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Original Article

R-twist gene expression during rat palatogenesis

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ABSTRACT Palatal clefting is often associated with premature fusion of cranial sutures in human craniosynostosis syndromes, many of which are characterised by mutations affecting the fibroblast growth factor receptor (*FGFR*) gene family. In palatal fusion, epithelio-mesenchymal transition (EMT) contributes to the dispersion of the midline epithelial seam. EMT has also been observed in neoplastic epithelial cells in relation to the acquisition of malignant characteristics where morphological changes are accompanied by rapid switching in the expression of *fgfr2* from the epithelial type (*kgfr*) to the mesenchymal type (*bek*). The *twist* gene codes for a basic helix-loop-helix transcription factor putatively involved in regulation of transcription of *fgfr2*. Mutations in the *TWIST* gene have been described as being responsible for the Saethre-Chotzen syndrome, an autosomal dominant craniosynostosis associated with cleft palate as well as other disturbances of the facial skeleton. In this study we have analysed the distribution of *twist* transcripts during rat palatogenesis *in vivo* from 14.5 to 17.5 days post coitum by *in situ* hybridisation with digoxygenin-labelled ssDNA probes. *twist* transcripts were found to be concentrated in mesenchymal cells beneath the epithelium at the tip of the palatal shelves immediately prior to, and during fusion as well as in a localised epithelial area at the tip of the shelves prior to fusion, thereby implicating *twist* gene expression in the process of palatogenesis. This pattern of expression illuminates the disturbances of maxillary growth that occur in human craniosynostotic syndromes.

KEY WORDS: *twist, palate development, in situ hybridisation, epithelial-mesenchymal transition, rat.*

Introduction

Formation of the secondary palate in normal facio-maxillary development in the foetus is dependent on successful integration of a complex sequence of events. Downgrowth, elevation, adhesion and fusion of the two palatal shelves must occur to achieve correct alignment, approximation and eventually fusion of embryonic structures initially covered by epithelium. Failure at any step of these events in the mid-palatal region can be responsible for development of cleft palate (Ferguson, 1988). Approximately 10 days are necessary to complete the normal fusion process in the Human (Greene and Pratt, 1976) whereas the same process in rodents is completed in approximately 24 hours. In the Human, the disappearance of the mid-palatal seam is often incomplete and associated with the persistence of epithelial nests or remnants called Epstein's pearls in the palatal midline (Wood and Kraus, 1962) whereas in rodents the seam disappears completely. Three mechanisms have been proposed as responsible for the disappearance of the midline epithelial seam required for fusion: apoptosis or programmed cell death (Shuler, 1995), epithelial-mesenchymal transition (EMT) (Fitchett and Hay, 1989; Griffith and Hay, 1992; Shuler *et al.*, 1992; Shuler, 1995; Sun *et al.*, 1998; Gibbins *et al.*, 1999) and migration and integration into the nasal or oral epithelium (Carette and Ferguson, 1992). Contraction-retraction of the epithelial sheet has also been proposed recently as a major contributor to the breakdown of the epithelial seam (Gibbins *et al.*, 2000).

The normal fusion process in the palate results in the formation of a suture-like structure that remains active almost until adulthood (Freng and Kvam, 1979). Normal growth of the mid-palatal region

Abbreviations used in this paper: b-HLH, basic helix-loop-helix; d.p.c., days post coitum; EMT, epithelial-mesenchymal transition; Fgfr, fibroblast growth factor receptor; ISH, *in situ* hybridisation; PBS, phosphate buffered saline; Tgfβ, transforming growth factor beta.

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is dependent on normal function of this mid-palatal suture and maxillary growth can be impeded by failure to form a functional suture (Friede, 1998).

Saethre-Chotzen syndrome is an autosomal dominant craniosynostosis (i.e. abnormal premature closure of cranial sutures). The diagnosis is based on the clinical findings of coronal craniosynostosis and characteristic facial features. Associated oral anomalies can include maxillary hypoplasia and a narrow or high arched palate, cleft palate on occasion, malocclusion, supernumerary teeth and enamel hypoplasia (Cohen, 1986). Goho, 1998 described also tooth shape abnormalities as well as the existence of pulp stones in the pulp chambers of all molars and premolars. El Ghouzzi *et al.*, (1997), Howard *et al.*, (1997), Krebs *et al.*, (1997), Rose *et al.*, (1997) demonstrated that Saethre-Chotzen syndrome (ACS III, OMIM 101400) results from mutation in *H-TWIST*. The human *H-TWIST* gene is located on chromosome 7p21 and the encoded protein presents 96% similarity with the murine Twist protein (Bourgeois *et al.*, 1996). *twist* was originally identified in *Drosophila* as one of the zygotic genes required for dorso ventral patterning, defining with *snail* the mid-ventral domain of the blastoderm (Simpson, 1983; Anderson, 1987). In *Drosophila*, *twist* gene expression is vital at gastrulation: in the homozygous *twist* mutant embryos, the mid ventral cells fail to invaginate to form the ventral furrow. The resulting "twist" embryo does not differentiate mesodermal derivatives and dies at the end of embryogenesis (Simpson, 1983). In *Drosophila*, *twist* codes for a nuclear protein essentially localised in the mesoderm layer (Thisse *et al.*, 1987a,b, 1988). The protein contains a basic helix-loop-helix (b-HLH) domain shared by several regulatory proteins and implicated in dimerization (Murre *et al.*, 1989). The basic DNA-binding domain is amino terminal to the HLH motif.

