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Twist functions in mouse development

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ABSTRACT The remarkable similarity in the profile of genetic activity and the frequent association of developmental defects of limb and craniofacial structures in mouse mutant and hereditary disorders point to the possibility that the development of the head and limb involves common morphogenetic mechanisms. Our recent studies on the impact of the loss of *Twist* function has highlighted the essential role of the basic helix-loop-helix transcription factor encoded by this gene on the development of both body parts. We have summarized in this review our findings on the molecular pathways that are disrupted in *Twist* mutant mouse embryos. Our results revealed an evolutionarily conserved function for *Twist* in mesodermal differentiation, and previously unrecognised effects of the loss-of-function mutation of this gene in the outgrowth and patterning of the limb and branchial arches, and neural crest cell migration. An important outcome of our study is the demonstration of a differential requirement for *Twist* in forelimb versus hindlimb development, and its functional interaction with *Gli3* in specifying anterior digit formation. Further evidence of the conservation of the function of *Twist* in different species is highlighted by similarity in the spectrum of potential downstream targets and interacting genes of *Twist* that have been identified by genetic, functional and microarray analysis.

KEY WORDS: *mouse, Twist, limb, branchial arch, morphogenesis, mouse embryo*

Twist and TWIST

The *Twist* gene was first identified in *Drosophila* by the presence of a twisted torso of embryos that lacked the activity of this gene (Simpson, 1983; Nusslein-Volhard *et al.,* 1984). In the fly, *twist* activity is crucial for the establishment of dorsoventral tissue pattern in the gastrulating embryo and the specification of mesodermal fates during cell differentiation (Thisse *et al.,* 1987). *twist* orthologs have subsequently been identified in other species including jellyfish (Spring *et al.,* 2000), *C. elegans* (Harfe *et al.,* 1998; Corsi *et al.,* 2000), leech (Soto *et al.,* 1997), lancelet (Yasui *et al.,* 1998), zebrafish (Kim and Chitnis, 1999 in Genbank), *Xenopus* (Hopwood *et al.,* 1989), chick (Spicer *et al.,* 1996), mouse (Wolf *et al.,* 1991), rat (Bloch-Zupan *et al.,* 2001) and human (Wang *et al.,* 1997).

The protein encoded by the *Twist* gene belongs to a diverse group of putative transcription factors that share a common basic helix-loop-helix (bHLH) configuration. Such bHLH structure was first recognised in the DNA binding proteins E12 and E47, and subsequently in the proteins encoded by many genes that are essential for cell fate specification, tissue differentiation and growth regulation, such as *Myod1, Hes7, Mash, Neurogenin* and *Mesp*. Amino acid sequences within the bHLH domains of *Twist* are conserved between vertebrates and invertebrates, along with other sequences at the amino and carboxy termini (Rose and Malcolm, 1997). Critical to the function of the bHLH factor is the ability to form dimerised complexes with other proteins via the helix domains, and the binding of the bipartite DNA-binding groove formed by the basic region of the complex to specific DNA sequences. TWIST can form dimers with itself or different protein partners and the different combinations have a significant impact on the action of the dimerized complex as either an activator or repressor of transcription. For example, heterodimerization with MYOD1 may suppress myogenesis by reducing the DNA-binding affinity of the complex to myogenic target genes, and dimerization with another factor that contains only the helix-loop-helix but no DNA-binding domains will potentially lead to down-regulation of transcriptional activity (Castanon *et al.,* 2001). Conversely, heterodimerization may generate a novel DNA-binding property that results in *de novo* activation of transcription that is not possible with TWIST homodimers (reviewed by Rose and Malcolm, 1997). The different functional properties of the various forms of dimerized complexes may underpin the diverse effect of *Twist* activity in different organisms.

Abbreviations used in this paper: AER, apical ectodermal ridge; bHLH, basic helix-loop-helix factor; BMP, bone morphogenetic protein; *C. elegans, Caenorhabditis elegans*; E, embryonic day for staging embryos; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HH, Hedgehog; NCC, neural crest cells; SHH, Sonic hedgehog; WNT, Wingless-related factor.

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Impact of *Twist* Activity on Cell Differentiation and Embryonic Development

The functional role of *Twist* in cell differentiation and development has been investigated using a combination of two well-established experimental paradigms: correlation of the expression pattern with events of differentiation and morphogenesis, and the elucidation of the effects of a gain- or a loss-of-function.

A Conserved Role for Twist *in Mesodermal Differentiation*

An early hint of a role for *Twist* in the specification of tissue fates came from analysis of *twist* expression patterns in the *Drosophila* embryo and the finding that the expression of several genes involved in myogenesis requires cooperating *twist* activity (Baylies and Bate, 1996; Castanon *et al.,* 2001). In *Drosophila*, *twist* is initially detected throughout the mesoderm. As the mesoderm separates into somatic and visceral components, *twist* expression is concentrated in the progenitors of the somatic muscles and dramatically decreases in the visceral region. Subsequently, expression rapidly disappears in differentiating embryonic muscle precursors but is retained in adult muscle precursors set aside during embryogenesis (Baylies and Bate, 1996), suggesting that *twist* may be required to maintain myoblasts in an undifferentiated state. The *C. elegans Twist* homolog, *hlh-8,* plays a critical role in the formation of non-striated muscles (Corsi *et al.,* 2000). The specification of subsets of myogenic tissues by *Twist*, may therefore be an evolutionarily conserved function. That such conservation of function occurs may be the result of conservation of the whole pathway, with the regulatory mesoderm genes that *Twist* controls being conserved in sequence and function between vertebrates and invertebrates, as well as *Twist* itself (Furlong *et al.,* 2001). The functional significance of this modulating expression pattern has been tested in the post-gastrulation fly embryo (Baylies and Bate, 1996). Maintaining high levels of *twist* expression in cells blocked heart and visceral muscle formation while development of somatic muscles proceeded normally. In addition, ectopic expression of *twist* in the ectoderm promoted a switch from epidermal and nervous system differentiation to a myogenic program, thus establishing a requirement for twist in cell-fate choice. Further evidence of a role for *twist* in mesoderm specification came from a study of *twist* function during development of the adult flight muscles in *Drosophila* (Anant *et al.,* 1998). Unlike the somatic muscles of the embryo, a reduction in *twist* levels was found to be required specifically for the differentiation of the indirect flight muscles but not the direct flight muscles.

The gene expression pattern in *Drosophila* and the effect of enforced expression of *Twist* in mammalian cells point to an apparent inconsistency in the role of *Twist* activity in muscle differentiation in different animal models. Whereas in *Drosophila,* a high level of *twist* activity enhanced the differentiation of somatic muscles, in mammalian cells, constitutive over-expression of *Twist* can inhibit muscle differentiation and down-regulate *Myf5* activity in mouse C_2C_{12} cells *in vitro* and suppresses myocyte and myotube differentiation in BLC6 embryonic stem cells (Hebrok *et al.,* 1994, 1997; Rohwedel *et al.,* 1995). However, it is not entirely clear whether the *twist*-expressing somatic muscle precursors of the fly are the functional equivalent of the non-expressing myotome of the vertebrate embryo. It is also possible that the TWIST factor may be interacting with different bHLH partner proteins in different species resulting in contrasting activating and repressing activities in myogenesis.

In the mouse and rat, *Twist* mRNA is present in the mesoderm of the gastrulating embryo (Fuchtbauer, 1995; Stoetzel *et al.,* 1995) and in the presomitic mesoderm, somites, cranial mesenchyme and the limb bud mesenchyme of the organogenesis stage embryo (Wolf *et al.,* 1991; our unpublished observations). It is also expressed in the mesenchyme of the palate, the tooth bud and the sutural tissues of the skull (Bloch-Zupan *et al.,* 2001; Rice *et al.,* 2000). However, appearance of the protein is not apparent in the paraxial mesoderm until E8.25 (Gitelman, 1997). As the somite matures, the *Twist* expression domain becomes confined to the dermomyotome (the precursor of muscles and dermis) and sclerotome in the ventral part of the somite (mouse: Wolf *et al.,* 1991; Fuchtbauer, 1995; chick: Tavares *et al.,* 2001). Of specific interest is the lack of *Twist* activity in the myotome where *MyoD* and *MEF2* are both expressed. This has led to the hypothesis that the normal function of *Twist* is the suppression of myogenic differentiation by counteracting the activity of myogenic regulators. *Twist,* however, is expressed in the lateral plate mesoderm of the mouse and chick embryo (Stoetzel *et al.,* 1995; Tavares *et al.,* 2001) from which the visceral muscles of the splanchnopleure are derived. *Twist* activity therefore highlights the compartmentalization of musculature development in the fly and vertebrates.

