with the fluorochrome, whereas little or no staining in somites was detected in the controls.

To assess the differentiation status of somites in the *twist*-null embryos, we examined the expression of the myotome-specific differentiation marker myogenin (Sassoon et al. 1989). In E9.5 wild-type embryos, myogenin expression was detected in the most rostral somites, specifically in myotome cells (Fig. 4*e*). The somites of mutant embryos also expressed myogenin in the same number of rostral somites, demonstrating that myoto-

Figure 4. Molecular marker analysis of E9.5 *twist*-null embryos. (a) Whole-mount immunocytochemical localization of engrailed protein in control (left) and *twist*-null (right) embryos. Note that the anterior and posterior limits of engrailed immunostaining in the ventral portion of the neural tube are identical in the control and *twist*-null embryo. The apparent expansion of area of engrailed protein staining in the dorsal region of the *twist*-null embryo is an illusion caused by the open cranial neural tube. (b) Whole-mount in situ hybridization of *AP-2* in control (left) and *twist*-null (right) embryos. Note the *AP-2* labeling in the streams of neural crest cells emigrating from rhombomeres 2 and 4 to branchial arches 1 and 2, respectively (arrowheads). (c,d) Whole-mount fluorescent analysis of apoptosis by acridine orange staining in control (c) and *twist*-null (d) embryos. Note that in the somites there is significantly

more cell death (arrow) than in the *twist*-null embryo in comparison to the wild-type control. (e) Whole-mount in situ hybridization of myogenin in control (left) and *twist*-null (right) embryos. Note the myogenin labeling in the myotome cells of the first seven somites of both the control and mutant (arrows).

mal cells were present and had formed at the appropriate time. Myogenin expression appeared to be reduced in the mutants, most likely because of the associated cell death. These results suggest that *twist* is not required for the initial steps of somite differentiation (i.e., the formation of the myotome) but may be required in somites for cell growth or survival.

Other mesoderm derivatives such as the allantois formed properly in the *twist*-null embryos and fused with the chorion to develop the placental connection with the mother. In addition, the notochord was morphologically normal in the mutants and extended anteriorly to its normal position. Furthermore, the expression of *Brachyury* (Herrmann 1991), which is expressed in notochord cells, showed no differences between mutants and controls at E9.5 (data not shown).

Generation of mouse chimeras composed of *twist*-null and wild-type cells

To better understand the mechanisms involved in the development of the complex phenotype of *twist*-null mice, we analyzed chimeric embryos generated from *twist*-null and wild-type cells. Our goals were to identify the target tissues for *twist* gene action and to determine whether *twist* acted in a cell autonomous manner. Therefore, ES cell lines were established from blastocysts derived from matings of *twist* heterozygotes. Southern blot analysis demonstrated that three ES cell lines (C1, C4, and C5) were homozygous for the *twist* mutation, two lines (C2 and C6) were heterozygous, and one (C3) was wild type (Fig. 1b). ES cells from two homozygous mutant lines (C1 and C5) were microinjected

into blastocysts that were wild type for *twist* and hemizygous for the ROSA 26 gene trap insertion that expresses β -gal ubiquitously during embryonic development (Friedrich and Soriano 1991). In parallel experiments, wild-type and heterozygous ES cells were also injected as controls. The ROSA 26 cellular marking system allowed us to distinguish ES-derived cells from the blastocyst-derived cells by staining for β -gal activity. E9.5 or E10.5 embryos were scored for morphological abnormalities, stained for β -gal activity, and then completely serially sectioned. E9.5 and E10.5 were chosen for analysis because the cranial neural tube defects of *twist*-null embryos are expressed fully after E9.0. The results of the microinjections are summarized in Table 2.

