ORAL DISEASES

Oral Diseases (2009) 15, 437–453. doi:10.1111/j.1601-0825.2009.01577.x

http://www.blackwellmunksgaard.com

INVITED MEDICAL REVIEW

The genetics of isolated orofacial clefts: from genotypes to subphenotypes

A Jugessur[1], PG Farlie[1], N Kilpatrick[1,2]

[1]*Musculoskeletal Disorders Theme, Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, Vic.;* [2]*Department of Dentistry, Royal Children's Hospital, Parkville, Vic., Australia*

Orofacial clefts are the most common craniofacial birth defects and one of the most common congenital malformations in humans. They require complex multidisciplinary treatment and are associated with elevated infant mortality and significant lifelong morbidity. The development of craniofacial structures is an exquisitely orchestrated process involving the coordinated growth of multiple, independently derived primordia. Perturbations impacting on the genesis or growth of these primordia may interfere with the proper morphogenesis of facial structures, resulting in clefting of the lip, the primary or secondary palate, or a combination of these sites. A variety of genetic approaches involving both human populations and animal models have greatly facilitated the search for genes involved in human clefting. In this article, we review the most prominent genes for orofacial clefts in the context of developmental pathways that shape the craniofacial complex. We highlight several Mendelian clefting syndromes that have provided valuable clues in identifying genes for the more common, isolated forms of clefting. Finally, we elaborate on a number of potential subclinical features (subphenotypes) associated with what have previously been diagnosed as 'isolated' clefts that may serve as additional markers for identifying individuals or families in whom there may be a greater risk of inheriting a cleft.

Oral Diseases (2009) **15**, 437–453

Keywords: congenital abnormalities; cleft lip; cleft palate; genetics; craniofacial development; *orbicularis oris;* dental anomaly; brain anomaly; speech pathology

Correspondence: Dr A Jugessur, Musculoskeletal Disorders Theme, Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road, 3052 Parkville, Vic., Australia. Tel: +613 9345 4109, Fax: +613 8341 6429, E-mail: anil.jugessur@mcri.edu.au
Received 20 April 2009; accepted 22 April 2009

Epidemiology of orofacial clefts

Orofacial clefts are the most common craniofacial birth defects in humans, with an average worldwide prevalence at birth of 1.2/1000 (Mossey and Little, 2002). They represent a significant public health burden in terms of the immediate and long-term medical costs as well as the social impact on patients and their families (Berk and Marazita, 2002). In the United States, for example, the lifetime cost for treating orofacial clefts has been estimated to be approximately $100 000 (Centers for Disease Control and Prevention, http://www.cdc.gov). Clinically, patients experience feeding difficulties in infancy; speech, hearing and dental problems as they grow older, and potentially life-long social and psychological sequelae from the facial deformity itself. Clefts also appear to be associated with a higher risk of cancer in later life (Zhu *et al*, 2002) and increased overall mortality well into adulthood (Christensen *et al*, 2004).

Orofacial clefts can be broadly divided into those that affect the lip only (CL), both the lip and palate (CLP), or the palate alone (CP) (Figure 1). Although CL and CLP are traditionally collapsed to form the single group of cleft lip with or without cleft palate (CL/P), recent data suggest that these two categories may have different genetic causes and should, when feasible, be analysed separately (Harville *et al*, 2005; Rahimov *et al*, 2008). Epidemiological data across different populations have shown that the prevalence of CP is generally lower than that of CL/P and families at high risk for one type of cleft are not at increased risk for the other type, reflecting the distinct developmental origins of each form of cleft (Jugessur and Murray, 2005). Occasionally, however, both CL/P and CP can occur within the same pedigree, suggesting at least some overlap in the aetiology of these two broader categories of clefts. Such instances of 'mixed clefting' are more commonly observed in clefting syndromes, such as Van der Woude syndrome (VWS) caused by mutations in the interferon regulatory factor 6 gene (*IRF6*) (Kondo *et al*, 2002) and CL/P with hypodontia caused by mutations in the muscle segment homeobox 1 gene (*MSX1*) (van den Boogaard *et al*, 2000).

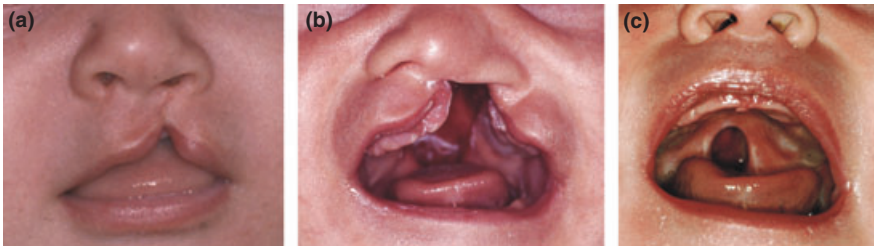

Figure 1 Common forms of orofacial clefts. **(a)** left-sided cleft lip (CL); **(b)** left-sided cleft lip and palate (CLP); and **(c)** cleft palate only (CP). The first two panels are usually collapsed to form the single group of cleft lip with or without cleft palate (CL/P)

A further subdivision of orofacial clefts into 'syndromic' *vs* 'isolated' forms depends on whether additional structural and/or developmental anomalies occur with the cleft. Whilst over 400 Mendelian disorders have been reported in OMIM (http://www.ncbi.nlm.nih.gov/Omim) in which clefting occurs as part of the overall clinical presentation, most studies suggest that around 70% of CL/P cases and half of all CP cases arise in the absence of other abnormalities and are thus collectively termed isolated defects (Jugessur and Murray, 2005). A finer subdivision of isolated clefts has recently been proposed based on the presence/absence of subtle subclinical features associated with the overt cleft, with the potential to improve the power of genetic analyses and the accuracy of risk estimates for genetic counselling (Weinberg *et al*, 2006b; Marazita, 2007).

Compared with other birth defects, orofacial clefts have a high rate of familial recurrence (Lie *et al*, 1994). In one study, the risk of cleft recurrence in first degree relatives was 32 for cleft lip and 56 for cleft palate alone compared to the reference populations, suggesting a stronger genetic basis for cleft palate compared with cleft lip (Sivertsen *et al*, 2008). In twin studies, the observed concordance rate of 40–60% in monozygotic (MZ) twins is much higher than the 3–5% concordance rate in dizygotic (DZ) twins. Although this weakens the case for either a purely genetic or purely environmental cause, the high concordance rate in MZ twins nevertheless provides compelling evidence for a strong genetic component to orofacial clefting.

The identification of genetic and environmental risk factors for clefting has been the subject of intensive research for several decades (Jugessur and Murray, 2005; Lidral and Moreno, 2005). The past few years in particular have witnessed major strides in the mapping of orofacial cleft loci, with the list of candidate genes rapidly expanding from the first reported association of transforming growth factor alpha (*TGFA*) gene variants with isolated CL/P in 1989 (Ardinger *et al*, 1989) to now include *IRF6*, *MSX1*, *TGFB3*, *FOXE1*, *FGFR1*, *FGFR2*, *FGF8*, *PDGFC*, *CRISPLD2*, *PVRL1*, *GABRB3*, *MSX2*, *SATB2*, *TBX10*, *TBX22*, *GLI2*, *JAG2*, *MTHFR*, *RARA*, *LHX8*, *SKI* and *SPRY2*, among the most prominent candidate genes for clefts (Ding *et al*, 2004; Lidral and Murray, 2004; Marazita *et al*, 2004; Jugessur and Murray, 2005; Lidral and Moreno, 2005; Vieira *et al*, 2005; Chiquet *et al*, 2007; Riley and Murray, 2007; Riley *et al*, 2007). Many of these genes are summarized in Table 1 and will be reviewed in detail in the sections below.

Lip and palate development

Facial clefting results from a failure of normal developmental processes. Understanding the developmental sequence leading to the formation of craniofacial structures clarifies why clefts occur in certain patterns and how disparate genetic influences can yield similar phenotypes. The details of human craniofacial development have been summarized in a number of reference texts [e.g. (Moore and Persaud, 2007)]. The maxillomandibular complex is derived from five facial primordia: the paired mandibular and maxillary prominences and the frontonasal prominence. The creation of a seamless, symmetrical facial structure depends on the coordinated growth of each of these independently generated prominences. Each facial prominence initially consists of an epithelial outgrowth populated by mesoderm and neural crest cells derived from the midbrain and hindbrain (O'Rahilly and Muller, 2007). The mandibular prominences are derived from the caudal domain of the first branchial arch and fuse directly with each other around the 4th week of gestation when the foetus is typically 3–5 mm in length. The extreme rarity of mandibular clefts indicates that this early fusion event is very robust, a fact that may be explained by the small size of the embryo at the stage when fusion occurs. The generation of the midfacial structures, including the maxilla, primary palate and secondary palate, involves a more complex series of fusion events between the lateral aspects of the frontonasal process and each of the maxillary processes (Figure 2). This three-way fusion occurs both later in development, starting during the 5th week of gestation and proceeds over an extended period of time, completing around the 7th week. The fusion events required for normal development of the secondary palate begin in the 8th week and are complete by the 10th, making formation of the secondary palate the latest fusion event when the foetus is typically 4 cm in length. Failure of fusion between these prominences or their derivatives is the cause of the majority of facial clefts. However, the factors that result in the failure of fusion are complex and varied, impacting on many different developmental events. While these developmental events are complex, a brief summary of the key stages illustrates why such a diverse array of genetic insults culminates in different types of orofacial clefts with broadly overlapping characteristics.

The maxillary prominence is derived from the rostral aspect of the first branchial arch and is populated by neural crest from the same axial level as those that

Table 1 Candidate genes for orofacial clefting

Gene name	*Gene ID*[a]	*Chromosome*	*Gene description*	*Molecular function* | *Biological process* | *Pathway*[b]
BMP4	652	14q22.2	Bone morphogenetic protein 4	Other signalling molecule | Skeletal development | TGF-beta signalling pathway → Transforming growth factor beta
FGF1	2246	5q31	Fibroblast growth factor 1	Growth factor | Cell surface receptor mediated signal transduction; MAPKKK cascade; Ligand-mediated signalling; Angiogenesis; Cell cycle control; Cell proliferation and differentiation | FGF signalling athway → fibroblast growth factor; Angiogenesis → Fibroblast Growth Factor
FGF10	2255	5p12	Fibroblast growth factor 10 precursor	Growth factor | Receptor protein tyrosine kinase signalling pathway | FGF signalling pathway → fibroblast growth factor
FGF12	2257	3q28	Fibroblast growth factor 12	Growth factor | Receptor protein tyrosine kinase signalling pathway | FGF signalling pathway → fibroblast growth factor
FGF2	2247	4q26-q27	Fibroblast growth factor 2	Growth factor | Cell surface receptor mediated signal transduction; MAPKKK cascade; Ligand-mediated signalling; Angiogenesis; Cell cycle control; Cell proliferation and differentiation | FGF signalling pathway → fibroblast growth factor; Angiogenesis → Fibroblast Growth Factor
FGF4	2249	11q13.3	Fibroblast growth factor 4	Growth factor | Receptor protein tyrosine kinase signalling pathway; Developmental processes | FGF signalling pathway → fibroblast growth factor
FGF5	2250	4q21	Fibroblast growth factor 5	Growth factor | Receptor protein tyrosine kinase signalling pathway; MAPKKK cascade; Ligand-mediated signalling; Neurogenesis; Cell cycle control; Cell proliferation and differentiation; Oncogene | FGF signalling pathway → fibroblast growth factor
FGF7	2252	15q15-q21.1	Fibroblast growth factor 7	Growth factor | Cell surface receptor mediated signal transduction; MAPKKK cascade; Ligand-mediated signalling; Ectoderm development; Cell cycle control; Cell proliferation and differentiation; Oncogene | FGF signalling pathway → fibroblast growth factor
FGF8	2253	10q24.32	Fibroblast growth factor 8	Growth factor | Cell surface receptor mediated signal transduction | FGF signalling pathway → fibroblast growth factor
FGF9	2254	13q11-q12	Fibroblast growth factor 9	Growth factor | Receptor protein tyrosine kinase signalling pathway | FGF signalling pathway → fibroblast growth factor
FGFR1	2260	8p12	Fibroblast growth factor receptor 1	Tyrosine protein kinase receptor; Protein kinase | Protein phosphorylation; Receptor protein tyrosine kinase signalling pathway; Neurogenesis; Cell proliferation and differentiation; Other oncogenesis | FGF signalling pathway → FGFR1-4; Angiogenesis → Fibroblast Growth Factor Receptor-1
FGFR2	2263	10q26.13	Fibroblast growth factor receptor 2	Tyrosine protein kinase receptor; Protein kinase | Protein phosphorylation; Receptor protein tyrosine kinase signalling pathway; Neurogenesis; Cell proliferation and differentiation; Other oncogenesis | FGF signalling pathway → FGFR1-4; Angiogenesis → Fibroblast Growth Factor Receptor-1
FGFR3	2261	4p16.3	Fibroblast growth factor receptor 3	Tyrosine protein kinase receptor; Protein kinase | Protein phosphorylation; Receptor protein tyrosine kinase signalling pathway; Neurogenesis; Cell proliferation and differentiation; Other oncogenesis | FGF signalling pathway → FGFR1-4; Angiogenesis → Fibroblast Growth Factor Receptor-1
FGFR4	2264	5q35.1-qter	Fibroblast growth factor receptor 4	Tyrosine protein kinase receptor; Protein kinase | Protein phosphorylation; Receptor protein tyrosine kinase signalling pathway; Neurogenesis; Cell proliferation and differentiation; Other oncogenesis | FGF signalling pathway → FGFR1-4; Angiogenesis → Fibroblast Growth Factor Receptor-1
FOXE1	2304	9q22	Forkhead box E1	Other transcription factor; Nucleic acid binding | Carbohydrate metabolism; mRNA transcription regulation; Other receptor mediated signalling pathway; Cell communication; Vision; Embryogenesis; Anterior/posterior patterning; Segment specification; Neurogenesis; Mesoderm development; Cell cycle control; Cell proliferation and differentiation; Cell structure | Insulin/IGF pathway-protein kinase B signalling cascade → Forkhead transcription factor; PI3 kinase pathway → FOXO; Interleukin signalling pathway → Forkhead in Rhabdomyosarcoma-like 1; TGF-beta signalling pathway → Co-activators or corepressors

