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Investigação da etiologia de malformações craniofaciais com uso de células derivadas de crista neural

Investigating craniofacial malformations with the use of neural crest-derived cell models

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ABSTRACT

Craniofacial malformations (CFMs) comprise a large and heterogeneous group of disorders in which tissues of the skull and face are affected. Affected subjects suffer from significant functional impairment and morbidity, and understanding the aetiology of these disorders is of great importance, as it may lead to the development or improvement of preventive and therapeutic strategies in the future. CFMs are largely considered to arise from developmental disturbances in the cranial neural crest and its cranioskeletal and cartilaginous mesenchymal derivatives. Neural crest-derived cell models have the potential to provide invaluable insight into the pathogenesis of CFMs, as functional studies can be to assess phenotypes in disease-relevant cell lineages. In this work, we applied this strategy to investigate three craniofacial disorders: non-syndromic cleft lip/palate (NSCL/P), Richieri-Costa-Pereira syndrome (RCPS), and Treacher Collins syndrome (TCS). NSCL/P was investigated through transcriptomic and functional assays on stem cells from human exfoliated deciduous teeth, which are neural crest-derived, adult mesenchymal cells. We identified a NSCL/P-specific dysregulated transcriptional signature involving a gene network responsible for DNA double-strand break repair that results in accumulation of DNA damage in patients' cells. These findings revealed a novel pathogenetic mechanism for NSCL/P and support previous observations pointing towards an aetiological overlap between this disease and cancer. RCPS and TCS were investigated with the use of a novel approach to generate neural crest cells from patient-specific induced pluripotent stem cells (iPSCs) as a means to recapitulate craniofacial development. We demonstrated that RCPS and TCS somatic cells can be successfully used to generate iPSCs and iPSC-derived neural crest cells and their mesenchymal derivatives. Phenotype screening showed that RCPS neural crest-derived mesenchymal cells display dysregulation of osteogenic differentiation, which was supported by confirmatory knockdown assays. Further, we report elevated apoptosis in TCS neural crest-derived mesenchymal cells, which was allied to alterations in chondrogenic and osteogenic differentiation. These results will aid in clarifying the pathogenic mechanism determining RCPS and TCS, revealing that neural crest mesenchymal cells are altered in these syndromes. In conclusion, we attested the applicability of NC-derived cell types to provide clues regarding the pathogenetic mechanisms leading to CFMs, and these novel findings will aid in dissecting the aetiology of CFMs by providing grounds to direct future efforts in craniofacial research.

RESUMO

As malformações craniofaciais (MCFs) compreendem uma vasta e heterogênea gama de doenças que envolvem o acometimento de tecidos do crânio e da face, sendo que indivíduos afetados enfrentam morbidade e deficiências funcionais relevantes. O entendimento da etiologia das MCFs é de extrema importância, pois poderá levar ao desenvolvimento ou melhoria de estratégias preventivas e terapêuticas. As MCFs são oriundas principalmente de distúrbios no desenvolvimento da crista neural cranial e seus derivados mesenquimais. Neste contexto, modelos baseados em células derivadas de crista neural são de grande potencial para o entendimento das MCFs, já que estudos funcionais podem ser realizados nestas células para averiguar fenótipos diretamente relacionados às doenças. Neste trabalho, nós empregamos esta estratégia na investigação de três tipos de MCFs: fissuras labiopalatinas não sindrômicas (FLP NS), síndrome de Richieri-Costa-Pereira (SRCP) e síndrome de Treacher Collins (STC). As FLP NS foram investigadas por meio de ensaios transcriptômicos e funcionais em células-tronco de polpa de dente decíduo, que são células mesenquimais adultas derivadas de crista neural cranial. Identificamos uma assinatura de expressão gênica específica às FLP NS, com desregulação de uma rede gênica responsável pelo reparo de quebras duplas no DNA, resultando no acúmulo deste tipo de lesão em células de indivíduos afetados pela doença. Estes achados revelam um novo mecanismo patogênico para as FLP NS e corroboram observações prévias que sugeriam sobreposição de etiologias entre esta doença e o câncer. A SRCP e a STC foram investigadas com o uso de uma nova metodologia para a geração de células de crista neural a partir de células-tronco pluripotentes induzidas (*induced pluripotent stem cells;* iPSCs) para recapitular o desenvolvimento craniofacial. Realizamos triagem de fenótipos celulares e identificamos desregulação de diferenciação osteogênica em células mesenquimais derivadas de crista neural de pacientes com SRCP, o que foi corroborado por ensaios de RNA de interferência. Além disso, mostramos que células mesenquimais de crista neural de pacientes com STC apresentam apoptose elevada aliada a alterações durante diferenciação osteogênica e condrogênica. Estes resultados revelam que células mesenquimais de crista neural estão alteradas na SRCP e STC, colaborando para o esclarecimento dos mecanismos patogênicos responsáveis por estas síndromes. Assim sendo, nós evidenciamos a aplicabilidade da modelagem de MCFs por meio de células oriundas da crista neural, e esses achados inéditos contribuirão para um melhor entendimento da etiologia das MCFs, e servem de base para futuras estratégias de pesquisa na área de doenças craniofaciais.