Seventeen control chimeras were generated from injections with the wild-type (C3) and one of the heterozygous (C6) ES cell lines and all were normal in phenotype (i.e., no neural tube defects) (Table 2). The spatial distribution of wild-type and heterozygous ES-derived cells and cells derived from the recipient blastocysts in the resulting chimeras was assessed. In one control chimera, >95% of the cells of the embryo were derived from the injected *twist* heterozygous ES cells and only a few wild-type cells were present (data not shown). The normal phenotype of this chimera indicated that the control ES cells behaved normally in vivo. More informative results regarding cell distribution were obtained from several control chimeras with ~50% ES-derived cells. Histological analysis revealed that no major clonal growth had occurred and that there was extensive mixing of cells derived from the injected wild-type or heterozygous ES cells and recipient blastocysts (Fig. 5A,C,E,G). That there had been no cell selection for or against these donor cells indicated that the injected ES cells were able to

Table 2. Summary of *twist* and control mouse chimera production and frequency of cranial neural tube defects

Cell line (<i>twist</i> genotype)	Blastocysts transferred (number)	Stage embryos recovered	Embryos recovered (number)	Chimeric embryos (number)	Chimeric embryos w/NTD ^a (number)
C1 (-/-)	77	9.5	41	33	9
C5 (-/-)	62	9.5	38	29	7
	32	10.5	19	7	0
Total	171		98	69	16
C3 (+/+)	17	9.5	13	10	0
C6 (+/-)	50	9.5	33	7	0
Total	67		46	17	0

Mutant ES cell lines C1 and C5 were injected into ROSA 26 blastocysts and transferred to foster mothers to generate *twist* chimeras. Wild-type (C3) and heterozygous (C6) ES cell lines were used to produce control chimeras. Only chimeras containing *twist*-null cells developed cranial neural tube defects.

^a(NTD) Neural tube defects.

contribute to any embryonic tissue equally as well as the host cells.

A correlation between the distribution of twist-null head mesenchyme cells and cranial neural tube defects

Sixty-two E9.5 *twist* chimeras were recovered from in-

jections of two of the *twist*-null ES cell lines (C1 and C5) and 16 had exencephaly (Table 2). The cranial neural tube defects of these chimeras varied in severity (Fig. 6). Four of 12 neural tube defect *twist* chimeras analyzed exhibited a complete failure of the cranial neural tube to close, mimicking the *twist*-null phenotype (Fig. 6d).

Figure 5. Transverse sections of β -gal-stained E9.5 control (A,C,E,G) and *twist* (B,D,F,H) chimeras. In the control chimeras, the donor and host cells readily mix together. (A, B) Forebrain region (fb). (B) A large percentage of *twist*-null cells (pink) is observed in the neural tube (nt) and ectoderm (ec, black curved arrow). In the mesenchyme region, mutant cells are present as a stripe (black arrow) tightly associated with the neural tube, whereas the wild-type cells (blue, white curved arrow) exhibit a typical mesenchymal phenotype. (C,D) Midbrain region. (D) *twist*-null mesenchyme cells (curved arrow) are segregated from wild-type cells (arrow) in large patches. (E,F) Mandibular arch. In F, *twist*-null cells form the core of the arch (white arrow) which is surrounded by wild-type cells. Note that a few wild-type cells are present in the core (open arrow). (G,H) Forelimb bud region. (p) Proximal; (d) distal. (H) *twist*-null cells are present only in the proximal mesenchyme region (arrow), and wild-type cells occupy the distal mesenchyme area. A few *twist*-null cells are present in the ectoderm (arrowhead).

Figure 6. Cranial neural tube defects in *twist* chimeras stained for β -gal activity. (a) The midbrain region shows exencephaly (arrow). (b) Portions of the midbrain and hindbrain regions are open (arrow). (c) Complete midbrain and partial hindbrain exencephaly are present (straight, solid arrows). Note that the morphology of the mandibular arch (open arrow) and forelimb bud (curved arrow) is normal. (d) An embryo composed of >95% *twist*-null cells, with a phenotype similar to that of *twist*-null embryos. A few wild-type cells are present (arrows).

β -Gal staining revealed that >95% of the cells of one of these four chimeras were derived from the injected *twist*-null ES cells (Fig. 6d). None of the other eight neural tube defect chimeras had forebrain closure defects (Fig. 6a–c), and all eight had wild-type mesenchyme cells in the forebrain region. Thus, closure defects in the forebrain appeared to occur only when no wild-type mesenchyme cells were present. In addition, analysis of 10 of the neural tube defect *twist* chimeras revealed a general correlation between the presence of *twist*-null mesenchyme cells and the development of midbrain and hindbrain exencephaly (Table 3). In contrast, the contribution of *twist*-null cells to the neural tube and surface ectoderm (tissues that do not express *twist*) did not correlate with the development of the cranial neural tube defects.