Table 1 Continued

Gene name	*Gene ID*[a]	*Chromosome*	*Gene description*	*Molecular function* | *Biological process* | *Pathway*[b]
GLI2	2736	2q14.2	GLI-Kruppel family member GLI2	Zinc finger transcription factor | mRNA transcription regulation; Embryogenesis | Hedgehog signalling pathway → Cubitus interruptus | Hedgehog signalling pathway → Cubitus interruptus repressor
IRF6	3664	1q32.2	Interferon regulatory factor 6	Other transcription factor; Nucleic acid binding | mRNA transcription regulation; Interferon-mediated immunity; Oncogenesis | N/A
MSX1	4487	4p16.3-p16.1	msh homeobox 1	Homeobox transcription factor; Other DNA-binding protein | mRNA transcription regulation; Skeletal development | N/A
MSX2	4488	5q34-35	msh homeobox 2	Homeobox transcription factor; Other DNA-binding protein | mRNA transcription regulation; Skeletal development | N/A
PDGFC	56034	4q32	Platelet derived growth factor C	Growth factor | Ligand-mediated signalling; Other developmental process; Cell proliferation and differentiation | Angiogenesis → Platelet-Derived Growth Factor
PDGFRA	5156	4q12	Platelet-derived growth factor receptor, alpha polypeptide	Tyrosine protein kinase receptor; Protein kinase | Protein phosphorylation; Receptor protein tyrosine kinase signalling pathway; Stress response; Gametogenesis; Embryogenesis; Cell proliferation and differentiation; Oncogenesis; Cell motility | Angiogenesis → Platelet-Derived Growth Factor Receptor
PTCH1	5727	9q22.3	Patched homolog 1	Molecular function unclassified | Biological process unclassified | Hedgehog signalling pathway → Patched
PVRL1	5818	11q23	Poliovirus receptor-related 1	Other receptor; Other defense and immunity protein | Cell adhesion-mediated signalling; Immunity and defense | Alzheimer disease-presenilin pathway
RARA	5914	17q21	Retinoic acid receptor, alpha	Nuclear hormone receptor; Transcription factor; Nucleic acid binding | mRNA transcription regulation; Cell communication; Developmental processes; Oncogenesis | Vitamin D metabolism and pathway → Retinoid X Receptor, Alpha
RUNX2	860	6p21	Runt-related transcription factor 2	Other transcription factor | Skeletal development; Oncogenesis | N/A
SMAD1	4086	4q31	SMAD family member 1	Other transcription factor | mRNA transcription regulation; Receptor protein serine/threonine kinase signalling pathway; Other intracellular signalling cascade; Ligand-mediated signalling; Developmental processes; Oncogenesis | Wnt signalling pathway → Mothers against decapentaplegic homolog 4; TGF-beta signalling pathway → RSmads
SUMO1	7341	2q33	SMT3 suppressor of mif two 3 homolog 1	Other miscellaneous function protein | Protein modification; Inhibition of apoptosis; Chromosome segregation; Miscellaneous | p53 pathway → Small ubiquitin-like modifier 1
TBX1	6899	22q11	T-box 1	Other transcription factor; Nucleic acid binding | mRNA transcription regulation; Developmental processes | N/A
TBX10	347853	11q13.1	T-box 10	Other transcription factor; Nucleic acid binding | mRNA transcription regulation; Developmental processes | N/A
TBX22	50945	Xq21.1	T-box 22	Other transcription factor; Nucleic acid binding | mRNA transcription regulation; T-cell mediated immunity; Cell proliferation and differentiation | N/A
TCOF1	6949	5q33.1	Treacher Collins-Franceschetti syndrome 1	Transporter | Apoptosis; Hearing; Neurogenesis | N/A
TFAP2A	7020	6p24	Transcription factor AP-2 alpha	Other transcription factor | mRNA transcription regulation; Ectoderm development; Oncogenesis | N/A
TGFA	7039	2p13	Transforming growth factor, alpha	Other cytokine | Cytokine and chemokine mediated signalling pathway; Ligand-mediated signalling; Cell cycle control; Cell proliferation and differentiation | EGF receptor signalling pathway → Epidermal growth factor
TGFB1	7040	19q13.2	Transforming growth factor, beta 1	Growth factor | Other receptor mediated signalling pathway; Developmental processes; Cell proliferation and differentiation | TGF-beta signalling pathway → Transforming growth factor beta
TGFB2	7042	1q41	Transforming growth factor, beta 2	Growth factor | Other receptor mediated signalling pathway; Developmental processes; Cell proliferation and differentiation | TGF-beta signalling pathway → Transforming growth factor beta
TGFB3	7043	14q24	Transforming growth factor, beta 3	Growth factor | Other receptor mediated signalling pathway; Developmental processes; Cell proliferation and differentiation | TGF-beta signalling pathway → Transforming growth factor beta

Table 1 Continued

Gene name	*Gene ID*[a]	*Chromosome*	*Gene description*	*Molecular function* | *Biological process* | *Pathway*[b]
TGFBR1	7046	9q22.33	Transforming growth factor, beta receptor I	Molecular function unclassified | Biological process unclassified | N/A
TGFBR2	7048	3p24.1	Transforming growth factor, beta receptor II	Other cytokine receptor; Serine/threonine protein kinase receptor; Protein kinase | Protein phosphorylation; Cytokine and chemokine mediated signalling pathway; Receptor protein serine/threonine kinase signalling pathway; Other developmental process | TGF-beta signalling pathway
TGFBR3	7049	1p33-p32	Transforming growth factor, beta receptor III	TGF-beta receptor | Cytokine and chemokine mediated signalling pathway; Receptor protein serine/threonine kinase signalling pathway | N/A
WNT3A	89780	1q42	Wingless-type MMTV integration site family, member 3A	Other signalling molecule | Ligand-mediated signalling; Developmental processes | Cadherin signalling pathway; Wnt signalling pathway
WNT9B	7484	17q21	Wingless-type MMTV integration site family, member 9B	Other signalling molecule; Extracellular matrix structural protein | Ligand-mediated signalling | Cadherin signalling pathway; Wnt signalling pathway

N/A, not available.
[a]Gene ID from NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/sites/entrez).
[b]Data collated from the PANTHER database (http://www.pantherdb.org).

populate the mandibular prominence. The entire facial skeleton is derived from the neural crest and any influence that impedes the growth or differentiation of this population will have a marked impact on orofacial development. The severity of malformation resulting from a deficiency in the neural crest population can be seen in Treacher Collins syndrome, where mutations in *TCOF1* result in death of neural crest cells prior to leaving the neural tube (Dixon *et al*, 2000). The derivatives of the maxillary prominence include the zygomatic complex, the lateral maxilla and the secondary palate, which forms as an outgrowth of the maxillary prominence. The frontonasal prominence is populated by midbrain neural crest cells that migrate laterally over the developing brain to coalesce over the medial forebrain. The frontonasal prominence subsequently extends in a caudal direction to produce the midfacial structures including the medial part of the nose, the philtrum, the intermaxillary segment and the primary palate. The left and right maxillary prominences fuse with the intermaxillary segment to form a continuous upper jaw including the primary palate. The palatal shelves (precursors of the secondary palate) initially grow vertically from the oral aspect of the maxillary prominence, but as the tongue flattens with increasing muscular development and the oral volume increases with overall growth, the palatal shelves elevate to eventually oppose each other as they become horizontal [for in-depth reviews of these processes, see (Dudas *et al*, 2007; Gritli-Linde, 2007; Meng *et al*, 2009)]. The ensuing growth of the palatal shelves eventually results in contact and fusion to form the secondary palate. In addition, the independently generated primary and secondary palates must fuse at their sites of contact, a process requiring a number of differentially expressed transcription factors, growth factors, and cell-signalling molecules (Gritli-Linde, 2008; Gu *et al*, 2008).

The fusion event itself is a multi-step process involving the initial adhesion of opposing epithelia via cell and extracellular matrix adhesion processes, consolidation of the epithelial junction through intercalation of epithelial cells, extension of the site of fusion and removal of the bi-epithelial seam through apoptosis and migration (Figure 3) (Tudela *et al*, 2002; Martinez-Sanz *et al*, 2008; Murillo *et al*, 2009). Facial clefts result from a failure of complete fusion between any of the facial primordia; hence, knowledge of the embryological sites of fusion is important for understanding how a particular cleft type arises. For example, failure of palatal shelf elevation results in cleft palate but does not affect lip and primary palate fusion, whereas a failure of fusion between a maxillary prominence and one side of the intermaxillary segment results in unilateral cleft lip and palate.

Genes and pathways implicated in orofacial clefting

A variety of genetic approaches have been used to identify multiple genes and genetic pathways critical for craniofacial development. These include informative mouse models for orofacial clefting (Juriloff, 2002; Juriloff and Harris, 2008), linkage and association scans (Marazita and Neiswanger, 2002; Marazita *et al*, 2004; Birnbaum *et al*, 2009), cytogenetics (Brewer *et al*, 1998, 1999; Higgins *et al*, 2008), studying rare Mendelian cleft syndromes that phenocopy isolated clefts (Kondo *et al*, 2002; Zucchero *et al*, 2004), and gene expression analyses in human and mouse embryonic tissues (Brown *et al*, 2003; Mukhopadhyay *et al*, 2004; Cai *et al*, 2005; Gong *et al*, 2005). The diversity of embryological events that contribute to the formation of the facial structures is reflected in the large number of genes known or suspected to be involved in clefting (Jugessur *et al*, 2009). While a complete discussion of each of these genes is beyond the scope of this review, we provide here a brief summary of some of the key candidate genes for clefts and their respective roles in genetic pathways known to shape the craniofacial complex.