INTRODUCTION AND OBJECTIVES

1. Overview: craniofacial malformations

Craniofacial malformations (CFMs) comprise a large and heterogeneous group of disorders in which tissues of the skull and face are affected. These disorders are largely considered to arise from disturbances in craniofacial morphogenesis during embryonic development, and they collectively represent over one-third of all congenital birth defects, or 0.3% of all stillbirths. CFMs can affect different craniofacial components with or without association with other clinical manifestations, including intellectual disability, limb defects, and others. Therefore, CFMs can be part of a clinically distinguishable syndrome, or they may emerge isolated, as is often the case for cleft lip and palate (Gorlin et al., 2001).

The development of craniofacial skeletal and connective tissues is particularly affected in CFMs. Craniosynostoses, for example, are determined by premature ossification of cranial sutures, resulting in abnormal skull shape and facial features, cranial growth restriction and increased intracranial pressure (Gorlin, 2001; Governale, 2015). Other craniofacial components are also compromised in CFMs, such as in facial dysostoses, which may involve hypoplasia of maxillary, mandibular, and zygomatic bones, in addition to ear anomalies (Trainor & Andrews, 2013). In the orofacial region, embryonically adjacent structures may fail to fuse, resulting in orofacial clefts that frequently consist of a gap affecting bone, muscle, and other tissues (De Mey et al., 1989; Wijayaweera et al., 2000; Farronato et al., 2014). By affecting a large variety of craniofacial tissues, CFMs ensue significant functional impairment and morbidity to patients, which often suffer from airway obstruction, feeding difficulties, and other problems (Trainor & Andrews, 2013).

CFMs entail significant burden to patients and health care systems. Since not only facial appearance is changed but also craniofacial tissues can be functionally affected, patients with CFMs face important psychological outcomes and require extensive treatment throughout several years of life. Functional rehabilitation and social integration of patients frequently involves diverse health professionals, such as otorhinolaryngologists, psychologists, surgeons, dentists, among others (Hamm & Robin, 2015). Considering these facts and the high incidence of CFMs, understanding the aetiology of these disorders is of great importance, as it

may lead to the development or improvement of preventive and therapeutic strategies in the future.

2. Development of the face and head

2.1 The neural crest

Craniofacial development is a spatio-temporally coordinated multi-stage process that begins with the emergence of the cranial neural crest (CNC) cells. Neural crest cells are transient cell populations that originate along the neuraxis, at the interface between the neural plate and non-neural ectoderm in the dorsal side of the embryo, around the time of neurulation (The ectodermal cells in this region, in response to molecular cues majorly determined by WNT, FGF and BMP signalling, undergo transdifferentiation through epithelial-mesenchymal transition (EMT) and migrate to colonise several sites within the embryonic body (Gong, 2014). In the foremost portion of the neuraxis, CNC cells migrate from the forebrain, the midbrain and hindbrain regions to populate the mesenchyme of the developing craniofacial complex. CNC cells emigrating from the forebrain and rostral midbrain colonise the frontonasal region, while those arising from the caudal midbrain populate the first pharyngeal arch; CNC cells from the hindbrain region emigrating from rhombomeres 1-6 populate the first, second, and third pharyngeal arches (Gong, 2014; Trainor, 2014; Fig. 1A). After migration, the CNC cell-derived mesenchyme within these structures undergoes intense proliferation to give rise to the facial prominences. Ultimately, differentiation of CNC-derived mesenchymal cells contributes to a range of craniofacial structures and cells, such as cartilage, pericytes, teeth, and most of the cranioskeleton (Szabo-Rogers et al., 2010; Twigg & Wilkie, 2015; Choe et al., 2014; Moody, 2015; Fig. 1A).

2.2 Craniofacial CNC cell derivatives

The facial prominences are subject to finely orchestrated morphogenetic events in order to make up the human face. The first pharyngeal arch originates the maxillary and mandibular prominences, which arise bilaterally and will constitute the upper and lower jaw tissues of the embryo. The maxillary prominences grow latero-medially and fuse with the frontonasal prominence-derived medial and lateral nasal prominences, constituting the upper lip region, maxilla, and primary palate (Fig. 1B). Likewise, inside the developing oral cavity,

bilaterally arranged extensions of the maxillary prominences (called palatine shelves) must also undergo re-orientation, growth and fusion to originate the secondary palate (Fig. 1B'). The fusion events taking place during orofacial development are reliant upon EMT and apoptosis of the epithelial cells lining the facial prominences to establish a seamless and continuum mesenchyme. CNC-derived mesenchymal cells within the maxillary prominences differentiate to create the zygomatic, maxillary, and palatine bones; the mandibular prominences, also derived from the first pharyngeal arch, develop into the lower jaw portion of the head, constituting the mandible, lower lip, and parts of the tongue (Fig. 1A, B). Akin to the maxillary prominences, the mandibular prominences must grow latero-medially and fuse through EMT; however, this process occurs prior to fusion of all the other facial prominences. During mandible development, CNC-derived mesenchymal cells differentiate into the Meckel's cartilage, a transient structure that supports the lower jaw. As the mandibular prominences fuse, the distal ends of the Meckel's cartilage also fuse, forming the mandibular symphysis, which undergoes ossification only after birth. As CNC-derived mesenchymal cells undergo osteogenic differentiation, the Meckel's cartilage is replaced by bone tissue to originate the mandible. Finally, CNC-derived cells within the remaining pharyngeal arches contribute to internal ear elements and the hyoid bone (Gong, 2014; Moody, 2015)

The series of morphogenetic events leading to the full assembly of the craniofacial structures is particularly sensitive to perturbations. Hence, insults to migration, growth, differentiation and fusion of cells or tissues that compose the facial prominences generate a variety of defects, in accordance with the high incidence of CFMs. Remarkably, the majority of cellular constituents of tissues taking part in these processes are of neural crest origin, further evidencing the pivotal role of these cells in the aetiology of CFMs.

