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Craniofacial Birth Defects: The Role of Neural Crest Cells in the Etiology and Pathogenesis of Treacher Collins Syndrome and the Potential for Prevention

Paul A. Trainor[1,2]*

[1]Stowers Institute for Medical Research, Kansas City, Missouri

[2]Department of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, Kansas

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Of all the babies born with birth defects, approximately one-third display anomalies of the head and face [Gorlin et al., 1990] including cleft lip, cleft palate, small or absent facial and skull bones and improperly formed nose, eyes, ears, and teeth. Craniofacial disorders are a primary cause of infant mortality and have serious lifetime functional, esthetic, and social consequences that are devastating to both children and parents alike. Comprehensive surgery, dental care, psychological counseling, and rehabilitation can help ameliorate-specific problems but at great cost over many years which dramatically affects national health care budgets. For example, the Center for Disease Control and Prevention estimates that the lifetime cost of treating the children born each year with cleft lip and/or cleft palate alone to be US$697 million. Treating craniofacial malformations, of which in excess of 700 distinct syndromes have been described, through comprehensive, well-coordinated and integrated strategies can provide satisfactory management of individual conditions, however, the results are often variable and rarely fully corrective. Therefore, better techniques for tissue repair and regeneration need to be developed and therapeutic avenues of prevention need to be explored in order to eliminate the devastating consequences of head and facial birth defects. To do this requires a thorough understanding of the normal events that control craniofacial development during embryogenesis. This review therefore focuses on recent advances in our understanding of the basic etiology and pathogenesis of a rare craniofacial disorder known as Treacher Collins syndrome and emerging prospects for prevention that may have broad application to congenital craniofacial birth defects. © 2010 Wiley-Liss, Inc.

Key words: Treacher Collins syndrome; Tcof1/Treacle; neural crest cells; craniofacial; ribosome biogenesis; p53

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INTRODUCTION

Craniofacial morphogenesis is a complex process and the blueprint for building the vertebrate head and face is established very early during embryonic development. In fact in humans, the most critical steps take place between about the third to eighth weeks of pregnancy. The facial region of the embryo starts out as a relatively blank slate, consisting of three distinct tissue layers known as ectoderm (outside), mesoderm (middle), and endoderm (inside) lying adjacent to one another. Shortly thereafter, the most critical step of head and facial development occurs; the formation of neural crest cells. Neural crest cells are derived from the neural ectoderm and migrate over long distances ultimately forming cartilage, bone, connective tissue, sensory neurons, glia, and pigments cells amongst many other cell types and tissues. Neural crest cells therefore generate the scaffold upon which the head and face are constructed and are largely responsible for facial shape and variation. The muscles of the face such as those required for jaw opening and closing (i.e., mastication) are generated from the mesoderm as are the endothelial cells that form the majority of the vasculature. The lining of the oral cavity of the mouth (the beginning of the gastro-intestinal tract), the esophagus, and organs in the neck such as the thyroid and parathyroids are derivatives of the endoderm. Differential cell proliferation, cell death, cell migration, and cell differentiation occurs in each of these layers ultimately shaping and

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*Correspondence to:
Paul A. Trainor, Stowers Institute for Medical Research, 1000 East 50th Street, Kansas City, MO 64110. E-mail: pat@stowers-institute.org
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transforming the blank tissue slate into a face with its characteristic myriad of contours and features [Gorlin et al., 1990].

NEURAL CREST CELL DEVELOPMENT

Craniofacial birth defects are typically recognized as abnormalities in the underlying structure of the face, that is, anomalies in bone and cartilage development. Therefore, craniofacial abnormalities are usually attributed to problems in neural crest cell development. Neural crest cell development can be divided into distinct stages (i) formation, (ii) migration, and (iii) differentiation. Neural crest cells are born at the interface between the neural plate (neural ectoderm) and surface ectoderm (presumptive epidermis/non-neural ectoderm) a region commonly referred to as the neural plate border. Cell lineage tracing has indicated that both neural plate and surface ectoderm give rise to neural crest cells [Selleck and Bronner-Fraser, 1995] and furthermore that induction requires planar interactions across the neural plate–surface ectoderm interface [Rollhauser-ter Horst, 1977; Moury and Jacobson, 1990].

Wingless/Int (Wnt) signaling emanating from the surface ectoderm has been shown to promote neural crest cell formation [Garcia-Castro et al., 2002; Lewis et al., 2004]. Similarly, bone morphogenetic protein (BMP) signaling is also involved. BMP4/7 have been shown to induce avian neural crest cell induction in naïve ectoderm explants [Liem et al., 1995; Selleck et al., 1998] and furthermore work performed in frog and fish embryos indicates that a precise threshold concentration gradient of BMP signaling within the dorsal neural plate is crucial for neural crest cell formation [Mayor et al., 1995; Morgan and Sargent, 1997]. The underlying mesoderm may play an important role in generating neural crest cells through its regulation of the BMP signaling gradient. The mesoderm produces BMP inhibitors such as follistatin that help to define low, intermediate, and high localized levels of BMP4/7 activity which induce the overlying neural plate, neural crest, and surface ectoderm, respectively [Marchant et al., 1998]. In addition, fibroblast growth factor (FGF) signaling from the underlying mesoderm has also been shown to be capable of independently inducing neural crest cell formation in frog embryos [Monsoro-Burq et al., 2003]. Thus, WNT, BMP, and FGF signaling have each been identified in species-specific contexts (avian, frog, and fish) as key signaling factors that govern neural crest formation. These signals may act in concert or in parallel but independent neural crest inducing pathways. Surprisingly however, to date, no mouse knockouts recapitulate a role for these signaling pathways in neural crest cell induction. Rather in mammalian embryos, the BMP, WNT, and FGF signaling pathways appear to regulate neural crest cell survival and lineage fate selection [Crane and Trainor, 2006].