Forty-six E9.5 and seven E10.5 *twist* chimeras had no cranial neural tube defects (Tables 2 and 4). About two-thirds of these *twist* chimeras were not informative because of low chimerism. We believe that the low level of

Table 3. Contribution of *twist*-null head mesenchyme cells in *twist* chimeras with NTD

<i>twist</i> chimera	Exencephaly ^a			Contribution of <i>twist</i> -null head mesenchyme cells ^a		
	fb	mb	hb	fb	mb	hb
1	+	+	+	++++	++++	++++
2	+	+	+	++++	++	+++
3	+	+	+	+++	++	+++
4	+	+	+	–	+++	+++
5	–	+	+	++	++	++
6	–	+	+	–	+	+
7	–	+	+	–	+	+
8	–	+	+	–	+	+
9	–	–	+	+++	++	+++
10	–	–	+	–	+	+
11	–	–	+	–	+	+
12	–	+	–	–	+	+

^a(fb) Forebrain; (mb) midbrain; (hb) hindbrain. (–) No *twist*-null cell contribution; (+) 1%–25% contribution; (++) 26%–50% contribution, 51%–75% contribution, 76%–100% contribution.

chimerism in these embryos was simply attributable to a low contribution of ES cells rather than selection for the host cells. From the remaining *twist* chimeras that had reasonably high ES contributions, we randomly chose 14 chimeras for further analysis (Table 4). In these chimeras with normal cranial neural tubes, the contribution of *twist*-null cells in the cranial neuroepithelium could be as high as 95%. However, in all of these chimeras (except chimera 1), the contribution of *twist*-null mesenchyme cells in the cranial regions never exceeded 25% (Table 4). Thus, the normal cranial phenotype in these *twist* chi-

Table 4. Contribution of *twist*-null neuroepithelial and head mesenchyme cells in *twist* chimeras without NTD

<i>twist</i> chimera	Neuroepithelium ^a			Head mesenchyme ^a		
	fb	mb	hb	fb	mb	hb
1	+++	++++	++++	++	+	+
2	+++	+++	+++	+	–	+
3	++	+++	+++	–	+	+
4	++	+++	+++	–	–	–
5	++	++	+++	+	+	–
6	++	++	+++	+	–	+
7	++	++	++	–	–	–
8	++	++	++	–	–	+
9	++	++	++	–	+	+
10	+++	++	+	–	–	–
11	+	+	+	–	+	+
12	+	+	+	+	–	–
13	+	+	+	–	–	+
14	+	+	+	+	–	–

^a(fb) Forebrain; (mb) midbrain; (hb) hindbrain. (–) No *twist*-null cell contribution; (+) 1%–25% contribution; (++) 26%–50% contribution, 51%–75% contribution, 76%–100% contribution.

meras correlated with a low percentage of *twist*-null head mesenchyme cells and not with the mutant contribution of the neuroepithelium. These results further suggest that *twist*-expressing head mesenchyme is required for closure of the cranial neural tube.

Differential behavior of twist-null and wild-type cells in the cranial region

Although no *twist*-null mesenchyme cells were present in the forebrain mesenchymal region of *twist* chimeras, *twist*-null cells were often found in a stripe intimately associated with the neural tube (Fig. 5B). These *twist*-null cells were tightly packed and lacked mesenchymal characteristics. Between this sheet of *twist*-null cells and the surface ectoderm were exclusively well organized wild-type mesenchyme cells (Fig. 5B). No such stripe of cells was ever found in control chimeras (Fig. 5A). In some *twist* chimeras, these cells had dispersed, but they retained their rounded morphology even in the presence of the wild-type mesenchyme (data not shown). Cell-marking studies in the mouse embryo have been used to map cranial neural crest cell migration pathways (Serbedzija et al. 1992; Osumi-Yamashita et al. 1994). In the forebrain, neural crest cells migrate in a contiguous stream ventrolaterally along the neural tube, whereas in midbrain and hindbrain, neural crest cells migrate as subectodermally dispersed cells. Therefore, based on the location of the *twist*-null stripes, these *twist*-null cells were probably composed of neural crest cells that failed to realize their mesenchymal phenotype despite their ability to migrate along the neural tube. However, recent cell marking and transplant studies suggest that cranial paraxial mesoderm also contributes to forebrain mesenchyme (Trainor et al. 1994), suggesting that both neural crest and paraxial mesoderm-derived mesenchyme are altered by the *twist* mutation. Unlike the *twist*-null cells in the forebrain that never exhibited a mesenchymal phenotype, the *twist*-null cells in the midbrain and hindbrain of *twist* chimeras were more mesenchymal in character (Fig. 5D). However, unlike the cell mixing in control chimeras, these *twist*-null cells were always segregated from wild-type cells in large patches (Fig. 5C, D).

