UNIVERSIDADE DE SÃO PAULO
INSTITUTO DE BIOCIÊNCIAS

CAMILA MANSO MUSSO

Investigação do mecanismo patogênico responsável pela síndrome Richieri-Costa-Pereira

Investigating the pathogenic mechanism underlying Richieri-Costa-Pereira syndrome

São Paulo
2019

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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Biologia/Genética.

Orientadora: Profa. Dra. Maria Rita dos Santos e Passos-Bueno

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Profa. Dra. Maria Rita dos Santos e Passos-Bueno
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À memória de Raúl Marcel González Garcia, amigo e professor.
A ele, que despertou em mim a paixão pelas Ciências Biológicas,
plantando a semente da profissional que me tornei.

“A vida é assim:
esquenta e esfria,
aperta e daí afrouxa,
sossega e depois
desinquieta.
O que ela quer da gente
é coragem.”

Guimarães Rosa

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NOTAS

Esta tese de doutorado compreende um trabalho inédito desenvolvido entre 2015 e 2019 no Laboratório de Genética do Desenvolvimento, gerido pela Dra. Maria Rita Passos-Bueno, do Centro de Estudos do Genoma Humano e Células Tronco, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brasil.

A tese foi redigida no modelo de artigos/capítulos, no idioma inglês. Dois artigos publicados (capítulos II e III) e dois artigos não publicados (capítulos IV e V) foram incluídos no corpo principal da tese. Esses capítulos estão precedidos por uma introdução geral (capítulos I) e seguidos de uma discussão geral e conclusões (capítulos VI). Publicações em co-autoria não relacionadas ao tema principal encontram-se sumarizadas nos apêndices.

Este trabalho foi importante para estabelecer uma colaboração internacional com a Dra. Debra Silver da Duke University, Carolina do Norte, EUA. O desenvolvimento da pesquisa envolveu um período de doutorado sanduíche, durante o qual alguns experimentos foram realizados no laboratório estrangeiro.

O projeto que resultou na presente tese foi cadastrado na Plataforma Brasil e contou com o parecer consubstanciado do Comitê de Ética em Pesquisa do Instituto de Biociências da Universidade de São Paulo (número 1.463.852/2016).

Este trabalho recebeu apoio financeiro da CAPES, CNPq e Company of Biologists.

NOTES

This doctoral thesis comprises an original work conceived and developed between 2015 and 2019 at the Laboratory of Developmental Genetics headed by Dr. Maria Rita Passos-Bueno within the Human Genome and Stem Cell Research Center, Institute of Biosciences, University of São Paulo, São Paulo, Brazil.

The thesis was organized into scientific articles that are presented as the core chapters. Two published articles (chapters II and III) and two unpublished articles (chapters IV and V) were included in the main body of the thesis. These chapters are preceded by a general introduction (chapter I) and followed by the general discussion and conclusions (chapter VI). Additional co-authored publications not related to the main subject of the thesis were assigned to an appendix section.

This work was important for establishing an international collaboration with Dr. Debra Silver from Duke University, North Carolina, USA. The development of this doctoral research involved an internship period, during which several experiments were carried out in the Silver lab.

The project that resulted in this thesis was registered on the Plataforma Brasil and approved by the Ethics Committee of the Institute of Biosciences at University of São Paulo (number 1.463.852/2016).

This work was financed by CAPES, CNPq and Company of Biologists.

LIST OF ABBREVIATIONS

A3SS	Alternative 3’ splice sites
A5SS	Alternative 5’ splice sites
ANOVA	Analysis of variance
AS	Alternative splicing
CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CEGH-CEL	Centro de Estudos do Genoma Humano e Células Tronco
CEPID	Centro de Pesquisa, Inovação e Difusão
CFM	Craniofacial malformations
CNCC	Cranial neural crest cells
CNPEM	Centro Nacional de Pesquisa em Energia e Materiais
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CNS	Central nervous system
CNV	Copy number variations
CRISPR	Clustered regularly interspaced short palindromic repeats
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEG	Differentially expressed genes
DEP	Differentially expressed proteins
DNA	Deoxyribonucleic acid
E	Embryonic stage
ECM	Extracellular matrix
EEP	Exclusively expressed proteins
EJC	Exon junction complex
EMT	Epithelial to mesenchymal transition
ENS	Enteric nervous system
ESC	Embryonic stem cells
FA	Focal adhesion
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FDR	False discovery rate
Fig.	Figure
g	Relative centrifuge force
GO	Gene ontology
GSEA	Gene set enrichment analysis
hiPSCs	Human induced pluripotent stem cells
hNCCs	Human neural crest cells
hnRNP	Heterogeneous nuclear ribonucleoproteins
HRAC	Hospital de Reabilitação de Anomalias Craniofaciais
IACUC	Institutional Animal Care and Use Committee
IB	Instituto de Biociências
IGV	Integrative Genomics Viewer

iNCCs	iPSC-derived neural crest cells
iPSCs	Induced pluripotent stem cells
JAX	The Jackson Laboratory
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCMS	Liquid chromatography mass spectrometry
LFQ	Label-free quantification
LNBio	Laboratório Nacional de Biociências
MC	Meckel's cartilage
min	Minutes
mNCCs	Mouse neural crest cells
mRNA	Messenger RNA
MXE	Mutually exclusive exons
n	Sample size
NBC	Neural plate border-like cells
NCCs	Neural crest cells
NCP	Neurocristopathies
NIH	National Institutes of Health
NMD	Nonsense-mediated RNA decay
nMSCs	NCC-derived mesenchymal stem cells
NPB	Neural plate border
NSCs	Neural stem cells
nt	Nucleotides
OMIM	Online mendelian inheritance in man
PCR	Polymerase chain reaction
PNS	Peripheral nervous system
RCPS	Richieri-Costa-Pereira syndrome
RI	Retained introns
RNA	Ribonucleic acid
RNAi	RNA interference
rRNA	Ribosomal RNA
RT	Room temperature
RT-qPCR	Real-time quantitative polymerase chain reaction
SE	Skipped exons
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SRCP	Síndrome Richieri-Costa-Pereira
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TALEN	Transcription activator-like effector nuclease
USP	Universidade de São Paulo
UTR	Untranslated region
ZFN	Zinc finger nuclease

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CHAPTER I

GENERAL INTRODUCTION

1. Vertebrate Craniofacial Development

A defining feature of the vertebrate lineage is the presence of a head that is both supported and protected by a robust skeleton (Kaucka and Adameyko 2019; Jandzik et al. 2015). Development of the head structure involved the acquisition of neural crest cells and ectodermal placodes, which was a crucial step and powerful propellant for vertebrate evolution (Gans and Northcutt 1983; Horie et al. 2018).

The head represents the most complex part of the body and its morphogenesis requires a sequence of spatio-temporally coordinated events. Craniofacial development encompasses an umpteen number of signaling molecules mainly belonging to the Wnt, fibroblast growth factor (FGF) and bone morphogenetic protein (BMP) pathways, which precisely coordinate the assembly of components from distinct sources including the ectoderm, endoderm and mesoderm as well as neural crest cells (Adameyko and Fried 2016).

1.1 Neural Crest Cells

Neural crest cells (NCCs) comprise a transient, multipotent population of migratory cells that originates at the border of the neural plate in the developing embryo (Bronner and LeDouarin 2012). They contribute to the formation of different systems, including the peripheral nervous system (PNS), enteric nervous system (ENS), and cardiovascular system while also playing a major role in the development of forebrain and midbrain structures. NCCs are capable

of giving rise to cell types as diverse as neurons, glia, melanocytes, endocrine cells, muscle, bone, cartilage and connective tissues (Fig. 1). Because of their striking plasticity, unique properties and importance in development, the neural crest has been dubbed the “fourth germ layer” of the vertebrate embryo (Cordero et al. 2011; Trainor 2013, Zhang et al. 2014).