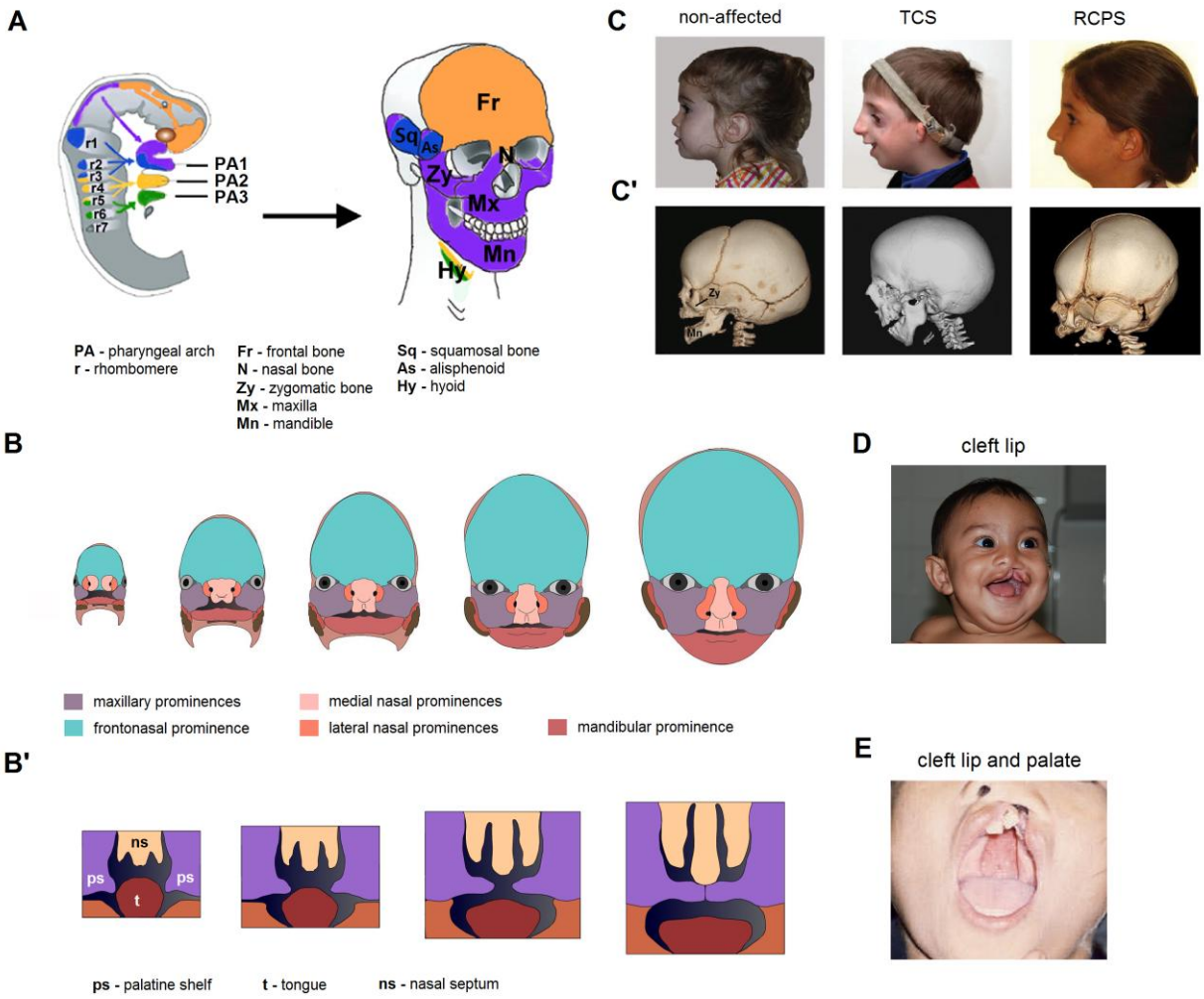

Figure 1: Craniofacial development and congenital malformations; **A)** Cranial neural crest migration streams and their skeletal fates; **B and B')** Orofacial development (left to right), showing **(B)** frontal view of the developing facial prominences during assembly of the maxillary (including upper lip) region, and **(B')** sagittal section of the developing secondary palate inside the oral cavity; **C and C')** Examples of craniofacial malformations and their respective cranioskeletal outcomes; subjects are shown in gross lateral view and lateral 3D computed tomography scans; **C)** Subjects affected by Treacher Collins syndrome (TCS) and Richieri-Costa-Pereira syndrome; **C')** Cranial CT scans; note zygomatic and mandibular bone hypoplasia (TCS), and mandibular agenesis (RCPS). The RCPS subjects in the CT scan and in the photograph are not the same; **D)** Subject with unilateral cleft lip; **E)** Subject with unilateral cleft lip and cleft palate. Modified from Gong, 2014; Clouthier et al., 2013; Raskin et al., 2013; Lehalle et al., 2015; Dixon et al., 2011; http://www.indiana.edu/~anat550/hnanim/face/face.html.