twist-null cells do not contribute to the neural crest mesenchyme of the branchial arches and distal forelimb bud mesenchyme

With the exception of the two *twist* chimeras that had the highest *twist*-null cell contribution, the *twist* chimeras had morphologically normal branchial arches. Histological examination of *twist* chimeras revealed that *twist*-null cells always populated the central cores of the arches, were mixed with a few wild-type cells and appeared morphologically normal (Fig. 5F). Strikingly, *twist*-null cells did not contribute to the mesenchyme surrounding these cores; they were exclusively populated by wild-type cells. The core of the branchial arch is derived from paraxial mesoderm, and the mesenchyme that surrounds the core of the arch is of cranial neural

crest origin (Le Douarin 1982; Trainor et al. 1994). Thus, *twist*-null cells were readily able to contribute to the mesodermal cores but were unable to contribute to the neural crest mesenchyme of the branchial arches in *twist* chimeras. As with the head mesenchyme, there were indications of altered cell-cell interactions in the branchial arches. In control chimeras, there was no histological evidence for morphological differences between the central cores and surrounding mesenchyme of the branchial arches (Fig. 5E). However, in the *twist* chimeras, there appeared to be a clear morphological delineation between the *twist*-null cell-containing cores and the exclusively wild-type arch mesenchyme (Fig. 5F).

Unlike *twist*-null embryos, the *twist* chimeras had normal forelimb bud morphology except for two chimeras with the highest *twist*-null ES contribution, indicating that growth arrest of the forelimb buds was rescued in the chimeric situation (Fig. 6c). Interestingly, *twist*-null mesenchyme cells were always located in the proximal region of the forelimb buds and were segregated from wild-type cells in patches (Fig. 5H); no *twist*-null mesenchyme cells were present in the distal part of the forelimb buds (Fig. 5H). In the limb buds of a few earlier stage E9.5 *twist* chimeras, mutant and wild-type cells were mixed together (data not shown). Thus, although *twist*-null cells could initially intermingle with wild-type cells in the forelimb bud mesenchyme, later in development *twist*-null cells were unable to contribute to the distal mesenchyme of the forelimb bud. In the lateral body wall region adjacent to the forelimb buds, *twist*-null mesoderm cells were also segregated from wild-type cells (data not shown).

Although there was a strong cell selection against *twist*-null mesenchyme cells in the branchial arch and forelimb bud regions, no selection was observed against the *twist*-null cells in other tissues. The two genotypes were usually well mixed in the somites (a *twist*-expressing tissue), neural tube, surface ectoderm, notochord, heart, and the region of the embryo caudal to the forelimb buds (data not shown).

Discussion

twist-expressing head mesenchyme is required for closure of the cranial neural tube

