ORIGINAL ARTICLE

Treacher Collins syndrome

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Structured Abstract
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Treacher Collins syndrome (TCS) is an autosomal dominant disorder of craniofacial development which results from loss-of-function mutations in the gene *TCOF1*. *TCOF1* encodes the nucleolar phosphoprotein, Treacle, which plays a key role in pre-ribosomal processing and ribosomal biogenesis. In mice, haploinsufficiency of *Tcof1* results in a depletion of neural crest cell precursors through high levels of cell death in the neuroepithelium, which results in a reduced number of neural crest cells migrating into the developing craniofacial complex. These combined advances have already impacted on clinical practice and provide invaluable resources for the continued dissection of the developmental basis of TCS.

Key words: apoptosis; facial development; ribosome; Treacher Collins syndrome

Introduction

Treacher Collins syndrome (TCS) is an autosomal dominant disorder of facial development which affects approximately 1 in 50 000 live births (1). More than 60% of cases do not appear to have a previous family history and are thought to arise as the result of a *de novo* mutation (2). On the basis that the tissues affected in TCS develop from the first and second branchial arches, which are populated extensively by cranial neural crest cells (3), several hypotheses were proposed to explain the cellular basis of this disorder. These theories included abnormal neural crest cell migration (4), improper cellular differentiation during development (5) or an abnormality of the extracellular matrix (6). The use of genetic, physical and transcript mapping techniques resulted in the identification of the gene mutated in TCS, designated *TCOF1*, which was found to encode a low complexity, serine/alanine-rich, nucleolar phosphoprotein that was named Treacle (7). More recently, the integration of molecular biology, cell biology, mouse genetics and experimental embryology has provided novel insights into the molecular pathogenesis of TCS.

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The Treacher Collins syndrome phenotype

The major clinical features of TCS, with their frequencies in parentheses (8), include abnormalities of the external ears (77%) and atresia of external auditory canals (36%). Radiographic analysis of the middle ears of affected

individuals has revealed malformation of the auditory ossicles with fusion between rudiments of the malleus and incus, partial absence of the stapes and oval window, or even complete absence of the middle ear and epitympanic space. As a result of these abnormalities, bilateral conductive hearing loss is common, whereas mixed or sensorineural hearing loss is rare (9). Audiological examination of individuals affected by TCS is therefore important as over 50% of patients may have significant hearing loss. Lateral downward sloping of the palpebral fissures (89%), usually with colobomas of the lower eyelids and a paucity of lashes medial to the defect (69%), are also commonly observed (Fig. 1). Hypoplasia of the facial bones, particularly affecting the mandible (78%) and zygomatic complex (81%) are also frequently seen in TCS (Fig. 1). In severe cases, the zygomatic arch may be absent and cleft palate (28%) may occur. These features are usually bilaterally symmetrical (Fig. 1). In most TCS patients a spectrum of affected features is observed; in fact, rarely is any single abnormality alone sufficient to lead to a diagnosis of TCS. More usually, the entire facial appearance is considered when trying to arrive at a diagnosis, particularly in mildly affected patients. Although the penetrance of the genetic mutations underlying TCS is high, the condition is characterized by marked inter- and intra-familial phenotypic variability (Fig. 1) (8, 10). On one hand, severe cases of TCS may result in perinatal death because of a compromised airway. On the other, some individuals may be so mildly affected that it can be difficult to establish an unequivocal clinical diagnosis. Indeed, a number of mildly affected TCS patients have been diagnosed retrospectively only after the birth of a severely affected child. These aspects of TCS often present the clinician with diagnostic and genetic counselling difficulties (see below).