Recently it was provocatively proposed that neural crest cells may be specified during gastrulation in avian embryos, which is much earlier during embryogenesis than previously thought and furthermore that this induction process is governed intrinsically by *Pax7* [Basch et al., 2006]. While *Pax* gene involvement in neural crest cell formation has also been observed in *Xenopus* embryos [Monsoro-Burq et al., 2005], conservation of this process and an essential role for *Pax* genes has not yet been demonstrated in mammals. Therefore, despite the importance of neural crest cells in craniofacial development and disease, we still have a poor understanding of the essential signals that govern neural crest cell formation in mammals.

Irrespective of which signals induce neural crest cell formation, neuroepithelial cells undergo an epithelial to mesenchymal conversion as part of the induction process. This requires tremendous changes in cytoskeletal architecture and cell adhesion and central to this transformation is the *Snail* family of transcriptional repressors. *Snail* genes directly repress cell adhesion molecules such as E-cadherin [Cano et al., 2000], thereby promoting the delamination or exit of neural crest cells from the neural plate which is concomitant with the commencement of their migration throughout the body. Thus, *Snail* gene expression is widely used as an indicator of neural crest cell formation.

Neural crest cells typically emerge from the neural tube in a wave that spreads from anterior to posterior along almost the entire neuraxis (Fig. 1A,B). The cranial neural crest cell population can be divided into forebrain, midbrain, and hindbrain domains of migrating cells. Rather than migrating randomly, neural crest cells appear to follow precise, region-specific pathways [Serbedzija et al., 1992; Osumi-Yamashita et al., 1994; Trainor and Tam, 1995; Kulesa et al., 2004]. The most striking aspect of cephalic neural crest cell migration is the apparent segregation of frontonasal, 1st pharyngeal arch, 2nd arch, and 3rd arch populations from one another, the patterns of which are highly conserved in vertebrate species as disparate as amphibians, teleosts, avians, marsupials, and mammals (Fig. 1A) [Noden, 1975; Trainor and Tam, 1995; Horigome et al., 1999; Epperlein et al., 2000; McCauley and Bronner-Fraser, 2003; Vaglia and Smith, 2003]. Briefly, forebrain and rostral midbrain neural crest cells colonize the frontonasal and periocular regions, while caudal midbrain-derived neural crest cells populate the maxillary component of the first pharyngeal arch [Osumi-Yamashita et al., 1994; Trainor and Tam, 1995]. Collectively, theses neural crest cells gives rise to the upper jaw, palatal mesenchyme, and extrinsic ocular muscles (Fig. 1D) [Noden, 1973, 1975; Le Lievre and Le Douarin, 1975; Couly and Le Douarin, 1990]. The hindbrain is transiently partitioned into seven contiguous segments called rhombomeres [Vaage 1969]. Neural crest cells from these rhombomeres migrate in discrete segregated streams into the first through sixth pharyngeal arches (Fig. 1A) [Osumi-Yamashita et al., 1994; Trainor and Tam, 1995], and produce the lower jaw, hyoid bone, and adjacent regions of the neck including the parathyroid glands and thymus together with the inner ear bones, cranial ganglia, and the pharyngeal and laryngeal parts of the tongue (Fig. 1C,D) [Le Lievre and Le Douarin, 1975; Noden, 1975; Couly and Le Douarin, 1990]. The segregation of distinct cranial neural crest cell populations is critical to prevent fusions of the cranial ganglia and skeletal elements and also to prevent mixing of neural crest cells with different genetic constitutions [Golding et al., 2000, 2002] and this is largely orchestrated by the ectoderm, mesoderm, and endoderm tissues with which the neural crest cells interact [Trainor and Krumlauf, 2001].

The neural crest is a discrete population being generated only transiently in the embryo, however, it is often considered the fourth germ layer due to the extraordinary array of embryonic and adult cell and tissue types that it generates. *In vivo* and *in vitro* clonal analyses have revealed that the majority of neural crest cells are bipotent or unipotent. However, a relatively small (1–3%)