Vertebrate homologues of *twist* were subsequently isolated in *Xenopus, X-twi* (Hopwood *et al.*, 1989) and *Mus Musculus, M-twist* (Wolf *et al.*, 1991). In post-implantation embryos, *M-twist* transcripts are detected in particular in subsets of the mesoderm layer such as somites (derived from paraxial mesoderm), limb buds (derived from lateral mesoderm), head mesenchyme and in mesenchymal cells of the first branchial arches derived from neural crest cells (Wolf *et al.*, 1991; Füchtbauer, 1995; Stoetzel *et al.*, 1995). This expression pattern suggests that in vertebrates Twist might regulate genes involved in the specification or differentiation of mesoderm and in the development of mesenchymal tissues in the cranial region.

M-twist is a vital gene. Homozygous mutant *twist*-null embryos die at 11.5 days p.c. with an obvious failure of neural tube closure (Chen and Behringer, 1995). *twist*-null heterozygous mice present skull and limb defects as well as defects in middle ear formation and the xyphoïd process thus mimicking the disorders observed in the human Saethre-Chotzen syndrome. The defects were observed in tissues derived from cells expressing *M-twist* and the expressivity of the phenotype was dependent on the genetic background. No cleft palate was described in these heterozygotic animals, however a delayed growth in the palatal bones was noticed (El Ghouzzi *et al.*, 1997; Bourgeois *et al.*, 1998).

In this study we have analysed the distribution of *R-twist* transcripts during rat palatogenesis *in vivo* from 14.5 to 17.5 days p.c. by *in situ* hybridisation with digoxygenin-labelled ssDNA probes. Our results show increased expression of *R-twist* concentrated in the mesenchyme in the region of palatal fusion during palatal elevation and the subsequent fusion events. The results indicate that *R-twist* expression plays a significant role in the fusion process and help to explain disturbances of maxillary growth observed in the Saethre-Chotzen syndrome.

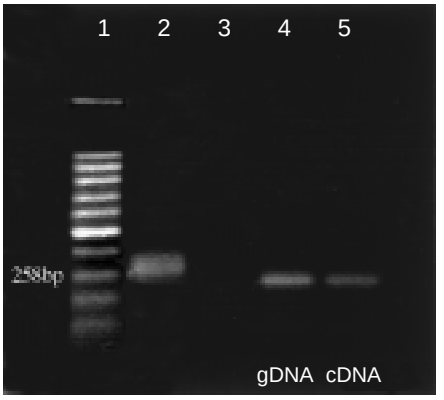

Fig. 1. Electrophoresis of QK1, QK3 PCR products. *Ethidium bromide stained agarose gel shows a single band between 250 and 300 bp. The same unique band is observed from genomic DNA or cDNA QK1, QK3 PCR products. Lanes: 1, 100 bp DNA ladder; 2, control GAPD; 3, no product; 4,5, 258 bp QK1, QK3 PCR products amplified from (4)genomic DNA or (5) complementary DNA*

Results

Rattus norvegicus twist cDNA bHLH domain and a 258bp fragment

A single band of approximately 250bp was visible at the same level both in genomic DNA PCR product amplification and in cDNA (Fig. 1). The amplified product was inserted into the pGEM®-T Vector and a 258bp fragment was sequenced between the QK1 and QK3 primers using SP6 and T7 primers to generate the total product from the insert. The region of interest was sequenced in both directions.

The sequence of the *twist* cDNA b-HLH domain between the primers (QK1 and QK3) used to generate probes for ISH is detailed in Fig. 2 and is identified by the GenBank accession number AF266260.

The fragment was aligned with mouse *M-twist* gene using the sequence comparison program NIH-BLAST. The sequence corresponded to the *twist* encoding b-HLH domain. It showed 98% similarity to the mouse counterpart with 255 identical nucleotides in a total of 258. The three different nucleotides resulted in codon changes of acc instead of acg, gca instead of gcg and ttt instead of ttc. These nucleotide differences would not produce amino acid modifications in protein, coding respectively for T, A, F (Fig. 2).

The sequence showed a similarity with the *Homo sapiens TWIST* gene of 96% or 248/258 nucleotides. Shared homology with *Rattus norvegicus dermo-1* cDNA is 88% (215/242).

These data confirmed the sequence specificity of the probe generated from the insert as the *Rattus norvegicus twist* b-HLH encoding domain.