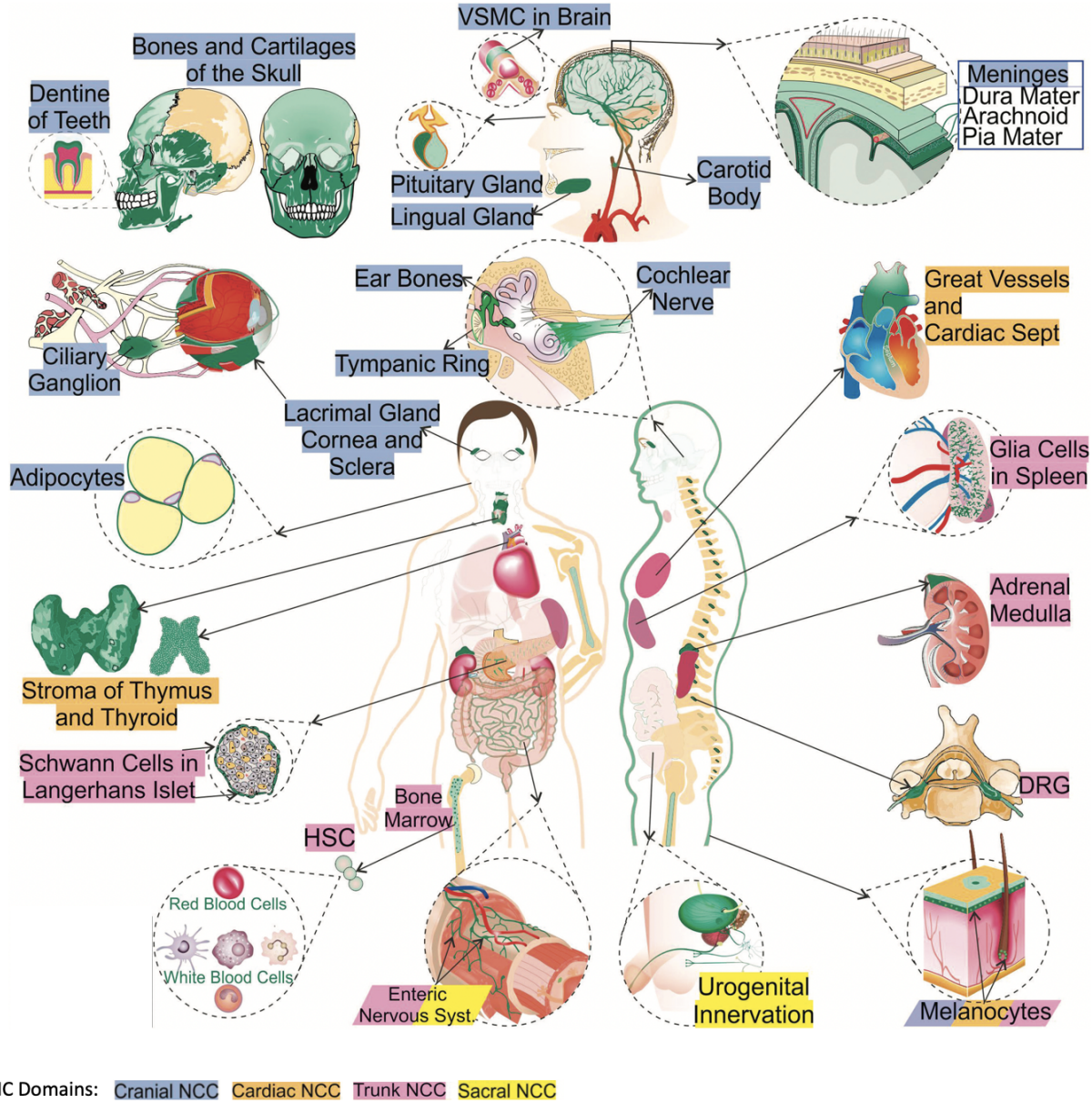

Figure 1. Schematic representation of the wide diversity of neural crest derivatives. The tissue, cell and organ names are highlighted with different colors according to its domain origin. Adapted from Vega-Lopez et al. 2018.

During late gastrulation and primary neurulation, the neural crest arises between the neural ectoderm that generates the central nervous system (CNS) and the adjacent non-neural ectoderm that generates the epidermis, a region known as neural plate border (NPB). The edges of the neural plate move upward to form the paired neural folds, which are brought together and fuse dorsally to form the neural tube in secondary neurulation. The neural crest cells reside at the dorsalmost portion of this hollow tube (Scott 2010).

After induction and specification, NCCs undergo a process designated as the epithelial to mesenchymal transition (EMT) and leaves the neuroepithelium territory through a delaminating process. The EMT orchestrates the transition from an epithelial to a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness and elevated resistance to apoptosis. This biological process encompasses a series of events regulated by a tightly controlled network requiring sequential activation and/or repression by molecular effectors (Theveneau and Mayor 2012).

After EMT, NCCs migrate along specific routes throughout the embryo. Once they arrive at their destination, they undergo intense proliferation and differentiation as derivative structures begin to take shape (Theveneau and Mayor 2012).

1.2 Cranial Neural Crest Cells

The NCCs can be divided into four domains i.e. cranial, cardiac, trunk, and vagal/sacral based on their axial level. The NCCs' fates vary depending upon their domain, where they migrate to and settle. Only the cranial or cephalic neural crest cells (CNCCs) have the potential to differentiate into cartilage and bone (Scott 2010).

There are differences in NCC development along the rostrocaudal axis of the embryo as well as between species. In mammals, the CNCCs delaminate all at once when the neural tube is still open, while in birds it coincides with the fusion of neural folds. Nevertheless, the trunk NCCs delaminate progressively after the neural tube closure in all

animals. After delamination, they migrate as a continuous wave moving into distinct streams (Bronner and LeDouarin 2012; Theveneau and Mayor 2012).

CNCCs can be further subdivided into forebrain-, midbrain- and hindbrain-derived populations. The rostral CNCCs originate the frontonasal process that generates the bones of the face, whereas the posterior cells migrate into the pharyngeal arches to produce the craniofacial mesenchyme. The cells that colonize the first (mandibular) pharyngeal arch give rise to the jawbones and middle ear bones. On the other hand, the cells from the second, third, fourth and sixth pharyngeal arches generate hyoid cartilage of the neck, thymus, parathyroid and thyroid glands (Cordero et al. 2011; Santagati and Rijli 2003) (Fig. 2B and C).

NCCs have specific segment identities according to their position along the anterior-posterior axis specified by a combination of *Hox* genes (Parker et al. 2018). However, CNCCs from the forebrain, midbrain and anterior hindbrain (rhombomeres 1 and 2) do not express any *Hox* genes (Fig. 2A). The patterning of CNCCs arising from Hox-negative regions are mainly controlled by distal-less (*Dlx*) genes (Depew et al. 2002).

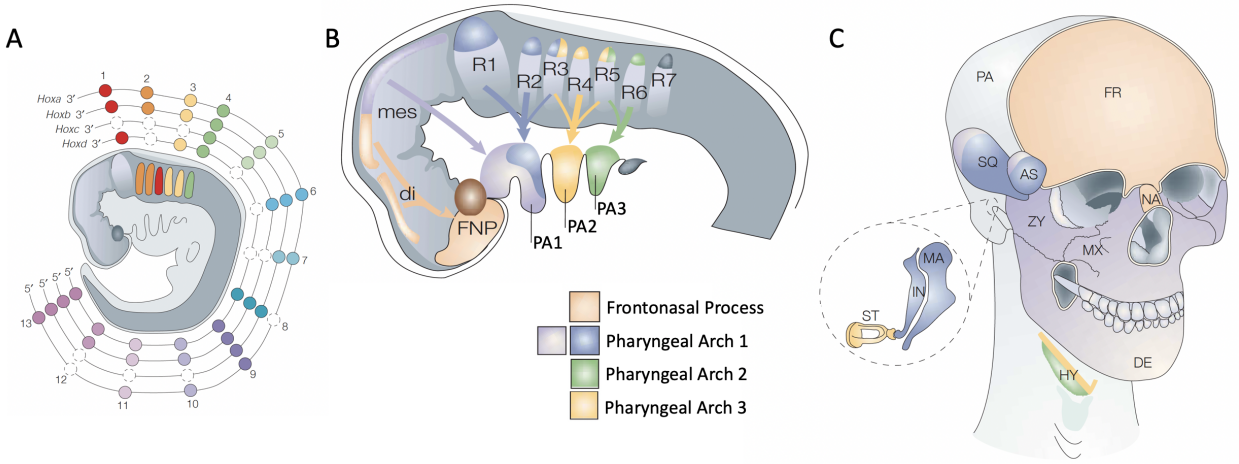

Figure 2. Scheme of the fate of cranial neural crest cells (CNCCs). (**A**) Representation of the Homeobox genes by color code, showing the four clusters and 13 paralogous groups present in humans. The physical position of each *Hox* in the cluster correlates with its expression along the anteroposterior axis of the embryo. (**B**) The CNCC migratory pathways and subsequent colonization of the frontonasal process and pharyngeal arches are shown in the embryo. (**C**) The skull indicates comparative contributions of CNCC populations to cranial skeletal elements. AS, alisphenoid; DE, dentary; di, diencephalon; FNP, frontonasal process; FR, frontal; HY, hyoid; IN, incus; MA, malleus; mes, mesencephalon; MX, maxillary; NA, nasal; PA,