3. Aetiology of CFMs

As stated above, the underlying causes of congenital CFMs can be traced back to disturbances during embryonic craniofacial development. These disturbances are thought to be exerted by genetic or environmental factors, acting separately in different cases, or in conjunction. Examples of environmental causes include maternal alcohol intake, smoking and nutritional deficiencies, which have been associated with several congenital malformations, including foetal alcohol syndrome and cleft lip/palate (Krapels et al., 2006; Smith et al., 2014;

Sabbagh et al., 2015). Amongst genetic factors, chromosomal alterations and monogenic variants are usually implicated in craniofacial syndromes or isolated CFMs segregating in a Mendelian fashion, whilst the interplay between environmental factors and susceptibility variants in different *loci* has been associated with multifactorial CFMs, such as non-syndromic cleft lip/palate (Brito et al., 2012). Considering the myriad of disorders affecting craniofacial tissues, only three types of CFMs have been selected to illustrate different aetiological mechanisms behind CFMs; these disorders are further explored in the core chapters of this work, and are briefly described below:

3.1 Facial dysostoses: Richieri-Costa-Pereira syndrome and Treacher Collins syndrome

Facial dysostoses are a heterogeneous group of CFMs in which tissues derived from the first and second pharyngeal arches are compromised. They can be classified into mandibulofacial dysostoses, presenting with normal extremities, and acrofacial dysostoses, when limb anomalies manifest in addition to CFMs (Wieczorek, 2013). Both groups of disorders are thought to originate from disturbances in development of CNC cells or CNC cell-derived tissues.

Richieri-Costa-Pereira syndrome (RCPS) is a rare acrofacial dysostosis characterised by midline mandibular cleft, mandibular hypoplasia, Robin sequence (micrognathia, glossoptosis, and cleft palate), radial and tibial defects, among other clinical findings (Favaro et al., 2011; Fig. 1C). So far, RCPS has only been diagnosed in Brazilian families and one French subject. It is caused by repeat expansions in the 5'UTR of the gene *EIF4A3*, leading to loss of function of the encoded protein eIF4AIII (Favaro et al., 2014). This protein plays important roles in RNA metabolism, including nonsense mRNA decay, splicing and translation (Chan et al., 2004; Singh et al., 2013; Wang et al., 2014). Currently, it is unclear how eIF4AII loss of function affects CNC cell development, resulting in the clinical manifestations of RCPS.

The mandibulofacial dysostosis Treacher Collins syndrome (TCS) is another rare condition, present in 1:50,000 live births. The main clinical findings in TCS include underdevelopment of facial bones, particularly the zygomatic bones and the mandible, external ear anomalies and conductive hearing loss (Gorlin, 2001; Fig. 1C). Mutations in *TCOF1* are responsible for the majority of TCS cases (Splendore et al., 2000) and lead to haploinsufficiency of the protein treacle, which is known to participate in ribosome biogenesis (Gonzales et al., 2005). Studies in animal models have shown that insufficient ribosome

production leads to proliferative stress and apoptosis of pre-migratory CNC cells at the neuroepithelial region of the embryo, reducing the amount of CNC cells migrating into the pharyngeal arches (Dixon et al., 2006; Weiner et al., 2012); such deficit of CNC-derived mesenchymal cells is thought to result in hypoplasia of the facial structures affected in the syndrome. However, it remains to be determined if this is the sole cause of TCS, as the functional consequences of treacle haploinsufficiency in post-migratory CNC cells have not been thoroughly investigated.

3.2 Non-syndromic cleft lip/palate

Non-syndromic cleft lip/palate (NSCL/P) is a multifactorial CFM in which clefts of the upper lip accompanied or not by cleft palate are the sole clinical finding in affected subjects (Fig. 1D, E). It is a prevalent congenital disorder, with incidences ranging between 3.4 and 22.9 per 10,000 births world-wide, depending upon factors such as ethnic background, geographical location, and socio-economic status (Mossey et al., 2009). Development, growth and fusion of the facial prominences and palatine shelves are reliant upon cell migration, proliferation, transdifferentiation and apoptosis; therefore, susceptibility factors acting on these processes are thought to be the underlying causes of NSCL/P (Jiang et al., 2006; Yu et al., 2009; Greene & Pisano, 2010). Although several susceptibility *loci* (e.g. 8q24, 10q25, *IRF6*; Brito et al., 2012a; Brito et al., 2012b; Ludwig et al., 2012) have been identified mainly through genetic association studies, the functional effects exerted on craniofacial development by the majority of them and how they interact with environmental factors remain poorly understood. Further, these variants do not explain the high heritability estimated for NSCL/P (45-85%) for several populations, including the Brazilian (Brito et al., 2011).