In this study we investigated the role of *twist* during mammalian development. That *twist* is required for *Drosophila* mesoderm formation suggested that a similar role in vertebrate embryogenesis might exist. However, in vertebrates *twist* is expressed after initial mesoderm formation. Thus, it is perhaps not surprising that mesoderm readily formed in our *twist*-null embryos. We found that *twist* was essential for embryonic viability and that before the *twist*-null embryos died, they developed very specific abnormalities. The most striking malformation was the failure of the neural tube to close specifically in the cranial region, whereas the posterior hindbrain and entire spinal cord formed normally. This was not predicted because *twist* is not expressed in the

neural tube. However, *twist* is abundantly expressed throughout the entire underlying head mesenchyme. We also found histological abnormalities in the head mesenchyme of *twist*-null embryos that preceded those detected in the neuroepithelium. Furthermore, in *twist* chimeras we found that cranial neural tube defects occurred when *twist*-null mesenchyme cells were present and that normal cranial neural tube closure took place when there were few *twist*-null mesenchyme cells present. The proportion of *twist*-null cells in other tissues such as the neuroepithelium and surface ectoderm was irrelevant to the development of the neural tube defects. Thus, from numerous lines of evidence, we conclude that head mesenchyme is a primary site of *twist* gene action.

What is the role of head mesenchyme in cranial neural tube formation? Minimally, the head mesenchyme must not interfere with the morphogenetic processes inherent to the neuroepithelium. However, the complex nature of head morphogenesis suggests that head mesenchyme has a more active role in cranial neural tube formation. One possibility is that head mesenchyme supports neural fold morphogenesis by maintaining the shape of the neural folds. This mechanical function could be a more active process; perhaps the mesenchyme provides a driving force that coordinates and balances the bending and apposition processes. It is also possible that head mesenchyme may itself mediate the process through secreted factors. Our results suggest that *twist* acts to regulate the supportive/mechanical aspects of head mesenchyme function.

The variable severity of the exencephaly observed in the *twist* chimeras suggests that the spatial and temporal distribution of *twist*-null and wild-type cells in any specific region or in areas critical for the initiation of cranial neural tube closure is important (Geelen and Langman 1977, Sakai 1989, Kaufman 1990). The absence of neural tube defects in some *twist* chimeras with higher proportions of *twist*-null head mesenchyme cells may be attributable to a balanced distribution of *twist*-null cells on both sides of the neural folds. In addition, the spatial relationships between two genetically distinct mesenchymes may change considerably from E8.5, when the apposition and fusion processes are under way, to E9.5, when we analyzed the chimeras.

The twist-null mouse: a novel model for neural tube defects

Cranial neural tube formation is a complex morphogenetic process that includes the formation and elevation of the neural plate and apposition and fusion of the bi-convex-shaped neural folds (Morris-Kay 1981). Aberrations in any aspect of this tightly regulated morphogenetic process can lead to neural tube defects such as exencephaly and spina bifida. Neural tube defects are among the most prevalent congenital malformations in humans and occur in ~1 in 1000 births worldwide (Lemire 1988). Ironically, they are among the least understood human birth defect. Numerous hypotheses

have been proposed to explain the cause of neural tube defects, and much research has focused on determining the role of the neuroepithelium in the development of this abnormality (Karfunkel 1974; Jacobson 1978; Gordon 1985). Currently, it is generally agreed that abnormalities in the neuroepithelium and non-neuroepithelial tissues can cause neural tube defects (Shoenwolf and Smith 1990). However, only correlative evidence suggesting that head mesenchyme is required for neural tube closure has been reported (Marin-Padilla 1966; Morriss 1972; Morriss and Solursh 1978a,b; Morris-Wiman and Brinkley 1990). Here, we provide direct evidence that head mesenchyme is required for cranial neural tube formation.

The variation in the severity of exencephaly observed in the *twist* chimeras demonstrates that region-specific inhibition of *twist* function in head mesenchyme can lead to regionally restricted neural tube defects. Thus, whereas all *twist*-null embryos die at midgestation, it seems likely that some of the *twist* chimeras with less severe neural tube defects would have been born with various forms of anencephaly (the absence of portions of the head). Numerous studies in rodents have demonstrated that various teratogens can cause neural tube defects (Copp et al. 1990). Perhaps some teratogens may act by altering, either globally or regionally, the expression of developmental genes such as *twist*. Thus, *twist* may be a genetic link between teratogens and a variety of neural tube defects. The identification of *twist* as a genetic factor in the development of neural tube defects is particularly important because the genetic basis for these very prevalent human congenital abnormalities are essentially unknown. Therefore, the *twist*-null mouse is a novel animal model for understanding the cellular and molecular mechanisms involved in neural tube formation and the development of neural tube defects.