parietal; PA1-P3, pharyngeal arches 1-3; R1-R7, rhombomeres 1-7; SQ, squamosal; ST, stapes; ZY, zygomatic. Adapted from Santagati and Rijli 2003.

Regarding the development of the mandible, after formation of the mandibular arch the CNCC-derived mesenchyme undergoes condensation. Subsequently the mesenchymal cells differentiate into chondrocytes and organize into a bilateral rod-shaped cartilage, i.e. Meckel's cartilage (MC), surrounded by the perichondrium. This cartilage comprises a primordial anlage of the skeletal element of the mandible, functioning as a morphogenetic template (Amano et al. 2010). Mesenchymal cells close to the MC start differentiating into osteoblasts and are surrounded by an osteogenic front. The MC lengthens ventromedially and dorsolaterally on both sides of the mandibular arch and fuses at the distal tip originating the mandibular symphysis. Therefore, formation of the bones of the mandible occurs in two different ways: the most distal region undergoes endochondral ossification in the symphyseal region, while the middle part undergoes intramembranous ossification. Whereas the proximal ends of Meckel's cartilage persist and form the basis of the middle ear bones, the intermediate part is degraded. In addition to the MC, mandibular development also involves other secondary cartilages e.g. coronoid and condylar cartilages (Parada and Chai 2015; Radlanski et al. 2003; Logjes et al. 2018) (Fig. 3).

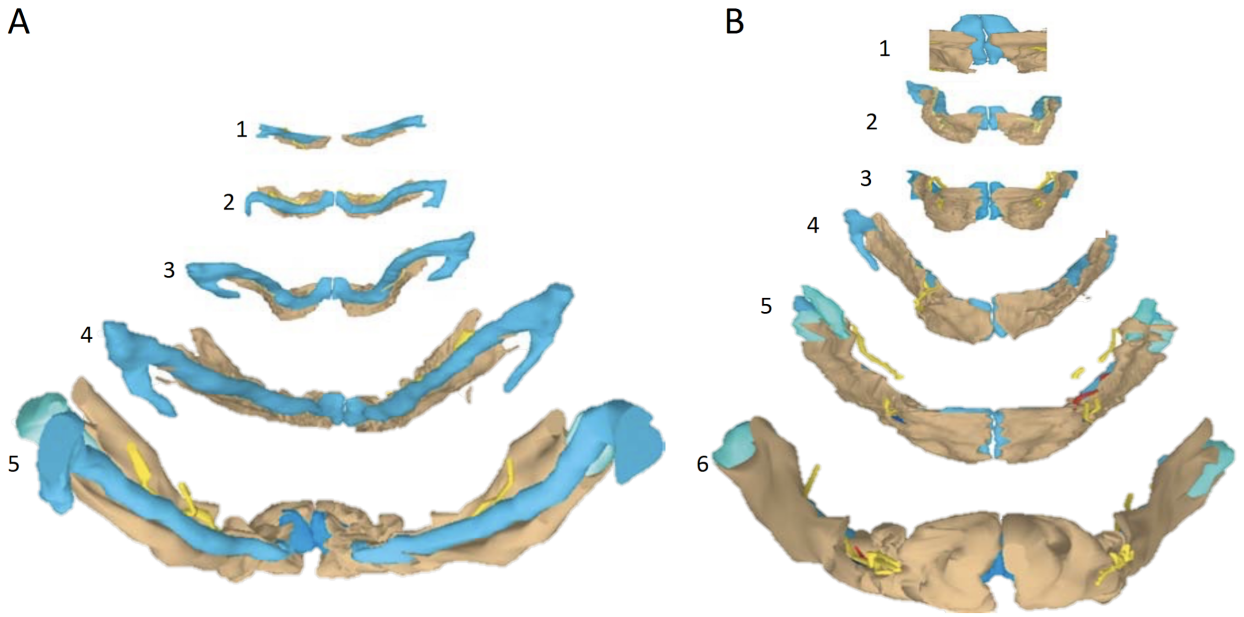

Figure 3. 3D reconstructions of human mandible at different stages of development. The mandible of human embryos is shown in dorsal views (**A**) and anterior views (**B**). (**A**) Figures I-V are arranged according to maturation at the same scales. CRL (crown-rump-length) sizes: **I** 22mm, **II** 19mm, **III** 25mm, **IV** 66mm, **V** 117mm. (**B**) Figures I-V are arranged according to maturation at the same scales, except **I**, which shows a clos-up view of the symphyseal region. CRL sizes: **I** 21mm, **II** 25mm, **III** 24mm, **IV** 66mm, **V** 76mm, **VI** 117mm. Bone is represented in brown, Meckel's cartilage in blue, cartilaginous core of condylar process in light blue, cartilaginous core of coronoid process in turquoise, inferior alveolar and lingual nerves in yellow, and inferior alveolar artery in red. Adapted from Radlanski et al. 2003.

Anomalies in any step of NCC development, such as neural crest specification, delamination, migration, proliferation, survival and differentiation are often associated with the pathophysiology of a variety of disorders known as neurocristopathies, which frequently encompass craniofacial abnormalities (Vega-Lopez et al. 2018; Knecht and Bronner-Fraser 2002).

A tightly controlled spatial and temporal signaling network is required for the multistep embryonic development, yet the molecular cascades that control human craniofacial development have until recently been little understood. The identification and functional studies of mutations that cause craniofacial malformations (CFMs) represent a promising approach for understanding craniofacial morphogenesis and have given significant insight into how the head develops. It is noteworthy that the intricate series of molecular signals orchestrating NCC development are distinct across organisms. However, there is a clear conservation of most

mechanisms leading to NCC formation and the transcriptional regulators of these processes largely overlap (e.g. *Snail*, *Twist* and *FoxD3* genes) (Barriga et al. 2015).

2. Craniofacial Malformations

As a highly social species, the human face is in many ways the most powerful and influential region of our anatomy. The face not only conveys our emotions to others, but also constitutes a fundamental aspect of any individual's identity. Indeed, no physical feature evokes more profound reactions when disease disfigures it than the face (Jack and Schyns 2015; Helms et al. 2005).

Craniofacial malformations comprise a heterogeneous group of disorders that collectively represent over one-third of all congenital birth defects. They arise from disturbances in craniofacial morphogenesis during embryonic development, affecting tissues of the skull and face (Gorlin et al. 2001).

CFMs can compromise a wide variety of craniofacial tissues at different levels of severity and can occur as isolated events or in association with other clinical manifestations (Gorlin et al. 2001). The most common craniofacial malformations, affecting 1:700 live-births worldwide, are the orofacial clefts, characterized by the discontinuity of the structures forming the lip and/or palate (Schutte and Murray 1999; Mossey et al. 2009). Another example are the craniosynostosis, a condition that involves premature ossification of cranial sutures resulting in abnormal skull shape as well as cranial growth restriction and increased intracranial pressure (Khanna et al. 2011).