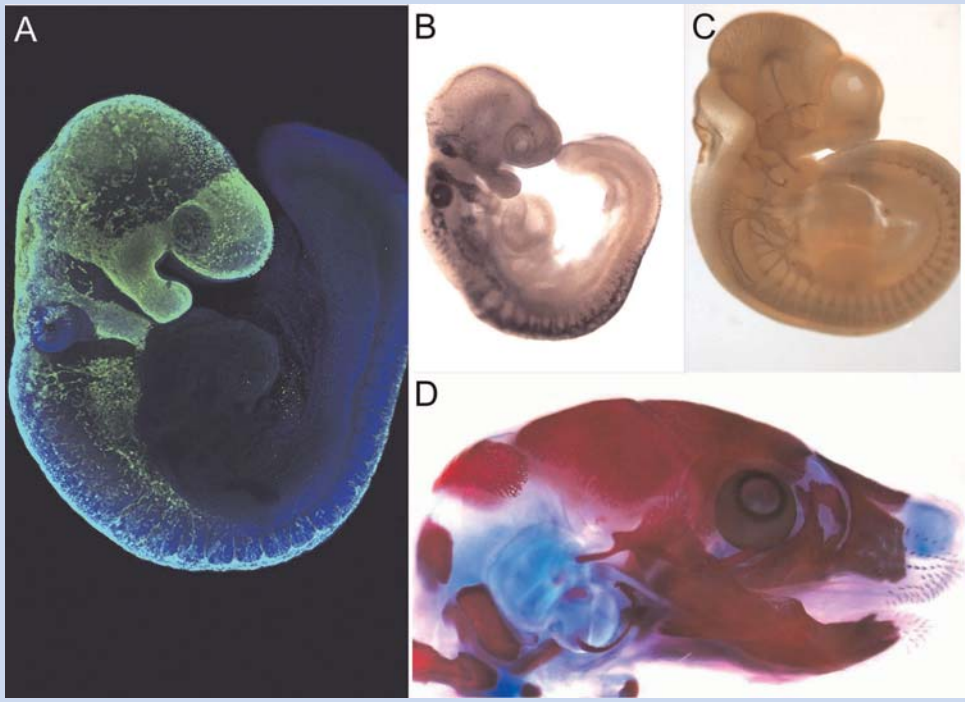

FIG. 1. Neural crest cell migration and differentiation. A: E9.5 mouse embryo (blue; DAPI stain) displaying migrating neural crest cells (GFP). B: Sox10 staining of neurogenic neural crest cells in an E9.5 mouse embryo. C: Neurofilament immunostaining of the peripheral nervous system in an E10.5 mouse embryo. D: E18.5 embryo stained with alizarin red (bone) and alcian blue (cartilage). Panel A courtesy of Amanda Barlow, Stowers Institute for Medical research. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

proportion of the neural crest cell population exhibits the characteristics of stem cells, being multipotent with the capacity for self-renewal. Interestingly multipotent neural crest stem cells may even persist throughout embryonic development and into adulthood. Similar to neural crest cell migration, the specific fate of an individual neural crest cell is determined by a balance between signals acquired within the neuroepithelium during their formation and from the ectoderm, mesoderm, and endoderm tissues with which the neural crest cells interact during their migration. For example, neural crest cells that underlie the surface ectoderm in the distal region of the mandibular prominence will differentiate into odontoblasts of the teeth [Lumsden, 1988], whereas neural crest cells located deeper within a pharyngeal arch will differentiate into cartilage and bone in response to signals from the endoderm [Couly et al., 2002]. Cranial neural crest cells contacting the mesoderm-derived musculature and vasculature generate connective tissue and smooth muscle cells, respectively [Noden, 1986]. One of the first major differentiation steps taken by a migrating neural crest cell is to become neurogenic or mesenchymal and *Sox10* is a good spatiotemporal indicator of this process (Fig. 1B). *Sox10* is initially expressed in all migrating neural crest cells and while it remains active in neural crest cells destined to contribute to the peripheral nervous system (Fig. 1C), it is switched off in neural crest cells that will ultimately form connective tissue, cartilage, and bone (Fig. 1D).

Proper peripheral nervous system development requires the differentiation of three distinct neuronal populations from the neural crest, including autonomic and sensory neurons and also glia [Sieber-Blum, 1989; Greenwood et al., 1999; White and Anderson, 1999]. *Notch* is a potent glial inducing cue which can cause an irreversible switch from neurogenesis to gliogenesis [Morrison et al., 2000] and *Notch* genes are expressed by subset of neural crest cells [Williams et al., 1995; De Bellard et al., 2002; Endo et al., 2002]. Similarly, glial growth factor, or *Neuregulin 1* (*Nrg1*), has also been implicated in regulating glial fate determination [Shah et al., 1994]. Although *Nrg1* is not essential for the formation of neural crest-derived glia [Garratt et al., 2000], *Nrg1* suppresses neuronal differentiation while simultaneously promoting glial cell fate in rat neural crest cells [Shah et al., 1994].

In contrast, BMP2 signaling induces neurogenesis and in particular the expression the basic-helix-loop-helix protein *Mash1*, an autonomic neuron marker, in neural crest cells [Shah et al., 1996]. *Neurogenin-2* (*Nrg2*) has also been shown to regulate neurogenesis, but specifically sensory neuron fate [Zirlinger et al., 2002], by promoting the expression of the tyrosine receptor kinases, *TrkB*, and *TrkC* [Ma et al., 1999]. In addition, *Wnt/β-catenin* signaling is also known to instruct neural crest cells to form sensory neurons. In conditional knockouts of *β-catenin*, a complete failure of sensory neurogenesis is observed in vitro and in vivo [Hari et al., 2002]. Conversely, sustained overexpression of *Wnt1/β-catenin* signaling enhances the derivation of sensory neurons from neural crest cells at the expense of all other neural crest derivatives [Lee et al., 2004].

Mesenchymal (non-neuogenic) neural crest cells generate cell types as diverse as chondrocytes, osteoblasts, vascular smooth muscle cells, and odontoblasts and their differentiation involves

the integration of many cell intrinsic and extrinsic signaling pathways. *Sox9* plays multiple roles in neural crest cell development, most critically as a key determinant of chondrogenesis. Conditional deletion of *Sox9* in the neural crest results in a complete absence of cartilage and endochondral bones in the head [Mori-Akiyama et al., 2003]. Chondrocyte differentiation through *Sox9* is achieved by the inhibition of osteoblast promoting genes such as β-*catenin* [Day et al., 2005]. Indeed, expression of a stable form of β-*catenin* inhibits chondrogenesis, mimicking the loss of *Sox9*. Consistent with this, the conditional deletion of β-*catenin* in chondrocytes mimics overexpression of *Sox9* [Akiyama et al., 2004]. Thus, an antagonistic relationship exists between *Sox9* and β-*catenin* in the regulation of cartilage and bone development [Mori-Akiyama et al., 2003]. Similar to *Sox9*, β-*catenin* plays multiple roles during ncc differentiation by influencing chondrogenesis as well as sensory neurogenesis. Collectively this illustrates the reiteration of the same signaling pathways during multiple stages of neural crest cell development and this is a common theme during embryogenesis.