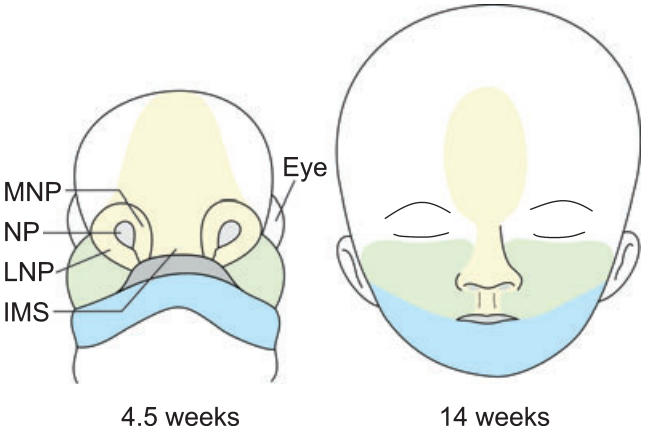

Figure 2 Diagrams representing a 4.5-week gestation human embryo and 14-week gestation human foetus illustrating the embryonic origins of the facial structures. The midfacial region is created by fusion of the frontonasal process at the top (yellow) and the paired maxillary processes in the middle portion of the developing face (green). The border between the lateral nasal process and the maxillary process is a common site of clefting. Failure of fusion at this site results in cleft of the lip and/or primary palate, which forms from the intermaxillary segment. The mandible is created by fusion of the paired maxillary processes (blue) at around 3.5 weeks of gestation. IMS, intermaxillary segment; LNP, lateral nasal prominence; MNP, medial nasal prominence; NP, nasal pit

Extracellular signalling factors

The transforming growth factor beta (TGF-β) superfamily. The TGF-β superfamily of growth factors regulate many aspects of skeletal development, including cartilage and bone formation, mesoderm patterning, and craniofacial and limb development (Wan and Cao, 2005). This group of structurally related growth and differentiation factors include TGF-βs, bone morphogenetic proteins (BMPs), and activins (Meng *et al*, 2009). One member of this superfamily, transforming growth factor alpha (TGF-α), binds to epidermal growth factor receptor (EGFR) and elicits responses similar to but more potent than EGF. The expression pattern of TGF-α in palatal tissues, especially in the midline seam and subjacent mesenchyme of the palatal shelves at the time of shelf fusion, supports a role for *TGFA* in clefting. A recent review of the accumulated evidence for *TGFA* concluded that this gene is a risk factor for orofacial clefts, possibly as a modifier gene rather than directly causing clefting (Vieira, 2006).

Although other members of the Tgf-β family are temporally and spatially expressed in the developing palate, only the *Tgfb3* knockout inhibits normal palatal shelf fusion in mice (Nawshad *et al*, 2004). The medial edge epithelium (MEE) in *Tgfb3*$^{-/-}$ mice exhibits a number of defects, including excess proliferation (Cui *et al*, 2003), less apoptosis (Martinez-Alvarez *et al*, 2000), morphological and adhesion deficiencies (Tudela *et al*, 2002), and a failure to degrade the basement membrane and undergo epithelial-mesenchymal transformation (Kang and Svoboda, 2002). Furthermore, in chickens, where the secondary palate is normally cleft, exogenous TGF-β3 can induce palatal fusion through a process that requires physical contact of the MEE and

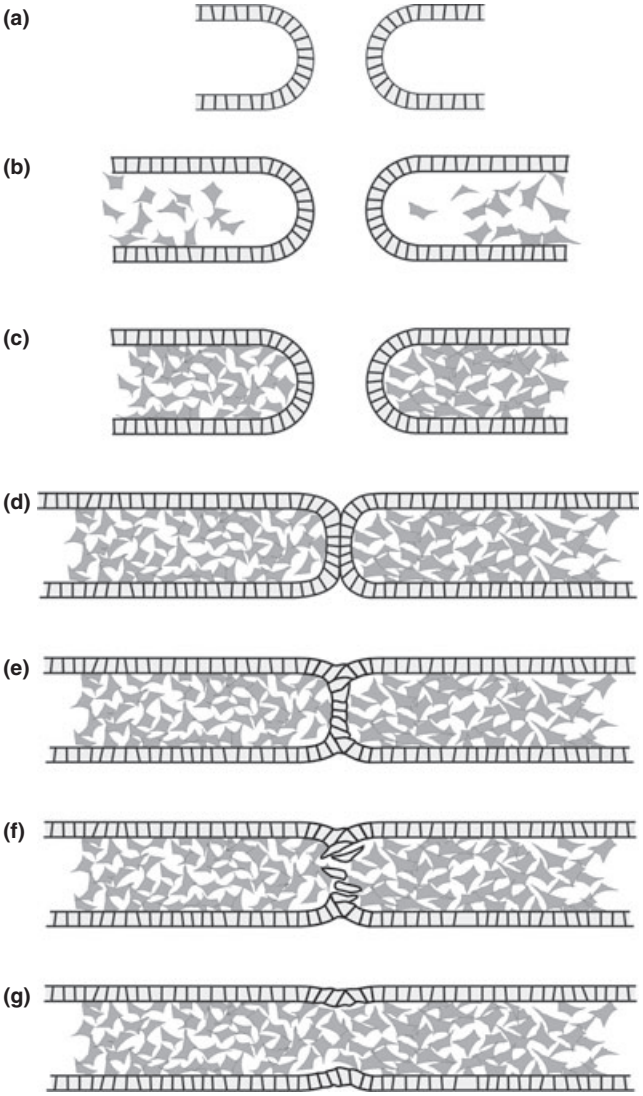

Figure 3 Diagrammatic representation of the steps of fusion in a theoretical pair of facial prominences. The facial prominences initially consist of a layer of epithelium **(a)**. The facial prominences are populated through the migration of mesenchymal cells **(b)** and early growth of the prominences is driven by proliferation of the mesenchyme **(c)**. Initial contact is made through opposing epithelia as the prominences grow towards each other **(d)** and this initial adhesion is consolidated through intercalation of epithelial cells **(e)**. The intervening epithelium is removed through a combination of apoptosis and migration **(f)**, resulting in complete fusion of the facial prominences (**g**). Failure of any of these stages of the fusion process can result in an orofacial cleft

formation of the midline seam (Nawshad *et al*, 2004). Thus, Tgf-β3 signalling is unequivocally a key pathway in palate development in the mouse and chicken. In humans, significant linkage to the *TGFB3* region was reported in a recent genome scan meta-analysis for isolated CL/P (Marazita *et al*, 2004), despite a lack of association with this gene in previous studies (Marazita and Neiswanger, 2002).

Like other TGF-β superfamily members, BMPs exert their effects by binding to two types (Type-I and -II) of membrane-bound receptor serine/threonine kinases

(Nohe *et al*, 2004). After binding of a BMP, the Type-II receptor phosphorylates the Type I receptor, resulting in recruitment of a group of transcriptional co-activators called Smads into the complex, followed by translocation of the complex into the nucleus and transcriptional activation of specific target genes (Greene and Pisano, 2004; Nohe *et al*, 2004).

Interactions between BMPs and MSX1 are important in orofacial development, as demonstrated in a study by Zhang *et al*, in which *Msx1* was required for the expression of *Bmp4* and *Bmp2* in the palatal mesenchyme and *Shh* in the MEE (Zhang *et al*, 2002). More importantly, transgenic expression of *Bmp4* in the palatal mesenchyme of $Msx1^{-/-}$ mutant mice rescued the cleft palate phenotype. The link between *Bmp4* and *Msx1* is particularly noteworthy in that mice lacking *Msx1* exhibit cleft palate and abnormalities of craniofacial and tooth development (Satokata and Maas, 1994). In humans, a nonsense mutation in *MSX1* was responsible for tooth agenesis and mixed clefting (van den Boogaard *et al*, 2000). *MSX1* is also deleted in patients with Wolf–Hirschhorn syndrome (WHS), caused by deletions in the 4p16.3 region (Nieminen *et al*, 2003). Among a range of clinical features, patients with WHS present with closure defects such as cleft lip and palate, coloboma of the eye, and cardiac septal defects. Finally, complete sequencing of *MSX1* revealed that 2% of patients with isolated CL/P carried mutations in this gene (Jezewski *et al*, 2003).

Bmp4 appears to be particularly important in lip and palate fusion. In a *Bmp4* conditional knockout mouse model, all embryos had bilateral cleft lip at 12-day post-conception, but by 14.5 days, only 22% still exhibited cleft lip (Liu *et al*, 2005a,b). Many of the initial clefts thus appeared to have been rescued or healed *in utero*, possibly through complementation or cross-regulation of other *Bmp* genes (Jiang *et al*, 2006). A mutation search in individuals with subepithelial *oris orbicularis* (OO) muscle defects found potentially deleterious *BMP4* mutations in a small proportion of cases (Marazita, 2007; Suzuki *et al*, 2009), indicating that these subtle defects may be part of a broader phenotypic spectrum of CL/P (Weinberg *et al*, 2006b).

Platelet-derived growth factor signalling. The gene for platelet-derived growth factor C (*PDGFC*) has a well-substantiated role in palatogenesis. Genetic linkage, association and cytogenetic deletions support the existence of a human CL/P locus in the 4q31-ter region containing the *PDGFC* locus [see (Choi *et al*, 2008) and references therein]. Mice in which the gene is knocked out die in the perinatal period, presumably due to feeding and respiratory difficulties from having a complete cleft of the secondary palate (Ding *et al*, 2004). In these mice, the palatal bones fail to extend across the roof of the oral–nasal cavity, suggesting that hypoplasia of palatal tissues combined with fusion defects of the MEE may contribute to the cleft palate seen in the $Pdgfc^{-/-}$ mice. Evidence from Zebrafish suggests that the Pdgf signalling system is important for chemoattraction of neural crest cells into the palatal shelves (Eberhart *et al*, 2008), suggesting that the hypoplasia in $Pdgfc^{-/-}$ mice could result from a neural crest migration deficiency.

In vitro PDGFC is downregulated by retinoic acid in mouse embryonic palatal mesenchymal cells (Han *et al*, 2006). A reduction in PDGFC activity by retinoic acid may inhibit proliferation in palatal shelves, thus resulting in cleft palate. More recently, Choi and colleagues performed sequence analysis and SNP genotyping on 1048 multiplex CL/P families and 1000 case–control samples from multiple geographic origins (Choi *et al*, 2008). Although no mutations were found in coding regions, one SNP in particular (rs28999109) was associated with CL/P in some but not all populations. The T-allele of this SNP disrupted potential transcription regulatory motifs and was associated with a significant decrease in *PDGFC* gene promoter activity (Choi *et al*, 2008).

Fibroblast growth factor signalling. The fibroblast growth factor (FGF) signalling pathway is highly conserved evolutionarily and plays an important role in several aspects of craniofacial development, including neural crest induction, skeletogenesis and epithelial-mesenchymal interactions (Nie *et al*, 2006). Multiple members of the FGF family are expressed in overlapping domains during development of the nasal and midfacial region (Bachler and Neubuser, 2001). Convincing evidence linking orofacial clefting to members of the FGF family and their receptors has been obtained from studies in transgenic mice. *Fgf8*, for example, is expressed within the developing pharyngeal arch ectoderm and endoderm during neural crest cell migration through the pharyngeal arches (Abu-Issa *et al*, 2002). *Fgf8* mouse mutants exhibit a range of defects involving the palate, mandible, middle ear bones, thyroid and external ear (Abu-Issa *et al*, 2002). Furthermore, mice homozygous for a hypomorphic allele of *Fgfr1* – resulting in reduced but not completely ablated Fgfr1 function – have craniofacial defects, including cleft palate (Trokovic *et al*, 2003). The rescue of these defects by reverting the hypomorphic *Fgfr1* allele back to wild type in neural crest cells demonstrates that *Fgf* signalling is essential for distribution and patterning of the neural crest within the facial primordia.