Expression pattern (ISH results)

The pattern of expression revealed by ISH showed *twist* transcripts concentrated in mesenchymal cells beneath the epithelium in the palatal shelves immediately prior to and during fusion thereby implicating *twist* gene expression in the process of palatal fusion (Fig. 3).

At 14.5 (Fig. 3 A,B,C) and 15.5 (Fig. 3 D,E) days p.c. the *twist* gene was transcribed in the mesenchyme in the forming palatal shelves that lie lateral to the tongue and extend in a vertical direction. The superficial mesenchyme underlying the epithelium is more intensely labelled. The side of the palatal shelves facing the tongue was more intensely labelled than the side facing the cheek. The mesenchyme

at the tip of the shelves was strongly stained indicating that these cells were expressing the gene at a high level. At 14.5 days p.c. some epithelial cells localised at the tip of the shelves demonstrated positive staining (Fig. 3C, arrow). Staining for transcripts was also detected in the mesenchyme underlying the tongue epithelium (Fig. 3 A through F) and in tooth mesenchyme (Fig. 4). The staining in the mesenchyme of the tongue was confined to a narrow subepithelial band opposite the palatal shelves.

At 16.5 (Fig. 3 F,G,H) days p.c. the palatal anlage began to elevate to reach a horizontal position rostral to the tongue and to come into contact in the midline area. The tongue was displaced from the developing nasal cavity, adopting a lower position. Transcripts were visible in the palate and maxillary area and were more abundant in the tip regions. The palatal shelves displayed strong mesenchymal staining prior to contact (Fig. 3G) and immediately following contact (Fig. 3F) as the process of fusion began. The staining was not uniform however, with some mesenchymal regions presenting a relatively weak signal, particularly around the larger palatine vessels.

At 17.5 (Fig. 3I) days p.c. the fusion proceeds with rapid and progressive disappearance of the midline epithelial seam and swelling of the palatal shelves. Mesenchymal cells in fusion area strongly expressed *twist*, especially the mesenchymal cells occupying the region of disappearance of the epithelial cells. Mesenchymal cells in the expanded mesenchyme more distant from the site of fusion showed relatively reduced levels of expression. The epithelia becoming the nasal and oral surfaces and the nasal and oral triangles formed as the epithelium of the seam breaks down do not show evidence of expression of *twist* gene, but are lined by a narrow subepithelial band of mesenchymal cells expressing the gene. Epithelial remnants detectable in the region of seam disintegration were devoid of any labelling.

During tooth development from the dental lamina to the bud and then cap (16.5 days p.c., Fig. 4A) stages, *twist* transcripts were detected in the whole dental papilla. At the bell stage (17.5 days p.c, Fig. 4D) they were confined to the mesenchyme facing the inner dental epithelium.