twist regulates head mesenchyme cell fate

Head mesenchyme cells are multipotent cells derived from the neural crest and the mesoderm that give rise to a variety of diverse tissue types (Le Douarin 1982; Noden 1988). Despite the importance of mesenchyme in head morphogenesis and its association with many craniofacial deformities, the cellular and molecular mechanisms that define this tissue have not yet been determined. In the forebrain of *twist* chimeras, *twist*-null cells were present as a tightly packed mass of rounded cells that had migrated along the neural tube, whereas wild-type cells were unequivocally mesenchymal in phenotype. These results suggest that *twist* is required for directing forebrain neural crest and perhaps paraxial mesoderm cells toward a mesenchymal cell fate. In the midbrains and hindbrains of *twist* chimeras, most *twist*-null cells were mesenchymal in character. Therefore, in the midbrain and hindbrain regions, *twist* may be one of many factors that together regulate the phenotype of head mesenchyme cells. Recent studies of the chick suggest that the zinc finger-containing gene called *slug* is one of the other factors (Nieto et al. 1994).

In *twist* chimeras, *twist*-null cells in the forebrain mesenchymal region did not mix with wild-type mesenchyme cells. In addition, in the midbrain and hindbrain *twist*-null mesenchyme cells were segregated from wild-type cells in large patches. In the forebrain region, cell segregation may be attributable to the different morphologies of the two types of cells. However, in the midbrain and hindbrain regions, the *twist*-null and wild-type cells were morphologically similar but still did not mix. One possible explanation is that the adhesive properties of the *twist*-null midbrain-hindbrain cells were altered.

That the phenotype of *twist*-null head mesenchyme cells did not change in *twist* chimeras suggests that in the cranial region, *twist* acts in a cell-autonomous manner. Because *twist* is a transcription factor, these findings suggest that the entire genetic cascade regulated by *twist* must reside within or very near *twist*-expressing cells. Therefore, certain classes of molecules are candidate direct or near downstream targets of *twist*. Judging by the phenotype of the *twist*-null cells in the cranial region, we suggest that genes encoding cytoskeletal proteins, surface molecules, and extracellular matrix may be targets of *twist*. Changes in the expression of these proteins could cause the cell morphology and behavior changes observed in the *twist*-null mice and *twist* chimeras. These observations are especially illuminating with respect to the role of *twist* in *Drosophila* and vertebrates. In the fly, the precise role of *twist* in mesoderm formation is to cause the cell shape changes that drive the morphogenetic movement of the presumptive mesoderm cells, thereby initiating gastrulation (Leptin and Grunewald 1990). Thus, that *twist* alters cell morphology and behavior in both flies and mice suggests that *twist* has the same primary function in insects and vertebrates. This in turn suggests that the downstream targets of *twist* that influence these changes are also the same.

Diverse actions of *twist*

In the *twist* chimeras, there was a strong selection against the *twist*-null neural crest-derived mesenchyme in the branchial arches and mesoderm-derived distal mesenchyme of the forelimb bud. In *twist*-null embryos, neural crest cells were able to migrate and populate the branchial arches, as judged by *AP-2* expression. Why did the *twist*-null crest cells fail to contribute to the arch region in the chimeras? One possible explanation is that differences in the proliferation rates of these mutant cells were uncovered in the chimeric situation and thus, *twist*-null crest cells were excluded from the neural crest-derived arch mesenchyme. An alternative explanation is that the ability of the neural crest cells to migrate was impaired, perhaps because of changes in their adhesive properties. Such deficiencies may only be revealed in the presence of competing wild-type cells. In the limb buds, the primary defect is probably in the *twist*-expressing mesoderm-derived cells, because *twist* is not expressed in the limb ectoderm. *twist*-null cells were found in the forelimb bud mesenchyme of the *twist* chi-