In humans, mutations in *FGFR1*, *2*, and *3* are associated with craniosynostosis and other facio-skeletal malformations (Pauws and Stanier, 2007). A good example is Apert syndrome, caused by mutations in *FGFR2*, where ~75% of patients present with CP or bifid uvula (Kreiborg and Cohen, 1992). Loss-of-function mutations in *FGFR1* on the other hand underlie KAL2, an autosomal dominant form of Kallmann syndrome associated with hypogonadism and anosmia, and clefting in around 5–10% of patients (Dode *et al*, 2003). As with VWS, some individuals with KAL2 may present with clefts as the only component of the phenotype, which further underscores the need for careful phenotypic assessments in gene-mapping efforts (Murray and Schutte, 2004).

More recently, diagnostic sequencing of 12 FGF-related genes in 184 individuals with isolated CL/P identified seven potential disease-causing mutations, including a nonsense mutation in *FGFR1*, a *de novo* missense mutation in *FGF8*, and other missense variants in *FGFR1*, *FGFR2*, and *FGFR3* (Riley *et al*, 2007). Interestingly, the nonsense mutation was identified both in a patient with Kallmann syndrome and her father with isolated CLP only, again highlighting non-penetrance issues in identifying mutations in isolated CL/P (Riley *et al*, 2007).

Sonic Hedgehog signalling. Sonic Hedgehog (SHH) is a member of the hedgehog family of secreted proteins regulating key developmental events during embryogenesis (Cohen, 2004). Shh signalling is essential for normal patterning and growth of the face, and analysis of *Shh–Fox* gene regulatory interactions indicate that *Fox* genes at least partially mediate the action of *Shh* in facial development (Jeong *et al*, 2004). In humans, loss of one *SHH* allele is sufficient to cause holoprosencephaly (abnormal forebrain and facial development), whereas both alleles need to be lost in the mouse knockout to produce a similar phenotype (Chiang *et al*, 1996; Roessler *et al*, 1996). In chick embryos, transient loss of SHH signalling in the embryonic face results in defects analogous to bilateral cleft lip and palate seen in humans and other characteristics reminiscent of the mild forms of holoprosencephaly (Hu and Helms, 1999).

Cell signalling is initiated through the binding of Shh to its cell surface receptor Ptch1 to relieve its basal repression of Smoothened (Smo) (Mullor *et al*, 2002). Smo then activates the GLI family of zinc-finger transcription factors to transduce the Shh signal to the nucleus. Human *PTCH* is a tumour suppressor gene that maps to chromosome 9q22.3, in the vicinity of a region highly likely to contain a strong candidate gene for orofacial clefts (Marazita *et al*, 2004). Mutations in various members of the SHH pathway (e.g. *SHH*, *PTCH*, *PTCH2*, *GLI2*, *GLI3*, *SMO*, and *CBP*) can lead to strikingly different diseases, including holoprocencephaly, Gorlin Syndrome (*a.k.a* nevoid basal cell carcinoma syndrome), Pallister–Hall syndrome, Rubinstein–Taybi syndrome and cancer (Cohen, 2004). Gorlin syndrome for example is caused by mutations in *PTCH* and is associated with cleft palate in 5% of cases (Evans *et al*, 1993). Because of this association with clefts, *PTCH* was examined for mutations in cases of isolated CL/P (Mansilla *et al*, 2006). Two missense mutations were found in the extracellular loops of *PTCH*, predicted to interfere with SHH binding. Missense mutations in *PTCH* may thus represent rare causes of isolated CL/P.

Shh and Bmp signalling has recently been linked to the function of transcription factor *Hand2* in murine palatogenesis. Mice lacking *Hand2* expression have cleft palate due to a severely hypoplastic mandible (Yanagisawa *et al*, 2003). Furthermore, *Hand2* controls cell proliferation via *Shh* and *Bmp2* in the anterior palate (Xiong *et al*, 2009). Using a conditional inactivation approach, the authors further showed that *Hand2* function is indispensable in the palatal epithelium, but not in the palatal mesenchyme during palatogenesis.

The Fgf-Shh signalling network may also play an important role in coordinating epithelial-mesenchymal interactions during the initial stages of palate development (Rice *et al*, 2004; Alappat *et al*, 2005). The Fgfr2b/Fgf10 signalling system has been demonstrated to be critical for normal development of the secondary palate (Rice *et al*, 2004). *Fgfr2b* is expressed in the oral epithelium, while *Fgf10* is expressed in the underlying mesenchyme facilitating signalling between the tissue layers. Mice deficient in either *Fgfr2b* or *Fgf10* exhibit cleft palate associated with a reduction in proliferation of epithelial and mesenchymal cell types and elevated cell death in the epithelium. Importantly, deletion of either the receptor or ligand results in loss of Shh and Ptc expression, indicating that Fgf and Shh signalling are components of the same pathway in palatogenesis.

WNT signalling. Several recent reports have drawn attention to the role of Wnt signalling in orofacial clefting. The Wnt signalling pathway consists of a large family of secreted molecules that play major roles in a range of developmental processes (Sheldahl and Moon, 2004). Many of the Wnt signalling components are shared by other signalling pathways. Signal transduction is initiated when Wnt proteins bind to Frizzled (Fz) cell surface receptors, resulting in the activation of the cytoplasmic protein Dishevelled (Dv) (Clevers, 2006). WNT3 in particular appears to be a key signalling molecule in human limb development. Mutations in this gene are associated with the rare recessive disorder Tetra-amelia, characterized by complete limb agenesis and other anomalies including clefts (Niemann *et al*, 2004). Mutations in *WNT7A* have been associated with Fuhrmann syndrome which also involves a range of limb anomalies and cleft lip and palate (Woods *et al*, 2006). Another *Wnt* family member, *Wnt9b*, while not yet implicated in clefting in humans, leads to an incompletely penetrant cleft lip and palate as well as defects in kidney morphogenesis when disrupted in mice (Carroll *et al*, 2005). Juriloff *et al* (2006) later confirmed *Wnt9b* as the mutated gene responsible for the CL/P seen in A/WySn mice, and proposed that *WNT9B* and the 3′ conserved non-coding region should be examined for a role in human isolated CL/P.

Transcription factors

IRF6. Of the large number of candidate genes thought to contribute to orofacial clefting, interferon regulatory factor 6 (*IRF6*) is among the few that have shown a convincing degree of consistency across studies (Jugessur *et al*, 2008; Rahimov *et al*, 2008). Mutations in this gene are known to cause two allelic autosomal-dominant clefting disorders**:** VWS and popliteal pterygium syndrome (PPS) (Kondo *et al*, 2002; de Lima *et al*, 2009). VWS is one of the best models for isolated CL/P in that ~15% of affected individuals are clinically indistinguishable from isolated clefts. This observation rapidly led to the hypothesis that genetic variants in

IRF6 might also be involved in the etiology of isolated clefts, which was subsequently confirmed in a large dataset of nearly 2000 families made up of 10 populations of diverse ancestry (Zucchero *et al*, 2004) and independently replicated in multiple studies [reviewed in (Jugessur *et al*, 2008)].

IRF6 belongs to a family of nine transcription factors that share a highly conserved winged-helix DNA-binding domain and a less conserved protein-binding domain (Kondo *et al*, 2002). It is strongly expressed in the leading edge ectoderm of the palatal shelves prior to and during formation of the secondary palate (Ben *et al*, 2005; Knight *et al*, 2006; Washbourne and Cox, 2006). IRF family members are known to regulate a variety of host defence mechanisms. Mice deficient in Irf1, 2, 3, 4, 5, 7, 8 or 9 have an impaired immune response (Ingraham *et al*, 2006). These mice, however, do not manifest any embryological abnormalities. In contrast, *Irf6*-null mice have abnormal skin, limb and craniofacial development (Kondo *et al*, 2002), consistent with the phenotypic distribution in VWS and PPS patients. A significant role for Irf6 in epidermal development was subsequently reported in two independent studies (Ingraham *et al*, 2006; Richardson *et al*, 2006), where *Irf6*-null mice were found to lack a normally stratified epidermis due to a defect in the keratinocyte proliferation-differentiation switch. More recently, mutations in the IRF6 transcriptional activation domain were shown to inhibit transcriptional activation (Little *et al*, 2008).

A breakthrough in our understanding of how *IRF6* affects the risk of isolated CL/P was the recent identification of a common SNP that disrupted the binding site for transcription factor AP-2α within a highly conserved *IRF6* enhancer element (Rahimov *et al*, 2008). The link between IRF6 and AP-2α is particularly noteworthy given the fact that previous data had demonstrated an essential role for AP-2α in cranial closure and craniofacial development (Schorle *et al*, 1996). Furthermore, mice carrying both wild-type and AP-2α-null cells have CLP and pronounced mandibular and maxillary dysmorphology, consistent with aberrant development of the facial prominences (Nottoli *et al*, 1998). Mutations in *TFAP2A* (the gene encoding AP-2α) cause Branchio-oculo-facial syndrome, characterized by some of the same features observed in VWS (occasional lip pits and orofacial clefts) (Milunsky *et al*, 2008). Lastly, *TFAP2A* maps to chromosome 6p24 where chromosomal anomalies have been associated with orofacial clefting (Lidral and Moreno, 2005). Taken together, these findings place IRF6 and AP-2α in a common developmental pathway in which disruptions may contribute to the pathogenesis of CL/P.

FOXE1. Forkhead box E1 (*FOXE1*) is a member of the forkhead/winged-helix domain transcription factor family the members of which are key regulators of embryogenesis. The palatal shelves in *Foxe1*-null mutant mice fail to fuse and result in an extensive cleft of the secondary palate (De Felice *et al*, 1998). In humans, a loss-of-function mutation in *FOXE1* is associated with Bamforth-Lazarus syndrome which includes thyroid agenesis, hair follicle defects, choanal atresia, and cleft palate among the clinical features (Clifton-Bligh *et al*, 1998; Castanet *et al*, 2002; Brancaccio *et al*, 2004). Three recent studies support a role for *FOXE1* in isolated clefting. First, direct sequencing of 184 isolated CL/P patients detected missense mutations in *FOXE1* in two unrelated patients, with none of the mutations detected in 186 matched controls (Vieira *et al*, 2005). Second, a meta-analysis of 13 genome-wide linkage studies produced the highest linkage signal on chromosome 9q21 (Marazita *et al*, 2004), which is near *FOXE1* (9q22) and *patched* (*PTCH;* 9q22.3). In addition, these genes map to the region homologous to the *clf2* locus in the mouse (Juriloff *et al*, 2006). A genome-wide screen for cleft loci in the cleft-susceptible A/WySn mouse strain had previously identified two epistatically interacting loci, *clf1* and *clf2*, that contribute to a cleft lip phenotype (Juriloff *et al*, 2004). Third, a recent genome-wide linkage scan of 820 multiplex CL/P families showed that the *FOXE1* region results were most significant in families in which some or all of the affected individuals have CL/P (Marazita *et al*, 2009). In the same paper, a follow-up fine-mapping SNP panel identified two genome-wide significant associations with SNPs in or near *FOXE1* and *IRF6*.

GLI2. The GLI family of zinc-finger transcription factors regulate the expression of downstream target genes in the SHH pathway. Loss-of-function mutations in *GLI2* are associated with holoprosencephaly-like features, pituitary anomalies, polydactyly, and clefting of the lip and palate (Roessler *et al*, 2003). Among a wide array of genes known to contribute to holoprosencephaly, *GLI2* has been specifically implicated in isolated clefting (Cohen, 2006). In a study of 104 cleft candidate genes spanning the length of chromosome 2, variants in *GLI2* were associated with CL/P (Beaty *et al*, 2006). Furthermore, potentially deleterious missense mutations in conserved amino acids were identified upon sequencing of DNA from individuals with isolated CL/P (Vieira *et al*, 2005).