Loss of Twist *Function in the Mouse causes Pleiotropic Defects in Growth and Patterning*

Further clues to the function of *Twist* come from the study of the effect of loss of gene function. Homozygous mutant fly embryos fail to form the ventral furrow at gastrulation resulting in absence of all mesoderm-derived internal organs (Thisse *et al.,* 1988). Embryos are also partially dorsalized indicating a function for *twist* in the establishment of dorsoventral pattern (Thisse *et al.,* 1987).

Ablation of the *Twist* gene in the mouse (Chen and Behringer, 1995) results in a dose-related mutant phenotype. Heterozygous mutant mice are viable but display abnormal craniofacial structures (such as delayed ossification, poor sutural growth and asymmetrical facial skeleton) and preaxial polydactyly specifically in the hindlimb (Bourgeois *et al.,* 1998). A strong indication of the relationship between haplo-insufficiency of *TWIST* and abnormal morphogenesis is revealed by the phenotype of the dominantly inherited Saethre-Chotzen syndrome (El Ghouzzi *et al.,* 1997). Loss-of-function mutation in one allele of the *TWIST* genes is associated with variable physical abnormalities including craniosynostosis (premature fusion of cranial sutures), facial anomalies and limb defects (syndactyly and polydactyly), but no evident muscular deficiency.

The homozygous mutant mouse embryo, in contrast to the fly mutant, undergoes normal gastrulation but dies at E10.5-11 (Chen and Behringer, 1995), consistent with the absence of *TWIST* homozygous individuals in the postnatal human population. This difference in mutant phenotype may reflect an earlier requirement for *Twist* in mesoderm specification in *Drosophila*, than in mice and worms (Borkowski *et al.,* 1995; Corsi *et al.,* 2000). *Twist*$^{-/-}$ mutant mouse embryos display severe defects in closure of the cephalic neural tube, deficient cranial mesoderm, malformed branchial arches and facial primordium, and retarded development of limb buds (Chen and Behringer, 1995). The timing of manifestation and the tissue-specificity of the mutant phenotypes are consistent with the localization of protein in the cranial neural crest, limb bud mesenchyme, lateral plate mesoderm and differentiating somites

of the mouse embryo. We have extended previous analyses of the *Twist* mutant phenotypes to study the impact of the loss of *Twist* function on the differentiation and patterning of tissues in the affected organs. Our findings (O'Rourke *et al.,* 2002; Soo *et al.,* 2002; Loebel *et al.,* 2002) are summarised below.

Defective dorso-ventral patterning of cranial neural tube. Analysis of the molecular characteristics of the malformed neural tube reveals that the brain is correctly regionalized along the anterior-posterior axis with all the major brain regions present in the appropriate order, but there is significant reduction in forebrain size and an expansion of the midbrain. In the forebrain and rostral midbrain, molecular markers that normally signify the tissues in the dorsal part of the neural tube are absent and ventral tissue markers are expressed in a much broader domain. Therefore, the loss of *Twist* function in the cranial mesenchyme has apparently led to the loss of dorsal tissue characteristics with more tissues acquiring a ventral phenotype, suggesting that the *Twist*-expressing paraxial mesoderm is critical to act in concert with the axial mesoderm in the dorsoventral patterning of the brain. Consistent with the impact of *Twist* on the patterning of the neural tube in the mouse, *twist* has been shown to be critical for the transcription of *single-minded*, which putatively is involved with the specification of the midline tissues in the nerve cord of the fly (Kasai *et al.,* 1998), and loss of *twist* leads to partial dorsalization of the body tissues (Thisse *et al.,* 1987).

Impaired growth and differentiation of the branchial arches. At E9.5, the mandibular arches of *Twist* null mutant embryos lack the normal curvature and appear foreshortened (Chen and Behringer, 1995). Histological abnormalities are detected in the arches half a day earlier with disorganization of the mesenchyme and expansion of the intercellular space in the branchial arch. The early lethality of the mutant precludes a complete analysis of the developmental defects of the branchial arches. However, analysis of gene activity reveals that markers for myogenic precursors (*RBP-lacZ*) and skeletogenic tissues (*Gsc*) are not expressed, *Sox10* activity fails to be consolidated to the neurogenic population of the neural crest cells, and *Dlx* and *Alx* genes in the mesenchyme are either significantly down-regulated or not expressed. Together with the poor ability of the arch tissues to differentiate into muscle and bone when they are grown as teratomas, this strongly suggests that *Twist* function may be critical for differentiation of the arch tissues that are derived from both the paraxial mesoderm and the cranial neural crest cells. Furthermore, the diminished potency to form teeth, especially molars, is concordant with the poor differentiation of the neural crest cells and the formation of a truncated arch in which only the proximal portion is formed properly.

Altered neural crest cell migration and differentiation. Analysis of expression of the neural crest cell (NCC) marker shows that *Ap2* expression is still detected in the craniofacial region of the E9.5