These disturbances lead to functional impairment such as airway obstruction, disorders of feeding, hearing, speech and psychosocial difficulties. Accordingly, CFMs demand extensive treatment from several distinct health professionals entailing a significant burden to both patients and the health care system overall (Hamm and Robin 2015).

Craniofacial malformations are caused by genetic and/or environmental factors including chromosomal alterations and maternal alcohol intake, smoking and nutritional deficits (Jones

2011; Brito et al. 2012; Sabbagh et al. 2015). CFMs are genetically and clinically heterogeneous and both monogenic (Mendelian) and multifactorial inheritances have been reported (Twigg and Wilkie 2015). We would like to highlight among monogenic forms the subject of this thesis, Richieri-Costa-Pereira syndrome, which is described in detail below.

3. Richieri Costa-Pereira Syndrome

Richieri-Costa-Pereira syndrome (RCPS; MIM #268305) is a rare acrofacial dysostosis initially described in five unrelated Brazilian females in 1992 (Richieri-Costa and Pereira 1992). In the subsequent years, the first male patients (Richieri-Costa and Pereira 1993) as well as additional cases have been described mainly in Brazilian individuals. To date, 40 individuals have been described in the literature with RCPS, including two non-Brazilian patients (Tabith Júnior and Gonçalves 1996; Walter-Nicolet et al. 1999; Richieri-Costa and Brandão-Almeida 1997; Golbert et al. 2007; Graziadio et al. 2009; Tabith and Bento-Gonçalves 2003; Guion-Almeida and Richieri-Costa 1998; Favaro et al. 2011; Vendramini et al. 2004; Bertola et al. 2018; Raskin et al. 2013). The fact that the majority of patients are from São Paulo state and a notable number of them are from an isolated geographical area named Vale do Ribeira have suggested a founder effect for this disorder (Favaro et al. 2011).

RCPS presents a wide phenotypic spectrum and is characterized by several craniofacial and limb abnormalities, including microstomia, Robin sequence (micrognathia, glossoptosis and cleft palate), abnormal fusion of the mandible, absence of central lower incisors, epiglottis hypoplasia/agenesis, laryngeal and ear anomalies, hypoplastic thumbs, mesomelic shortening of upper and lower limbs, clubfeet, learning and speech impairments, and microcephaly, which has been recently included (Bertola et al. 2018) (Fig. 4). The phenotype expressivity is variable, ranging from only mild defects in fusion of the mandible to severe cases with agenesis of the mandible and craniofacial pansynostosis (Raskin et al. 2013). RCPS affects multiple structures derived from neural crest cells (NCCs), suggesting that these cells are compromised during embryonic development.

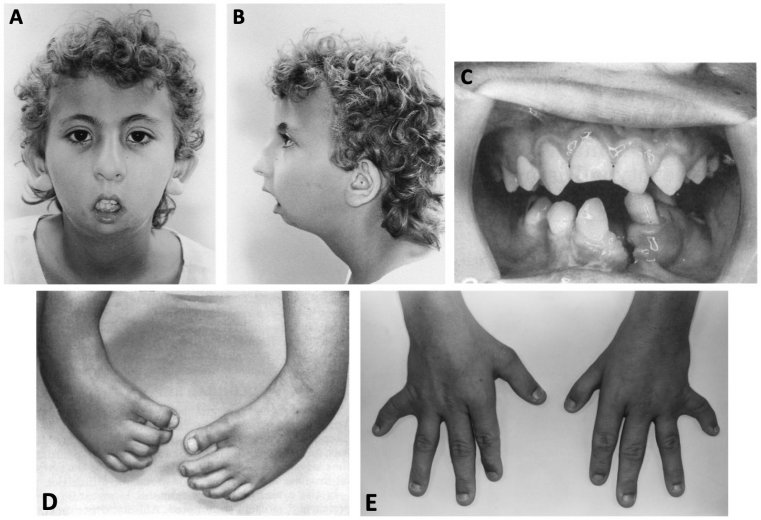

Figure 4. RCPS patients illustrating typical facial features of the syndrome, including microstomia, micrognathia (**A**, **B**) and cleft of lower alveolar ridge (**C**), as well as limb defects, including clubfeet (**D)** and hand anomalies (**E**). Patients reported by Richieri-Costa and Pereira 1993; Richieri-Costa Pereira and Brandão-Almeida 1997; Tabith and Bento-Gonçalves 2003.

3.1 Etiology

In 2014, our group identified the causative mutation of this syndrome by homozygosity mapping and targeted sequencing. RCPS is mainly caused by an increased number of repeat motifs in the 5'UTR of the *EIF4A3*, which encodes a core component of the exon junction complex (EJC). The *EIF4A3* 5'UTR is complex, presenting multiple allele patterns varying in size and organization of three types of motifs: (1) CA-18nt, TCGGCAGCGGCAGCGAGG; (2) CACA-20nt, TCGGCAGCGGCACAGCGAGG and (3) CGCA-20nt, TCGGCAGCGGCGCAGCGAGG (Favaro et al. 2014).

RCPS patients show a distinct allelic pattern, determined not only by the larger number of motifs but also by the presence of a unique motif containing an A-to-G substitution. The most prevalent allelic pattern among controls is an initial CACA-20nt repeated 2 to 9 times, followed by one CA-18nt, one CACA-20nt, and a final CA-18nt. However, RCPS patients present an initial CACA-20nt, followed by 12 to 13 repeats of CGCA-20nt, another CACA-20nt, and one final CA-

18nt (Favaro et al. 2014). Therefore, control alleles carry up to 12 repeats whereas disease-associated alleles carry 15 or 16 repeats (Fig. 5).

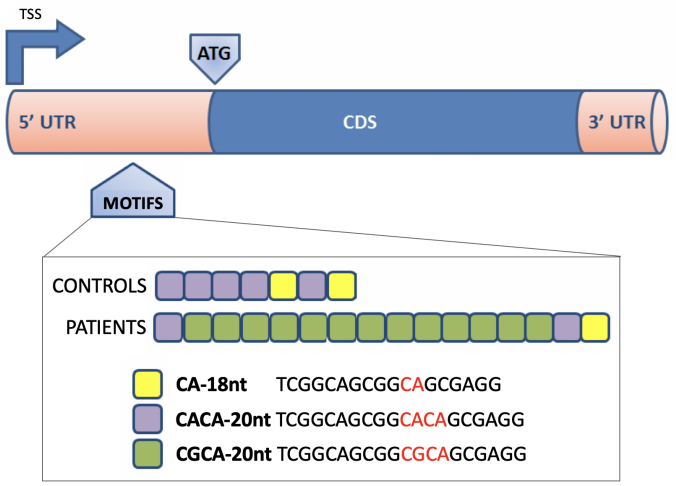

Figure 5. Cartoon of the repeat motifs within the *EIF4A3* 5'UTR, ending 43 bases upstream the first ATG. The different colors represent the motif types: CA-18nt, CACA-20nt and CGCA-20nt. TSS, transcription start site; UTR, untranslated region; CDS, coding sequence. Adapted from Alvizi 2017.

The most part of affected individuals are homozygous for the 16-repeat allele. However, a compound heterozygote patient was reported by Favaro et al. carrying a 14-repeat allele *in trans* with a likely pathogenic non-synonymous point mutation (c.809A>G; p.Asp270Gly), who interestingly presents a milder phenotype than 16-repeat allele homozygotes (Favaro et al. 2014).

EIF4A3 transcript abundance is 30-40 % lower in affected individuals than in controls (Favaro et al. 2014). However, the functional effects of the 5'UTR motifs on gene expression and whether both the number and sequence of the motifs are involved in *EIF4A3* downregulation remain to be uncovered. In addition, the origin of the pathogenic alleles (e.g. meiotic instability or unequal crossing-over events) is still unknown. Insights into the origin and effect of these complex alleles will contribute to a better understanding of regulatory features of 5'UTR regions and their role in craniofacial development.