Intriguingly, there is a general axial registration between the neural crest cells, mesodermal cells, and ectoderm that persists during their migration and differentiation [Noden, 1991; Trainor and Tam, 1995]. For example, the neural crest cells that arise from the caudal midbrain and anterior hindbrain are always associated with the mesoderm and ectoderm cells that arise at the same axial level. Together they occupy the first pharyngeal arch in a characteristic fashion with the mesoderm forming the central core or muscle plates of the pharyngeal arches. These muscle plates are enveloped by neural crest cells which are in turn surrounded by the surface ectoderm. These relations and the tissue boundaries they create are maintained through later stages of development when the muscles and their connective tissues may have moved to other parts of the head [Kontges and Lumsden, 1996]. Furthermore, this congruence and axial registration also includes the cranial motor nerves and precursors of epipharyngeal placodes [D'Amico-Martel and Noden, 1983; Baker and Bronner-Fraser, 2001], which will innervate-specific craniofacial muscles. These interactions are essential for generating a fully functioning jaw and indicate that the early registration between different tissues in the head during early embryogenesis is critical for the establishing the blueprint or foundations of vertebrate craniofacial development. Thus, craniofacial anomalies are not always the consequence of defects autonomous or intrinsic to the neural crest cells. Abnormal neural crest cell patterning can also arise secondarily as a consequence of non-cell autonomous or extrinsic defects in the mesoderm, ectoderm, and endoderm tissues with which the neural crest cells interact.

Cranial neural crest cells thus give rise to an extraordinary array of distinct cell and tissue types (Fig. 1) but are only transiently generated. Therefore, it is critical that the embryo generates and maintains a sufficient pool of neural crest progenitors that survive, proliferate, migrate, and differentiate appropriately as deficiencies in these processes underlie a number of congenital craniofacial malformation disorders. In fact, depending on which phase of neural crest cell development is disrupted (i.e., formation vs. differentiation), very different craniofacial anomalies can manifest. For example, if neural crest cell formation or migration is perturbed such that too few neural crest cells are produced or they fail to migrate to their final destinations, this can result in babies with small noses, jaws, and ears as well as cleft palate. These phenotypes are characteristic of Treacher Collins syndrome [Dixon et al., 2006; Jones et al., 2008]. In contrast if neural crest cell differentiation is disrupted conditions known as craniosynostosis can arise which are characterized by dysmorphic cranial shape, midface hypoplasia, seizures and mental retardation [reviewed in Morriss-Kay and Wilkie, 2005]. The suture mesenchyme separating the individual bony plates in the skull is derived from neural crest [Jiang et al., 2002] and this tissue should stay undifferentiated to facilitate birth of the fetus as well as accommodate postnatal brain growth. Aberrant neural crest cell differentiation results in pre-mature ossification of the suture mesenchyme which fuses the individual skull bones (craniosynostosis) consequently restricting skull growth and impacting upon facial and brain growth, development, and maturation.

Determining the precise mechanisms that govern neural crest cell development during normal head and facial morphogenesis is essential for furthering our understanding of the etiology and pathogenesis of individual congenital craniofacial malformation disorders. Furthermore, it can also provide the basis for designing potential therapeutic avenues to prevent and rescue craniofacial abnormalities, as has been the case recently in animal model studies of Treacher Collins syndrome [Jones et al., 2008].

TREACHER COLLINS SYNDROME

Treacher Collins syndrome (TCS, OMIM 154500) is a congenital disorder of craniofacial development first described by Treacher Collins [1900]. The same condition has also been called mandibulofacial dysostosis [Franceschetti and Klein, 1949]. Characteristic TCS abnormalities include hypoplasia of the facial bones, particularly the maxilla, mandible, and zygomatic complex and in severe cases the zygomatic arches may be absent (Fig. 2) [Poswillo, 1975]. Hypoplasia of the facial bones often results in dental malocclusion, with anterior open bite. The teeth may be widely spaced, malpositioned, or reduced in number. In a large proportion of cases the palate is high, arched, and often cleft. Alterations in the size, shape, and position of the external ears are common and usually associated with atresia of the external auditory canals and anomalies of the middle ear ossicles. Radiographic analyses of the middle ears of TCS patients has revealed irregular or absent auditory ossicles with fusions between the rudiments of the malleus and incus, partial absence of the stapes and oval window, complete absence of the middle ear and epitympanic space [Stovin et al., 1960]. As a result bilateral conductive hearing loss is common, whereas mixed or sensorineural hearing loss is rare [Phelps et al., 1981] Ophthalmic abnormalities include downward slanting of the palpebral fissures with colobomas of the lower eyelids. Other clinical features of TCS may include defects in brain development such as microcephaly, mental retardation, and psychomotor delay [Milligan et al., 1994; Cohen et al., 1995; Teber et al., 2004].

CLINICAL MANAGEMENT

The care of individuals affected by TCS requires a multidisciplinary approach and may involve intervention from a number of healthcare professionals both pre- and postoperatively [Arndt et al., 1987]. Of