meras, suggesting that they had migrated from their mesodermal origins, but only to the proximal region. The observation that the proximally located *twist*-null mesenchyme cells failed to proliferate even in the presence of wild-type cells suggests that *twist* also acts in a cell-autonomous manner in this tissue. The development of the limb involves a series of epithelial-mesenchymal interactions that occur in a proximal-to-distal sequence (Saunders 1948; Saunders and Reuss 1974). It is possible that *twist*-null limb bud mesenchyme may fail to produce a short range signal that is necessary for differentiation of the ectoderm. Alternatively, *twist*-null limb mesenchyme cells may not be competent to respond to signals produced by the overlying ectoderm. In *twist*-null embryos, somites formed, became disorganized, and somitic cell death was observed before embryo growth slowed. In the somites of *twist* chimeras, *twist*-null and wild-type cells mixed extensively and appeared histologically normal. Therefore, in contrast with the cell-autonomous nature of *twist* in head and limb mesenchyme, these observations lend support to the hypothesis that in somites *twist* acts through non-cell-autonomous mechanisms.

The widespread expression of *twist* and the various phenotypes we found suggest that *twist* regulates diverse biological processes (Fig. 7). Perhaps *twist* acts upon a restricted set of diverse downstream target genes, depending upon the tissue, that can act in a cell-autonomous manner as in the case of head, branchial arch and limb bud mesenchyme, or act in a non-cell-autonomous manner as in somites, to elicit a variety of cellular responses. The chimeric nature of b-HLH proteins such as *twist* and the possibility of heterodimeric combinations with other b-HLH proteins expressed in specific tissues suggests that the activation or repression of diverse downstream targets may provide a mechanism for generating different types of biological responses from a single transcription factor (Jones 1990).

Materials and methods

Deletion of the *twist* gene in mouse ES cells

A 129/SvEv mouse genomic library was screened with a probe containing nucleotides +4 to +864 of the mouse *twist* genomic sequences (Wolf et al. 1991). The probe is a subfragment of the *twist* locus generated by PCR amplification of mouse genomic DNA. One phage clone that hybridized with the probe was subcloned into pBluescript, and its *twist* identity was verified by DNA sequencing. A 5.0-kb *Bam*HI upstream fragment and a 1.0-kb downstream region from the *Sac*II-*Nco*I sites, containing the *twist* intron and exon 2 from the phage insert were used to construct a replacement vector (Fig. 1A). A PGKneobpA resistance expression cassette was inserted in reverse orientation relative to the direction of *twist* transcription between the two *twist* regions (Soriano et al. 1991). A MC1tkpA herpes simplex virus thymidine kinase expression cassette was added onto the short arm of homology to enrich for homologous recombinants using negative selection with 1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-s-iodouracil (FIAU) (Mansour et al. 1988). The targeting vector can be linearized at a unique *Sal*I site outside of the homology. Twenty-five micrograms of linearized targeting

Figure 7. Summary of the phenotypes observed in the *twist* chimeras. The relevant regions of the embryos are listed in the left column. The distribution of wild-type and *twist*-null cells is diagrammed in the second column. (■) Wild-type chimeras; (□) *twist*-null cells. In the forebrain mesenchyme region, *twist*-null cells lacked mesenchymal characteristics (○) and were segregated from wild-type mesenchyme cells. In the midbrain and hindbrain mesenchyme regions, *twist*-null cells were mesenchymal in phenotype but were segregated from wild-type cells in large patches. The neural crest-derived mesenchyme of the branchial arch was exclusively wild type, as was the mesoderm-derived distal forelimb bud mesenchyme. In the somites, *twist*-null and wild-type cells readily mixed. A priori, because *twist* is most likely a transcription factor, it must act within the cell. However, downstream target genes that are regulated by *twist* could act cell-autonomously or non-cell-autonomously.

vector was electroporated into 10^7 AB-1 ES cells that were subsequently cultured in the presence of G418 and FIAU (McMahon and Bradley 1990; Soriano et al. 1991). Seven hundred eighty G418/FIAU-resistant ES clones were initially screened by *Xba*I digestion and hybridization with a unique 5' probe external to the region of vector homology. Correctly targeted clones were then expanded for further Southern blot analysis by *Hind*III digestion and hybridization with a unique 3' probe external to the vector homology. Twenty-four correctly targeted ES clones were identified.