Despite the fact that these repeat motifs are CG rich and their increased number in the disease-associated alleles results in a gain of 37 CpG sites, they are not hypermethylated in RCPS

patients compared to controls (Alvizi 2017). Therefore, DNA hypermethylation does not explain *EIF4A3* downregulation in RCPS. Further investigation of potential transcriptional or post-transcriptional mechanisms is needed to unveil how these motifs interfere with *EIF4A3* expression.

4. The Exon Junction Complex

The EJC plays a central role in gene expression control (Le Hir et al. 2016). It contributes at many levels to mRNA metabolism, including splicing, intra-cellular mRNA localization and nuclear export, mRNA stability control by nonsense-mediated decay (NMD), translation efficiency and rRNA biogenesis (Wang et al. 2014; Hachet and Ephrussi 2004; Chazal et al. 2013; Gehring et al. 2003; Le Hir et al. 2001).

Pre-mRNAs are often associated with nuclear proteins, collectively referred to as heterogeneous nuclear ribonucleoproteins (hnRNP). mRNA processing, including capping, splicing, and polyadenylation, are accompanied by profound changes in the composition and arrangements of these protein complexes (Dreyfuss et al. 2002).

In particular, the exon junction complex (EJC) is required by the splicing machinery and is assembled 20 to 24nt upstream of spliced junctions onto mature spliced mRNAs. EJC binds to RNA without sequence specificity and acts as a link between nuclear splicing and cytoplasmic processes, as it remains associated on mRNAs when transported to the cytoplasm (Le Hir et al. 2016). This multiprotein complex is organized around four central proteins that constitute the EJC core: eIF4A3, MAGOH, Y14 (also known as RBM8A) and MLN51. MAGOH and Y14 form a tight heterodimer and contact the two globular domains (RecA1 and RecA2) of eIF4A3. The MLN51 is a long protein that surrounds eIF4A3, binds to MAGOH and contacts one nucleotide, stabilizing the complex (Fig. 6). This tetrameric core serves as a platform for multiple peripheral factors during different post-transcriptional processes (Andreou and Klostermeier 2013).

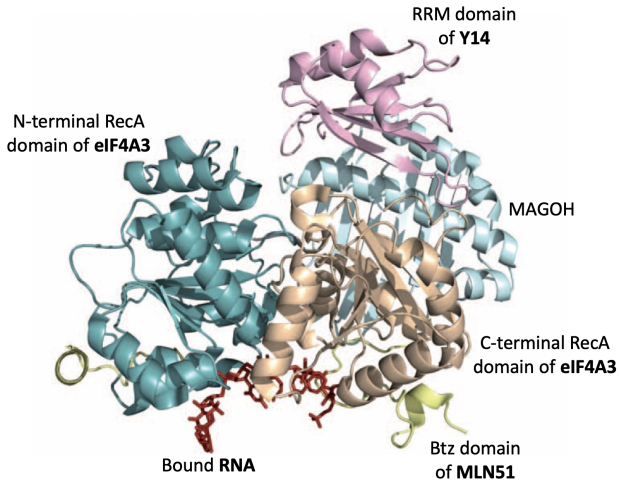

Figure 6. Organization of the domains of the exon junction complex (EJC) core elements. The N- and C-terminal RecA-domain of eIF4A3 are depicted in teal and brown, respectively, the RRM domain of Y14 in pink, MAGOH in cyan, the Btz of MLN51 in yellow, and the RNA is represented in red. Adapted from Andreou and Klostermeier 2013.

4.1 Eukaryotic Translation Initiation Factor 4A3

There are three paralogues of eIF4A in mammals: eIF4A1, eIF4A2 and eIF4A3. Despite sharing 60% sequence identity, the eukaryotic translation initiation factor 4A3 (eIF4A3) is functionally distinct from the others, is localized mostly in the nucleus, and is the only one incorporated into EJC. eIF4A1 and eIF4A2 are both cytoplasmic proteins and present higher structural and sequence similarity, sharing 90% identity to each other. However, they are not functionally redundant *in vivo*, and are involved in translation by the eIF4F translation initiation complex (Lu et al. 2014).

eIF4A3 is a member of the DEAD-box protein family of RNA helicases, which is characterized by a number of conserved motifs on a helicase core formed by two RecA-domains. The helicase core can unwind short RNA duplexes and also comprise an RNA-stimulated ATPase, acting as a nucleotide-dependent molecular switch with different affinities for RNA. In other words, in the absence of RNA or ATP, the helicase core adopts an open conformation, and binding

of RNA and ATP triggers a transition to a close conformation. It retains the closed conformation upon ATP hydrolysis, returning to an open state after phosphate release which in turn leads to release of the RNA strand. According, eIF4A3 serves as an RNA clamp (Ballut et al. 2005; Andreou and Klostermeier 2013).

4.2 EJC Components and Developmental Disorders

EJC components are mutated in several pathologies, such as in thrombocytopenia with absent radii (TAR) syndrome, for example, which is caused by *RBM8A* mutations and can present with microcephaly or Autism spectrum disorder (ASD) (Addington et al. 2011; Laumonnier et al. 2010; Nguyen et al. 2012; Tarpey et al. 2007; Albers et al. 2012). Copy number variations (CNVs) of either *EIF4A3* or *RBM8A* are associated with intellectual disability and brain malformations (Nguyen et al. 2013). Furthermore, haploinsufficiency for each of the EJC components (*Eif4a3*, *Magoh* and *Rbm8a*) in neural stem cells (NSCs) leads to aberrant neurogenesis and causes p53-mediated microcephaly in mice (Mao et al. 2016). Altogether, these genetic studies indicate disruption of *EIF4A3* impairs neurodevelopment.

It is known that biallelic hypomorphic mutations in *EIF4A3* leads to RCPS (Favaro et al. 2014), postulating *EIF4A3* as an essential element during craniofacial development. However, the pathogenetic mechanisms responsible for this syndrome are completely unknown. Ultimately, the requirements of *EIF4A3* for NCC functions is still obscure, and the molecular mechanisms by which *EIF4A3* mutation causes RCPS remain unclear.

5. Investigating the Pathogenetic Mechanisms of CFMs

The understanding of the molecular and cellular mechanisms governing craniofacial morphogenesis is critical to clarify how these CFMs arise. To date, several studies in animal and

cellular models have contributed to our understanding of normal or abnormal (disease-associated) craniofacial development.

5.1 Cellular Models

The use of primary cell cultures cannot cover the study of conditions in which embryonic tissues are primarily affected, such as in craniofacial malformations. In addition, the use of embryonic stem cells (ESCs) raises major concerns and controversies, mainly sharp ethical and political considerations, which have hindered their use for research and clinical applications (Lo and Parham 2009). Therefore, the advent of an alternative technology that allowed for reprogramming of somatic cells back to a pluripotent state was a remarkable event in the history of biological research. Indeed, the development of induced pluripotent stem cell (iPSC) method resulted in the 2012 Nobel Prize in Physiology or Medicine being awarded to its pioneer Shinya Yamanaka. iPSCs are derived from mature cells that have been genetically reprogrammed to an embryonic stem cell-like state and subsequently can be differentiated into multiple cell types (Takahashi et al. 2007).

Since the emergence of iPSCs, enormous progress has been made in stem cell biology. Reprogrammed cells have been widely used for drug discovery and testing, cell therapy development, and regenerative medicine (Shi et al. 2017). Furthermore, this powerful tool offers great promise for understanding basic mechanisms of human development and presents opportunities for modeling, and potentially treating human diseases. An increasing number of novel pathological mechanisms have been elucidated by using patient-derived iPSCs for disease modeling (Bellin et al. 2012; Ishiy 2016; Kobayashi 2016). Cellular models offer a unique approach to study cellular phenotypes and molecular mechanisms underlying human disorders, in particular those in which is difficult to obtain the tissue of interest such as developmental disorders. Assessing cells from patients carrying disease-specific genomic mutations has been a promising instrument to conduct functional studies.

iPSCs can be differentiated into disease-relevant cell types, such as neural crest cells in order to study craniofacial disorders. Recent protocols allow for the efficient generation of iPSC-derived NCCs by Wnt pathway activation and Smad blockade with the use of small molecules, producing homogeneous NCC populations (Fukuta et al. 2014; Menendez et al. 2013). However, the available methods for generation NCCs lack a NPB stage and subsequently do not recapitulate important steps involved in NCC development such as neural crest specification and EMT.