T-box transcription factors

The T-box transcription factor gene *TBX22* was among the first genes to be identified for a major CP syndrome. Specifically, mutations in *TBX22* were found to cause X-linked cleft palate (CPX), usually associated with ankyloglossia in which the lingual fraenulum is too short and limits the normal mobility of the tongue (Braybrook *et al*, 2001). Subsequently, *TBX22* mutations were also found to account for a significant proportion of isolated CP cases (Marcano *et al*, 2004; Suphapeetiporn *et al*, 2007). *TBX22* functions as a transcriptional repressor and the small ubiquitin-like modifier SUMO-1 is required for repressing its activity (see further below for SUMO) (Andreou *et al*, 2007). In mice, *Tbx22* expression is localized to the developing palatal shelves and the base of the tongue where the ankyloglossia is observed (Braybrook *et al*, 2002; Bush *et al*, 2002). Expression of *Tbx22* in the palatal shelves is at least partially regulated by the *Meningioma* gene

(*Mn1*) and *Mn1* knockout mice also develop a cleft palate, suggesting that *Mn1* may also be a good candidate for cleft palate in humans (Liu *et al*, 2008).

Two additional members of the T-Box family, *TBX1* and *TBX10*, have been implicated in CL/P pathogenesis. The genetics of 22q11.2 deletion syndrome are complex, but mutations in *TBX1* have been identified in a small number of patients with a 22q11.2 deletion syndrome phenotype (Yagi *et al*, 2003; Paylor *et al*, 2006; Zweier *et al*, 2007). A significant proportion of patients with 22q11.2 deletion syndrome have palatal anomalies, including cleft palate in 9–11% of cases (Kobrynski and Sullivan, 2007). Deletion of *Tbx1* in mice phenocopies a number of the features of the 22q11.2 deletion syndrome, including cleft palate (Jerome and Papaioannou, 2001). *Tbx1* is strongly expressed in the pharyngeal endoderm and mice deficient for *Tbx1* have pharyngeal hypoplasia, and as a consequence, disrupted neural crest cell migration (Vitelli *et al*, 2002), supporting the hypothesis that the common developmental issue with 22q deletion syndrome patients is a defect in neural crest cell development. Additionally, ectopic expression of *TBX10* results in cleft lip and palate in transgenic mice (Bush *et al*, 2004) and mutations in this gene were also reported to be rare causes of CL/P in humans (Vieira *et al*, 2005).

TP63. Mutations in the tumour protein p63 (*TP63*) gene are implicated in five distinct human developmental disorders, characterized by various degrees of limb abnormalities, ectodermal dysplasia, and orofacial clefts (van Bokhoven and Brunner, 2002). Whilst the phenotypes of these conditions are complex, cleft lip and/or palate is a feature in four of the five p63 syndromes. In addition, *TP63* mutations have also been identified in isolated cleft lip and palate (Leoyklang *et al*, 2006). Interestingly, the distribution of mutations over the different p63 protein domains shows a clear pattern of genotype-phenotype correlation (Rinne *et al*, 2007), suggesting that specific mutations alter the function of p63 in subtly different ways. The *TP63* gene is expressed in the branchial arch ectoderm in mice, is required for induction of factors involved in epithelial-mesenchymal signalling and has been demonstrated to be essential for limb and craniofacial development (Yang *et al*, 1999).

Cell adhesion

PVRL1. Poliovirus receptor-related 1 (*PVRL1*) encodes nectin-1, an immunoglobulin-related transmembrane cell-cell adhesion molecule, which, in the mouse embryo, is expressed primarily in the MEE of the palatal shelves, the ectodermal component of tooth buds, the olfactory epithelium and the skin surface epithelium (Suzuki *et al*, 2000). Homozygosity for a common nonsense mutation in this gene (W185X) results in an autosomal recessive clefting disorder known as CL/P-ectodermal dysplasia syndrome (CLPED1) (Suzuki *et al*, 2000). The indigenous population of Margarita Island is remarkable in that it has a high incidence of CLPED1 (1/2000) and a high prevalence of isolated CL/P (5.4/1000). As a large enough sample of isolated CL/P could not be accrued from the limited indigenous population of the island, Sozen *et al* used a larger sample from neighbouring Venezuela to assess whether heterozygosity for the W185X mutation might also be a risk factor for isolated CL/P (Sozen *et al*, 2001). The results showed a highly significant association between heterozygosity for this mutation and isolated CL/P. Significant associations between variants in *PVRL1* and isolated CL/P were subsequently confirmed (Avila *et al*, 2006; Neiswanger *et al*, 2006). Taken together, these findings suggest that both rare and common mutations within *PVRL1* make minor contributions to the pathogenesis of isolated CL/P.

Protein modification

SUMO. Small ubiquitin-like modifier (SUMO) proteins posttranslationally modify numerous cellular proteins and participate in a number of cellular processes such as nuclear transport, transcriptional regulation, apoptosis and protein stability (Su and Li, 2002). This gene was interrupted by a balanced reciprocal translocation between chromosome 2q and 8q in a patient born with unilateral CLP, and further mapping of the breakpoint on chromosome 2 confirmed *SUMO1* haploinsufficiency (Alkuraya *et al*, 2006). In the same paper, strong *Sumo1* expression was observed in the upper lip, primary palate, and MEE of the secondary palate at embryonic day 13.5. Furthermore, deletions involving *SUMO1* were recently identified in a search for microdeletions among a large number of candidate genes for orofacial clefts (Shi *et al*, 2009).

It has newly been proposed that synergistic interactions may exist between the FGF signalling pathway, SUMO modification, and environmental risk factors in the causation of CL/P (Pauws and Stanier, 2007). Given protein sumoylation is influenced by environmental stress (Bossis and Melchior, 2006), external stress stimuli on the SUMO pathway might affect development of the lip and palate. Interestingly, several genes previously found to be strongly associated with orofacial clefting are also targets of SUMO modification (e.g. *TBX22*, *MSX1*, *SATB2*, *TP63*, *PAX9*, *TRPS1*, and *EYA1*) (Pauws and Stanier, 2007). Thus, aberrant SUMO modification of these genes during early pregnancy is likely to provide a high-risk environment for the pathogenesis of both Mendelian and idiopathic forms of orofacial clefts (Andreou *et al*, 2007).

Phenotypic aspects of isolated orofacial clefting

In many of the examples of clefting syndromes cited above, there is often a clear correlation between genetic mutation and cleft phenotype. However, as a consequence of genetic and allelic heterogeneity, incomplete penetrance, and the spectrum of phenotypic severity resulting from identical mutations, there is often no such clear-cut correlation for isolated clefts. Although multicentre collaborations should enhance the capacity to identify such correlations through an increase in sample size, there remains the more fundamental question of when is an unaffected individual truly unaffected? The

answer may not be as clear as previously thought with contemporary research focusing on refining the clinical descriptors of the cleft phenotype.

Until recently, understanding the genetic aetiology for orofacial clefts has been based upon the notion that an individual either has or does not have a cleft. However, whilst severe clefting is unmistakable (Figure 1), milder expressions such as a submucous cleft palate, a bifid uvula or forme fruste lip (also known as microform cleft) are harder to detect. Traditional definitions of 'affected/unaffected' relying solely on the basis of 'no overt cleft = unaffected' may be too simplistic in a way that might have hampered gene identification (Weinberg *et al*, 2006b). Currently, all unaffected family members are treated as though their genetic risks are equivalent. However, the range of phenotypic variation may extend beyond the externally visible, with increasing evidence that even without an overt CL/P an individual may have other features that fall under the 'spectrum' of clefting. Such 'unaffected' individuals may represent gene-carriers that are currently overlooked. The identification of unaffected individuals within cleft families who may be harbouring susceptibility factors is important and could lead to significant progress not only in improved recurrence risk estimations, genetic counselling and overall cleft management, but also in more targeted gene-mapping efforts.

Whilst there are a large number of potential clinical/subclinical features associated with the so-called 'expanded cleft phenotype' (Weinberg *et al*, 2006b), the strength of evidence for each varies. Three features in particular, namely subepithelial defects of the superior *orbicularis oris* (OO) muscle (Marazita, 2007), dental anomalies (specifically hypodontia) (Letra *et al*, 2007), and craniofacial morphology (Weinberg *et al*, 2008b) have received the greatest attention. Although additional features such as dermatoglyphic patterns, non-right-handedness and directional asymmetry have also been discussed in the context of clefting (Weinberg *et al*, 2006b), they appear to lack the strong theoretical basis that is associated with other features such as structural brain anomalies and neuropsychological deficits, and speech anomalies.

OO muscle defects

It has been hypothesized that subepithelial defects of the superior OO muscle result from the incomplete development of the OO sphincter, and as such may represent the mildest phenotypic expression of the clefting spectrum (Martin *et al*, 1993; Suzuki *et al*, 2009). Such defects have been identified (using high-resolution ultrasound) significantly more frequently in apparently 'unaffected' relatives of individuals with isolated CL/P than in unrelated controls with no family history of clefting (Martinez-Alvarez *et al*, 2000; Neiswanger *et al*, 2007). Furthermore, despite the traditional view that CL/P is etiologically distinct from CP, defects of the OO muscle have recently been reported in individuals previously diagnosed with isolated CP (Weinberg *et al*, 2008a). This in itself suggests that, in some cases at least, the diagnosis of isolated CP may also not be entirely accurate and further supports the hypothesis of 'mixed clefting' discussed earlier.

Dental anomalies

Dental anomalies, particularly hypodontia, microdontia and supernumerary teeth, occur more frequently in individuals with CL/P. Indeed, it has been suggested that, as with subepithelial defects of the OO muscle, the presence or absence of dental anomalies should be included in an overall assessment of the cleft phenotype (Letra *et al*, 2007). Given the close temporal and spatial development of the lip, palate and teeth, the most commonly reported anomaly—and one that warrants closer evaluation—is the congenitally absent lateral incisor. It is well recognized that in individuals with CL/P, the lateral incisor in the line of the cleft is often missing (Figure 4a) (Ribeiro *et al*, 2003). Likewise, in those same individuals there is an increased incidence of missing teeth outside the cleft area, particularly premolars and the contralateral lateral incisor (Figure 4b) (Menezes and Vieira, 2008). However, there is also some evidence that hypodontia is more common in the 'unaffected' siblings of individuals with a cleft (11.1%) than in unrelated controls (3.4%) (Eerens *et al*, 2001).

Given the fact that there are a number of genes associated with both clefting and tooth agenesis [e.g. *MSX1*, *TGFA*, *PAX9*, *TGFB3*, *FGFR1* and *IRF6* (Slayton *et al*, 2003; Vieira *et al*, 2004, 2007)], pursuing the hypothesis that dental anomalies contribute to the

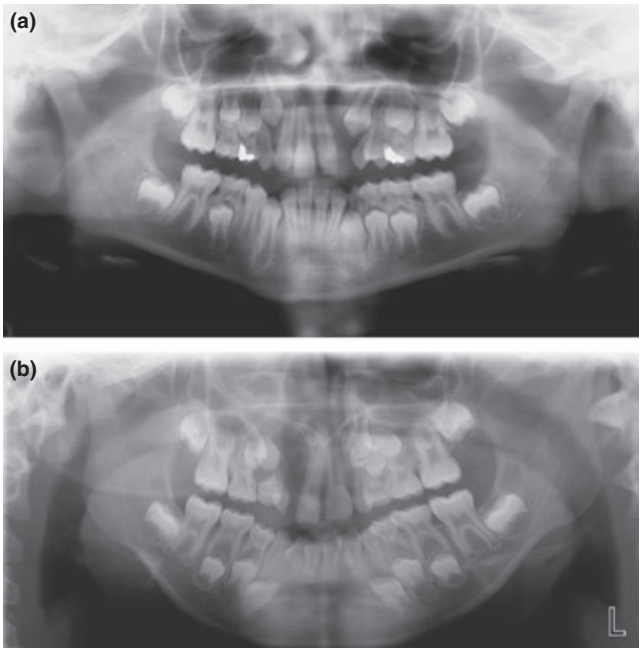

Figure 4 Examples of dental agenesis in two patients diagnosed with cleft lip and palate. **(a)** An orthopantogram (OPG) radiograph of a 10-year-old individual with a left-sided unilateral cleft of the lip and palate just prior to secondary alveolar bone grafting. Note the agenesis of the permanent upper left lateral incisor (tooth 22). **(b)** OPG of an 8-year-old individual with a right-sided unilateral cleft of the lip and palate prior to secondary alveolar bone grafting. Note the agenesis of both permanent upper lateral incisors (teeth 12 and 22) and the upper right second premolar (tooth 15)

cleft phenotype has the potential to make a significant contribution to contemporary understanding. However, validating the aetiology of a missing tooth can be challenging. Most studies use retrospective review of dental radiographs which, when screening for hypodontia, does not allow confirmation of the reason for a tooth to be missing (for example, whether the missing tooth is due to extraction or is congenitally missing). Furthermore, it is possible that the primary cleft-repair surgery itself may disrupt the developing tooth germs, resulting in agenesis or at least in some degree of tooth malformation. Therefore, detailed long-term clinical as well as radiological data on tooth development and dental intervention should be collected prospectively from affected families to facilitate more accurate subphenotyping.