RCPS, TCS and NSCL/P are examples of how disruption of basic cellular mechanisms imparts such drastic outcomes in craniofacial development. The mechanisms through which genetic and/or environmental insults affect such important developmental pathways have yet to be completely dissected for most CFMs. Although genetic variants and environmental factors possibly involved in CFMs can be identified through various approaches (e.g. association studies, gene mapping, and next-generation sequencing), their biological effects must still be demonstrated through functional studies. In this regard, cellular and animal models are of great use to perform these analyses, having so far provided important contributions to the understanding of craniofacial development and the pathogenesis of a number of CFMs.

4. Investigating the pathogenesis of CFMs with the use of in vivo and in vitro models

4.1 Animal models

Animal models have greatly contributed to our understanding of normal and abnormal craniofacial development. By targeting specific genes involved in CFMs, it is possible to examine the outcomes in craniofacial morphogenesis of developing embryos. This approach includes the use of dominant-negative constructs, RNA interference and anti-sense morpholinos particularly in *Xenopus*, zebrafish, and chick embryos (Barriga et al., 2015). Since mice are presumptively more likely to reflect mammalian development, they are considered to be more appropriate to model human disorders, and gene targeting/loss-of-function approaches in this organism have also led to important advances in understanding human CFMs such as TCS, 22q11.2 deletion syndrome, an craniosynostoses (Dixon et al., 2006; Jones et al., 2008; Holmes, 2012; Trainor and Andrews, 2013; He and Soriano, 2013; Fantauzzo and Soriano, 2014; Meechan et al., 2015). Recent technical advances in genome editing have enabled the production of improved knockout animals or animals harbouring specific mutations, opening new possibilities for disease modelling (Seruggia & Montoliu, 2014).

Despite being thoroughly explored as a mammalian model for craniofacial development, the mouse embryo may not completely represent embryonic development across species. Knockout mice for important genes involved in neural crest development often do not show results comparable to other model organisms, sometimes turning out without any detectable phenotype. This discrepancy has been attributed to technical limitations in generating mutant mouse lines or species-specific differences within the neural crest gene regulatory network (Thyagarajan et al., 2003; Barriga et al., 2015). Despite these limitations, the mouse model still stands as an important tool to investigate human craniofacial phenotypes, and, in conjunction with non-mammalian models and human cell culture systems, it holds great potential for discovery.

4.2 Cellular models

Cell culture systems offer a unique opportunity to study cellular phenotypes and pathways involved in human disease. Given that a genetic defect or disorder does not necessarily produce symptoms in animal models, cell cultures from human tissues are the most appropriate complement to *in vivo* studies. Furthermore, the phenotype of patients' cells

carrying disease-specific genomic elements can be examined under culture conditions that can be controlled and experimentally manipulated *in vitro* (Unternaehrer & Daley, 2011; Srijaya et al., 2012; Sterneckert et al., 2014). However, access to disease-relevant primary cells is hindered for conditions in which embryonic tissues are primarily affected, such as in craniofacial and neurodevelopmental disorders. Circumventing this drawback, stem cells have emerged as a promising instrument with which to conduct functional studies.

4.2.1 Stem cells from human exfoliated deciduous teeth

Stem cells from human exfoliated deciduous teeth (SHED) are an easily accessible, multipotent stem cell population (Miura et al., 2003). These cells possess osteogenic, chondrogenic, adipogenic, myogenic and dentinogenic capacity, which can be studied *in vitro* if these differentiation properties are of interest to the disease under investigation. More importantly, SHED comprise CNC-derived mesenchymal cells, and therefore they share the same origin with the mesenchymal cells within the embryonic craniofacial structures affected in many CFMs (Cordero et al., 2011; Janebodin et al., 2011; Komada et al., 2012).

SHED have been successfully employed to identify altered cellular pathways involved in developmental disorders, including NSCL/P (Bueno et al., 2011; Kobayashi et al., 2013; Grisei-Oliveira et al., 2013; Suzuki et al., 2014). In 2011, our group performed transcriptome profiling of SHED from NSCL/P patients, identifying dysregulation of co-expressed gene networks involved in DNA damage response and repair. These networks displayed aberrant expression of key genes involved in these cellular processes, including *BRCA1*, *MSH2*, and *RAD51*, which were predicted to be regulated by transcription factor E2F1 (Kobayashi, 2011). These dysregulation patterns are in accordance with the proposed aetiological overlap between cleft lip/palate and several types of cancer, which frequently co-occur in patients and families (Zhu et al., 2002; Bille et al., 2005; Taioli et al., 2010; Vieira et al., 2012); also, these findings raised the possibility that susceptibility to DNA damage in addition to environmental insults during embryonic development could lead to alterations in lip and palate development. If that hypothesis is true, DNA repair gene networks must be important for proper craniofacial development, and tissues from NSCL/P subjects should display increased DNA damage when exposed to genotoxic factors, in comparison to controls.

4.2.2 Induced pluripotent stem cell-derived neural crest cells

Induced pluripotent stem cells (iPSCs) are an invaluable asset to study human disorders. iPSCs can be generated from somatic cells by forced expression of pluripotency-associated factors (Takahashi et al., 2007), and differentiated into a multitude of cell types. With this technology, it is possible to isolate patients' cells, induce pluripotency, and generate the specific cell type afflicted with the disease.