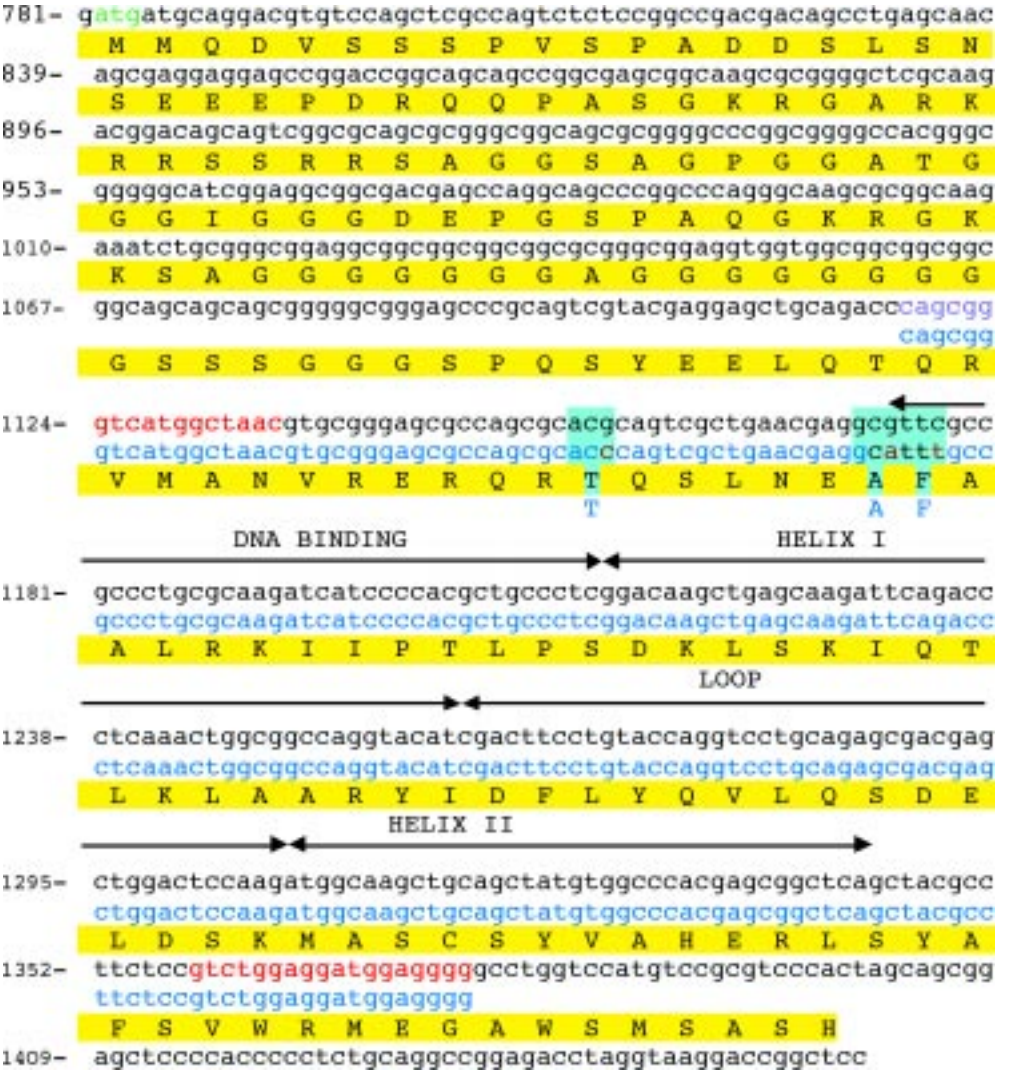

Fig. 2. Sequence comparison of the rat 258bp QK1, QK3 DNA fragment, with the mouse *twist* gene basic helix-loop helix domain. *The differences concern 3 nucleotides of the basic domain. The translated protein is identical in both species. Mus Musculus (black sequence); Rattus Norvegicus (blue sequence); primers QK1, QK3 (red); atg, initiation codon, (green); protein sequence in yellow box).*

Discussion

The rat b-HLH domain encoding sequence of the rat *twist* gene identified with the QK1 and QK3 primers differed in only three base pairs from the mouse counterpart, resulting in 98% homology between the two sequences. The homology with *Rattus norvegicus* sequence for the Twist related b-HLH Dermo1 protein was rated as 88%. The constructed sscDNA probe thus can be considered as being specific under the high stringency conditions used in this study and legitimately used to determine rat *twist* gene expression during palatogenesis.

The specificity of the ISH reaction was also assessed using the rat cytokeratin K5/6 control probe specific for epithelium (Gibbins *et al*., 1999) to indicate a relative level of expression of *twist*. The pattern of hybridisation of the PCR probe for *R-twist* in the developing rat tooth was identical to the pattern of expression of *M-twist* described in Bourgeois *et al.,* (1998) using the *Xba*I-*Eco*RI 420bp *M-twist* exon 2 fragment as probe providing additional validation that the probe was identifying *twist* mRNA.

The pattern of expression detected with this probe is consistent with a role for the *twist* gene in mesenchyme regionalization during palatogenesis. The transcripts were localised in the mesenchyme