5.2 Animal Models

Many different vertebrates including amphibians, birds, teleosts and mice have been used to study craniofacial development, mainly by gene targeting/loss of function strategies. Targeting specific genes by using RNA interference (RNAi), anti-sense morpholinos or dominant-negative constructs enable assessment of their role in embryonic development (Barriga et al. 2015). In recent years, novel approaches have been developed as technical advances in genome editing, including CRISPR/Cas9 system, Zinc Finger Nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) technologies, have opened new possibilities for the study of embryonic development as well as disease modeling (Wang and Sun 2019).

Over the years, one approach to control gene expression that has become increasingly widespread is the Cre/loxP system. It is a simple and powerful tool which allows researchers to create a variety of transgenic mice (McLellan et al. 2017). Briefly, the c*re* gene encodes a site-specific DNA recombinase, which recognizes *loxP* sequences and recombines these sites leading to the excision of the DNA between them. Cre/loxP recombination stands as an important strategy to investigate gene function *in vivo* and has greatly expanded the versatility with which biologic questions can be addressed in mouse models, particularly because the desired gene modification can be restricted to specific cell types or developmental stages using a spatial and/or temporal control of gene expression (conditional mouse mutants) (Kühn and Torres 2002).

Since mice closely reflect many aspects of human development, they have served as the major mammalian model system and have been extensively explored to study gene function in

craniofacial development as well as the etiology of disorders. Indeed, many mouse models have led to relevant discoveries regarding the pathogenetic mechanisms responsible for CFMs (Chai and Maxson 2006; Sakai and Trainor 2009; Fantauzzo and Soriano 2014; Mao et al. 2016). However, some discrepancies in NCC development across species should be taken into consideration (Barriga et al. 2015). A valid approach is the combination of human cell culture and mouse model systems, which may provide a paradigm to study craniofacial development and CFM pathogenesis, address reproducibility and provide validation to data.

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OBJECTIVES

The main objective of this study was to investigate the cellular and molecular pathogenetic mechanisms by which *EIF4A3* mutations cause Richieri-Costa-Pereira syndrome (RCPS). We carried out genomic and functional studies to test the following hypothesis: (**1**) the RCPS-associated allelic patterns arose by unequal crossing-over events; (**2**) the increased number of motifs in the *EIF4A3* 5'UTR decrease gene expression; (**3**) the decreased expression of *EIF4A3* impairs NCC functions; (**4**) the *EIF4A3* deficiency in NCCs disrupts splicing and/or expression of transcripts related to craniofacial development.

The specific objectives were as follows:

a) To investigate the origin of the pathogenic alleles associated with RCPS;

b) To evaluate the functional effects of the 5'UTR repeat motifs on gene expression;

c) To identify dysfunctions related to RCPS in *EIF4A3* depleted hiPSC-derived NCCs;

d) To access differentially expressed genes/proteins and pathways by studying the transcriptome/proteome of patient-derived and control NCCs;

e) To validate human transcriptomic data using NCCs from conditional *EiF4a3* haploinsufficient mouse embryos;

In parallel with the aforementioned main objective, we also aimed to develop a simple and efficient *in vitro* method for NCC generation to recapitulate developmental events involved in NCC specification and epithelial to mesenchymal transition (EMT).

The specific objective was:

f) To derivate hiPSC-derived NCCs through a neural plate border (NPB)-like stage.

CHAPTER VI

GENERAL DISCUSSION

Richieri-Costa-Pereira syndrome (RCPS) is a rare autosomal-recessive disorder that affects craniofacial and limb development and is reported almost entirely in Brazilian patients (Favaro et al. 2011). RCPS is caused by an increased number of repeat motifs within the *EIF4A3* 5'UTR (Favaro et al. 2014). Specifically, the noncoding 5' region presents a complex pattern of three distinct motifs varying in both size and organization among individuals.

In chapter II, variability in the *EIF4A3* 5'UTR was characterized in a large cohort in order to investigate the mechanism that originated the RCPS-associated allelic pattern. This approach was important not only to clarify the origins of the pathogenic alleles but also to provide clues on the chance of RCPS arising in other populations. Despite advances in genome sequencing, repetitive regions remain difficult to sequence and noncoding regions lack detailed analysis in routine NGS pipelines. Indeed, the 5'UTR of *EIF4A3* is not covered in GnomAD database. Notably, RCPS is described in only two non-Brazilian patients to date, which may potentially be due to missing diagnoses of this condition leading to an underestimation of affected individuals, reinforcing the importance of characterizing this region and publishing results in an international journal.

We have found 43 different variations amongst unaffected individuals, which shows the polymorphic nature and structural complexity of this region (Hsia et al. 2018). Based on these allele patterns and haplotype analyses, we suggest that RCPS-associated alleles have arisen from independent unequal crossing-over events between ancient alleles. The fact that the number of repeats in the *EIF4A3* 5'UTR seems to be stable across generations (Favaro et al. 2014) supports this hypothesis. RCPS etiology is comparable to synpolydactyly, in which polyalanine expansion may have resulted from unequal crossing over of *HOXD13* (Warren 1997), contradistinguishing from several neurological disorders caused by unstable dynamic expansion mutations that have

arisen only once and increase in size during meiotic divisions (Cummings and Zoghbi 2000; Gatchel and Zoghbi 2005; Mirkin 2007; La Spada and Taylor 2010; McMurray 2010; Haeusler et al. 2016; DeJesus-Hernandez et al. 2011; Renton et al. 2011; Usdin et al. 2015; Virtaneva et al. 1997).

Furthermore, considering the restricted inclusion of CGCA-20nt motifs in the largest alleles along with the difference in allele pattern between Brazilian patients and the sole UK patient (Hsia et al. 2018), we propose that the pathogenic alleles have originated more than once and that RCPS can potentially occur in any population containing alleles with the disease-associated CGCA-20nt motif.

Additionally, we demonstrated a direct association between the number of motifs and *EIF4A3* expression, revealing a potential cis-acting regulatory mechanism for these repeat motifs (Hsia et al. 2018). The inverse correlation between number of motifs and expression levels suggests that the 5'UTR structure plays a central role in phenotypic modulation. Another interesting finding is that the disease-associated CGCA-20nt motif showed a reduction in expression compared to CACA-20nt and CA-18nt motifs, albeit not statistically significant, suggesting that both the number and sequence of the motif regulate *EIF4A3* expression. Indeed, a broad clinical variability is observed in RCPS patients. Specifically, individuals homozygous for the 16-repeat allele present more severe phenotypes than both the compound heterozygote carrying a 14-repeat allele *in trans* with a nucleotide change (Favaro et al. 2014) and the homozygote carrying the 14-repeat alleles, which was recently reported with a very mild phenotype (Bertola et al. 2018). Therefore, RCPS phenotypic variability may depend on the number and sequence of the motifs in the *EIF4A3* 5'UTR.

Since hypermethylation is not the mechanism by which the motifs repress *EIF4A3* expression in RCPS (Hsia et al. 2018), there could be alternative mechanisms responsible for this decreased expression that may involve creation of binding sites for repressors or post-translational events. Further studies are needed to better understand the mechanisms behind *EIF4A3* downregulation.