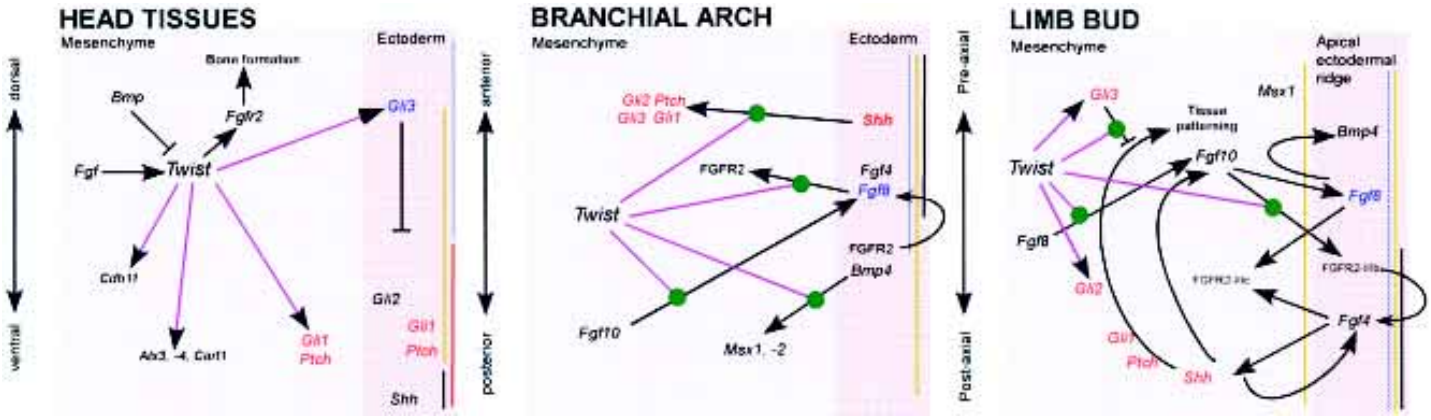

Fig. 1.The putative functional relationship of *Twist* and the signalling pathways in head tissues, the limb bud and the branchial arch implied by changes in downstream gene activity in *Twist* mutant mouse embryos *(O'Rourke* et al., *2002; Soo* et al., *2002; Rice* et al., *2000; Xu* et al., *1998). Potential interactions between the genes or molecules involve the activation or maintenance of the expression of downstream genes, the positive modulation of the signalling activity, the process of gene regulation (line with end-dot), and the suppression of signalling or gene activity (line with end-bar; interactions involving* Twist *are coloured purple). Expression of some genes in the ectodermal compartment of the three embryonic structures is regionalized (expression domains are indicated by bars for genes of matching colour) along one of the major axes of asymmetry (double-ended arrows). In the head tissues* **(Left)**, Twist *plays a central role in the induction of several transcription factors and signalling receptors in both the paraxial mesenchyme and the neural tube. The effect of* Twist *on the dorsoventral patterning of the fore- and midbrain may be mediated through its regulation of* Gli3 *activity, which counteracts the ventralising activity of SHH and its downstream factors.* Twist *may influence the migration of the cranial neural crest by affecting the level of* Cdh11*-mediated interaction between the cranial mesenchyme and the neural crest cells. The FGF-*Twist*-FGFR pathway has been shown to be essential for the formation of calvarial bone and suture development (Rice* et al., *2000). In the branchial arch* **(Middle)**, Twist *function is essential for regulating the activity of several transcription factors encoded by the* Alx, Dlx *and* Pitx *family (not shown).* Twist *activity may be involved in the modulation of the activity of the FGF, SHH and BMP-signalling cascades which mediate the epithelial-mesenchymal interactions responsible for arch outgrowth and tissue patterning. . In the limb bud* **(Right)**, Twist *activity in the mesenchyme is required to sustain FGF8-FGF10-FGFR2 signalling. This signalling activity induces apical ectodermal ridge (AER) formation and initiates limb bud outgrowth. FGF signalling from the AER specifies regionalization of the activity of Bmp4 and its downstream target, Msx1, and induces and/or maintains SHH expression in the zone of polarizing activity. The positive feedback loop between SHH and AER-FGFs is established either directly, or indirectly via FGFR2 activity in the mesenchyme, and maintains the AER to support limb growth. SHH activity, which is mediated via downstream activators/repressors such as* Ptch, Gli1, *-2 and -3, is essential for anterior-posterior patterning. Twist is involved with the regulation of the expression of* Gli2 *and* Gli3*, and influences the repressor activity of* Gli3. Twist *may therefore play a key role in establishing a pattern of graded strength of SHH signalling across the anterior-posterior dimension of the limb bud.*

TABLE 1

MUTATIONS THAT AFFECT CRANIOFACIAL AND LIMB DEVELOPMENT

Genes	Nature of mutation	Phenotype	
		Craniofacial	**Limb**
Fgfr1	Mouse: Pro250Arg substitution	Abnormal skull shape, premature sutural fusion	Not described
	Human: Pfeiffer	Craniosynostosis involving several cranial sutures resulting in short tower-shaped head, widely spaced eyes, small nose and underdeveloped midface	Broad thumbs, varying degrees of cutaneous syndactyly, shortened fingers, medially deviated broad toes
Fgfr2	Mouse: Targeted mutation by deletion of Ig domain III	Not described	Failure to form limb bud
	Human:	Craniosynostosis and maxillary hypoplasia (Antley-Bixler, Apert, Jackson-Weiss), also hypoplastic zygomatic arch and nasal bones (Apert)	hand and foot anomalies (Antley-Bixler)
	1. Antley-Bixler (affects membrane proximal (MP) domain)		
	2. Apert (missense - affects LII-III domain)		hand and foot anomalies: syndactyly often include bone and soft tissue fusions (Apert)
	3. Jackson-Weiss (missense-affects Ig-IIIa, Ig-IIIc and MP domains)		
	4. Pfeiffer (missense - affects LII-III, Ig-IIIa, Ig-IIIc; splice site deletion affects MP domains)	Pfeiffer: see *FGFR1* mutant phenotype Saethre-Chotzen: see *TWIST* mutant phenotype	foot anomalies: enlarged and sideways-pointing great toes (Jackson-Weiss)
	5. Saethre-Chotzen (affects Ig-IIIa domain)		Pfeiffer: see *FGFR1* mutant phenotype Saethre-Chotzen: see *TWIST* mutant phenotype
Fgfr3	Mouse: Targeted mutation by deleting the Ig-like domain II to transmembrane domain	Reduced skull size	Lengthening of long bone and increased curvature, and isolated pocket of hypertrophic chondroctyes in the trabecular bone
	Human: Achondroplasia (single amino acid change) and thanatophoric dysplasia (TD) type I (mostly missense mutation or a base change in the stop codon) and type II (a single known missense substitution in the tyrosine kinase domain)	Macrocephaly (Achondroplasia) Cloverleaf-shaped skull (TD type I) Marked cloverleaf-shaped skull (TD type II)	Shortening of the proximal long bones of the limbs (Achondroplasia) Curved short femurs (TD type I) Straight femurs (TD type II)
Fgf8	Mouse: Conditional inactivation of floxed alleles by Nes1-cre (branchial arch) and Msx1-Cre (limb bud)	Disruption of the growth of the first branchial arch, loss of maxilla and mandible and associated middle ear ossicles	Disruption in the forelimb results in aplasia of the radius and first digit, hypoplastic or absent humerus, thickened bowed ulna, fused and/or absent carpal bones; hindlimb less affected due to compensation by *Fgf4*
	Human: No evidence for a role for *Fgf8* in craniosynostosis/limb defect syndromes		
Shh	Mouse: Targeted null mutation	Reduced size, lack of a distinct midline, abnormal forebrain morphology, long proboscis, single optic vesicle	Stunted limbs, hindlimbs have one distal digit, forelimbs have one distal cartilage element, both limbs have fusion of intermediate elements
	Human: holoprosencephaly (HPE)	Cerebral hemispheres of forebrain fail to separate into distinct halves (HPE); also associated with solitary maxillary central incisor, cyclopia, proboscis-like nasal structure, midline cleft palate and premaxillary agenesis	Not described
Gli3	Mouse: Spontaneous mutation due to deletion of at least a 30kb fragment containing the *Gli3* gene	*Heterozygous*: enlarged interfrontal bone and sometimes hydrocephaly	Postaxial nubbin on forelimb, preaxial polydactyly of hindlimbs
		Homozygous: microphthalmia, open neural tube in midbrain region or overt exencephaly, enlarged maxillary arch, reduced external nasal process, poorly developed eyes, misplaced ears, abnormal growth of mystacial and supra-orbital hair	Paddle-shaped limbs with up to 8 digits (polysyndactyly)
	Human: Greig cephalopolysyndactyly syndrome (chromosomal translocations and microdeletions involving 7p13)	Macrocephaly, broad nasal root, occasionally ear anomalies and hydrocephaly	Postaxial polydactyly of hands, preaxial polydactyly of feet
Ptch	Mouse: Targeted mutation deleting part of exon 1 and all of exon 2	*Heterozygous*: Brain tumour (medulloblastoma)	Poly- and syndactyly in the hindlimb (1% of cases)
		Homozygous: Defective closure and loss of dorsal tissue markers in the neural tube	Mutation is lethal (E9.5) before limbs develop
	Human: Gorlin syndrome caused by haploinsufficiency	Craniofacial defects include cleft lip and palate, hypertelorism, intracranial calcifications (falx cerebri), macrocephaly and tumours (medulloblastoma, jaw cysts and basal cell carcinomas)	Polydactyly
Wnt5a	Mouse: Targeted mutation by inserting a PGKneo cassette into exon 2	Abnormally shaped head, reduced outgrowth of the external ear,foreshortened snout, tongue and mandible	Foreshortened limbs with no digits
Dkk1	Mouse: Targeted null mutation	Loss of forebrain and rostral midbrain, truncation of the skull and face anterior to the inner and parietal bone	Defects range from widening of the limb buds to fusion of the distal-most skeletal elements and ectopic pre-axial and post-axial digits and split hand and foot
Msx2	Mouse: Targeted null mutation	Defects of skull ossification, persistent calvarial foramen	Enlarged nails
	Human		
	1. Parietal foramina (PFM) caused by loss of *Msx2* function or haploinsufficiency	Oval defects of the parietal bones, may be associated with scalp defects and structural or vascular malformations of the brain (PFM).	Not described
	2. Boston-type craniosynostosis caused by gain of *Msx2* function: CA substitution resulting in a missense mutation		
Twist	Mouse: Targeted null mutation deleting the entire coding region	*Heterozygous*: Nasal septum deviation, facial asymmetry, accelerated or delayed ossification of specific skull bones	Preaxial polydactyly of hindlimb
		Homozygous: Exencephaly, cranial haemorrhages, malformation of the branchial arches	Retarded limb growth especially of the forelimb, altered patterning of the forelimb mesenchyme
	Human: Saethre-Chotzen syndrome caused by insertions, nonsense or missense mutations, premature termination, or deletions, all resulting in haploinsufficiency	Craniosynostosis of coronal suture resulting in brachycephaly; also facial dysmorphism (inc. flat supraorbital ridges, ptosis, deviated nasal septum, low frontal hairline, small ears with prominent crura), cleft palate, maxillary hypoplasia	Brachydactyly, partial cutaneous syndactyly of the second interdigital space (hands), broad halluces (feet)