Craniofacial morphology
It is widely recognized that craniofacial features of individuals with clefts are distinctive compared to those of unaffected people. Consequently, craniofacial shape and form of first-degree relatives of cleft patients might be expected to differ from that of the general population. Meta-analysis of cephalometric studies involving 'unaffected' parents of individuals with CL/P suggests that these adults 'are characterized by a suite of consistent, yet subtle, craniofacial differences, which could indicate an underlying genetic liability' (Weinberg *et al*, 2006a). The introduction of 3-dimensional photogrammetry has further refined and confirmed these differences (Weinberg *et al*, 2008b). Whilst potential embryological mechanisms exist that might explain why aberrations in specific craniofacial features may represent a risk marker for clefting, these mechanisms are complex and are yet to be fully explored. In addition, the accuracy, reproducibility and validity of these measures also require further investigation.

Neuropsychological deficits and brain anomalies
Given the close inter-relationship between the development of craniofacial structures and the brain, it is not surprising that neurological deficits have been reported in otherwise healthy individuals with isolated CL/P (Conrad *et al*, 2008). The aetiology of these deficits in isolated CL/P remains poorly understood. They were historically considered secondary to other factors, such as hearing or speech deficits. More recently, however, it has been suggested that these deficits could be a primary problem related to abnormal brain structure (Boes *et al*, 2007; Nopoulos *et al*, 2007a). Several candidate genes associated with CL/P (e.g. *PVRL1*, *MSX1*, and *IRF6*) are also associated with brain abnormalities and mental impairment (Nopoulos *et al*, 2007b). Therefore, it is feasible that these genes may have a more direct role in abnormal brain development and associated cognitive impairment in individuals with CL/P. The identification of milder neuropsychological deficits and brain anomalies in 'unaffected' relatives is yet to be reported, but given the close inter-relationship between face and brain development, this area requires further investigation. Such studies are likely to be hampered by the availability of large enough samples, low participation rates, the time-consuming nature of neuropsychological testing, access to and ethical considerations surrounding magnetic brain imaging of large numbers of 'unaffected' relatives of cleft patients and unrelated controls.

Speech pathology
Normal speech requires that the muscles that make up the velopharyngeal sphincter work in a coordinated fashion. Defects in any aspect of the nasopharyngeal anatomy and/or physiology may lead to velopharyngeal incompetence (VPI), which is characterized principally by aberrations in nasality (hyper- or hypo-nasality and nasal air emission). Whilst VPI is not uncommon in individuals with overt clefts of the palate, it can also occur in the absence of an overt cleft although its population prevalence is unknown. In addition to a variety of neuromuscular deficits, there are several potential causes of this VPI that may represent a subclinical feature or a risk marker for clefting. These include the presence of a submucous defect, occult anatomical defects of the *levator palatini* or *musculus uvulae* and an anatomic disproportion between the size of the nasopharynx and the length of the palate. Whilst there is a lack of strong evidence, anecdotal clinical experience suggests that these milder forms of deficit may be present, but often pass unnoticed in 'unaffected' siblings. It is possible that these deficits remain undiagnosed because clinical assessment of VPI (particularly relatively mild VPI) can be very subjective even to trained, experienced clinicians and definitive diagnosis often requires relatively invasive diagnostic testing such as nasendoscopy.

Summary

Every 2½ min, somewhere in the world, a child is born with an orofacial cleft. Not surprisingly, this birth defect is the single most commonly treated craniofacial malformation in a paediatric hospital setting. Although surgery can correct most of the structural defects, patients still face a lifetime of functional, social, and aesthetic challenges. Developmental studies in various animal models have been particularly insightful in unravelling the complex processes and mechanisms involved in clefting. The past few years have witnessed great advances in gene-identification for this complex birth defect, providing an unprecedented opportunity to identify genetically susceptible subgroups in the population. Whether through variant growth patterns or through variant metabolic pathways, genetically susceptible subgroups offer a rich opportunity for research by providing a more sensitive means of identifying substances that are teratogenic in humans. Ultimately, a clearer identification of genetic risk factors and subgroups of the population who are at risk will allow the generation of plausible new biological mechanisms for cleft causation, and new insights into the developmental biology of the face will help nurture the translation of scientific findings into clinical care.

To facilitate ongoing gene-identification efforts, more attention needs to focus on refining the isolated cleft phenotype. There is a growing body of evidence to suggest that the spectrum of isolated orofacial cleft phenotypes may be far more complex than traditionally considered. Detailed evaluation has revealed a range of subclinical features such as defects in the OO muscle, differences in craniofacial morphology, dental and brain anomalies, and neurological deficits in individuals with so-called 'isolated' clefts. Furthermore, by broadening the scope of clinical screening to include non-cleft first degree relatives, these and indeed other anomalies (e.g. speech) may well be confirmed as risk markers for isolated clefting. Revealing the full spectrum of affected individuals within cleft families through such detailed subphenotyping holds the promise of providing a useful benchmark for discriminating at-risk relatives and identifying specific risk genes. To achieve this, standardized protocols and data-sharing between cleft centres worldwide should be encouraged.

Acknowledgements

We thank Brian Schutte and Fedik Rahimov for many insightful comments on an earlier draft of the manuscript. We are especially grateful to the many families who have contributed to our cleft research over the years, and to our colleagues Jeff Murray, Rolv Terje Lie, Allen Wilcox, Håkon Gjessing, Min Shi, Clarice Weinberg, Mary Marazita, Kaare Christensen, Camilla Bille, Abee Boyles, Truc Trung Nguyen, Roy Nilsen, Sandra Daack-Hirsch, Michael Dixon, David Fitzpatrick, Andrew Lidral, Satoshi Suzuki, and Temis Felix for many helpful discussions.

Author contributions

All three authors were involved in the literature search, analysis of available evidence and the writing and editing of the manuscript.

References

Abu-Issa R, Smyth G, Smoak I, Yamamura K, Meyers EN (2002). Fgf8 is required for pharyngeal arch and cardiovascular development in the mouse. *Development* **129:** 4613–4625.

Alappat SR, Zhang Z, Suzuki K *et al* (2005). The cellular and molecular etiology of the cleft secondary palate in Fgf10 mutant mice. *Dev Biol* **277:** 102–113.

Alkuraya FS, Saadi I, Lund JJ, Turbe-Doan A, Morton CC, Maas RL (2006). SUMO1 haploinsufficiency leads to cleft lip and palate. *Science* **313:** 1751.

Andreou AM, Pauws E, Jones MC *et al* (2007). TBX22 missense mutations found in patients with X-linked cleft palate affect DNA binding, sumoylation, and transcriptional repression. *Am J Hum Genet* **81:** 700–712.

Ardinger HH, Buetow KH, Bell GI, Bardach J, VanDemark DR, Murray JC (1989). Association of genetic variation of the transforming growth factor- alpha gene with cleft lip and palate. *Am J Hum Genet* **45:** 348–353.

Avila JR, Jezewski PA, Vieira AR *et al* (2006). PVRL1 variants contribute to non-syndromic cleft lip and palate in multiple populations. *Am J Med Genet A* **140:** 2562–2570.

Bachler M, Neubuser A (2001). Expression of members of the Fgf family and their receptors during midfacial development. *Mech Dev* **100:** 313–316.

Beaty TH, Hetmanski JB, Fallin MD *et al* (2006). Analysis of candidate genes on chromosome 2 in oral cleft case-parent trios from three populations. *Hum Genet* **120:** 501–518.

Ben J, Jabs EW, Chong SS (2005). Genomic, cDNA and embryonic expression analysis of zebrafish IRF6, the gene mutated in the human oral clefting disorders Van der Woude and popliteal pterygium syndromes. *Gene Expr Patterns* **5:** 629–638.

Berk NW, Marazita ML (2002). Costs of cleft lip and palate: personal and societal implications. In: Wyszynski DF, ed. *Cleft lip and palate: from origin to treatment*. Oxford University Press, Inc: New York, NY, pp. 458–467.

Birnbaum S, Ludwig KU, Reutter H *et al* (2009). Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. *Nat Genet* **41:** 473–477.

Boes AD, Murko V, Wood JL *et al* (2007). Social function in boys with cleft lip and palate: relationship to ventral frontal cortex morphology. *Behav Brain Res* **181:** 224–231.

van Bokhoven H, Brunner HG (2002). Splitting p63. *Am J Hum Genet* **71:** 1–13.

van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK (2000). MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. *Nat Genet* **24:** 342–343.

Bossis G, Melchior F (2006). SUMO: regulating the regulator. *Cell Div* **1:** 13.

Brancaccio A, Minichiello A, Grachtchouk M *et al* (2004). Requirement of the forkhead gene Foxe1, a target of sonic hedgehog signaling, in hair follicle morphogenesis. *Hum Mol Genet* **13:** 2595–2606.

Braybrook C, Doudney K, Marcano AC *et al* (2001). The T-box transcription factor gene TBX22 is mutated in X-linked cleft palate and ankyloglossia. *Nat Genet* **29:** 179–183.

Braybrook C, Lisgo S, Doudney K *et al* (2002). Craniofacial expression of human and murine TBX22 correlates with the cleft palate and ankyloglossia phenotype observed in CPX patients. *Hum Mol Genet* **11:** 2793–2804.

Brewer C, Holloway S, Zawalnyski P, Schinzel A, FitzPatrick D (1998). A chromosomal deletion map of human malformations. *Am J Hum Genet* **63:** 1153–1159.

Brewer C, Holloway S, Zawalnyski P, Schinzel A, FitzPatrick D (1999). A chromosomal duplication map of malformations: regions of suspected haplo- and triplolethality – and tolerance of segmental aneuploidy – in humans. *Am J Hum Genet* **64:** 1702–1708.

Brown NL, Knott L, Halligan E, Yarram SJ, Mansell JP, Sandy JR (2003). Microarray analysis of murine palatogenesis: temporal expression of genes during normal palate development. *Dev Growth Differ* **45:** 153–165.

Bush JO, Lan Y, Maltby KM, Jiang R (2002). Isolation and developmental expression analysis of Tbx22, the mouse homolog of the human X-linked cleft palate gene. *Dev Dyn* **225:** 322–326.

Bush JO, Lan Y, Jiang R (2004). The cleft lip and palate defects in Dancer mutant mice result from gain of function of the Tbx10 gene. *Proc Natl Acad Sci U S A* **101:** 7022–7027.

Cai J, Ash D, Kotch LE *et al* (2005). Gene expression in pharyngeal arch 1 during human embryonic development. *Hum Mol Genet* **14:** 903–912.

Carroll TJ, Park JS, Hayashi S, Majumdar A, McMahon AP (2005). Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell* **9:** 283–292.

Castanet M, Park SM, Smith A *et al* (2002). A novel loss-of-function mutation in TTF-2 is associated with congenital hypothyroidism, thyroid agenesis and cleft palate. *Hum Mol Genet* **11:** 2051–2059.

Chiang C, Litingtung Y, Lee E *et al* (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383:** 407–413.

Chiquet BT, Lidral AC, Stal S *et al* (2007). CRISPLD2: a novel NSCLP candidate gene. *Hum Mol Genet* **16:** 2241–2248.

Choi SJ, Marazita ML, Hart PS *et al* (2008). The PDGF-C regulatory region SNP rs28999109 decreases promoter transcriptional activity and is associated with CL/P. *Eur J Hum Genet* 2009 Dec 17. Epub ahead of print.