Generation of chimeric mice and germ-line transmission of the *twist* deletion allele

Four of the *twist* mutant ES clones were microinjected into C57BL/6J blastocysts and the resulting chimeric embryos were transferred to the uterine horns of day 2.5 pseudopregnant foster mothers (Bradley 1987). Chimeras were identified among the resulting progeny by their agouti fur (ES derived) and were subsequently bred with C57BL/6 mates. Two of the mutant ES clones (G8 and E9) were found to be capable of contributing to the germ line of chimeric mice. Tail DNA from the agouti pups that resulted from those matings was analyzed by Southern blotting with either of the probes used to identify *twist* het-

erozygotes. E9.0 and older embryos from heterozygote matings were genotyped by Southern blotting of yolk sac DNA. Younger embryos were genotyped by PCR amplification of yolk sac DNA with the *twist* specific primers: 5'-ATCTGGCGGCCAGGTA-CATCG-3' and 5'-GGCTGTTTTCTATGACCGCT-3' and a neo primer 5'-TTGTGCCCAGTCATAGCCGAAT-3'. Chimeras were also bred with 129/J females to establish the *twist* deletion on the 129 inbred genetic background. The phenotype of *twist*-null mice on the C57BL6×129 mixed genetic background and the 129 inbred genetic background was identical.

Scanning electron microscopy

Embryos were washed in 0.125 M sodium cacodylate buffer, (pH 7.3), 310 mOsm at 37°C, and fixed in Karnovsky's fixative (pH 7.5), for at least 30 min. The embryos were then rinsed in the buffer, postfixed in 2% osmium tetroxide, rinsed, dehydrated in graded alcohols, transferred to Peldri II (Ted Pella, Inc., Redding, CA) for critical dehydration, and placed in a vacuum desiccator for 24 hr. The embryos were then mounted onto stubs and sputter-coated with a 200 Å coating of gold-palladium (80:20) in a Hummer VI sputter coater (Technics, Springfield, VA), and examined with a Hitachi model S520 scanning electron microscope.

Histological analysis

Embryos were processed for histological analysis as described by Kaufman (1990). Briefly, the embryos were fixed in Bouin's fixative, dehydrated, and embedded in paraffin. Six-micrometer-thick sections were cut and stained with hematoxylin and eosin.

Whole-mount in situ hybridization, immunocytochemistry, and apoptosis analysis

Whole-mount in situ hybridization was performed by the protocol described by Conlon and Rossant (1992). The following probes were used for the in situ hybridization studies: *AP-2* (Mitchell et al. 1991), *myogenin* (Sassoon et al. 1989), and *Brachyury* (Herrmann 1991). Whole-mount immunocytochemistry was performed by the protocol of Davis et al. (1991). Acridine orange staining of whole embryos to detect apoptosis was performed as described by Graham et al. (1993).

Generation of *twist*-null ES cell lines

ES cell lines were generated by the method of Robertson (1987) from blastocysts isolated from matings between mice heterozygous for the *twist*-null mutation. Each cell line was genotyped by Southern blot analysis with the probes described above.

Generation and characterization of *twist* chimeras

twist-null, heterozygous, and wild-type ES cells at passage 4 or 5 were injected into *twist* wild-type blastocysts generated by mating males homozygous for the ROSA 26 retroviral gene trap insertion (Friedrich and Soriano 1991) with Swiss females. Therefore, all *twist* wild-type recipient blastocysts were hemizygous for the ROSA 26 gene trap allele and ubiquitously expressed β -gal during embryonic development. This was confirmed by completely serially sectioning E9.5 and E10.5 ROSA 26 hemizygous β -gal-stained embryos. Chimeric embryos were stained for β -gal activity (Behringer et al. 1993) and subsequently processed for histological analysis. Stained embryos were postfixed in 4% paraformaldehyde, dehydrated, embedded

in paraffin, cut into 6- μ m-thick sections, and counterstained with 1.0% eosin. Thus, in the histological sections, ES-derived cells (wild type, heterozygous, or *twist*-null) appeared pink, whereas recipient blastocyst cells (wild type for *twist*) were blue. Five consecutive histological sections per tissue per chimera were examined to assess the average contribution of ES-derived cells in a particular region. It should be noted that this average value does not reflect the spatial distribution of donor and recipient cells in a given tissue.

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