References: *Fgfr1:* De Moerlooze and Dickson, 1997; Zhou *et al.,* 2000, *Fgfr2:* Xu *et al.,* 1998; Nuckolls *et al.,* 1999; Cohen and Kreiborg, 1996; De Moerlooze and Dickson, 1997; Revest *et al.,* 2001, *Fgfr3:* De Moerlooze and Dickson, 1997; Deng *et al.,* 1996; Colvin *et al.,* 1996, *Fgf8:* Lewandoski *et al.,* 2000; Yoshiura *et al.,* 1997; Trumpp *et al.,* 1999, *Shh:* Chiang *et al.,* 1996; Nanni *et al.,* 2001; Kraus *et al.,* 2001, *Gli3:* Hui and Joyner, 1993, *Wnt5a:* Yamaguchi *et al.,* 1999, *Dkk1:* Mukhopadhyay *et al.,* 2001, *Ptch:* Goodrich *et al.,* 1997; Milenkovic *et al.,* 1999; Saldanha, G., 2001, *Msx2*: Jabs *et al.,* 1993; Wu *et al.,* 2000; Cohen, 2000; Wuyts *et al.,* 2000; Satokata *et al.,* 2000; Wilkie *et al.,* 2000, *Twist:* Chen and Behringer, 1995; O'Rourke *et al.,* 2002; Soo *et al.,* 2002; Rose and Malcolm, 1997; El Ghouzzi *et al.,* 1997; Bourgeois *et al.,* 1998.

Twist$^{-/-}$ embryos, suggesting that *Twist*$^{-/-}$ NCC are formed and may be able to migrate to the various craniofacial regions (Chen and Behringer, 1995). However, our study on the pattern of *Sox10* activity has revealed some previously unrecognised defects of the neural crest cells in the mutant embryo. *Sox10*-expressing neural crest cells that are migrating from the 2nd and 4th rhombomeres of the hindbrain to the first two branchial arches fail to remain segregated as discrete streams, leading to the filling of the NCC-free gap normally found at the level of rhombomere 3 by *Sox10*-expressing cells. Cell transplantation studies have revealed that the *Twist*-deficient paraxial mesoderm fails to restrain the neural crest cells along specific paths as they migrate to the branchial arches, and although *Twist*-deficient neural crest cells are able to home in to the correct branchial arch, they are unable to colonize the correct tissue compartment within the arch. It may be significant that neural tissues are absent in the teratomas derived from somites of *Twist*$^{-/-}$ embryos, pointing to a lack of neural crest cells in the somite. This may be due to the inability of the neural crest cells to colonise the *Twist*-deficient somite because of the lack of guidance signal, or to the failure of the neural crest cells to undergo neurogenic differentiation. A possible cause of this abnormal pattern of NCC migration and regionalization is the alteration of cell adhesion properties. This hypothesis is supported by the fact that (1) there are significant overlaps in the expression domains of *Cdh11* and *Twist* in the paraxial mesoderm and the branchial arches (Kimura *et al.,* 1995), (2) loss of *Twist* activity is associated with down-regulation of *Cdh11* expression in the cranial mesenchyme and the branchial arches, and (3) over-expressing *Cdh11* in the *Xenopus* embryo inhibits the migration of the neural crest cells (Borchers *et al.,* 2001).

Abnormal somite differentiation. Consistent with the presumptive role of *Twist* in mesoderm differentiation, genes that are associated with the differentiation of the dermomyotome, myotome and sclerotome are expressed at a lower level, or not at all, in mutant mice. The architecture of the somites is disorganised. Mutant somites show poor capacity to differentiate into bone and striated muscle when tested in teratoma studies. Extensive cell death is observed at the time of the demise of the embryo, especially in the sclerotome, indicating a possible role for *Twist* in cell growth or survival (Chen and Behringer, 1995). Evidence of a potential role in cell viability has since been suggested by another study where *Twist* was shown to promote colony formation of Ras-transformed cells and can inhibit *myc*-induced apoptosis (Maestro *et al.,* 1999).

Limb defects are dose-dependent and limb type-specific. In the limb bud of the mouse embryo, *Twist* is expressed in a dynamic manner during development. Early on the transcript is distributed widely throughout the limb bud mesenchyme and in the adjacent lateral mesoderm. Expression is then down-regulated in the core mesenchyme and becomes restricted to the apical mesenchyme and to the pre- and postaxial mesenchyme flanking the prospective constriction that develops later at the base of the paddle-shape limb bud. Late expression is restricted to the interdigital tissues and the perichondrium of the digital cartilage. With such a dynamic and protracted expression pattern, which is similar to that in chick limbs (Tavares *et al.,* 2001), it is not surprising that a major impact of the loss of *Twist* activity in the mesoderm is an effect on limb growth and patterning. What is perhaps surprising is the differential role that *Twist* appears to play in forelimb and hindlimb development considering its identical expression domain in both limb types.

Forelimb development appears to be less sensitive to *Twist* gene dosage than hindlimb development. *Twist*$^{+/-}$ forelimbs are morphologically normal. In contrast, loss of just one functional copy of the *Twist* gene is sufficient to result in polydactyly of the hindlimb, which is heralded by an ectopic *Shh* domain in the anterior limb mesenchyme. *Twist*$^{-/-}$ forelimbs are severely retarded and have a diminished histogenetic capacity for bone and muscle differentiation. The defective development of homozygous mutant forelimbs is associated with the loss of a morphologically identifiable apical ectodermal ridge, and altered expression of many mesodermal and ectodermal patterning genes. Intriguingly, in the complete absence of *Twist* activity, the early development of the hindlimb bud is less affected than that of the forelimb bud. Because of embryonic lethality of the homozygous mutant, it is not known whether the hindlimb bud may exhibit defective tissue patterning similar to the polydactyly of the *Twist*$^{+/-}$ hindlimb, however the observation of expanded *Fgf4* expression to the anterior AER indicates that this might be the case should these embryos survive longer.

Since *Twist* homozygosity causes embryonic lethality at a later stage in mouse than in *Drosophila*, additional insight has been gained into the diverse functions of *Twist* through analysis of the specific variety of organs affected in the mutant mice. However, despite the divergent features, it is clear that the primary action of *Twist* is to regulate the differentiation of mesodermal tissue, which in turn influences the ability of the mesoderm to impose morphogenetic controls on other embryonic tissues during organogenesis.