This possibility has recently embraced craniofacial diseases, as directed generation of neural crest cells (NCCs) from iPSCs is now attainable. iPSC-derived NCCs express several neural crest markers and are able to migrate under appropriate cues *in vitro* and *in vivo* (Kreitzer et al., 2013; Menendez et al., 2013). Moreover, akin to their embryonic counterparts, they are able to generate multipotent mesenchymal progenitors that can be differentiated into craniofacial cell types affected in CFMs, thus expanding possibilities for investigating disease-related phenotypes (Menendez et al., 2013; Fukuta et al., 2014, Matsumoto et al., 2015). Albeit methods for differentiation of NCCs from pluripotent cells have existed since 2007 (Lee et al., 2007), they required laborious procedures involving embryoid body and neural rosette cultures, often resulting in only a small proportion of NCCs (Menendez et al., 2011; Trainor, 2014). Recent approaches for differentiation of NCCs from iPSCs rely on WNT pathway activation and TGF-β pathway blockade with the use of small molecules, producing more homogeneous NCC populations (Menendez et al., 2011; Menendez et al., 2013; Fukuta et al., 2014). Given the novelty of this approach, so far only mesenchymal, non-craniofacial phenotypes of fibrodysplasia ossificans progressiva have been modelled with these cells (Matsumoto et al., 2015; Hino et al., 2015), and reports exploring the properties of iPSC-derived NCCs to unravel the pathogenesis of CFMs are still scarce.

5. Objectives

In face of the necessity to better understand the pathogenesis of CFMs, in this work we proposed functional studies to investigate three selected disorders: NSCL/P, RCPS, and TCS. These studies were conducted in neural crest-derived cell cultures from affected patients, as a means to assess disease-specific phenotypes in a cell type presumptively affected in these disorders. The main objectives of this thesis were as follows:

a) To perform functional studies in SHED from NSCL/P patients in order to evaluate if the dysregulated patterns of expression previously observed (Kobayashi, 2011) result in observable cellular phenotypes and ;

b) To produce patient-specific RCPS and TCS iPSCs and differentiate them into NCCs through standardisation of current differentiation methodology;

c) To identify a possible pathogenetic mechanism responsible for RCPS by screening for altered cellular phenotypes in iPSC-derived NCCs from affected subjects;

d) To evaluate if Treacle haploinsufficiency in post-migratory CNC derivatives could play a role in the aetiology of TCS, through investigation of cellular properties of NCC-derived mesenchymal cells.

DISCUSSION AND CONCLUSIONS

This work consisted in the functional evaluation of neural crest (NC)-derived cell models in order to shed light on the pathogenesis of three congenital craniofacial malformations (CFMs): non-syndromic cleft lip/palate (NSCL/P), Richieri-Costa-Pereira syndrome (RCPS), and Treacher Collins syndrome (TCS). First and foremost, our results demonstrate the applicability of patient-derived cell cultures in investigating the pathogenesis of craniofacial phenotypes. Moreover, they show the importance of examining disease-relevant cell types, in this case, post-natal stem cells from exfoliated deciduous teeth (SHED), which are derived from the cranial NC, and iPSC-derived NC cells (iNCCs) and their mesenchymal derivatives.

In Chapter II, we confirmed that NSCL/P SHED have an expression signature and that gene networks governing cellular defences against DNA damage may play a role in the aetiology of NSCL/P. SHED cultures from NSCL/P subjects exhibited transcriptional dysregulation of gene networks responsible for DNA double-strand-break (DSB) repair and cell cycle control, a phenomenon that, if biologically relevant, should be coupled with observable cellular phenotypes (Kobayashi, 2011). Indeed, we showed that, upon exposure to H_2O_2, NSCL/P SHED accumulate DSBs and display increased cell death, in comparison to controls; furthermore, we detected co-expression of key genes involved in DSB repair (*Brca1*, *Rad51* and *E2f1)* in similar domains within the facial prominences of murine embryos, in critical stages of lip and palate morphogenesis. These findings thus confirm a functional effect for the observed NSCL/P-specific transcriptional profile, and importantly, they suggest that, during craniofacial development, the action of environmental insults allied to genetic or epigenetic factors upstream of the observed transcriptional dysregulation could be involved in the aetiology of NSCL/P. Finally, our results are in accordance with the proposed aetiological overlap between NSCL/P and cancer, as a number of dysregulated genes detected here have also been implicated in tumourigenesis (e.g. *CDC6*, *BLM*, *BRCA1*, *RAD51*, *MSH2*; Borlado & Mendes, 2008; Calin et al., 1998; Gao et al., 2011; Seifert & Reichrath, 2006; Rosen et al., 2006).

Bearing in mind the morphogenetic events necessary for proper craniofacial development, the relationship between a CFM such as NSCL/P and disrupted cellular DNA repair

mechanisms/cancer is not unexpected. As intense NC-derived mesenchymal cell proliferation is required for growth and differentiation of the facial prominences (Twigg & Wilkie, 2015), and therefore increased DNA damage and cell death are likely to negatively affect this process. Such assumption is supported by the existence of craniofacial alterations associated with a number of disorders specifically caused by mutations in DNA repair genes, such as Nijmegen breakage syndrome, *ERCC1*-associated cerebro-oculo-facio-skeletal syndrome, and Cornelia de Lange syndrome-4 and TCS (Berardinelli et al., 2007; Jaspers et al., 2007; Deardoff et al., 2012; Ciccia et al., 2014). Moreover, recent studies have reported association between genetic variants within DNA repair genes and NSCL/P, namely *BLM*, *E2F1*, *BRIP1*, and *RAD51* (Mostowska et al., 2014; Machado et al., 2015), further supporting our findings and strengthening the relationship between NSCL/P and impairment of cellular defences against DNA damage.