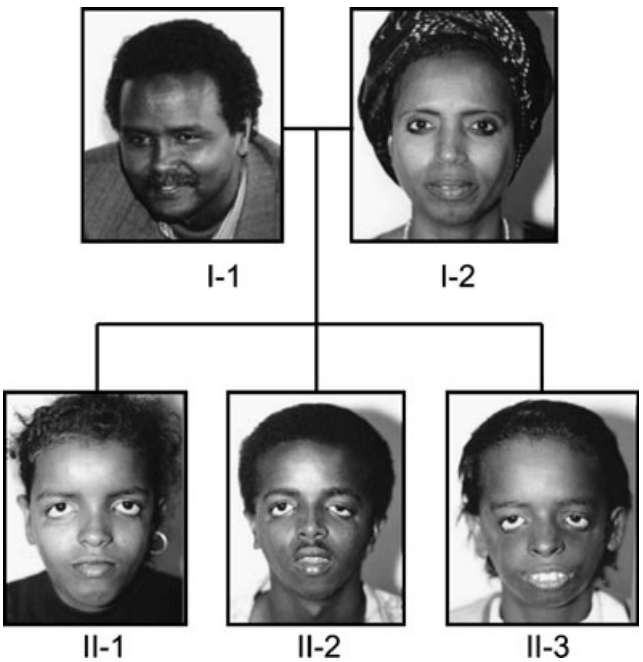

Fig. 1. The clinical features of Treacher Collins syndrome (TCS). Frontal views of the members of a single family illustrate the intra-familial variability characteristic of TCS. The mother (individual I-2) is very mildly affected, while her children (individuals II-1, II-2 and II-3) are all more severely affected. The father (I-1) is unaffected.

Molecular genetics of Treacher Collins syndrome

The combined facts that TCS exhibits a well-defined mode of inheritance and the majority of cases can be diagnosed clinically facilitated the identification of the gene mutated in this condition, designated *TCOF1*, using a positional cloning strategy (reviewed in Ref. 11). *TCOF1* was originally described as having an open reading frame of 4233 base pairs, encompassed by 25 exons encoding a predicted protein of 1411 amino acids with a theoretical molecular weight of 144 kDa (12, 13); however, So *et al.* (14) recently described two alternatively spliced exons, designated 6A and 16A, the inclusion of which would result in a protein of 1488 amino acids, with a calculated molecular mass of 152 kDa. Indeed, exon 6A–containing variants are the most common *TCOF1* species detected; however, only minor isoforms contain exon 16A (14).

Mutation analysis of *TCOF1* has resulted in the identification of over 120 mutations that are spread throughout the gene (7, 13, 15–23). The combined results of these studies have indicated that there does not appear to be a genotype/phenotype correlation in TCS and that the clinical variability observed in this condition does not depend upon the type or location of the underlying mutation, on the sex of the patient or on whether the case is sporadic or familial (17, 21). While the mutations detected to date include splicing mutations, insertions and non-sense mutations, by far the majority are deletions, which range in size from one to 40 nucleotides. The result of all these mutations is to introduce a premature termination codon into Treacle, which suggests that the mechanism underlying TCS is

haploinsufficiency. Although the majority of mutations tend to be family-specific, the recurrent mutation nt4135 del(GAAAA) [now renamed as c.4366_4370del-GAAAA, relative to the longer cDNA isoform (23)], accounts for approximately 17% of cases.

Remarkably, very few mis-sense mutations in *TCOF1* have been documented; those that have been identified, Y50C, W53C, W53R, reside in the amino terminus of the protein (16, 18, 19). This region of the protein is the most highly conserved between man and mouse with the region encoded by exons 1 and 2 displaying 92.6% identity, in comparison with 54.8% identity for the entire protein (24). In this regard, it is interesting to note that the consensus sequence of a putative nuclear export signal lies between amino acid positions 40 and 49 (Fig. 2A). Nevertheless, no functional data have been generated to suggest that the protein is exported from the nucleus.

Analysis of Treacle, the protein encoded by *TCOF1*

TCOF1 encodes the low complexity protein Treacle, in which serine, alanine, lysine, proline and glutamic acid account for over 60% of the amino acid residues (12, 13). Bioinformatics analyses indicated that Treacle contains three domains; unique amino and carboxy termini, and a characteristic central repeat domain (Fig. 2A) (12, 13). A single repeat consists of a cluster of acidic amino acid residues, containing numerous consensus sites for casein kinase II (CKII) phosphorylation, separated by basic amino acids comprising a majority of lysine, alanine and proline residues. Further analysis of Treacle identified a number of potential nuclear localization signals (NLSs), clustered in the lysine-rich carboxy terminus of the protein.