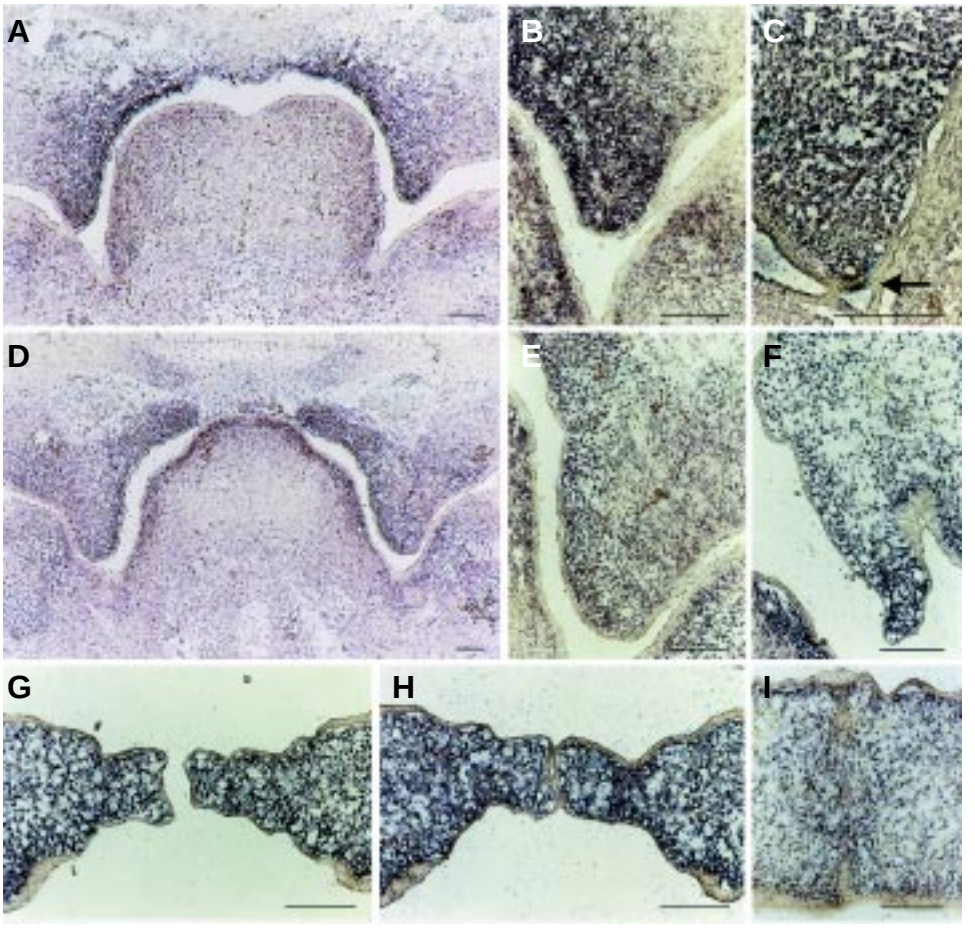

Fig. 3. Expression pattern of the rat *twist* gene during palatogenesis. *At 14.5 d.p.c., the palatal shelves were growing downward from the maxillary prominences, expending vertically towards each lateral side of the tongue.* twist *transcripts were detected in the palatal mesenchyme and their distribution was highest in subepithelial mesenchyme; the mesenchyme facing the tongue was more labelled than the outer mesenchyme. The mesenchyme underlying the tongue epithelium displayed also a signal for twist* **(A)**. *The tips of the shelves were strongly labelled* **(B)**. *In some specimen, an epithelial expression for the twist gene was discovered in a few cells at the tip of the shelves* **(C)**. *At 15.5 d.p.c., the palatal shelves have developed considerably. The transcription of the twist gene was similar to that at the precedent developing stage* **(D)**. *The labelling was more intense on the palatal shelve side facing the tongue. No epithelial signal was detected* **(E)**. *16.5 d.p.c. demonstrated the elevation* **(F)** *and junction* **(G)** *of the palatal shelves on the midline to form the two layer midline epithelial seam* **(H)**. *The mesenchyme of the palatal shelves was strongly labelled during these events. The midline epithelial seam was devoid of any signal. [At 17.5 d.p.c.* **(I)**, *for the fusion process to be complete, the dispersion of the medial epithelial seam is necessary]; twist transcripts were observed in the fusion area in the mesenchyme. The epithelial remnants did not express twist gene. Mesenchymal cells underlying the nasal or oral epithelium showed positive staining. The lateral internal mesenchymal area seemed less labelled. Bar, 100 μm.*

of the palatal processes during all the events leading to palate formation and closure. Subepithelial focus of expression was evident with strongest expression in the tip of the shelves, in the mesenchyme underlying the oral epithelium facing the tongue, and then as the fusion proceeds, in the mesenchyme underlying the oral and nasal epithelium and in the area of fusion. Beside indicating a role of *twist* expression during the morphogenetic process, these gradients could also reflect a heterogeneity in embryonic origin within the mesenchyme. Palatal mesenchyme is derived from the cephalic neural crest (Hall and Ekanayake, 1991; Le Douarin *et al.*, 1994). However, mesoderm derived from somitomere II and III and neural crest cells have been shown to colonise the maxillary prominences of the first branchial arch (Trainor *et al.*, 1994). The neural crest cells colonise the periphery of the branchial arches and envelop the somitomere-derived core tissues (Trainor and Tam, 1995). Somitomeres contributed frequently to the endothelium of branchial arch arteries (Trainor *et al.*, 1994). The lower level of expression observed in the central mesenchyme of the palatal shelves around the palatine vessels at 16.5 and 17.5 days p.c. may reflect this difference in the embryonic origins of the mesenchymal cells populating these regions.

An epithelial signal corresponding to a brief transient expression of *R-twist* gene in a few cells was discovered in the epithelium at the tip of the palatal shelves at 14.5 days p.c. The epithelial cells of the medial edge epithelium are specifically primed prior to integration into and breakdown of the medial epithelial seam to upregulate expression of mesenchymal genes (Gibbins *et al.*, 1999). These epithelial cells in the region of presumptive contact express keratins K8 and K5 prior to elevation of the palatal shelf and also dispaly the presence of α-smooth muscle actin protein prior to and during fusion (Gibbins *et al.*, 2000). Vimentin, an intermediate filament protein found in mesenchymal cells is upregulated in the same epithelial area before shelf contact indicating that the epithelial cells of the tip of the palatal shelves are primed before fusion to express genes consistent with an epithelio-mesenchymal transformation (Gibbins *et al.*, 1999).