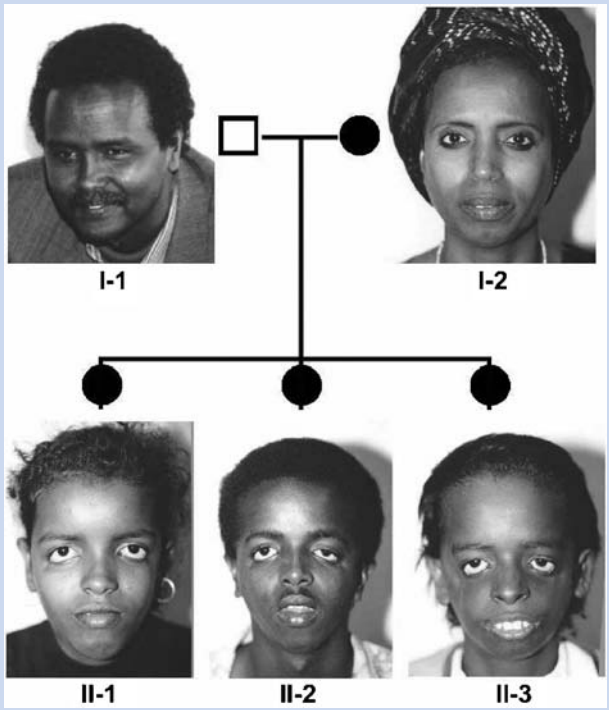

FIG. 2. Clinical photographs and partial pedigree of a Somalian family. Individual I-2, who has an extensive family history of TCS, exhibits no apparent clinical features of mandibulofacial dysostosis. In contrast all three children exhibit severe craniofacial anomalies consistent with TCS and furthermore share the same mutation (c.2259delA) as their mother. Adapted from Dixon et al. [2008]. By permission of Oxford University Press. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

primary concern are breathing and feeding problems that present at birth as a consequence of micrognathia and tongue obstruction of the hypopharynx. Emergency surgery in the form of a tracheostomy may be essential to maintain an adequate airway. Subsequently, at defined ages or when specific developmental milestones have been reached, extensive reconstructive surgery can help to restore the structure of the face. Management of the hard and soft tissues typically requires multiple surgeries and initially, depending on severity, palatal clefting is corrected in the earliest years of life, preferably around 12 months of age. Although the results can be variable, excellent outcomes are achievable through a comprehensive, well co-ordinated and integrated treatment plan incorporating craniofacial surgeons, orthodontists, ophthalmologists, otolaryngologists, and speech pathologists. The complexity of clinical management combined with results that may not be fully corrective implore more effort to be invested in exploring therapeutic avenues for prevention. However, this can only come from a better appreciation of the genetic, cellular, and biochemical basis of TCS.

GENETIC BASIS OF TCS

Treacher Collins syndrome is a rare autosomal dominant disorder, occurring with an incidence of 1 in 50,000 live births. Genetic, physical, and transcript mapping techniques have demonstrated that TCS is caused by mutations in the *TCOF1* gene, located on chromosome 5, which encodes a low complexity, serine/alanine-rich, nucleolar phosphoprotein known as Treacle [TreacherCollinsSyndromeCollaborativeGroup, 1996]. *TCOF1* consists of a 4,233 bp open reading frame spanning 26 exons in which over 200 largely family-specific mutations have been documented throughout the gene including deletions, insertions, splicing, mis-sense and nonsense mutations (http://genoma.ib.usp.br/TCOF1_database/). Deletions which range in size from 1 to 40 nucleotides are by far the most common and within that group a reoccurring 5 bp deletion in exon 24 accounts for 17% of TCS cases. The majority of mutations in *TCOF1* lead to truncations of the C-terminal end of Treacle [Dixon et al., 2007] and since truncated proteins have not been detected in patient fibroblasts this suggests that RNAs with premature termination codons are degraded by nonsense-mediated mRNA decay.

Although the causative mutations in a subset of patients have not been identified, TCS is thought to be genetically homogeneous because all the multigenerational families analyzed to date exhibit linkage to the human chromosome 5q32 locus. Intriguingly however, 50% of cases do not appear to have a previous family history and are thought to arise as the result of a de novo mutation [Jones et al., 1975]. Penetrance of the genetic mutations underlying TCS is high yet inter- and intra-familial variation in the severity of the phenotype (Fig. 2) is a striking feature of the condition [Dixon et al., 1994; Marres et al., 1995]. Individuals can be so mildly affected that it can be difficult to establish an unequivocal diagnosis and it is not uncommon for mildly affected TCS patients to be diagnosed retrospectively after the birth of a more severely affected child; this observation implies that the frequency of non-penetrance is underreported. In contrast, at the other end of the clinical spectrum, severe cases of TCS have resulted in perinatal death [Edwards et al., 1996]. Thus, no genotype–phenotype correlation has been observed in TCS and similarly no clear evidence of an association between disease severity and parental origin or type of pathogenic mutation, male or female, sporadic or familial [Gladwin et al., 1996; Edwards et al., 1997; Isaac et al., 2000; Splendore et al., 2000; Teber et al., 2004]. However, more recent cephalometric analyses of the craniofacial skeleton in age- and sex-matched TCS individuals has suggested that craniofacial deficiencies may be more significant in females [Chong et al., 2008]. Collectively the variable severity indicates that genetic background, environmental factors, and stochastic events may contribute to the clinical variation observed in TCS patients [Dixon and Dixon, 2004].

CELLULAR BASIS OF TCS

Since the initial description of TCS, several hypotheses have been proposed to explain the cellular basis of the craniofacial anomalies. These include abnormal patterns of neural crest cell migration [Poswillo, 1975], abnormal domains of cell death [Sulik et al., 1987; Sulik et al., 1988], improper cellular differentiation [Wiley et al., 1983], or an abnormality of the extracellular matrix [Herring et al., 1979]. However, until recently, there was scant experimental evidence to support any of these hypotheses. The first clue came from analyses of *Tcof1* gene expression during mouse embryogenesis.

Although not ubiquitous, *Tcof1* is broadly expressed in both embryonic and adult tissues. Interestingly, between E8.5 and 10.5, elevated levels of *Tcof1* expression were observed in the neuroepithelium and facial mesenchyme. Thus, the spatiotemporal expression of *Tcof1* coincides with the formation and migration of neural crest cells implying that *Tcof1* plays an important role in their development [Dixon et al., 2006].