Next, the chapters III and IV described the functional evaluation of neural crest cells (NCCs) in order to unravel the pathological mechanisms of RCPS. Foremost, this work reinforced

the applicability of induced pluripotent stem cell (iPSC)-based disease modeling in deciphering the etiology and pathogenetic mechanisms underlying human craniofacial disorders. Patient iPSC-derived cell cultures have proven to be a very powerful and effective approach in contributing to our knowledge of embryonic development in both normal and disease contexts. Likewise, haploinsufficient mouse mutants were valuable for investigating *Eif4a3* functions in craniofacial development. The integrative use of iPSC technology with mouse models establishes a foundation for studying craniofacial malformations such as RCPS.

iPSCs highly expressed pluripotency markers, did not present aneuploidies, and demonstrated the ability to generate teratomas *in vivo*. iPSCs were successfully differentiated into NCCs which in turn exhibited p75(NRT)/HNK1 double-positive staining and high expression of NCC markers. RCPS patient-derived NCCs showed decreased *EIF4A3* mRNA and protein levels compared to controls. All NCC lineages were able to generate mesenchymal stem cells (nMSCs) with the typical immunophenotype, which gave rise to osteoblasts, chondrocytes and adipocytes (Miller et al. 2017). Taken together, these data demonstrated that our strategy efficiently yielded disease-relevant cell types, which allowed us to assess cellular and molecular phenotypes associated with RCPS *in vitro*. The mouse models recapitulated several anomalies seen in RCPS patients including mandibular and craniofacial abnormalities, indicating essential requirements of *EIF4A3* for NCC development. Both iPSC-derived cells and haploinsufficient mouse embryos contributed to clarify the NCC dysfunctions behind RCPS pathogenesis.

The cellular mechanisms by which reduced *EIF4A3* causes RCPS have been elucidated in chapter III (Miller et al. 2017). Patient-derived iPSCs differentiated toward a NCC lineage revealed defective migration and those differentiated into mesenchymal derivatives exhibited premature ossification and dysregulated chondrogenesis. Albeit some neurocristopathies have shown increased apoptosis and/or impaired proliferation involved in their pathogenesis (Devotta et al. 2016; Dixon et al. 2006; Brugmann et al. 2010), neither NCCs, nMSCs nor mouse models displayed commitment of either process. Cell death and cell cycle alteration was detectable only in extremely severe mouse mutants. Therefore, aberrant apoptosis, proliferation or mitosis delay are not major causes of RCPS. Depletion of *Eif4a3* in mice disrupted formation of Meckel's

cartilage that is critically important for proper mandible morphogenesis and led to precocious clavicle ossification, which parallels observations made with human nMSCs.

We provided evidence that impaired NCC migratory capacity is a key cellular dysfunction involved in RCPS pathogenesis. Delayed migration at early developmental stages can reduce mesenchymal progenitors populating the pharyngeal arches and subsequently impair cartilage and bone formation leading to abnormal or absence of mandibular fusion, which is amongst the most relevant RCPS phenotypes. Migration defects seem to be unique to RCPS etiology, since they have not been observed in other craniofacial disorders (Lehalle et al. 2015; Mills and Green 2017; Jones et al. 2008; Dixon et al. 2006) despite the clinical overlap. Additionally, chondrogenesis and osteogenesis dysregulation have been demonstrated in both *in vitro* and *in vivo* models and may contribute to the mandibular and clavicular anomalies seen in RCPS patients.

Despite presenting phenotypes comparable to humans, whose *EIF4A3* levels are varied depending on cell type, *Eif4a3* haploinsufficient mice also displayed severe phenotypes not so far described in humans (Miller et al. 2017). These phenotypic differences could be due to Cre efficiency or because humans are genetically diverse whereas mice are inbred, which exposes phenotypes that are normally masked in humans. In addition, the reported high rates of spontaneous abortion in RCPS families (Favaro et al. 2011) suggest that severe phenotypes in humans, particularly brain malformations, may be embryonic lethal.

One interesting fact that deserves our attention is that while *EIF4A3* exerts basic and ubiquitous functions as a core component of exon junction complex (EJC) which is involved in many aspects of mRNA metabolism, mutations in this gene lead to very specific phenotypes. Indeed, this paradox does not happen only in RCPS, but also in other disorders such as Diamond-Blackfan anemia, Hoyeraal-Hreidarsson and Bowen-Conradi syndromes (Boria et al. 2010; Armistead et al. 2009; Heiss et al. 1998). Accordingly, these diverse disorders may potentially be related at a molecular level.

Chapter IV described the study of the molecular mechanisms by which *EIF4A3* depletion impairs NCC functions. To assess these mechanisms, we employed transcriptomic and proteomic analyses of RCPS patient-derived NCCs in order to expose candidate targets and pathways

controlled by *EIF4A3* at the onset of craniofacial development. In addition, we used conditional mouse models for validation and further understanding of transcriptome data.

Gene set enrichment analysis (GSEA) of both human and mouse data revealed enrichment of extracellular matrix (ECM)-receptor interaction and focal adhesion pathway elements in response to decreased *EIF4A3* expression. Closer inspection demonstrated enrichment for GO categories of extracellular matrix and plasma membrane components. Optimal cell migration requires dramatic changes in cell shape and interaction with the ECM, which involves actin polymerization and continuous formation and disassembly of adhesions (Gardel et al. 2010). Focal adhesions (FA) are macromolecular assemblies that function as a physical connection between the cell and the ECM through interaction of integrin receptors and the actin cytoskeleton, mediated by numerous FA-associated proteins (Wu 2007). The interaction with ECM and focal adhesion features e.g. size, shape, and turnover dynamics, have long been associated with cell migration (Ridley et al. 2003; Kim and Wirtz 2013; Perris and Perissinotto 2000). Moreover, overexpression of CAV1, an identified DEG upregulated in RCPS patients, has been linked to decreased migration in tumor cells (Zhang et al. 2000). These findings suggest that impairment of cell adhesion dynamics is implicated in the cell migration defects underlying RCPS pathogenesis. However, future experiments will be needed for further clarifying the involvement of *EIF4A3* on migration.

Several human genetic conditions that manifest as tissue-specific phenotypes restricted to craniofacial structures are caused by mutations affecting mRNA splicing and ribosome biogenesis, known collectively as spliceopathies and ribosomopathies, respectively (Danilova and Gazda 2015; Yelick and Trainor 2015; Armistead and Triggs-Raine 2014; Mills and Green 2017; Lehalle et al. 2015). Treacher Collins and Bowen-Conradi syndromes are examples of such conditions (Choesmel et al. 2007; Sakai and Trainor 2009; Armistead et al. 2009; Jones et al. 2008), and notably both have phenotype overlap with RCPS. Ribosomes appear to be especially sensitive to deficits in EJC components, since we identified aberrant splicing as well as alterations at the proteomic level of ribosomal components in *EIF4A3* deficient hNCCs. Furthermore, haploinsufficiency for each of the core EJC components (i.e. Magoh, Eif4a3 and Rbm8a) resulted in altered expression of ribosomal components in mouse brains (Mao et al. 2016). These data

suggest that ribosome defects contribute to RCPS pathogenesis, reinforcing the idea that the EJC is a strong regulator of protein homeostasis machinery.

Bearing in mind that *EIF4A3* is super-stoichiometric to other EJC members in mammalian cells (Singh et al. 2015; Singh et al. 2012) and ribosome composition is heterogeneous and may reflect tissue requirements to translate specific mRNAs (Shi et al. 2017), we hypothesized that the tissue-specific defects in RCPS may be caused by insufficient *EIF4A3* expression in tissues with high EJC demand, leading to aberrant splicing of specific riboproteins. However, how *EIF4A3* influences ribosomal components and whether this is a direct effect is still unknown and may be an important question for future studies. In fact, NSC-specific *EiF4a3* haploinsufficiency alters expression of ribosomal components and triggers aberrant p53 activation leading to microcephaly in mouse model (Mao et al. 2016). Interestingly, microcephaly has recently been included in the phenotypic spectrum of RCPS (Bertola et al. 2018). Given these connections, it is fascinating to consider that *EIF4A3* regulates ribosome biogenesis and consequently contributes to RCPS pathology.

This work was designed with a focus on neural crest cells and craniofacial development although RCPS is also characterized by limb anomalies. The limb bud has different origins than craniofacial structures (Zuniga 2015) and *Wnt1*-Cre mouse models are not useful in the investigation of limb development since Cre is not active in the developing limb (Lewis et al. 2013). In the future, it will be of interest to employ other Cre drivers to investigate additional, relevant RCPS phenotypes.

Finally, owing to the importance of neural crest cells in embryonic development, in parallel with aforementioned studies we developed a simple method to recapitulate developmental events involved in NCC specification from neural plate border (NPB)-like cells.