Twist expression by some epithelial cells could then reflect an early priming signal for the cells towards a future mesenchymal fate. Epithelial *twist* gene expression occurs in other sites during development. Hopwood *et al.,* 1989 demonstrated expression of *Xtwi* in *Xenopus* cephalic neural crest and mentioned possible expression in non-mesodermal tissues. A brief transient expression is also observed in mouse neural crest cells prior to migration (Perrin-Schmitt, unpublished observations) and these epithelial cells are known to undergo an epithelio-mesenchymal transition process (Newgreen and Minichiello, 1995). The limited epithelial zone showing *twist* gene expression could represent relatively few cells primed to complete the epithelio-mesenchymal transition in the region of fusion. Indeed, evidence has been presented for the potential of other mechanisms (apoptosis, migration, contraction) to contribute to the disappearance of the medial epithelial seam (Carette and

Ferguson, 1992; Shuler, 1995; Gibbins *et al*., 2000). Alternatively, the findings could be explained by transient expression only within a particular epithelial cell as noted for pre-migratory neural crest cells (see above).

A confirmation of the role of *twist* gene during palatal development would require further identification and localisation of the corresponding protein.

Epithelio-mesenchymal transition has also been observed in neoplastic epithelial cells which acquire malignant characteristics where morphological changes are accompanied by rapid switching in the expression of *fgfr2* from the epithelial type (*kgfr*) to the mesenchymal type (*bek*) (Thiery and Chopin, 1999). These two different receptor molecules are generated from the one gene transcript by alternate splicing to produce the specific mRNAs. This switch between the two isoforms is very rapid as it does not require a change in transcription of the *fgfr2* gene. *twist* might regulate the transcription of target genes such as *fgfr2* (Shishido *et al*., 1993; Harfe *et al*., 1998) and might be involved in the upregulation of *fgfr2* expression prior to the *kgfr* to *bek* transition thus contributing to the specification of the mesenchymal fate of the cells.

Craniosynostosis syndromes, sharing the same common general clinical features with Saethre-Chotzen syndrome can be correlated with mutations affecting the fibroblast growth factor receptor (FGFR) gene family. Paznekas *et al*., 1998 screened unrelated patients with features of Saethre-Chotzen syndrome and found mutations in *TWIST*, *FGFR2* and *FGFR3* suggesting that *TWIST* and *FGFRs* might be components of the same molecular pathway involved in the modulation of craniofacial and limb development.

FGFR2 and fgfr2 expression have been described in the craniofacial region by (Orr-Urtreger *et al*., 1991; Peters *et al*., 1992; Wilke *et al*., 1997; Chan and Thorogood, 1999). *fgfr2* is expressed in chick embryos only at the lateral edges of the maxillary prominences, whereas *fgfr3* is expressed at the medial edge region corresponding to the medial outgrowth of the maxillae to form the palatal shelves (Wilke *et al*., 1997). It is important to notice that avian species display a physiological cleft palate. The human data reported by (Chan and Thorogood, 1999) focused on cranial vault, brain, mandible and salivary gland development but omitted any information on the palate. It could be interesting to determine precisely the expression pattern of *fgfr2 (k-sam or bek*) during rat palate development to unravel any possible interaction between these two genes during palatogenesis.

Following fusion of the palatal processes, plates of membrane bone form bilaterally in the palatal shelves, grow towards the midline and meet to form a structure analogous to a suture (Del Santo Junior *et al*., 1998) that remains active almost until adulthood (Freng and Kvam, 1979). The maxillary suture shares common features with the cranial sutures at the level of bone formation but differs significantly from them in that the pre-existing and potentially obstructive band of epithelial cells between the shelves when they contact must be removed before suture approximation can be achieved (Persson, 1995). Normal growth of the mid-palatal region is dependent on normal function of this suture and maxillary growth can be impeded by failure to form a functional suture (Friede, 1998). *Fgfr2* expression is described in the cranial suture in the mouse (Iseki *et al*., 1997) and is considered to be a marker of proliferative, uncommitted cells of the suture being switched to osteogenic differentiation (Wilkie, 1997). Kim *et al,* 1998 also demonstrated a strong expression of *fgfr2* in the osteogenic front of the mouse cranial suture. *FGFR2 (BEK)* transcripts, but also *KGFR* at a much lower level of expression were present in sheets of condensed mesenchyme before overt chondrogenic differentiation in the developing human cranial skeleton (Chan and Thorogood, 1999). Recently, expression patterns of *twist* and *fgfrs* were compared during mouse calvarial sutures development (Johnson *et al*., 2000; Rice *et al*., 2000). *Twist* expression preceded that of *fgfr* genes and was localised in the midsutural mesenchyme cells with highest expression in the osteoprogenitors. Establishing the expression pattern of *fgfr2* in the palatal suture and comparing it with the cranial suture could contribute to the characterisation of the features of this unique palatal medial suture.

Interestingly, cranial and palatal suture morphogenesis seems to share the requirement for *tgfβ3* activity. *Tgfβ3* is involved in cranial suture obliteration; deletion of this activity leads to the obliteration of fetal rat coronal sutures *in vitro* (Opperman *et al*., 1999). *Tgfβ3* expression is also necessary for the secondary palate fusion and dispersion of the medial epithelial seam (Brunet *et al*., 1995) as *tgfβ3-/-* transgenic mouse displays a cleft palate (Kaartinen *et al*., 1995, 1997; Proetzel *et al*., 1995) .