Database searches failed to reveal any strong homologies with other polypeptides suggesting that Treacle is not part of a protein family; however, weak similarities with other classes of nucleolar phosphoproteins such as *Xenopus* and rat nucleolar phosphoprotein 140 have been detected (25, 26), predominantly due to the low complexity repeat region present in these proteins. These results suggested that Treacle may also be a nucleolar phosphoprotein (12, 13). This hypothesis was verified by sub-cellular localization studies using green fluorescent protein (GFP) reporter assays and immunolabeling which demonstrated that Treacle localizes to the dense fibrillar component of the nucleolus (Fig. 2C,D) (27–30). The phosphorylation sites within the central repeat domain of Treacle have also been shown to be functional as the protein migrates at an abnormally high position (*c.* 220 kDa) on SDS-PAGE gels; phosphatase treatment results in a *c.* 40 kDa mobility shift reduction, suggesting a very high degree of phosphorylation (Fig. 2B) (29). It has also been determined that CKII and Treacle co-immunoprecipitate from HeLa cell lysates and that CKII is likely to be the kinase responsible for the post-translational modification of Treacle (29). This is consistent with the results of Jones *et al.* (31) who showed that avian

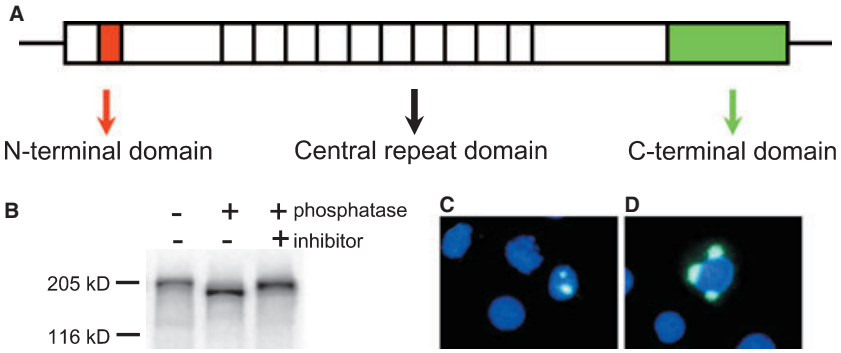

Fig. 2. Treacle is a nucleolar phosphoprotein. (A) An ideogram to illustrate the positions of the N-terminal domain which contains the putative nuclear export signal, the central repeat domain and the C-terminal domain, which contains numerous nuclear localization signals (NLSs). (B) The phosphorylation sites within the central repeat domain are functional as *in vitro* translated Treacle migrates at approximately 220 kDa on SDS-PAGE gels. Treatment with phosphatase (+) results in a *c.* 40 kDa mobility shift reduction, which is abrogated in the presence (+) of phosphatase inhibitor. (C, D) GFP sub-cellular localization studies confirm that, in the presence of the C-terminal NLSs, Treacle localizes to the nucleus (green staining in C); however, in the absence of these signals, Treacle fails to enter the nucleus (green staining in D). Blue staining, DAPI nuclear counterstain.

branchial arches contain a kinase activity that can phosphorylate Treacle peptides consistent with CKII site recognition.

Treacle functions in the ribosome biogenesis pathway

The demonstration that Treacle localizes to the dense fibrillar component of the nucleolus strongly implicated Treacle as playing a role in pre-ribosomal RNA processing. Subsequently, Valdez *et al.* (30) established that while Treacle co-localized with the pre-rRNA processing proteins Guα, C23 and B23 during interphase, the protein localized to punctate regions that overlap with the condensed rDNA genes during mitosis, suggesting that Treacle may co-localize with the RNA polymerase I complex (Fig. 3A). Further support for this hypothesis was generated by the demonstration that both Treacle and upstream binding factor (UBF; an RNA I transcription factor) associate with the rDNA transcriptional machinery on chromosomes 13, 14, 15, 21, and 22 (Fig. 3B) (30). The suggested interaction with UBF was substantiated by yeast-two hybrid analysis and immunoprecipitation assays in the presence of RNase and DNase, confirming a genuine protein–protein interaction rather than an RNA- or DNA-mediated association (30). A further role for Treacle in ribosome biogenesis was demonstrated by Hayano *et al.* (32) who identified Treacle as a component of human Nop56p-associated pre-ribosomal ribonucleoprotein (pre-rRNPs) complexes. Nop56p is a component of the box C/D small RNP complexes that direct 2′*O*-methylation of pre-rRNA during its maturation (33). As with the interaction between Treacle and UBF, that between Treacle and Nop56p was found to be a direct interaction independent of rRNA integrity (32, 34). These data suggest that the function of Treacle in pre-rRNA methylation is most likely mediated by its direct physical interaction with Nop56. Although Treacle co-localizes with UBF throughout mitosis, it co-localizes with Nop56 and fibrillarin, a putative methyl transferase, only during telophase when rDNA gene transcription and pre-rRNA methylation are known to commence (34).