Chapters III and IV comprehend the employment of iNCCs derived from iPSCs to investigate the pathogenesis of RCPS and TCS, two facial dysostoses in which disturbances in cranial NC cell development are thought to have an aetiological role. The successful derivation of iPSC lines and iNCCs from RCPS and TCS patients displaying reduced amounts of *EIF4A3* and *TCOF1* transcripts, respectively, shows that somatic cell reprogramming to a pluripotent state and neural crest induction are not constrained by expression of these genes, at least in the context of patient's cells or under the method reported herein. Importantly, the adaptation of a methodology to easily produce iPSC-derived iNCCs (Menendez et al., 2013; Fukuta et al., 2014) to our cell culture conditions enables the study of other neural crest-related diseases for which biological material is available in our centre.

In Chapter III, the assessment of iNCC-derived mesenchymal stem-like cells (nMSCs) revealed augmented osteogenic potential of RCPS cells in comparison to controls. This behaviour was clearly accompanied by transcriptional alteration of genes important for osteogenesis, such as *RUNX2*, *ALPL* and *BGLAP*, during initial stages of nMSC differentiation, a dysregulation that was also observed in *EIF4A3* knockdown nMSCs. Although additional confirmatory differentiation assays with *EIF4A3* knockdowns are needed, the results reported here suggest that aberrant expression of the aforementioned genes in early differentiation account for the increment in the osteopotential of RCPS nMSCs. Since craniofacial development is sensitive to perturbations, alterations in osteopotential of cranial NC-derived mesenchymal cells could be accountable for the

clinical manifestations of patients. These cells are responsible for the development and ossification of the craniofacial skeleton, which is promoted by the BMP pathway (Graf et al., 2015). Mouse models showing augmented *Bmp* signalling (bearing constitutive expression of *Bmp4, Bmpr1a* or loss of *Bmp* antagonists Chordin and Noggin) manifest a range of craniofacial defects, including craniosynostosis, cleft palate, and mandibular hypoplasia or agnathia, thus reproducing craniofacial characteristics of RCPS (Stottman et al., 2001; Bonilla-Claudio et al., 2012; Li et al., 2013; Komatsu et al., 2013; Favaro et al., 2011; Raskin et al., 2013). Consequently, an otherwise expected increment in bone formation can ultimately lead to developmental bone deficits. If ossification disturbances are responsible for the cranioskeletal findings in RCPS patients, our results can arguably be transposed to their limbs, which frequently show radial/tibial defects, hand anomalies and club feet; therefore, low amounts of *EIF4A3* transcripts could affect ossification of those structures, although a neural crest component in appendicular skeleton development has yet to be confirmed (Isern et al., 2014; Favaro et al., 2011). Alternatively, given the role of endochondral ossification in limb skeletal development, it will be important to further assess the chondrogenic potential of RCPS nMSCs, as discussed next.

Since the craniofacial phenotype of RCPS revolves around mandible development, alterations in chondropotential would also be expected in nMSCs from affected patients. Lower jaw formation relies upon proper ontogenesis of the Meckel's cartilage (Moody, 2014), so fluctuations in chondrogenesis could ultimately result in mandibular ossification defects. The fact that we were unable to produce chondrocyte pellets from any RCPS patients raises the attractive possibility that chondrogenesis can be disrupted by decreased *EIF4A3* expression, as previously observed in a zebrafish model (Favaro et al., 2014). However, this could not be confirmed in the two independent nMSC replicates under *EIF4A3* knockdown, which showed inconsistent patterns of expression for chondrogenesis markers *SOX9*, *ACAN*, and *COL2A1*. Further attempts to quantify chondrogenic potential of RCPS and *EIF4A3* knockdown nMSCs are therefore worthwhile, and should be carried out in the near future.

The cellular functions attributed to the protein encoded by *EIF4A3* highly indicate that RCPS cells could indeed display dysregulated patterns of mRNA expression. The protein eIF4AIII belongs to the exon junction complex (EJC), playing important roles in RNA post-transcriptional control, including mRNA splicing through EJC-spliceosome interactions (Michelle et al., 2012;

Wang et al., 2014; Steckelberg et al., 2015). Recently, depletion of eIF4AIII in HeLa cells has been reported to produce a generalised shift in mRNA splicing patterns (Wang et al., 2014), thus suggesting that reduced *EIF4A3* expression could lead to aberrant splicing of specific mRNA species, as observed in *Xenopus* embryos (Haremaki & Weinstein, 2012); accordingly, aberrant transcript expression along the osteogenesis molecular hierarchy could be responsible for the cellular phenotypes detected in RCPS nMSCs. Mutations in spliceosomal genes are responsible for a number of clinically overlapping CFM syndromes: RCPS, mandibulofacial dysostosis Guion-Almeida type, Nager syndrome, Burn-Mckeown syndrome, and cerebrocostomandibular syndrome (Lehalle et al., 2015). This staggering observation implies that splicing defects are likely to result in impairment of similar developmental processes during craniofacial morphogenesis, and therefore the results generated in this work should prime other researchers to concentrate efforts in examining NC-derived mesenchymal cell differentiation properties in tissues or animal models relevant for the aforementioned syndromes. Finally, the dissection of the splicing profile of RCPS should benefit from RNA sequencing (RNAseq) technology, with which accurate quantification of alternative or novel mRNA variants is achievable. In this regard, iNCCs from RCPS and control subjects have already been subjected to RNAseq, and a comparative transcriptome analysis is currently underway. These results will contribute to further clarifying the pathogenesis of RCPS and possibly of other spliceosome-related syndromes.