Palatal clefting is not a common feature shared by all the *TWIST-FGFR2* pathway associated craniosynostoses. Only the autosomal dominant Apert craniofacial synostosis and more significantly the variant named S252W (Slaney *et al*., 1996) and the

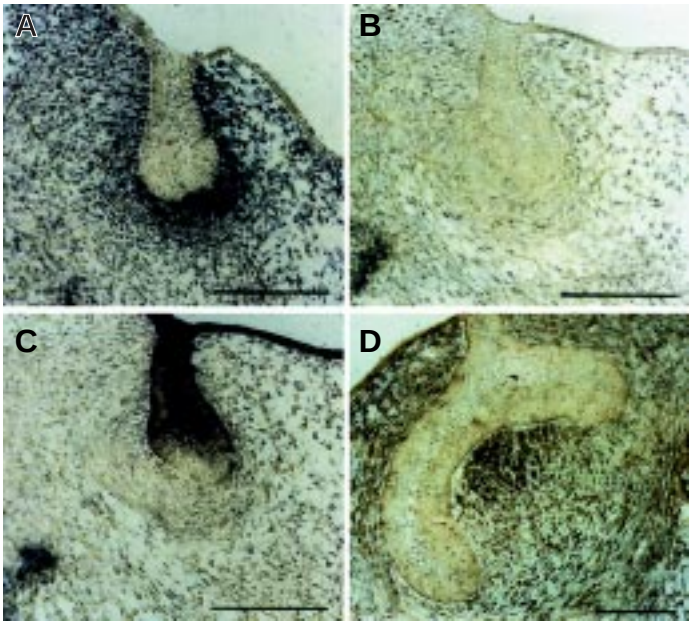

Fig. 4. Expression pattern of rat *twist* during odontogenesis. *Strong twist expression was detected in tooth mesenchyme at the cap stage (16.5 d.p.c.)* **(A)**. *A section from the same region processed identically following hybridisation with the sense ssDNA probe for twist as a control for the antisense ssDNA probe, showed no reaction product in the mesenchyme or epithelium* **(B)**. *The signal for expression of cytokeratin K5/6, used as a positive control of hybridisation in equivalent sections, was intense in the enamel organ at the cap stage* **(C)**. *At the bell stage (17.5 d.p.c.), the expression of twist became restricted to specific mesenchymal regions facing the inner dental epithelium prior the terminal differentiation of odontoblasts* **(D)**. *Bar, 50 μm.*

Saethre-Chotzen syndrome display a cleft palate as a possible component of the clinical synopsis. Gripp *et al.*, (1999) also pointed out this genetic heterogeneity and the difficulties of making a correct diagnostic linking between genotype and phenotype by finding a mutation in *TWIST* gene in a child with cranial synostosis and unilateral radial aplasia presenting as Baller-Gerold syndrome. In the light of this genetic diagnosis further investigations pointed out facial asymmetry and a high palate. The variable expressivity and the incomplete penetrance of these clefts might be associated with the genetic background as demonstrated in the mouse for *twist* mutation and its variable phenotypes (Bourgeois *et al.*, 1998). The absence of palatal clefting in the heterozygote *twist*–null mutant mice (Bourgeois *et al.*, 1998) versus the possible association of a cleft palate in Saethre-Chotzen syndrome could reveal some major differences in palatogenesis between rodents and human.

Understanding whether these clefts result from a defect of palatal fusion or from a defect of craniofacial skeletal formation may provide useful information on the pathogenesis of the clinical presentation of defects in normal craniofacial development.

Materials and Methods

1. Isolating Rattus norvegicus twist cDNA b-HLH domain

Tissue collection for DNA/RNA preparation:

Day 14.5 *Rattus norvegicus* heads were collected under aseptic conditions and stored frozen at –70°C. Total genomic DNA and total RNA were extracted using QIAGEN® RNA/DNA extraction kit. cDNA was synthesised from extracted RNA using reverse transcription with RNA template extracted at 65°C for 10 min, chilled on ice, and incubated at 42°C for 30 min in 25 µl Reverse transcription solution (5X RT buffer (5 µl), Reverse primer $p(dT)_{15}$ 20 pmol (1 µl), RNAse inhibitor 40 U (0.5 µl), RT, AMV (Boehringer Mannheim Cat N°109 118) 25 U (0.5 µl), dNTP mix 1.0 mM of each (1 µl), RNA template (5 µl), DEPC-H_2O (12 µl)).

Primers

Oligonucleotides:

QK1 forward sense strand: F: QK1 CAGCGGGTCATGGCTAAC

QK3 reverse antisense strand: R: QK3 CCCCTCCATCCTCCAGAC

These primers were used previously by (Bourgeois *et al.*, 1996) to isolate human *H-TWIST* gene fragments through PCR amplification of genomic DNA. The primers were chosen from *M-twist* cDNA sequence of *Mus Musculus* (Wolf *et al.*, 1991) within and bordering the entire b-HLH coding sequence. The isolated DNA fragment between the primers is 258 bp. Primers were used at a 20 pmol/µl concentration.