Christensen K, Juel K, Herskind AM, Murray JC (2004). Long term follow up study of survival associated with cleft lip and palate at birth. *BMJ* **328:** 1405.

Clevers H (2006). Wnt/beta-catenin signaling in development and disease. *Cell* **127:** 469–480.

Clifton-Bligh RJ, Wentworth JM, Heinz P *et al* (1998). Mutation of the gene encoding human TTF-2 associated with thyroid agenesis, cleft palate and choanal atresia. *Nat Genet* **19:** 399–401.

Cohen MM Jr (2004). An introduction to Sonic Hedgehog Signaling. In: Epstein CJ, Erickson RP, Wynshaw-Boris A, eds *Inborn errors of development*. Oxford University Press: New York, pp. 210–228.

Cohen MM Jr (2006). Holoprosencephaly: clinical, anatomic, and molecular dimensions. *Birth Defects Res A Clin Mol Teratol* **76:** 658–673.

Conrad AL, Canady J, Richman L, Nopoulos P (2008). Incidence of neurological soft signs in children with isolated cleft of the lip or palate. *Percept Mot Skills* **106:** 197–206.

Cui XM, Chai Y, Chen J *et al* (2003). TGF-beta3-dependent SMAD2 phosphorylation and inhibition of MEE proliferation during palatal fusion. *Dev Dyn* **227:** 387–394.

De Felice M, Ovitt C, Biffali E *et al* (1998). A mouse model for hereditary thyroid dysgenesis and cleft palate. *Nat Genet* **19:** 395–398.

Ding H, Wu X, Bostrom H *et al* (2004). A specific requirement for PDGF-C in palate formation and PDGFR-alpha signaling. *Nat Genet* **36:** 1111–1116.

Dixon J, Brakebusch C, Fassler R, Dixon MJ (2000). Increased levels of apoptosis in the prefusion neural folds underlie the craniofacial disorder, Treacher Collins syndrome. *Hum Mol Genet* **9:** 1473–1480.

Dode C, Levilliers J, Dupont JM *et al* (2003). Loss-of-function mutations in FGFR1 cause autosomal dominant Kallmann syndrome. *Nat Genet* **33:** 463–465.

Dudas M, Li WY, Kim J, Yang A, Kaartinen V (2007). Palatal fusion - where do the midline cells go? A review on cleft palate, a major human birth defect. *Acta Histochem* **109:** 1–14.

Eberhart JK, He X, Swartz ME *et al* (2008). MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nat Genet* **40:** 290–298.

Eerens K, Vlietinck R, Heidbuchel K *et al* (2001). Hypodontia and tooth formation in groups of children with cleft, siblings without cleft, and nonrelated controls. *Cleft Palate Craniofac J* **38:** 374–378.

Evans DG, Ladusans EJ, Rimmer S, Burnell LD, Thakker N, Farndon PA (1993). Complications of the naevoid basal cell carcinoma syndrome: results of a population based study. *J Med Genet* **30:** 460–464.

Gong SG, Gong TW, Shum L (2005). Identification of markers of the midface. *J Dent Res* **84:** 69–72.

Greene RM, Pisano MM (2004). Perspectives on growth factors and orofacial development. *Curr Pharm Des* **10:** 2701–2717.

Gritli-Linde A (2007). Molecular control of secondary palate development. *Dev Biol* **301:** 309–326.

Gritli-Linde A (2008). The etiopathogenesis of cleft lip and cleft palate usefulness and caveats of mouse models. *Curr Top Dev Biol* **84:** 37–138.

Gu S, Wei N, Yu X, Jiang Y, Fei J, Chen Y (2008). Mice with an anterior cleft of the palate survive neonatal lethality. *Dev Dyn* **237:** 1509–1516.

Han J, Xiao Y, Lin J, Li Y (2006). PDGF-C controls proliferation and is down-regulated by retinoic acid in mouse embryonic palatal mesenchymal cells. *Birth Defects Res B Dev Reprod Toxicol* **77:** 438–444.

Harville EW, Wilcox AJ, Lie RT, Vindenes H, Abyholm F (2005). Cleft lip and palate versus cleft lip only: are they distinct defects? *Am J Epidemiol* **162:** 448–453.

Higgins AW, Alkuraya FS, Bosco AF *et al* (2008). Characterization of apparently balanced chromosomal rearrangements from the developmental genome anatomy project. *Am J Hum Genet* **82:** 712–722.

Hu D, Helms JA (1999). The role of sonic hedgehog in normal and abnormal craniofacial morphogenesis. *Development* **126:** 4873–4884.

Ingraham CR, Kinoshita A, Kondo S *et al* (2006). Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). *Nat Genet* **38:** 1335–1340.

Jeong J, Mao J, Tenzen T, Kottmann AH, McMahon AP (2004). Hedgehog signaling in the neural crest cells regulates the patterning and growth of facial primordia. *Genes Dev* **18:** 937–951.

Jerome LA, Papaioannou VE (2001). DiGeorge syndrome phenotype in mice mutant for the T-box gene, Tbx1. *Nat Genet* **27:** 286–291.

Jezewski PA, Vieira AR, Nishimura C *et al* (2003). Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. *J Med Genet* **40:** 399–407.

Jiang R, Bush JO, Lidral AC (2006). Development of the upper lip: morphogenetic and molecular mechanisms. *Dev Dyn* **235:** 1152–1166.

Jugessur A, Murray JC (2005). Orofacial clefting: recent insights into a complex trait. *Curr Opin Genet Dev* **15:** 270–278.

Jugessur A, Rahimov F, Lie RT *et al* (2008). Genetic variants in IRF6 and the risk of facial clefts: single-marker and haplotype-based analyses in a population-based case-control study of facial clefts in Norway. *Genet Epidemiol* **32:** 413–424.

Jugessur A, Shi M, Gjessing HK *et al* (2009). Genetic Determinants of Facial Clefting: Analysis of 357 Candidate Genes Using Two National Cleft Studies from Scandinavia. *PLoS ONE* **4:** e5385.

Juriloff DM (2002). Mapping studies in animal models. In: Wyszynski DF, ed. *Cleft Lip and Palate: From Origin to Treatment*. Oxford University Press: New York, NY, pp. 265–282.

Juriloff DM, Harris MJ (2008). Mouse genetic models of cleft lip with or without cleft palate. *Birth Defects Res A Clin Mol Teratol* **82:** 63–77.

Juriloff DM, Harris MJ, Dewell SL (2004). A digenic cause of cleft lip in A-strain mice and definition of candidate genes for the two loci. *Birth Defects Res A Clin Mol Teratol* **70:** 509–518.

Juriloff DM, Harris MJ, McMahon AP, Carroll TJ, Lidral AC (2006). Wnt9b is the mutated gene involved in multifactorial nonsyndromic cleft lip with or without cleft palate in A/WySn mice, as confirmed by a genetic complementation test. *Birth Defects Res A Clin Mol Teratol* **76:** 574–579.

Kang P, Svoboda KK (2002). PI-3 kinase activity is required for epithelial-mesenchymal transformation during palate fusion. *Dev Dyn* **225:** 316–321.

Knight AS, Schutte BC, Jiang R, Dixon MJ (2006). Developmental expression analysis of the mouse and chick orthologues of IRF6: the gene mutated in Van der Woude syndrome. *Dev Dyn* **235:** 1441–1447.

Kobrynski LJ, Sullivan KE (2007). Velocardiofacial syndrome, DiGeorge syndrome: the chromosome 22q11.2 deletion syndromes. *Lancet* **370:** 1443–1452.

Kondo S, Schutte BC, Richardson RJ *et al* (2002). Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. *Nat Genet* **32:** 285–289.

Kreiborg S, Cohen MM Jr (1992). The oral manifestations of Apert syndrome. *J Craniofac Genet Dev Biol* **12:** 41–48.

Leoyklang P, Siriwan P, Shotelersuk V (2006). A mutation of the p63 gene in non-syndromic cleft lip. *J Med Genet* **43:** e28.

Letra A, Menezes R, Granjeiro JM, Vieira AR (2007). Defining subphenotypes for oral clefts based on dental development. *J Dent Res* **86:** 986–991.

Lidral AC, Moreno LM (2005). Progress toward discerning the genetics of cleft lip. *Curr Opin Pediatr* **17:** 731–739.

Lidral AC, Murray JC (2004). Genetic approaches to identify disease genes for birth defects with cleft lip/palate as a model. *Birth Defects Res A Clin Mol Teratol* **70:** 893–901.

Lie RT, Wilcox AJ, Skjaerven R (1994). A population-based study of the risk of recurrence of birth defects. *N Engl J Med* **331:** 1–4.

de Lima RL, Hoper SA, Ghassibe M *et al* (2009). Prevalence and nonrandom distribution of exonic mutations in interferon regulatory factor 6 in 307 families with Van der Woude syndrome and 37 families with popliteal pterygium syndrome. *Genet Med* **11:** 241–247.

Little HJ, Rorick NK, Su LI *et al* (2008). Mis-sense mutations that cause Van der Woude syndrome and popliteal pterygium syndrome affect the DNA-binding and transcriptional activation functions of IRF6. *Hum Mol Genet* **18:** 535–545.

Liu W, Selever J, Murali D *et al* (2005a). Threshold-specific requirements for Bmp4 in mandibular development. *Dev Biol* **283:** 282–293.

Liu W, Sun X, Braut A *et al* (2005b). Distinct functions for Bmp signaling in lip and palate fusion in mice. *Development* **132:** 1453–1461.

Liu W, Lan Y, Pauws E *et al* (2008). The Mn1 transcription factor acts upstream of Tbx22 and preferentially regulates posterior palate growth in mice. *Development* **135:** 3959–3968.

Mansilla MA, Cooper ME, Goldstein T *et al* (2006). Contributions of PTCH gene variants to isolated cleft lip and palate. *Cleft Palate Craniofac J* **43:** 21–29.

Marazita ML (2007). Subclinical features in non-syndromic cleft lip with or without cleft palate (CL/P): review of the evidence that subepithelial orbicularis oris muscle defects are part of an expanded phenotype for CL/P. *Orthod Craniofac Res* **10:** 82–87.

Marazita ML, Neiswanger K (2002). Association studies. In: Wyszynski DFE, ed. *Cleft lip and palate: from origin to treatment*. Oxford University Press: New York, NY, pp. 240–254.

Marazita ML, Murray JC, Lidral AC *et al* (2004). Meta-analysis of 13 genome scans reveals multiple cleft lip/palate genes with novel loci on 9q21 and 2q32-35. *Am J Hum Genet* **75:** 161–173.

Marazita ML, Lidral AC, Murray JC *et al* (2009). Genome scan, fine-mapping, and candidate gene analysis of non-syndromic cleft lip with or without cleft palate reveals phenotype specific differences in linkage and association results. *Hum Hered* In press.

Marcano AC, Doudney K, Braybrook C *et al* (2004). TBX22 mutations are a frequent cause of cleft palate. *J Med Genet* **41:** 68–74.

Martin RA, Jones KL, Benirschke K (1993). Extension ofo the cleft lip phenotype: the subepithelial cleft. *Am J Med Genet* **47:** 744–747.

Martin RA, Hunter V, Neufeld-Kaiser W, Flodman P, Spence MA, Furnas D (2000). Ultrasonographic detection of orbicularis oris defects in first degree relativesl of isolated cleft lip patients. *Am J Med Genet* **90:** 155–161.

Martinez-Alvarez C, Tudela C, Perez-Miguelsanz J, O'Kane S, Puerta J, Ferguson MW (2000). Medial edge epithelial cell fate during palatal fusion. *Dev Biol* **220:** 343–357.

Martinez-Sanz E, Del RioA, Barrio C *et al* (2008). Alteration of medial-edge epithelium cell adhesion in two Tgf-beta3 null mouse strains. *Differentiation* **76:** 417–430.

Menezes R, Vieira AR (2008). Dental anomalies as part of the cleft spectrum. *Cleft Palate Craniofac J* **45:** 414–419.