These biochemical assays provided the first evidence that haploinsufficiency of Treacle not only affects the transcription of the ribosomal DNA genes, but also affects the production of properly modified mature ribosomal RNA. It is thought that these combined factors must significantly affect the proliferation and differentiation of certain cell types during development; indeed, it has been estimated that in a proliferating cell up to 95% of transcription and metabolism are dedicated to ribosomal biogenesis which implies a cell growth and proliferation function for Treacle (30). Significantly, down-regulation of Treacle expression using specific short interfering RNA inhibited ribosomal DNA transcription and cell growth, a similar correlation being observed in mouse embryos heterozygous for a loss-of-function mutation in Treacle which exhibit craniofacial defects and growth retardation (30).

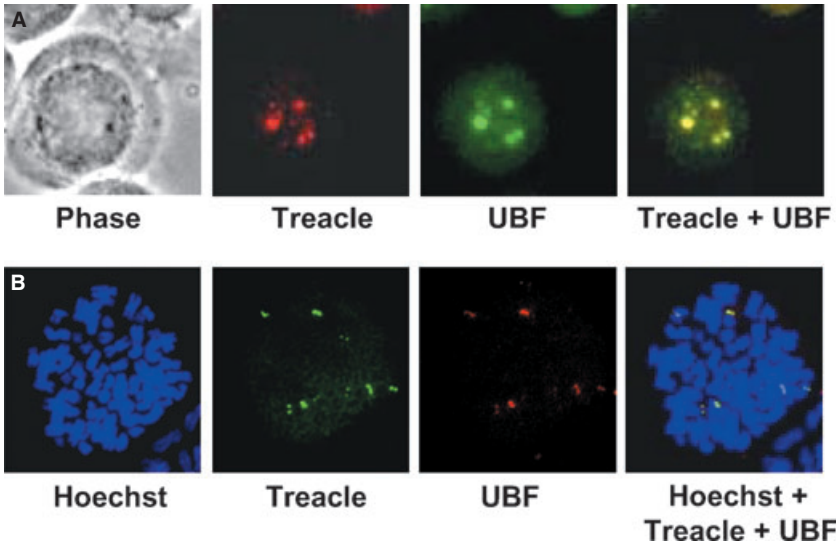

Fig. 3. Co-localization of Treacle and the RNA I transcription factor UBF. (A) HeLa cells were stained using antibodies directed against Treacle (red staining) and UBF (green staining) which demonstrated that the two proteins co-localize. (B) HeLa cells were blocked in mitosis using colchicine and chromosome spreads were stained for Treacle (green) and UBF (red). Both Treacle and UBF localize to the nucleolar organizer regions on chromosomes 13, 14, 15, 21 and 22. Blue staining, Hoechst nuclear counterstain.