Amplification PCR

DNA was amplified on a Corbett Research Thermal Sequencer (FTS-320) after 95°C denaturation for 5 min with 37 cycles of 95°C for 40 s, 54°C for 20 s, 72°C extension for 10 min and held at 4°C in a 100 µl reaction mix of 10x PCR buffer (10 µl); F primer QK1 (2 µl); R primer QK3 (2 µl); dNTP mix 1.0 mM of each (2 µl), DNA (10 µl), Taq DNA polymerase (Boehringer Mannheim Cat #M1861) (1 µl), DEPC water (73 µl).

GAPD primers were used as controls in all PCR reactions. An aliquot from each PCR reaction was loaded in a loading solution consisting of PCR product (5 µl) TAE (5 µl), loading dye buffer (Promega Blue/Orange 6x Loading Dye Cat N° G188A) (1.8 µl) on to 1.5% agarose gel in 1xTAE tris-acetate buffer and electrophoresis run at 90V for about 1 h. A 100 bp DNA ladder (Promega Cat N°G201) was used to estimate the size of the double-stranded DNA PCR products.

The gel was stained with ethidium bromide solution, destained and observed and recorded under UV light.

Sequencing

Double-stranded PCR products were purified using QIAquick PCR Purification Kit Protocol (QIAGEN®) using a microcentrifuge and the DNA samples sequenced using the Applied Biosystems (ABI) 373A DNA Sequencing System using Taq/Terminator reaction with 100 ng of DNA per µl ddH_2O and 5 pmol/µl primers.

Cloning the PCR fragment product QK1QK3 into pGEM

The insert was cloned using the Promega pGEM®-T and pGEM®-T Easy Vector systems with the SP6 RNA polymerase transcription initiation site as forward or sense and T7 RNA polymerase transcription initiation site as reverse or antisense. SP6 and T7 promoter sites were used as primers and the 258 bp fragment generated between primers QK1 and QK3 was sequenced.

2. *In situ* hybridisation

Synthesising twist PCR single strand DNA probe for in situ hybridisation

Digoxygenin (DIG)-labelled ssDNA probes were generated using the method described in (Paine *et al.*, 1995) using the plasmid containing *twist* cDNA as template with primers QK1 to generate the sense 258bp probe and QK3 to generate the antisense 258bp probe.

The *twist* template generated by PCR from the plasmid using QK1 and QK3 primers was gel purified from an agarose gel (QIAGEN® QIAEX II gel extraction kit) and then used to synthesise via PCR (DIG)-labelled ssDNA sense and antisense probes.

Labelling cycle: (cycle 1: 95°C, 5 min; cycles 2-50: 95°C, 40 s; 54°C, 20 s; 72°C 1min; cycle 51: 72°C 10 min; 52: 4°C 1 min).

Tissue collection

Individual male Sprague-Dawley rats were added to the cages of female Sprague-Dawley rats in prime breeding conditions (aged between 70 and 120 days) at 5 pm for mating and removed next morning at 8 am. Gestation time was determined, from morning following removal of the male, as being half a day post coïtum (d.p.c.). Embryos were harvested by surgical delivery from the pregnant dams under fluothane anaesthesia and heads were dissected under sterile conditions.

Tissue processing for frozen sections

Rat embryonic heads were dissected at different developmental stages (from 14.5 to 17.5 days post coïtum) and were fixed for 2 h at 4°C in Karnovsky's fixative (2% paraformaldehyde, 2.5% gluteraldehyde in sterile phosphate-buffered saline (PBS)), then washed 3 times in cold sterile PBS and soaked overnight in 30% sucrose, 2 mM $MgCl_2$ in sterile PBS. The heads were then embedded in O.C.T. compound and frozen in liquid nitrogen vapour. The material was stored at -80°C prior to hybridisation.

In situ *hybridisation on cryosections (ISH)*

The procedure was performed as described in (Gibbins *et al.*, 1999). During permeabilization treatments, sections were digested in proteinase K solution at 37°C for 8 minutes (PK (4 µg/ml)/50 mMTris (pH 7.4)). Pre-hybridisation, hybridisation treatments and post-hybridisation washes were performed at 55°C. Levamisole was omitted in the final coloration step with BCIP/NBT for the detection of hybridised probes.

The rat cytokeratin K5/6 control probe specific for epithelium (Gibbins *et al.*, 1999) was used as positive control of hybridisation and indicator of the relative level of expression of *twist* and the specificity of the reaction.

Note

All procedures involved in the collection of rat embryos were approved by the Animal Ethics Review Committee for Central Sydney Area Health Services of the New South Wales Health Department and the laboratory procedures involving manipulation of DNA were approved by the Genetic Manipulation Advisory Committee of the University of Sydney.

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