Meng L, Bian Z, Torensma R, Von den Hoff JW (2009). Biological mechanisms in palatogenesis and cleft palate. *J Dent Res* **88:** 22–33.

Milunsky JM, Maher TA, Zhao G *et al* (2008). TFAP2A mutations result in branchio-oculo-facial syndrome. *Am J Hum Genet* **82:** 1171–1177.

Moore K, Persaud T (2007). *The developing human*. Saunders/Elsevier: St Louis.

Mossey PA, Little J (2002). Epidemiology of oral clefts: an international perspective. In: Wyszynski DFE, ed. *Cleft lip and palate: from origin to treatment*. Oxford University Press: New York, NY, pp. 127–144.

Mukhopadhyay P, Greene RM, Zacharias W *et al* (2004). Developmental gene expression profiling of mammalian, fetal orofacial tissue. *Birth Defects Res A Clin Mol Teratol* **70:** 912–926.

Mullor JL, Sanchez P, Ruiz i Altaba A (2002). Pathways and consequences: Hedgehog signaling in human disease. *Trends Cell Biol* **12:** 562–569.

Murillo J, Maldonado E, Barrio MC *et al* (2009). Interactions between TGF-beta1 and TGF-beta3 and their role in medial edge epithelium cell death and palatal fusion in vitro. *Differentiation* **77:** 209–220.

Murray JC, Schutte BC (2004). Cleft palate: players, pathways, and pursuits. *J Clin Invest* **113:** 1676–1678.

Nawshad A, LaGamba D, Hay ED (2004). Transforming growth factor beta (TGFbeta) signalling in palatal growth, apoptosis and epithelial mesenchymal transformation (EMT). *Arch Oral Biol* **49:** 675–689.

Neiswanger K, Deleyiannis FW, Avila JR *et al* (2006). Candidate genes for oral-facial clefts in Guatemalan families. *Ann Plast Surg* **56:** 518–521. Discussion 521.

Neiswanger K, Weinberg SM, Rogers CR *et al* (2007). Orbicularis oris muscle defects as an expanded phenotypic feature in nonsyndromic cleft lip with or without cleft palate. *Am J Med Genet A*. **143:** 1143–1149.

Nie X, Luukko K, Kettunen P (2006). FGF signalling in craniofacial development and developmental disorders. *Oral Dis* **12:** 102–111.

Niemann S, Zhao C, Pascu F *et al* (2004). Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. *Am J Hum Genet* **74:** 558–563.

Nieminen P, Kotilainen J, Aalto Y, Knuutila S, Pirinen S, Thesleff I (2003). MSX1 gene is deleted in Wolf–Hirschhorn syndrome patients with oligodontia. *J Dent Res* **82:** 1013–1017.

Nohe A, Keating E, Knaus P, Petersen NO (2004). Signal transduction of bone morphogenetic protein receptors. *Cell Signal* **16:** 291–299.

Nopoulos P, Langbehn DR, Canady J, Magnotta V, Richman L (2007a). Abnormal brain structure in children with isolated clefts of the lip or palate.[see comment]. *Arch Pediatr Adolesc Med* **161:** 753–758.

Nopoulos P, Richman L, Andreasen NC, Murray JC, Schutte B (2007b). Abnormal brain structure in adults with Van der Woude syndrome. *Clin Genet* **71:** 511–517.

Nottoli T, Hagopian-Donaldson S, Zhang J, Perkins A, Williams T (1998). AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. *Proc Natl Acad Sci U S A* **95:** 13714–13719.

O'Rahilly R, Muller F (2007). The development of the neural crest in the human. *J Anat* **211:** 335–351.

Pauws E, Stanier P (2007). FGF signalling and SUMO modification: new players in the aetiology of cleft lip and/or palate. *Trends Genet* **23:** 631–640.

Paylor R, Glaser B, Mupo A *et al* (2006). Tbx1 haploinsufficiency is linked to behavioral disorders in mice and humans: implications for 22q11 deletion syndrome. *Proc Natl Acad Sci U S A* **103:** 7729–7734.

Rahimov F, Marazita ML, Visel A *et al* (2008). Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. *Nat Genet* **40:** 1341–1347.

Ribeiro LL, Teixeira Das Neves L, Costa B, Ribeiro Gomide M (2003). Dental anomalies of the permanent lateral incisors and prevalence of hypodontia outside the cleft area in complete unilateral cleft lip and palate. *Cleft Palate Craniofac J* **40:** 172–175.

Rice R, Spencer-Dene B, Connor EC *et al* (2004). Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. *J Clin Invest* **113:** 1692–1700.

Richardson RJ, Dixon J, Malhotra S *et al* (2006). Irf6 is a key determinant of the keratinocyte proliferation-differentiation switch. *Nat Genet* **38:** 1329–1334.

Riley BM, Murray JC (2007). Sequence evaluation of FGF and FGFR gene conserved non-coding elements in nonsyndromic cleft lip and palate cases. *Am J Med Genet A* **143:** 3228–3234.

Riley BM, Mansilla MA, Ma J *et al* (2007). Impaired FGF signaling contributes to cleft lip and palate. *Proc Natl Acad Sci U S A* **104:** 4512–4517.

Rinne T, Brunner HG, van Bokhoven H (2007). p63-associated disorders. *Cell Cycle* **6:** 262–268.

Roessler E, Belloni E, Gaudenz K *et al* (1996). Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. *Nat Genet* **14:** 357–360.

Roessler E, Du YZ, Mullor JL *et al* (2003). Loss-of-function mutations in the human GLI2 gene are associated with pituitary anomalies and holoprosencephaly-like features. *Proc Natl Acad Sci U S A* **100:** 13424–13429.

Satokata I, Maas R (1994). Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. *Nat Genet* **6:** 348–356.

Schorle H, Meier P, Buchert M, Jaenisch R, Mitchell PJ (1996). Transcription factor AP-2 essential for cranial closure and craniofacial development. *Nature* **381:** 235–238.

Sheldahl LC, Moon RT (2004). Wnt signaling pathways. In: Epstein CJ, Erickson RP, Wynshaw-Boris A, eds *Inborn errors of development*. Oxford University Press: New York, pp. 272–281.

Shi M, Mostowska A, Jugessur A *et al* (2009). Identification of microdeletions in candidate genes for cleft lip and/or palate. *Birth Defects Res A Clin Mol Teratol* **85:** 42–51.

Sivertsen A, Wilcox AJ, Skjaerven R *et al* (2008). Familial risk of oral clefts by morphological type and severity: population based cohort study of first degree relatives. *BMJ* **336:** 432–434.

Slayton RL, Williams L, Murray JC, Wheeler JJ, Lidral AC, Nishimura CJ (2003). Genetic association studies of cleft lip and/or palate with hypodontia outside the cleft region. *Cleft Palate Craniofac J* **40:** 274–279.

Sozen MA, Suzuki K, Tolarova MM, Bustos T, Fernandez Iglesias JE, Spritz RA (2001). Mutation of PVRL1 is associated with sporadic, non-syndromic cleft lip/palate in northern Venezuela. *Nat Genet* **29:** 141–142.

Su HL, Li SS (2002). Molecular features of human ubiquitin-like SUMO genes and their encoded proteins. *Gene* **296:** 65–73.

Suphapeetiporn K, Tongkobpetch S, Siriwan P, Shotelersuk V (2007). TBX22 mutations are a frequent cause of non-syndromic cleft palate in the Thai population. *Clin Genet* **72:** 478–483.

Suzuki K, Hu D, Bustos T *et al* (2000). Mutations of PVRL1, encoding a cell-cell adhesion molecule/herpesvirus receptor, in cleft lip/palate-ectodermal dysplasia. *Nat Genet* **25:** 427–430.

Suzuki S, Marazita ML, Cooper ME *et al* (2009). Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. *Am J Hum Genet* **84:** 406–411.

Trokovic N, Trokovic R, Mai P, Partanen J (2003). Fgfr1 regulates patterning of the pharyngeal region. *Genes Dev* **17:** 141–153.

Tudela C, Formoso MA, Martinez T *et al* (2002). TGF-beta3 is required for the adhesion and intercalation of medial edge epithelial cells during palate fusion. *Int J Dev Biol* **46:** 333–336.

Vieira AR (2006). Association between the transforming growth factor alpha gene and nonsyndromic oral clefts: a HuGE review. *Am J Epidemiol* **163:** 790–810.

Vieira AR, Meira R, Modesto A, Murray JC (2004). MSX1, PAX9, and TGFA contribute to tooth agenesis in humans.[see comment]. *J Dent Res* **83:** 723–727.

Vieira AR, Avila JR, Daack-Hirsch S *et al* (2005). Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. *PLoS Genet* **1:** e64.

Vieira AR, Modesto A, Meira R, Barbosa AR, Lidral AC, Murray JC (2007). Interferon regulatory factor 6 (IRF6) and fibroblast growth factor receptor 1 (FGFR1) contribute to human tooth agenesis. *Am J Med Genet A* **143:** 538–545.

Vitelli F, Morishima M, Taddei I, Lindsay EA, Baldini A (2002). Tbx1 mutation causes multiple cardiovascular defects and disrupts neural crest and cranial nerve migratory pathways. *Hum Mol Genet* **11:** 915–922.

Wan M, Cao X (2005). BMP signaling in skeletal development. *Biochem Biophys Res Commun* **328:** 651–657.

Washbourne BJ, Cox TC (2006). Expression profiles of cIRF6, cLHX6 and cLHX7 in the facial primordia suggest specific roles during primary palatogenesis. *BMC Dev Biol* **6:** 18.

Weinberg SM, Maher BS, Marazita ML (2006a). Parental craniofacial morphology in cleft lip with or without cleft palate as determined by cephalometry: a meta-analysis. *Orthod Craniofac Res* **9:** 18–30.

Weinberg SM, Neiswanger K, Martin RA *et al* (2006b). The Pittsburgh Oral-Facial Cleft study: expanding the cleft phenotype. Background and justification. *Cleft Palate Craniofac J* **43:** 7–20.

Weinberg SM, Brandon CA, McHenry TH *et al* (2008a). Rethinking isolated cleft palate: evidence of occult lip defects in a subset of cases. *Am J Med Genet A* **146A:** 1670–1675.

Weinberg SM, Neiswanger K, Richtsmeier JT *et al* (2008b). Three-dimensional morphometric analysis of craniofacial shape in the unaffected relatives of individuals with nonsyndromic orofacial clefts: a possible marker for genetic susceptibility. *Am J Med Genet A* **146A:** 409–420.

Woods CG, Stricker S, Seemann P *et al* (2006). Mutations in WNT7A cause a range of limb malformations, including Fuhrmann syndrome and Al-Awadi/Raas-Rothschild/ Schinzel phocomelia syndrome. *Am J Hum Genet* **79:** 402–408.

Xiong W, He F, Morikawa Y *et al* (2009). Hand2 is required in the epithelium for palatogenesis in mice. *Dev Biol* (Epub ahead of print).

Yagi H, Furutani Y, Hamada H *et al* (2003). Role of TBX1 in human del22q11.2 syndrome. *Lancet* **362:** 1366–1373.

Yanagisawa H, Clouthier DE, Richardson JA, Charite J, Olson EN (2003). Targeted deletion of a branchial arch-specific enhancer reveals a role of dHAND in craniofacial development. *Development* **130:** 1069–1078.

Yang A, Schweitzer R, Sun D *et al* (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398:** 714–718.

Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y (2002). Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. *Development* **129:** 4135–4146.

Zhu JL, Basso O, Hasle H, Winther JF, Olsen JH, Olsen J (2002). Do parents of children with congenital malformations have a higher cancer risk? A nationwide study in Denmark. *Br J Cancer* **87:** 524–528.

Zucchero TM, Cooper ME, Maher BS *et al* (2004). Interferon regulatory factor 6 (IRF6) gene variants and the risk of isolated cleft lip or palate. *N Engl J Med* **351:** 769–780.

Zweier C, Sticht H, Aydin-Yaylagul I, Campbell CE, Rauch A (2007). Human TBX1 missense mutations cause gain of function resulting in the same phenotype as 22q11.2 deletions. *Am J Hum Genet* **80:** 510–517.