Twist Function is Critical for Cell Signalling in the Branchial Arch and the Limb Bud

A significant outcome of the analysis of the *Twist* mutant phenotype is that both haplo-insufficiency and complete loss of function impact on the development of the craniofacial structures and the limb. *Twist* mutation therefore joins the growing list of targeted and spontaneous mutations in mice and clinical syndromes in humans that show strong association between developmental defects of the skull and the limb. Some of the mutations are associated with genes encoding factors which mediate the signalling activity of the FGF (fibroblast growth factors), HH (Hedgehog) and WNT (Wingless-related factor) families (Table 1). A survey of the types of genes that are expressed in both the branchial arches and the limb buds has indeed identified many genes that are involved with FGF, SHH, WNT and BMP signalling in both embryonic structures. In addition, the arch and limb tissues also express genes encoding transcription factors whose activities may interact with one another and may regulate the activity of the afore-mentioned signalling pathways (Table 2). The pleiotropic nature of the mutant phenotype and the expression of common genes suggests that the development of these two distinctly different organs may utilize similar molecular mechanisms which are deployed in an organ-specific manner to generate structural diversity. Development of lateral outgrowths of the body, such as those of the limb and branchial arch primordia, is accomplished by the same morphogenetic events of initiation, proximo-distal extension, and tissue patterning. Development of both the vertebrate limb and arches is regulated by complex reciprocal interactions between the mesenchyme and the epithelium (Tickle and Eichele, 1994; Wedden

et al., 1988; Ferguson *et al.,* 2000; Moerlooze *et al.,* 2000; Martin, 2001), which is mediated by cell-cell signalling. The response to these signals appears to be interchangeable between the limb and arch tissues. Recombination experiments in chick have shown that the epithelium is essentially interchangeable. Limb bud ectoderm allows reasonable development and differentiation of craniofacial mesenchyme while craniofacial ectoderm can sustain outgrowth of limb mesenchyme in some cases (Richman and Tickle, 1992). A further demonstration of the overlap of the signals involved in limb and face development has come from grafting experiments in which

TABLE 2

GENES THAT ARE EXPRESSED IN BOTH THE BRANCHIAL ARCH AND THE LIMB BUD DURING MORPHOGENESIS

Gene	Expression pattern		Reference
	Branchial arch	**Limb bud**	
FGF signalling			
Fgf4	Anterior 1/2 of the 1st (mandibular) and 2nd (hyoid) arches	Posterior 2/3 of apical ectodermal ridge (AER)	Niswander and Martin, 1992
Fgf8	Surface ectoderm of maxillary and mandibular components of the 1st arch	Throughout AER	Crossley and Martin, 1995
Fgf9	Oral epithelium of 1st arch	Throughout AER	Kettunen and Thesleff, 1998; Martin, 1998
Fgf10	Mesenchyme of maxillary component and rostral half of the mandibular process, core of 2nd arch	Initially throughout mesenchyme, later confined to posterior and distal mesenchyme	Xu *et al.,* 1998
Fgfr2	Surface ectoderm of maxillary and mandibular components of the 1st arch	Surface ectoderm, low levels in mesenchyme	Orr-Urtreger *et al.,* 1991, 1993; Peters *et al.,* 1992
Shh signalling			
Shh	Lateral and medial domains in the oral epithelium of the mandibular processes	ZPA in posterior mesenchyme	Riddle *et al.,* 1993; ten Berge *et al.,* 2001
Gli1	branchial arch mesenchyme	Posterior limb mesenchyme but not in ZPA	Hui *et al.,* 1994; Gli limb expression reviewed in Theil *et al.,* 1999
Gli2	branchial arch mesenchyme	Diffusely throughout limb mesenchyme but not in ZPA	Hui *et al.,* 1994; Gli limb expression reviewed in Theil *et al.,* 1999
Gli3	branchial arch mesenchyme	Anterior limb bud mesenchyme	Hui *et al.,* 1994; Gli limb expression reviewed in Theil *et al.,* 1999
Ptch	Mesenchyme of mandibular arch	Posterior half limb bud mesenchyme but not in ZPA	Goodrich *et al.,* 1996
Wnt signalling			
Wnt5a	Maxillary and mandibular components of 1st arch	Distal mesenchyme (PZ), later in the distal 2/3 of the digit perichondrium, ventral limb ectoderm, AER	Parr *et al.,* 1993; Yamaguchi *et al.,* 1999
Wnt11	Maxillary components of 1st arch	Ectoderm on dorsal surface then in the mesenchyme at the rostral and caudal margins of the developing digits and developing perichondrium of each digit	Christiansen *et al.,* 1995
BMP signalling			
Bmp2	Mesenchyme of the lateral region of the mandibular arch	AER; initially also in posterior mesenchyme then around the digit rudiments and later in the joints and footpads	Hogan, 1996, Lyons *et al.,* 1995; Neubuser *et al.,* 1997
Bmp4	Epithelium covering the medial ends of the maxillary and mandibular processes, at E12.5 in the mesenchyme of the developing molars	AER; posterior and anterior mesenchyme and in the PZ; later in the developing joints and ventral footpads.	Hogan, 1996, Lyons *et al.,* 1995; Wang *et al.,* 1999
Bmp7	In the entire epithelium covering the maxillary and mandibular processes, at E13 in the dental lamina of the developing molars	AER; initially diffusely throughout mesenchyme, then concentrated around the digit rudiments and in the interdigital mesenchyme	Hogan, 1996, Lyons *et al.,* 1995; Wang *et al.,* 1999
Transcription factors			
Twist	Most strongly in maxillary and mandibular components of 1st arch, weaker in 2nd arch, weakest in 3rd arch	Limb mesenchyme with stronger expression anteriorly at E10.5, later in the interdigital tissues then the perichondrium of the phalanges	Fuchtbauer, 1995; O'Rourke *et al.,* 2002
Dlx1	Mesenchyme of the proximal and distal domains of the 1st and 2nd branchial arches	AER	Qiu *et al.,* 1997; Kraus and Lufkin, 1999
Dlx2 (Tes-1)	Mandibular component of 1st arch, distal region of 2nd arch. *Dlx2* is expressed at higher levels in the 1st arch ectoderm than is *Dlx1*.	AER	Simeone *et al.,* 1994a, b; Qiu *et al.,* 1997; Bulfone *et al.,* 1993; Kraus and Lufkin, 1999
Dlx3	Distal tips of branchial arch mesenchyme (1st and 2nd arches), later restricted to the caudal portion of the mandibular process	AER	Qiu *et al.,* 1997; Kraus and Lufkin, 1999; Beanan and Sargent, 2000
Dlx5	Distal branchial arch mesenchyme (1st and 2nd arches)	AER and underlying mesenchyme, later (E14.5) in the progress zone at the digit tips and in all skeletal elements	Qiu *et al.,* 1997; Kraus and Lufkin, 1999; Merlo *et al.,* 2000
Dlx6	Distal branchial arch mesenchyme (1st and 2nd arches)	Perichondrial areas of limbs	Qiu *et al.,* 1997; Kraus and Lufkin, 1999; Simeone *et al.,* 1994a
Msx1	Distal mesenchyme of maxillary and mandibular processes with an A-P gradient of expression	Distally restricted expression in the mesenchyme with highest expression immediately under the AER and lower expression more proximally	MacKenzie *et al.,* 1991; Brown *et al.,* 1993
Msx2	Distal epithelium and mesenchyme of maxillary and mandibular processes (more distally restricted than *Msx1*) with an A-P gradient of expression	Limb ectoderm and anterior mesenchyme	MacKenzie *et al.,* 1992; Carlson *et al.,* 1998
Alx3	Distal mesenchyme of 1st and 2nd arches; medially in the mesenchyme of the mandibular processes	Anterior mesenchyme, later extending to distal and distal-posterior regions	ten Berge *et al.,* 1998; ten Berge *et al.,* 2001
Alx4	Mesenchyme of 1st arch along the anterior aspect	Anterior mesenchyme	Qu *et al.,* 1997, 1999
Cart1	Mesenchyme of 1st arch	Anterior mesenchyme	Zhao *et al.,* 1994; Qu *et al.,* 1999
Pitx1	Mesenchyme in the middle of the 1st arch	Hindlimb mesenchyme	Lanctot *et al.,* 1997
Gsc	Posterior portion of the 1st arch, anterior portion of the 2nd arch	Proximal limb buds	Gaunt *et al.,* 1993
Mtsh	Distal mesenchyme of 1st and 2nd arches then confined to the distal-posterior portion of the mandibular arch; also in a limited part of the antero-proximal epithelium of the 1st arch	Distal mesenchyme subjacent to AER, presumptive anterior and posterior necrotic zones, and near ZPA; later in a subset of cells at the lateral border of the developing digits and at the distal tip of each digit	Long *et al.,* 2001

Shh-expressing facial epithelia were able to induce digit duplications when transplanted to the anterior region of early limb buds (Helms *et al.,* 1997). Our study on the expression of genes associated with FGF, SHH and BMP signalling activity in the *Twist*$^{-/-}$ embryo (O'Rourke *et al.,* 2002; Soo *et al.,* 2002) reveals that the loss of *Twist* function impacts on similar signalling pathways in the head tissues, branchial arch and the limb bud (Fig. 1).