The role of *TCOF1*/Treacle in craniofacial development

Recently, the integration of mouse genetics and experimental embryology has provided unique insights into the developmental basis of the disorder. Expression analyses in mouse embryos have indicated that the peak levels of *Tcof1* expression are detected in the neuroepithelium lining the neural folds immediately prior to fusion, and in the developing frontonasal process and branchial arches (24, 35). These observations suggested that high levels of Treacle are required in the developing craniofacial complex during times of critical morphogenetic events. To investigate the role of Treacle *in vivo*, our laboratory generated mice carrying a loss-of-function mutation in one allele of *Tcof1*. On a C57BL/6 × 129 genetic background, although *Tcof1*$^{+/-}$ mice exhibited a number of features reminiscent of TCS including abnormalities of the maxilla and mandible, additional abnormalities not observed in TCS patients, including severe developmental delay, anophthalmia and exencephaly, were found (Fig. 4) (36). The extreme nature of this phenotype resulted in premature neonatal death, thereby precluding breeding and expansion of the mutant mouse line. Subsequent studies therefore investigated the contribution of different genetic backgrounds to the phenotype of *Tcof1*$^{+/-}$ mice. The penetrance and severity of facial defects was found to be dependent upon the genetic background on which the mutation was placed with *Tcof1*$^{+/-}$ embryos exhibiting a highly reproducible, mouse strain-dependent phenotype (37). For example, when placed on CBA/Ca or C3H/HEN backgrounds, the *Tcof1* mutation resulted in death in the immediately postnatal period; however, on the DBA genetic background, *Tcof1* heterozygosity was compatible with postnatal life (37). These data indicated that modifying genes present in the different genetic backgrounds make a significant contribution to the phenotypic outcome of *Tcof1* heterozygous mice. Importantly, these studies enabled congenic lines that carried the *Tcof1* mutation to be generated (37).

The subsequent observation that backcrossing congenic DBA mice carrying the *Tcof1* mutation to C57BL/6 mice generated *Tcof1*$^{+/-}$ mice that phenocopy TCS was central to the delineation of the molecular pathogenesis of TCS (Fig. 4C,F). *Tcof1*$^{+/-}$ mice resulting from this breeding strategy were characterized by a reduction in the size of the head, which was shortened in the anterior to posterior direction. Skeletal analyses revealed that the cranial vault was domed and that the nasal and frontal bones were abnormal. The premaxilla, maxillary and palatine bones were also abnormal (Fig. 4F). Histological analysis confirmed that the palatal shelves had failed to fuse and indicated that the nasal passages were poorly formed, with no evidence of a nasal septum or conchae. These defects are reminiscent of the mid-facial abnormalities associated with severe cases of TCS in man (35).

To establish whether a neural crest cell migration defect was responsible for these abnormalities, as originally proposed by Poswillo (4), lineage tracing experiments were undertaken which indicated that segmental migration of cranial neural crest cells was unaffected in *Tcof1*$^{+/-}$ embryos. These results demonstrated that abnormal neural crest cell migration is not the cause of the craniofacial abnormalities characteristic of TCS. Nevertheless, fewer neural crest cells appeared to migrate from the neural folds into the developing craniofacial complex in *Tcof1*$^{+/-}$ embryos compared with wild-type littermates. To quantify the degree of neural crest cell reduction, flow cytometric

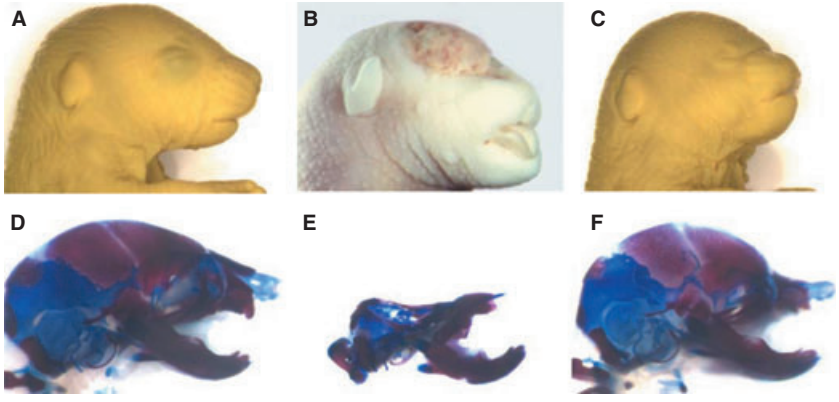

Fig. 4. Genetic background influences the phenotype of *Tcof1*$^{+/-}$ mice. (A–C) Gross morphology, (D–F) whole mount alcian blue (cartilage) and alizarin red (bone) stained skull preparations. (A, D) Wild-type mice. (B, E) On a mixed 129 × C57BL/6 background, *Tcof1*$^{+/-}$ mice exhibit severe craniofacial skeletal anomalies, anophthalmia and exencephaly. (C, F) In contrast, on a mixed DBA × C57BL/6 background, *Tcof1*$^{+/-}$ mice display abnormalities of the premaxilla, maxillary, palatine, nasal and frontal bones reminiscent of the clinical features observed in Treacher Collins syndrome.