Results reported in Chapter IV suggest that NC-derived mesenchymal cell characteristics are impacted by *TCOF1*/Treacle haploinsufficiency. TCS nMSCs showed augmented apoptosis, and their osteogenic and chondrogenic differentiation properties were altered in comparison to controls. Although the pathogenetic mechanism so far ascribed to TCS involves apoptosis of pre-migratory cranial NC cells (Dixon et al., 2006), our findings indicate that disturbances in NC-derived mesenchymal cells after migration could also be involved in the aetiology of the syndrome. Consequently, the cranial bone and cartilage deficits observed in TCS patients could arise from a combination of dysregulated osteo-chondrogenic differentiation and apoptosis of post-migratory NCs, in addition to the already established pre-migratory NC cell apoptosis; importantly, these results have potential implications for the surgical rehabilitation of TCS patients, in which mandibular distraction osteogenesis frequently lacks long-lasting results (Stelnicki et al., 2002; Gursoy et al., 2008), a limitation that could be related to anomalous differentiation and/or survival of mesenchymal cells residing in the mandible. Finally, further assays to quantify osteo-

chondrogenic potential with the use of *TCOF1*-deficient isogenic cells (such knockdown cells) will aid in the corroboration of the aforementioned findings.

If the changes in differentiation potential observed in TCS nMSCs are caused by reduced Treacle expression, the molecular pathways controlling osteogenic/chondrogenic differentiation and ribosome biogenesis must be functionally coupled. Indeed, chondrogenesis-associated regulators *Runx2* and *Runx3* are known to associate with rDNA to regulate transcription through interaction with UBF1, an important factor involved in ribosome synthesis. In addition, *Runx2*, a master regulator of osteogenesis, inhibits UBF1 to attenuate rRNA production and control cell division during osteodifferentiation (Trainor & Merril, 2014). These observations establish a connection between Treacle function, ribosome biogenesis, and osteo-chondrogenic differentiation, as Treacle has been shown to co-localise with UBF1 and RNA Polymerase I, being essential for rDNA transcription (Valdez et al., 2004). However, with the present data we cannot demonstrate how the increased osteopotential of TCS nMSCs reflects the craniofacial alterations of the syndrome, to which reduced bone and cartilage formation has been attributed in TCS animal models (Dixon et al., 2006; Weiner et al., 2012). Therefore, we hypothesise that the deviant osteo-chondrogenic behaviour of TCS nMSCs rather represents a dysregulation of these processes, which, during embryonic development, could lead to corruption of cartilage and bone production. Additional studies focusing on the phenotype and differentiation properties of cranial NC-derived mesenchymal cells with the use of TCS *in vivo* models will be essential to clarify this issue.

It is currently unknown whether iNCCs derived from iPSCs comprise a mixed population of cranial and non-cranial NCs or a non-specified population capable of acquiring multiple NC fates (Fukuta et al., 2014; Kreitzer et al., 2014). Such uncertainty regarding the nature of *in vitro*-produced NC cells is exemplified by the $HNK1^{+}$ and HNK^{-} iNCCs generated from TCS iPSCs, which could represent migratory and non-migratory NC cells. Nonetheless, the presence of cranial NC cells (or progenitors capable of giving rise to cranial NC cells) in the iNCC populations reported here is unquestionable given their ability to originate mesenchymal derivatives (e.g. osteoblasts and chondrocytes), and it will be interesting to assess expression profiles specifically ascribed to cranial NC cells (such as expression patterns of *HOX* transcripts) to better characterise the iNCCs generated in this work. Moreover, irrespective of their nature, these iNCCs are invaluable tools for a preliminary screening for possible pathogenic mechanisms involved in CFMs, which was the

objective of this thesis. We acknowledge that complementary *in vivo* experimentation will be paramount to corroborate our findings; in this sense, our group is currently undertaking RCPS modelling with the use of Crispr/Cas9 technology to generate zebrafish lines harbouring the *eif4a3* 5' UTR expansion mutations present in affected patients, which will enable a thorough *in vivo* phenotypical confirmation and characterisation of the alterations observed in RCPS nMSCs.

In conclusion, we demonstrated the applicability of NC-derived cell types to provide clues regarding the pathogenetic mechanisms leading to CFMs. Since CFMs are consequence of disturbances in NC-derived tissue development, the cell types explored in this study constitute adequate *in* vitro models to investigate craniofacial phenotypes. We identified a possible aetiological determinant of susceptibility to cleft lip/palate, and we provided evidence establishing a relationship between altered mesenchymal differentiation and the craniofacial phenotypes of RCPS and TCS. These novel findings will aid in dissecting the aetiology of CFMs by providing grounds to direct future efforts in craniofacial research.

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