Tcof1$^{+/-}$ mice exhibit profound craniofacial anomalies including frontonasal hypoplasia, particularly of the maxilla and mandible, together with cleft palate and agenesis of the nasal passages. The zygomatic arch, tympanic ring, and middle ear ossicles are all hypoplastic and misshapen [Dixon et al., 2000]. At birth these mice gasp for breath and display abdominal distension and consequently die due to asphyxia and an inability to feed. The mouse *Tcof1*$^{+/-}$ phenotype mimics the severe form of TCS observed in humans, and similarly the penetrance and severity of facial defects in mice is also dependent upon genetic background. In contrast to the lethal phenotype described above for C57BL/6 *Tcof1*$^{+/-}$ mice, the vast majority of DBA *Tcof1*$^{+/-}$ mice are viable and fertile postnatally and exhibit minor if any craniofacial anomalies [Dixon and Dixon, 2004]. The generation of *Tcof1*$^{+/-}$ haploinsufficient mouse models provided a unique opportunity to use experimental embryology to decipher the in vivo cellular basis of TCS together with the biochemical function of Treacle.

Cell lineage tracing performed in E8.5 wild-type and *Tcof1*$^{+/-}$ mouse embryos revealed no migratory nor path finding defects in cranial neural crest cell migration [Dixon et al., 2006] which was contrary to previous hypotheses [Poswillo, 1975]. This observation therefore indicated that *Tcof1* does not play a role in neural crest cell migration and, furthermore, that aberrant neural crest cell migration is not the underlying cause of TCS. However, despite the absence of a migration defect, 25% fewer migrating neural crest cells were reproducibly observed in TCS embryos compared to their wild-type littermates [Dixon et al., 2006]. The deficiency in neural crest cell number arises due to extensive neuroepithelial apoptosis from E8.0 to 10.5, which diminishes the neural stem cell pool from which neural crest cells are derived. This process is p53 dependent as nuclear activation and stabilization of p53 is observed in the neuroepithelium of *Tcof1*$^{+/-}$ embryos [Jones et al., 2008]. As a corollary to the elevated levels of apoptosis observed specifically in the neuroepithelium of TCS embryos, rates of proliferation were also examined. This surprisingly revealed that not only was proliferation reduced in the neuroepithelium but it was also compromised in the migrating neural crest cells. Thus, the deficiency in the number of neural crest cells formed is compounded by their reduced proliferation capacity. Therefore, the general cranioskeletal hypoplasia observed in individuals with TCS arises not because of a neural crest cell migration defect, but rather a deficiency in neural crest cell number. Hence *Tcof1*/treacle plays a critical role in neural crest cell formation and is required for neuroepithelial survival and neural crest cell proliferation [Dixon et al., 2006].

BIOCHEMICAL BASIS OF TCS

To precisely understand the pathogenetic mechanism of TCS, it is essential to elucidate the biochemical function of treacle, the protein encoded by *TCOF1*. However, compared to other developmentally important genes, *TCOF1* is very poorly conserved among mammals and even less so compared to non-mammalian species. For example, *TCOF1* exhibits only 62% amino acid identity to the mouse protein [Dixon et al., 1997b] and a very low 19% identity to the frog protein [Gonzales et al., 2005]. Treacle is a relatively simple 144 kDa protein that consists of three distinct domains, including unique amino and carboxy termini and a characteristic central repeat domain [Dixon et al., 1997a; Wise et al., 1997]. Putative nuclear export and import signals are seen at the N-terminus and C-terminus, respectively. Interestingly, the amino terminus of the protein which encompasses exons 1 and 2 of *TCOF1* is the most highly conserved region displaying 92.6% identity between mouse and human [Dixon et al., 1997a]. However, there is no functional data to indicate that treacle is exported from the nucleus. In contrast, it has been reported that the C-terminal domain is important for nucleolar localization of treacle [Marsh et al., 1998], and that perhaps the intracellular localization of treacle is very dynamic. Within the central domain, treacle contains multiple casein kinase II and protein kinase C phosphorylation site repeats. This is consistent with the fact that treacle is highly phosphorylated and associates with casein kinase II in vitro [Isaac et al., 2000]. However, to date it has not been determined if phosphorylation is required for normal treacle function nor if it plays an important role in its subcellular localization.

Immunofluorescence studies have shown that treacle co-localizes with upstream binding factor (UBF) and RNA polymerase I in the nucleolus [Valdez et al., 2004]. Furthermore, biochemical analyses of treacle via in vitro siRNA-mediated knockdown demonstrated that treacle is essential for the proper transcription of rDNA, which is consistent with its structural homology to Nopp140, another nucleolar protein which also regulates rDNA transcription [Chen et al., 1999]. Treacle has also been identified as a constituent of human Nop56-associated pre-ribosomal ribonucleoprotein (pre-rRNPs) complexes [Hayano et al., 2003] that 2′-*O*-methylate pre-ribosomal RNA during the early stages of pre-RNA processing in the nucleolus [Valdez et al., 2004]. These data imply that treacle is contained within an RNP complex in the nucleolus and may be specifically involved in governing the ribosome biogenesis process.