FGF signalling. To date, the FGF family in mammals consists of 23 members that signal through four transmembrane tyrosine kinase receptors, *Fgfr1-4* (Ornitz and Itoh, 2001). At least 4 FGFs are expressed in both the branchial arches and the limbs: three in the ectoderm (*Fgf4, -8, -9*) and one (*Fgf10*) in the mesenchyme (Table 2). In addition to these four FGFs, *Fgf1, -2, -5* and *–12* are also expressed in the branchial arches (Francis-West *et al.,* 1998). Two isoforms of *Fgfr2* (*Fgfr2b* in the apical ectoderm ridge (AER) and *Fgfr2c* in the mesenchyme) are expressed in the limb bud. Three FGFRs (*Fgfr1, -2, -3*) are expressed in the facial primordia and one (*Fgfr2*) specifically in the branchial arch ectoderm (Table 2). *Fgfr2b* can bind *Fgf2*, *-3* and *-10*, suggesting that some of these ligands are involved in facial development (Francis-West *et al.,* 1998). FGF signalling plays a critical role in the initiation of limb bud outgrowth by the inductive activity of *Fgf10* in the lateral mesoderm mediated through a signalling loop involving reciprocal activation in the AER and mesenchyme of *Fgfr2-IIIb* to *Fgf4/Fgf8* to *Fgfr2-IIIc* (Xu *et al.,* 1998; Revest *et al.,* 2001). In the developing limb bud, *Fgf4* expression is confined to the posterior (post-axial) part of the AER, whereas *Fgf8* is expressed in the entire AER with *Fgf10* in the distal mesenchyme. In the forelimb bud, complete loss of *Twist* activity does not significantly alter *Fgfr2* expression but leads to the down-regulation of *Fgf10* in the posterior mesenchyme and the absence of *Fgf4* and *Fgf8* expression in the posterior AER. *Twist* therefore is likely to act downstream of *Fgfr2-IIIc* in the mesenchyme but is critical for the activation of *Fgfr2-IIIb*, *Fgf4* and *–8* in the posterior AER and *Fgf10* in the posterior mesenchyme. Expression of *Fgf8* in the anterior AER and *Fgf10* in the anterior mesenchyme is apparently independent of *Twist*. Interestingly, a much less dramatic change in FGFR/FGF activity is found in the hindlimb bud with *Fgf4* the only gene significantly affected, showing expansion of expression anteriorly. This limb-specific response of FGF signalling to *Twist* absence may underpin the differential effect of the mutation on the extent of limb bud outgrowth. The complete loss of *Fgf4* and the more restricted expression of *Fgf8* in the forelimb bud may lead to a more drastic arrest in growth. Conditional gene inactivation experiments have shown that in the absence of *Fgf8* activity in the AER, forelimb bud growth is more affected than that of the hindlimb because of the longer duration that the forelimb bud is subjected to lack of FGF signalling before *Fgf4* is activated to restore the inductive function in the AER (Lewandowski *et al.,* 2000). A more refractory response of the forelimb bud tissues to activate *Shh* expression in response to *Fgf4* stimulation is found in the avian embryo, again highlighting the difference in competence of limb bud mesenchyme to respond to FGF signalling (Wada and Nohno, 2001).

Similar down-regulation of FGF signalling activity may also occur in the *Twist*-deficient branchial arches. Analogous to its function in the limb, *Fgf8* is one of the key players in the oral epithelium which is capable of inducing and/or maintaining expression of mesenchymal genes in the mandibular arch (Neubuser *et al.,* 1997; Kettunen and Thesleff, 1998; Ferguson *et al.,* 1998; Tucker *et al.,* 1999), where it is also responsible for establishing and maintaining rostral-caudal polarity (Tucker *et al.,* 1999; Francis-West *et al.,* 1998). The mutant embryo completely loses the expression of *Fgfr2, Fgf4* and *Fgf10* in both first and second arches. *Fgf8* expression is lost in the distal anterior ectoderm of the mandibular component of the first arch and is markedly reduced in the second arch. That truncated arches can be formed in the *Twist*$^{-/-}$ embryo suggests that the residual *Fgf8* activity is sufficient to initiate the outgrowth of the arch (Trumpp *et al.,* 1999). The failure to sustain more growth could be due to the reduction in overall signalling activity in the absence of *Twist* function similar to that in the limb bud. Thus of all the FGFs, FGF8 function is particularly critical for the development of both the branchial arch and the limb (Fig. 1).

***Shh* activity and downstream factors.** *Shh* is expressed in the zone of polarising activity in the posterior mesenchyme of the limb buds where it is responsible for anterior-posterior patterning of the digits (Laufer *et al.,* 1994; Niswander *et al.,* 1994). A similar role for *Shh* in pattern formation of the branchial arches has been proposed (Wall and Hogan, 1995; Helms *et al.,* 1997). *Shh* expression is localized to the distal anterior epithelium of the mandibular component of the first branchial arch (Table 2) where it is required for the regionalization of *Prx1* and *–2* activities in the mesenchyme (ten Berge *et al.,* 2001). Similar to its morphogenetic role in the limb, *Shh* has been found to stimulate growth and shaping of the mandibular processes by its regionalized mitogenic activity on the mesenchyme (ten Berge *et al.,* 2001) and the maintenance of cell viability (Ahlgren and Bronner-Fraser, 1999). Null mutation of *Shh* in mice results in almost complete absence of craniofacial skeletal elements despite apparently normal development of the branchial arches until E9.5 (Chiang *et al.,* 1996), indicating that *Shh* is needed to sustain but not to initiate outgrowth. Akin to its role in imparting positional information in the limb, *Shh* activity also specifies the dental pattern in the mesial part of the jaw well before the initiation of incisor development (ten Berge *et al.,* 2001).

Shh activity is completely lost in the posterior mesenchyme in the *Twist*$^{-/-}$ forelimb bud and is markedly diminished in the branchial arch. The expression of some of the downstream molecules that modulate SHH signalling is also altered. In the forelimb bud, *Gli3* activity in the anterior mesenchyme and *Gli2* activity in the proximal mesenchyme are both lost. *Gli1* and *Ptch* expression, which are normally localised in the posterior mesenchyme, are found ectopically in the anterior mesenchyme. In the branchial arch, expression of *Gli1* and *Ptch* are markedly down-regulated, and *Gli2* and *Gli3* are no longer expressed. Contrary to observations in the forelimb bud, expression of *Shh* and four of its downstream genes is maintained in the appropriate tissue domains in the hindlimb bud. The loss or reduction of *Shh* expression in the arch and forelimb may be related to the suppression of FGF activity, rather than a direct effect of *Twist* deficiency. In the limb bud, activation of *Fgfr2* initiates the expression of *Fgf4* in the AER (Revest *et al.,* 2001). This FGF activity induces the expression of SHH, which then establishes a positive feedback control on further *Fgf4* activity. SHH may also act via *Fgf10/Ffgr2b* to maintain the activity of FGF4 in the posterior AER (Revest *et al.,* 2001). The retention of FGF activity in the hindlimb bud may have resulted in the apparently normal SHH activity.

The three GLI zinc-finger genes, *Gli1, -2* and *-3*, are implicated in transduction of SHH signals: *Gli1* and *-2* are thought to be the activators of SHH signalling while *Gli3* is the major repressor which acts in a SHH-independent manner (Aza-Blanc *et al.,* 2000). The loss of *Gli3* expression and the ectopic expression of *Gli1* and *Ptch* in the anterior mesenchyme is coincidental to the loss of *Twist* in the same tissue. That *Gli1* and *Ptch* are expressed in the absence of *Shh* suggests that both factors are activated by default widely in the limb mesenchyme but their final pattern of activity is maintained by *Shh* in the posterior mesenchyme and repressed by *Gli3* in the anterior mesenchyme. One of the roles of *Twist* is likely to activate or maintain a graded pattern of *Gli3* activity to suppress SHH signalling in the anterior mesenchyme and thereby pattern the limb mesenchyme. A possible disruption in tissue pattern of the mutant forelimb is revealed by the loss of *Bmp4* and *Msx1* expression in the posterior limb tissue and the shift of *Hoxd13* expression to the anterior mesenchyme. The changes in activity of the SHH signalling pathway in the *Twist*$^{-/-}$ branchial arch may also be related to the loss of FGF activity and the lack of repression of downstream SHH signalling by *Gli3*, and may be responsible for the loss of distal jaw structures in the truncated first arch. Whilst *Gli2* and *-3* are expressed in migrating neural crest and all three *Gli* genes are found in the mesenchyme of the branchial arch (Hui *et al.,* 1994; Walterhouse *et al.,* 1993), their effect on SHH signalling in the craniofacial tissues remains to be clarified (Francis-West *et al.,* 1998). The experimental evidence, however, has strongly supported the postulation that *Twist* influences SHH signalling in both the arch and the limb indirectly via its effect on FGF activity and the upstream regulation of *Gli2* and *Gli3* expression (Fig. 1).