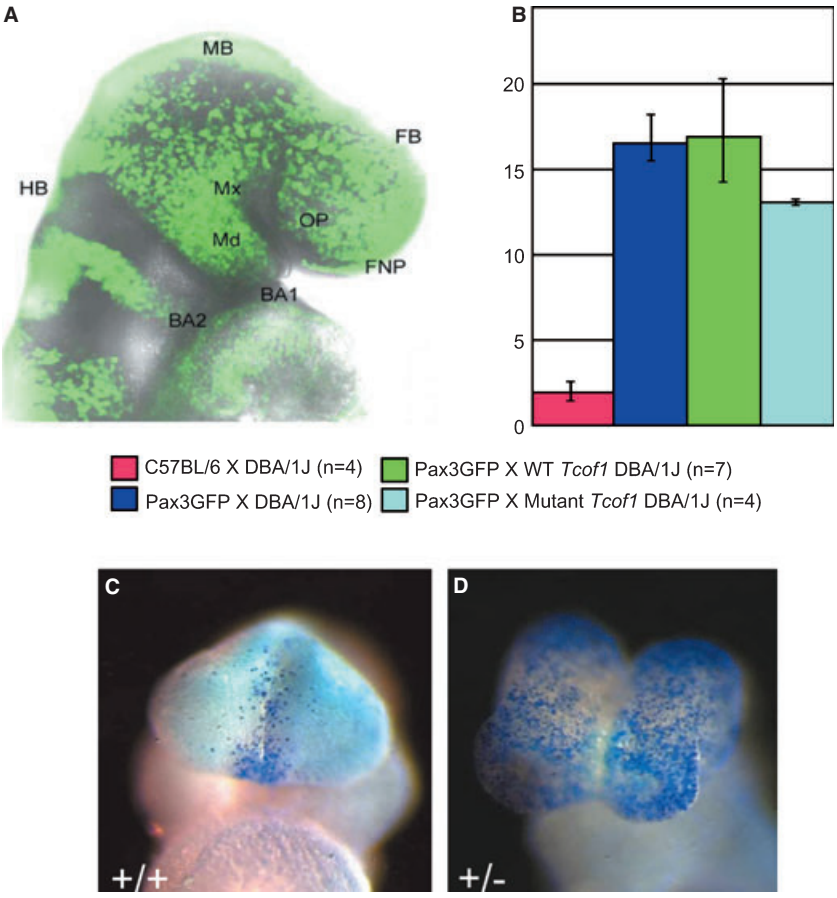

Fig. 5. Neural crest cell reduction in *Tcof1*$^{+/-}$ mice. (A) Green fluorescent protein-labelled neural crest cells (green) migrate into the developing craniofacial complex. HB, hindbrain; MB, midbrain; FB, Forebrain; FNP, frontonasal process; OP, optic placode; Mx, maxilla; Md, mandible; BA1, first branchial arch; BA2, second branchial arch. (B) Quantification of neural crest cells using flow cytometry reveals that there are *c.* 22% fewer migrating cells in *Tcof1*$^{+/-}$ mutant (light blue bar) compared with wild-type littermate (green bar) and positive control (dark blue bar) embryos. A negative control to show the background fluorescence (red bar) is also shown. (C, D) High levels of neuroepithelial cell death (blue staining) in *Tcof1*$^{+/-}$ mutant embryos (D) compared with wild-type littermate controls (C), reduce the number of neural crest cell precursors and underlie the diminished number of migrating neural crest cells.

analyses of GFP-labelled neural crest cells were performed. These experiments demonstrated that there were approximately 25% fewer migrating cranial neural crest cells in *Tcof1*$^{+/-}$ embryos compared with wild-type littermates (Fig. 5A,B) (35).