Recently an essential role for treacle, in ribosome biogenesis was demonstrated in vivo [Dixon et al., 2006]. Mice haploinsufficient for *Tcof1* exhibit diminished mature ribosome production as measured by the levels of 28S rRNA. In addition, *Tcof1* mutant embryos exhibit a significant reduction in methylation at specific residues of 18S rRNA [Gonzales et al., 2005]. These ribosome biogenesis deficiencies correlated with decreased proliferation in both the neural ectoderm and neural crest cells in *Tcof1* mutants. Consequently it has been hypothesized that deficient ribosome biogenesis is insufficient to meet the cellular and metabolic needs of these highly proliferative cell populations during embryogenesis and more specifically it is directly responsible for the high levels of cell death observed in the neural ectoderm at the time of neural crest formation [Dixon et al., 2006]. In support of this idea, deficient ribosome biogenesis is known to trigger nucleolar stress activation of p53 [Rubbi and Milner, 2003] and p53 in turn transcriptionally activates numerous pro-apoptotic effector genes. Increased p53 activity together with increased *Cdkn1a*, *Eda2r*, *Ccng1*, *Trp53inp1*,

Noxa, *Perp*, and *Wig1* expression is observed within the neuroepithelium of *Tcof1*$^{+/-}$ embryos. Collectively this accounts for the high levels of neural ectoderm-specific cell death observed in the pathogenesis of TCS [Jones et al., 2008]. Since neuroepithelial cells are the precursors of the neural crest, it is not surprising that the apoptosis impairs their generation. Moreover, this directly links deficiencies in ribosome biogenesis to p53-dependent cell death impairment of neural crest cell formation which mechanistically accounts for the cranioskeletal hyoplasia observed in TCS individuals (Fig. 2). Interestingly, the majority of mutations identified in humans are predicted to result in 3′ truncations of treacle and loss of the nuclear import signals. This strongly implies that the nuclear and nucleolar subcellular localization of treacle is critical to its ribosome biogenesis functions and moreover that ribosome biogenesis and consequently neural crest cell formation are similarly impaired in TCS patients.

PREVENTION OF TCS IN THE MOUSE

The correlation between *Tcof1* haploinsufficiency, deficient ribosome biogenesis, nucleolar stress, and p53-dependent neuroepithelial apoptosis, satisfactorily accounts for the neural crest cell hypoplasia and craniofacial phenotype observed in mouse models of TCS [Jones et al., 2008]. More importantly however, this simplistic mechanism raised the intriguing possibility that suppressing neuroepithelial apoptosis through inhibition of p53 function might be sufficient to prevent the onset and pathogenesis of TCS. Indeed, *Tcof1*$^{+/-}$ embryos treated in utero from E6.5 onwards with pifithrin-α as a specific inhibitor of p53 activity, exhibit a dose-dependent inhibition of neuroepithelial apoptosis and rescue of cranioskeletal development [Jones et al., 2008] A similar but more efficient rescue was observed when p53 activity was blocked genetically (Fig. 3A,B). Removal of one or two copies of p53 from the *Tcof1*$^{+/-}$ background revealed a dose-dependent inhibition of neuroepithelial apoptosis, restoration of the neural crest cell population and prevention of cranioskeletal anomalies characteristic of TCS [Jones et al., 2008]. Remarkably this also restored postnatal viability (Fig. 3C). However, a major surprise arising from the p53 inhibition experiments, was that the prevention of TCS craniofacial anomalies occurred without altering or restoring ribosome production [Jones et al., 2008]. 28S levels were essentially equivalent in *Tcof1*$^{+/-}$; *p53*$^{-/-}$ as in *Tcof1*$^{+/-}$ embryos. Thus, rescued embryos can develop normally with reduced 28S ribosome production, which raises the probability of non-ribosome-associated functions for treacle in activating p53-dependent apoptosis in the pathogenesis of TCS.

FUTURE DIRECTIONS FOR TCS

The major challenges facing the TCS clinical and research community in terms of improving the prognosis of affected or at risk individuals reside in three key areas; detection, repair, and prevention. Since the identification of the TCS locus, both pre- and postnatal molecular diagnoses have been possible. In this regard, pre-natal diagnosis in families with a history of TCS represents a key approach towards ameliorating the consequences of being born with TCS. However, the low incidence (1:50,000) of mutations compounded with 50% of the mutations arising spontaneously makes routine genetic screening for *TCOF1* mutations during early gestation economically prohibitive except in families with a known history of TCS.

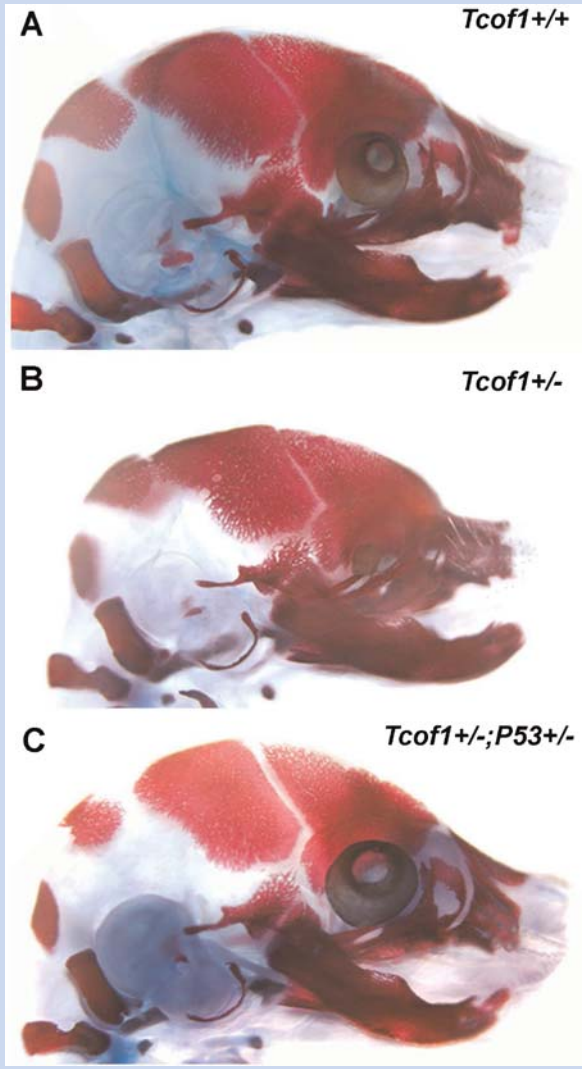

FIG. 3. Prevention of Treacher Collins syndrome craniofacial anomalies. Alizarin red (bone) and alcian blue (cartilage) staining of E18.5 *Tcof1*$^{+/+}$ (A), *Tcof1*$^{+/-}$ (B), and (C) *Tcof1*$^{+/-}$;*P53*$^{+/-}$ mouse embryos. Adapted from Jones et al. [2008]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com]

Consequently, the majority of individuals with craniofacial anomalies are detected during mid to late gestation via ultrasound screening. However, caution still needs to be exercised because a number of conditions exhibit phenotypic overlap with TCS, particularly hemifacial microsomia as well as Nager and Miller syndromes. Thus, confirmation that any craniofacial malformation is consistent with TCS still requires genetic testing and even then, the extreme variability in the degree to which individuals can be affected, together with the high rate of de novo mutations makes the outcome uncertain and the provision of genetic counseling extremely complicated.