BMP signalling. Three vertebrate bone morphogenetic protein (BMP) family members, *Bmp2*, *-4*, and *-7*, are expressed in both the ectoderm and mesenchyme of the limbs and the 1st branchial arch (Table 2). In the limb their broad expression domains encompass the mesenchyme of the anterior, posterior, and interdigital necrotic zones, which subsequently undergoes apoptosis, contributing to the widely-held belief that BMPs are the signals responsible for cell death in the limb (Zou and Niswander, 1996). BMPs also promote the regression of the AER at the end of the outgrowth phase (Ganan *et al.,* 1998; Pizette and Niswander, 1999), regulate chondrogenic differentiation (Duprez *et al.,* 1996a; Macias *et al.,* 1997; Merino *et al.,* 1998; Enomoto-Iwamoto *et al.,* 1998), control muscle formation (Duprez *et al.,* 1996b; Amthor *et al.,* 1998), and influence the morphological identity of the digits (Dahn and Fallon, 2000). Loss of *Twist* activity results in the down-regulation of *Bmp4* in the surface ectoderm of the mandibular component of the first arch and in the posterior AER and subjacent mesenchyme of the forelimb bud (Fig. 1). These changes in *Bmp4* expression are accompanied by the down-regulation of the putative downstream *Msx1* gene in the mesenchyme of the branchial arches and the limb bud. *Twist*-deficiency, however, has no effect on the expression of *Bmp4* and *Msx1* in the hindlimb. Attempts to assess the specific function of *Bmp2*, *-4* and *-7* by knockout mutation have been hampered by early embryonic lethality (*Bmp2, -4*: Winnier *et al.,* 1995; Zhang and Bradley, 1996) or functional redundancy (*Bmp7:* Dudley *et al.,* 1995; Luo *et al.,* 1995; Dudley *et al.,* 1995). However examination of *Bmp4*$^{+/-}$ mice has revealed shorter frontal and nasal bones, and hindlimb polydactyly (Dunn *et al.,* 1997). Whether *Twist* activity may be altered by the loss of BMP in these mice has not been investigated.

Downstream and Interacting Factors

Analysis of downstream targets of *Twist* in the worm and the fly has revealed that they are predominantly associated with the specification and patterning of the mesodermal tissues. In *C. elegans*, *ceTwist* in conjunction with another bHLH E-protein, Daughterless, can activate two target genes, *ceh-24* and *egl-15*, which encode an NK-2 class homeodomain and a homolog of FGF receptor (Harfe *et al.,* 1998). Both genes are expressed in the M

TABLE 3

MUTANT PHENOTYPES OF GENES THAT ARE DOWNSTREAM OF *TWIST* ACTIVITY REPRESENT SUBSETS OF THE *TWIST* PHENOTYPE

Gene	Craniofacial defects	Limb defects	References
Twist	**Skull asymmetry and synostosis (+/-)** **Neural tube closure defect (-/-)** **Abnormal branchial arches (-/-)**	**Preaxial polydactyly (+/-)** **Hypoplasia and patterning defects (-/-)**	Chen and Behringer, 1995; Bourgeois *et al.,* 1998; O'Rourke *et al.,* 2002
Fgf10	None	Limb buds initiate but do not develop (-/-)	Sekine *et al.,* 1999
Shh	Holoprosencephaly (-/-)	Hypoplasia of distal components (-/-)	Chiang *et al.,* 1996
Ptch	Neural tube closure defect (-/-)	Preaxial polydactyly 1% (+/-)	Goodrich *et al.,* 1997; Milenkovic *et al.,* 1999
Gli2	Microcephaly, shortened jaws, missing incisors, cleft palate (-/-)	Shortened long bones of the limbs (-/-) Postaxial nubbin on forelimb (*Gli1*$^{-/-}$;*Gli2*$^{-/-}$)	Mo *et al.,* 1997; Park *et al.,* 2000 Mo *et al.,* 1997; Dunn *et al.,* 1997
Gli3^{xtJ}	Exencephaly (-/-)	Preaxial polydactyly of hindlimbs (enhanced in *Gli3*$^{+/-}$;*Bmp4*$^{+/-}$) and postaxial nubbin on forelimb (+/-) Polysyndactyly (-/-)	
Alx4	Mild skull bone defects (reduced size of parietal bone) (-/-)	Preaxial polydactyly of hindlimbs (*Alx4*$^{+/-}$ F1 progeny of a 129/Sv x C57BL/6 mating) Preaxial polydactyly of all 4 limbs (*Alx4*$^{-/-}$) which is enhanced in *Alx4*$^{-/-}$;*Cart*$^{+/-}$ mice and enhanced further in *Alx4*$^{-/-}$;*Cart1*$^{-/-}$	Qu *et al.,* 1997, 1998, 1999
Cart1	Exencephaly with secondary defects in the formation of craniofacial bones and cartilages, including absent skull vault; absent eyes (-/-) Hypoplastic jaw	None	Zhao *et al.,* 1996
Alx3;Alx4	Broader and shorter cranium and variable facial clefting comprising the entire nose region (*Alx3*$^{-/-}$; *Alx4*$^{1st\text{-}J/1st\text{-}J}$, *Alx3*$^{-/-}$; *Alx4*$^{1st\text{-}J/+}$ and *Alx3*$^{+/-}$; *Alx4*$^{1st\text{-}J/1st\text{-}J}$). Malformations of most facial bones increasing in severity from *Alx3*$^{+/+}$; *Alx4*$^{1st\text{-}J/1st\text{-}J}$, to *Alx3*$^{-/-}$; *Alx4*$^{1st\text{-}J/+}$ to *Alx3*$^{+/-}$; *Alx4*$^{1st\text{-}J/1st\text{-}J}$ to *Alx3*$^{-/-}$; *Alx4*$^{1st\text{-}J/1st\text{-}J}$.	Preaxial polydactyly of fore- and hindlimbs similar to that described for *Alx4*$^{-/-}$. Absent deltoid crest in forelimb *(Alx3*$^{-/-}$; *Alx4*$^{-/-}$*)*	Beverdam *et al.,* 2001

blast cell that gives rise to subsets of non-striated muscles and the activation of these genes is brought about by response of the cis-acting element in the promoter to *ceTwist*. A conserved molecular pathway has been described in the fly, where *twist* activity is mediated by activation of the FGFR homologue, *heartless*, and the NK domain gene, *tinman* (also called *NK-4* and *msh-2*; Yin *et al.,* 1997). In the fly, *twist* acts as a positive regulator of mesoderm differentiation by promoting the expression of *PS2* and *how* (held out wings) in the invaginating mesoderm and limiting that of *crumbs* in the lateral ectoderm (Leptin, 1991; Zaffran *et al.,* 1997). *twist* activates the *NK-2* gene in the posterior portion of the embryo and acts with *dorsal* to activate *snail* expression (Mellerick and Nirenberg, 1995; Ip *et al.,* 1992). *Tinman* is a direct transcriptional target for *twist* and its own gene product in visceral mesodermal cells, supporting the idea that *twist* and *tinman* participate in the subdivision of the mesoderm during embryogenesis (Lee *et al.,* 1997). The *tinman* gene is activated by a number of discrete enhancer elements respectively for the early mesoderm, the dorsal mesoderm and the cardioblasts. Early *tinman* expression can only be achieved by a direct interaction of *twist* on the early-acting enhancer element (Bodmer *et al.,* 1990; Yin *et al.,* 1997). *MEF2*, a myogenic regulatory gene, is also a direct target for transcriptional activation by *twist* during myogenesis of the somatic mesoderm (Lilly *et al.,* 1994; Taylor *et al.,* 1995; Cripps *et al.,* 1998). In zebrafish, expression of *zfh-1*, a zinc-finger- and homeobox-containing gene that is expressed in the early mesoderm and later in the forming heart mesoderm, requires activity of *twist* and *snail* (Lai *et al.,* 1991). Whether this myogenic gene is a direct target is not known.