Further studies using whole-mount TUNEL analysis, a technique that identifies cells undergoing apoptosis, revealed that the number of apoptotic cells was markedly elevated in the neuroepithelium of the cranial neural folds and the neural tube in E8 and E9 *Tcof1*$^{+/-}$ embryos (Fig. 5C,D) (35, 36). The high levels of neuroepithelial cell death resulted in a depleted premigratory neural crest stem cell population. In addition, BrdU incorporation assays demonstrated a significant reduction in the numbers of proliferating cells in the neuroepithelium and neural crest-derived cranial mesenchyme in *Tcof1*$^{+/-}$ embryos. These data led to the hypothesis that Treacle may regulate cell proliferation through its role in ribosome biogenesis. To assay for ribosomal integrity in *Tcof1*$^{+/-}$ embryos, Dixon and coworkers performed immunolabeling using an antibody which specifically recognized the 28S subunit of RNA and observed significantly reduced immunoreactivity in the neuroepithelium and neural crest-derived craniofacial mesenchyme of E8.75-E9 *Tcof1*$^{+/-}$ embryos compared to wild-type littermates (35). These combined experiments demonstrated that Treacle is essential for the formation and proliferation of neural crest cells through the regulation of ribosome biogenesis and the generation of mature ribosomes. In *Tcof1*$^{+/-}$ embryos, although neural crest cell induction appears to occur normally, it does so from a depleted precursor cell pool resulting in a diminished number of migrating cranial neural crest cells. The reduction in neural crest cell number is compounded further by reduced proliferative capacity. Currently, it therefore appears as though this is the developmental mechanism that underlies the clinical features observed in TCS (35).

Clinical utility and implications

Although most cases of TCS can be diagnosed clinically, the variable expression observed in this condition,

together with the high rate of new mutations may present the clinician with diagnostic and genetic counselling difficulties. Moreover, prior to the identification of *TCOF1*, prenatal diagnosis was only possible using foetoscopy or ultrasound imaging (38, 39). While the quality of ultrasound imaging has improved markedly in recent years, allowing non-invasive prenatal diagnosis to be performed, it may still be difficult to make a positive diagnosis, particularly where the foetus is mildly affected. Given these circumstances the procedure is usually not diagnostic for apparently unaffected foetuses. Moreover, prenatal diagnosis using either foetoscopy or ultrasound imaging is not possible until the second trimester of pregnancy whereas molecular diagnosis can be undertaken in the first trimester of pregnancy. Recently, molecular analysis has been used to facilitate both pre- and postnatal diagnoses in families with a history of TCS (40). In a subset of these families, TCS was diagnosed in the proband, but the status of the parents could not be established unequivocally on the basis of clinical examination alone. In these families, molecular analysis determined that one parent was also affected, with a 50% recurrence risk in future pregnancies. Several additional families were referred for molecular verification that TCS had arisen as the result of a *de novo* mutation in *TCOF1*; in each case, this was shown to be the case and a low recurrence risk for the families confirmed. Dixon *et al.* (40) also reported the results of four prenatal molecular diagnoses. Happily, in each case the foetus was predicted (correctly) to be unaffected and the parents were reassured accordingly. Nevertheless, molecular diagnosis for TCS is complicated by the fact that, in general, mutations between families are different. Consequently, the specific mutation must first be identified within a family before diagnosis and counselling can be performed. As this may require analysis of multiple exons of *TCOF1*, this can be a time-consuming process. In addition, where prenatal molecular testing is performed and a positive diagnosis made, no conclusions can be drawn about the severity with which the child will be affected. Therefore, parents may opt to delay their decision-making until ultrasound can be used to provide further information on the extent of the abnormalities. However, for those cases in which the foetus does not carry the mutation, parents can be reassured that their child will be unaffected.

In summary, TCS results from loss-of-function mutations in *TCOF1*. Treacle, the protein encoded by *TCOF1*, is a nucleolar phosphoprotein that plays a central role in the regulation of ribosome biogenesis. In mice, haploinsufficiency of *Tcof1* results in a depletion of neural crest cell precursors through neuroepithelial apoptosis, which results in a reduced number of neural crest cells migrating into the developing craniofacial complex. These combined advances have already impacted on clinical practice and provide invaluable resources for the continued dissection of the developmental basis of TCS and for understanding of the clinical variability observed in this disorder.

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