Therefore, gestational diagnosis of TCS leaves postnatal surgery as the only available treatment option. Most craniofacial treatment centers have established timetables for facial reconstruction, however, individuals with TCS are known to have problems in the long-term stability of surgical outcomes following mandibular distraction [Karp et al., 1992; Stelnicki et al., 2002; Gursoy et al., 2008]. This suggests there may be consequences for *TCOF1* haploinsufficiency even during adult life. Thus, despite the multiple rounds of surgery that a TCS patient typically endures, rarely are they fully corrective. One possibility for improving surgical outcomes might be the incorporation of stem cells in craniofacial reconstructive surgery. Mesenchymal stem cells can be bioengineered to form bone and cartilage and potentially be used to reconstitute the head and facial tissues so severely disrupted in craniofacial syndromes such as TCS.

The numerous limitations in detection and repair of craniofacial malformation syndromes such as TCS leave prevention as an optimistic therapeutic goal. However, prevention is not without its own caveats. It is clear in animal models that chemical and genetic inhibition of p53 function can prevent neuroepithelial apoptosis and the occurrence of craniofacial anomalies characteristic of TCS. However, p53 performs many critically important cellular functions during embryogenesis and adulthood, most notably suppressing cancer and tumorigenesis [Levine, 1997]. Thus, blocking p53 function to prevent craniofacial malformation syndromes carries a substantial cancer and tumor inducing risk. Hence in developing alternative avenues for preventative intervention of TCS, it is critical to pursue downstream targets of p53, specifically those that can prevent apoptosis but have no links to tumor susceptibility nor abnormal embryonic or postnatal development. Therefore, with respect to craniofacial malformation syndromes, prevention is potentially better than cure, but still a long way off!

Central to a better understanding of TCS and improved management in the future is (i) the identification and characterization of novel (non-ribosome biogenesis) functions for *TCOF1*/treacle and (ii) a deeper appreciation of the mechanisms underlying the viability in phenotypic severity observed throughout the TCS community. With respect to non-ribosome biogenesis-associated functions for Treacle, it is interesting that treacle has been shown to possess a LisH (Lis1—homolgous motif) in its N-terminal region [Emes and Ponting, 2001]. LisH motif-containing proteins are associated with microtubule binding and have been localized at centrosomes implicating them in microtubule dynamics, chromosome segregation, and cell migration [Feng et al., 2000; Sasaki et al., 2000]. However, to date no functional data has demonstrated that treacle protein is exported from the nucleus. Nonetheless, the identification of TCS-affected individuals with mutations solely in the LisH domain implies that treacle may shuttle between the nucleolus and cytoplasm. Disruptions to this shuttling process or interference with as yet unknown cytoplasmic functions for *TCOF1*/treacle are thus potentially critical factors in the pathogenesis of characteristic TCS craniofacial abnormalities.

As described earlier, a striking feature of TCS is the inter- and intra-familial variation in the phenotypic severity [Dixon et al., 1994; Marres et al., 1995]. This is also reflected in the different strains of mice harboring mutations in *Tcof1*. There are three principal mechanisms which could account for the phenotypic variation. Firstly, background-dependent differences in *Tcof1*/treacle itself. TCS results from haploinsufficiency of *Tcof1*/treacle and subtle spatiotemporal differences in *Tcof1*/treacle activity or perhaps endogenous level of activity from the DBA versus C57BL/6 allele may be critically relevant to the severity of the phenotype. Secondly, background differences in p53 could influence the phenotype. One of the distinctive features of p53 is its ability to elicit both cell-cycle arrest and apoptosis. The different fates are orchestrated through activation of distinct subsets of target genes and this is intimately associated with posttranslational modification of specific p53 residues [Aylon and Oren, 2007], which may be background dependent. Thirdly, there may be multiple genetic modifiers that contribute to the severity of TCS. The identification of both positive and negative genetic modifiers will provide further opportunities for therapeutic intervention and an improvement in the prognosis of at risk or affected individuals.

CONCLUSIONS

The integration of human and mouse genetics, cell biology, biochemistry, and experimental embryology has recently provided novel insights into the etiology and pathogenesis of TCS and furthermore, potentially an avenue for intervention. Given the extraordinary variety of craniofacial anomalies, it is essential to understand genetically and morphologically, the distinct mechanisms that regulate the formation, migration, and differentiation of neural crest cells as a prelude to understanding the origins of congenital craniofacial defects and their prevention or repair. Of central importance to this process is a more profound understanding of the specific tissue interactions that occur between the neural crest cells and the endoderm, mesoderm, and ectoderm that they contact during their migration in establishing the foundations of craniofacial morphogenesis. The prevention of TCS craniofacial anomalies represents one of the first successful animal model rescues of a congenital neurocristopathy. Consequently, it provides an attractive model for the prevention of TCS and other craniofacial birth defects of similar etiology and pathogenesis. However, the challenges of translating these approaches into the clinic still remain.

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