A recent genome-wide surveillance of the transcriptional profile of *Drosophila* embryos has identified numerous genes whose activity has been significantly altered in response to either loss of *twist* function or constitutive up-regulation of *twist* by expressing a gain-of-function $Toll^{10B}$ allele (Furlong *et al.,* 2001). These genes encode transcription factors, signal transduction molecules, kinases and pioneer proteins that are required for stage-related mesoderm specification and differentiation. However, there are also non-mesodermal factors among the responding genes.

That the expression of *myogenin* and *Myf5* is maintained (Chen and Behringer, 1995; our unpublished observations) in the absence of *Twist* strongly suggest that *Twist* is not required to activate myogenic factors in the muscle precursor cells. Ectopic expression of *Twist* inhibits myogenesis by blocking DNA binding by *MyoD*, by titrating E proteins, and by inhibiting trans-activation by *MEF2* by heterodimerization with E proteins. Thus, *Twist* may act directly on the myogenic factors by repressing their transcriptional activity in non-myogenic tissues (Spicer *et al.,* 1996).

Our approach of analysing expression of candidate downstream target genes in craniofacial tissues, somites and limb buds reveals that *Twist* activity may influence SHH and FGF signalling activity. In the forelimb bud, loss of *Twist* is accompanied by the loss of *Shh*, *Gli2* and *Gli3* expression. Expression of *Gli1* and *Ptch* is down-regulated in the post-axial mesenchyme, but is found ectopically in the pre-axial mesenchyme. SHH signalling is known to be modulated by a combination of positive and negative factors, and in the limb bud, the processing of the GLI3 factor establishes a pre- to post-axial repressor gradient that constrains the SHH signalling activity to the post-axial mesenchyme. The loss of GLI3 and GLI2 activity may cause the failure to suppress the default SHH-activating GLI1 and PTCH activity in the pre-axial mesenchyme and the initiation and/or maintenance of SHH signalling activity in the post-axial mesenchyme. It is not known whether *Twist* has a direct effect on transcription of the *Gli* genes, however the microarray analysis in *Drosophila* has identified an invertebrate *Gli* homologue (*gleeful*) that is critical for muscle differentiation (Furlong *et al.,* 2001). Furthermore, the demonstration of an enhanced polydactylous phenotype in the limbs of compound *Twist;Gli3* mutant mouse embryos (O'Rourke *et al.,* 2002) has provided direct evidence of a genetic interaction between the activity of *Twist* and *Gli3* in limb patterning, and therefore that *Twist* may be acting synergistically with SHH signalling.

In contrast to *Drosophila*, a direct interaction between vertebrate TWIST and FGFR genes has not yet been established. Analysis of the pattern of gene expression in the sutural mesenchyme of the skull shows that *Twist* expression precedes that of FGFR genes when suture formation initiates, and that there is subsequently some overlap in expression domains of *Twist* and *Fgfr2* (Johnson *et al.,* 2000; Morriss-Kay *et al.,* 2001). The precise position of *Twist* in the FGF signalling cascade is not fully known. In the $Twist^{-/-}$ mutant forelimb bud, expression of *Fgf4* in the apical ectoderm ridge is absent, while that of *Fgf8, Fgf10* and *Fgfr2* is much reduced. In branchial arches and brain, *Fgf8* and *Fgfr2* expression is found in ectopic sites, but *Fgf8* and *Fgfr2* activity in the paraxial mesoderm is unchanged. In the chick wing bud, removal of the apical ectoderm ridge leads to loss of *Twist* expression but replenishing *Fgf4/8* maintains *Twist* in the AER-ablated limb (Tavares *et al.,* 2001). *Fgf2/8* can also induce ectopic *Twist* expression in the flank of the body (Isaac *et al.,* 2000). A similar inductive effect of *Fgf2* on *Twist* expression is found in fetal calvarial tissues (Rice *et al.,* 2000). These findings are consistent with the notion that *Twist* and FGF are integrated in a reciprocally interactive pathway whereby FGF induces *Twist,* which in turn regulates FGFR activity (Fig. 1).

Functional interactions between FGFs and WNTs have been shown to take place in a variety of organs including development of the tooth, kidney, brain, and limb (reviewed in Moon *et al.,* 1997), and initiation of the inner ear (Ladher *et al.,* 2000). In chick limb, *Wnt2b* and *Wnt8c* induce *Fgf10* activity in the mesoderm, an event that is essential to initiate limb bud outgrowth. *Fgf10* acting in concert with *Wnt3a* then induces formation of the AER (Kawakami *et al.,* 2001; Huelsken and Birchmeier, 2001; reviewed in Martin, 2001). *Wnt7a* in the dorsal ectoderm and *En1* in the ventral ectoderm are responsible for dorso-ventral subdivision of the limb bud (Yang and Niswander, 1995; Parr and McMahon, 1995). The impact of TWIST on WNT signalling has yet to be investigated but it is of interest to note that *Wnt6* expression in the dorsal myotome is altered in the absence of *Twist* activity (O'Rourke and Tam, unpublished).

Loss of *Twist* function disrupts the expression of genes encoding the ALX transcription factors (Loebel *et al.,* 2002). A comparison of the effects of loss of function of these genes shows that the pleiotropic phenotype of the *Twist* mutant might represent the summation of the phenotypic consequences of these individual gene mutations (Table 3), suggesting that they are potential downstream genes. The overlapping phenotypic outcome of *Alx* and *Twist* mutations, the universal repression of *Alx* gene activity in the $Twist^{-/-}$ mutant, and the presence of possible E-box like HLH binding sites in conserved regions of the upstream region of mouse

and human *Alx4* orthologues (Loebel *et al.,* 2002) argue that the *Alx* genes may even be direct targets. *Twist* and another bHLH factor (*Dermo1*) can inhibit oncogene- and p53-dependent cell death (Maestro *et al.,* 1999). This effect correlates with an ability of *Twist* to interfere with activation of a p53-dependent reporter and to suppress the induction of p53 target genes in response to DNA damage. It has been postulated that *Twist* may interfere with the p53 tumor-suppressor pathway indirectly through modulation of the ARF/MDM2/p53 activity (Maestro *et al.,* 1999).

The investigation of the effects of gain or loss of gene activity on embryonic development and mesodermal differentiation has provided significant insight into the function of the *Twist* gene. It also reveals that the translation of the transcriptional regulation to the control of morphogenesis and tissue differentiation requires the activation or repression of molecular pathways that are downstream of, and/or interacting with, *Twist*. The identification of downstream factors is achieved by assessing whether the expression of specific genes may be altered by ectopically expressing or over-expressing *Twist*, or by interference with *Twist* activity. The analysis has taken two different approaches: first, specific sets of genes that are known to be involved with tissue morphogenesis and differentiation may be examined for being candidates of the downstream genes affected by *Twist* expression; second, a genome-wide screening without any predilection of the response of all the expressing genes to the alteration of *Twist* activity (Furlong *et al.,* 2001). Neither approach, however, could distinguish between direct target genes and genes that are secondarily regulated by *Twist* downstream factors, without additional validation of transcriptional interaction. Further elucidation of the differential transcriptional activity of wild type and *Twist* mutant embryonic tissues coupled with functional tests of the candidate genes is crucial for a proper understanding of the molecular pathways regulated by TWIST, and to ascertain whether this transcription factor acts primarily as a repressor or as an activator.

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