NCC development depends upon a tightly controlled spatial and temporal molecular program involving a sequential activation and/or repression of multiple molecules (Knecht and Bronner-Fraser 2002). Anomalies in these developmental steps cause neurocristopathies (NCP), frequently encompassing craniofacial abnormalities (Vega-Lopez et al. 2018). A central process which orchestrates transitioning from an epithelial to a mesenchymal cell phenotype during NCC development is designated as the epithelial to mesenchymal transition (EMT). EMT drives the

onset of NCC migration and subsequent tissue morphogenesis and is associated with the segregation of homogeneous precursors into distinct fates (Kalcheim 2015; Theveneau and Mayor 2012).

In view of the limited access to human embryonic tissues and the fact that animal embryos may not completely recapitulate human embryonic development (Thyagarajan et al. 2003; de Bakker et al. 2016; Barriga et al. 2015), human iPSC-based strategies have been largely used as an alternative approach to study NCC biology in pathological conditions. The available methods for generating NCCs rely on BMP pathway modulation (Jiang et al. 2009; Lee et al. 2010; Pomp et al. 2005; Lee et al. 2007; Curchoe et al. 2010) and may or may not be combined with WNT pathway activation (Menendez et al. 2013; Fukuta et al. 2014; Miller et al. 2017; Menendez et al. 2011; Noisa et al. 2014; Huang et al. 2016; Liu et al. 2012; Lee et al. 2009; Mica et al. 2013; Kreitzer et al. 2013; Leung et al. 2016; Thier et al. 2019; Tchieu et al. 2017). However, many of these methods lack a NPB stage or are unable to fully convert neuroepithelial cell populations into NCCs, potentially hindering investigation of the various stages of neural crest specification. Thus, in chapter V we showed that neuroepithelial induction of hiPSCs without altering BMP signaling (Lippmann et al. 2014) confers a NPB-like transcriptional state to differentiating cells, which can be directed towards the neural crest fate with near 100% efficiency, recapitulating for the first time the sequential steps that orchestrate NCC specification and EMT.

This method provided an efficient *in vitro* human model to investigate the functional effects of candidate genetic variants and a promising approach to better understand human development and neural crest-related disorders.

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CONCLUSIONS

In conclusion, we unraveled novel mechanisms by which *EIF4A3* hypomorphic mutations lead to craniofacial phenotypes observed in RCPS patients.

In summary, we revealed that:

- RCPS-associated alleles have arisen from unequal crossing-over events between ancient alleles at least twice;
- Both the number and sequence of repeat motifs within *EIF4A3* 5'UTR regulate gene expression;
- *EIF4A3* is required for NCC development and its deficiency leads to decreased NCC migratory capacity and compromised osteogenic and chondrogenic differentiation of NCC-mesenchymal derivatives;
- *EIF4A3* deficient NCCs present altered expression of cell-ECM interaction and FA components, which can be implicated in the defective NCC migration;
- Aberrant splicing of ribosomal components contributes to RCPS pathogenesis;

Furthermore, we explored previously published protocols to establish a simple method for studying early NCC development.

- iPSC-derived NCCs were efficiently generated in a manner that recapitulates developmental events involved in NCC specification and EMT from the NPB onward.

CHAPTER VII

ABSTRACT

Richieri-Costa-Pereira syndrome (RCPS) is a rare autosomal-recessive disorder characterized by craniofacial abnormalities, including mandible cleft, microstomia, Robin sequence and microcephaly, as well as limb defects and learning impairments. RCPS is mainly caused by an increased number of repeat motifs within the *EIF4A3* 5'UTR, which in turn leads to decreased expression of the gene product. The DEAD-box RNA helicase eIF4A3 is a core component of the RNA-binding exon junction complex (EJC), which is involved in post-transcriptional events such as alternative splicing, nonsense-mediated mRNA decay (NMD), translation initiation and mRNA localization. The *EIF4A3* 5'UTR varies in both number and organization of three types of motifs between individuals. However, the origin of the RCPS-associated allelic pattern, as well as the functional effects of these motifs on *EIF4A3* expression, remain to be uncovered. Although a relationship between *EIF4A3* hypomorphic biallelic mutations and RCPS has been established, the pathogenetic mechanisms by which decreased levels of *EIF4A3* lead to craniofacial malformation are unknown. To address these gaps, we first characterized the variation in the *EIF4A3* 5'UTR at a populational level. This analysis demonstrated that this noncoding region displays a polymorphic nature and structural complexity presenting multiple patterns. The RCPS-associated allele patterns may have arisen from independent unequal crossing-over events between ancient alleles and can potentially emerge in any population containing alleles with the CGCA-20nt motif. Furthermore, there is a direct association between the number of motifs and *EIF4A3* expression, revealing a potential cis-acting regulatory mechanism for these motifs and suggesting that the 5'UTR structure plays a central role in phenotypic modulation. Next, we unraveled the cellular and molecular mechanisms responsible for RCPS using two complementary models, patient-derived induced pluripotent stem cells (iPSCs) and *EIF4A3* haploinsufficient mouse models. The craniofacial structures compromised in RCPS patients are suggestive of

disturbances in neural crest cells (NCCs), a transient cell population that originates at the neural plate border (NPB) in the developing embryo and gives rise to multiple cell types, generating most of the cranioskeleton. Accordingly, we differentiated iPSCs from RCPS patients and control individuals into NCCs and demonstrated that *EIF4A3* deficiency impairs NCC development, leading to defective migration, premature osteogenic and dysregulated chondrogenic differentiation of NCC mesenchymal derivatives. Besides pinpointing that impaired NCC migratory capacity is a key cellular dysfunction underlying RCPS pathogenesis, we provided evidence by transcriptome analyses that impairment to cell adhesion dynamics is implicated in this dysfunction and involves alteration of cell-extracellular matrix (ECM) interaction components. Additionally, we suggest that ribosome defects contribute to RCPS pathogenesis, since aberrant splicing as well as alterations at the proteomic level of ribosomal components were identified in patient-derived NCCs. Elucidating the pathogenetic mechanism underlying RCPS will also aid in clarifying the etiology of other craniofacial syndromes and how mutations in genes with basic and ubiquitous functions such as *EIF4A3* lead to specific phenotypes. Finally, we provided a simple protocol for quickly deriving human NCCs *in vitro* in a manner that recapitulates multiple stages from the early NPB onward. This method represents a promising approach to better understand human craniofacial development.

RESUMO

A síndrome Richieri-Costa-Pereira (SRCP) é uma doença autossômica-recessiva rara caracterizada por anomalias craniofaciais, incluindo fissura de mandíbula, microstomia, sequência Robin e microcefalia, assim como malformação dos membros e dificuldade de aprendizado. A SRCP é principalmente causada por um aumento no número de motivos repetitivos na região 5' não traduzida (5'UTR) do gene *EIF4A3*, o que por sua vez leva à diminuição da expressão do produto gênico. A DEAD-box RNA helicase eIF4A3 é um dos principais componentes do complexo de junção de éxons (EJC) que se liga ao RNA e está envolvido em eventos pós-transcricionais incluindo *splicing* alternativo, decaimento de RNAm mediado por mutações sem sentido (NMD), iniciação da tradução e localização de RNAm. A 5'UTR do gene *EIF4A3* varia em número e organização de três tipos de motivos entre indivíduos. Entretanto, a origem do padrão alélico associado à SRCP assim como o efeito funcional desses motivos na expressão de *EIF4A3* continua a ser descoberto. Embora a relação entre mutações bialélilcas hipomórficas em *EIF4A3* e SRCP foi estabelecida, os mecanismos patogênicos pelos quais a diminuição de *EIF4A3* leva a malformação craniofacial são desconhecidos. Para responder essas questões, nós primeiramente caracterizamos a 5'UTR do *EIF4A3* em nível populacional. Essa análise demonstrou que essa região não codificante apresenta uma natureza polimórfica e estruturalmente complexa, exibindo múltiplos padrões. O padrão alélico associado à SRCP parece ter surgido a partir de eventos independentes de *crossing-over* desigual entre alelos ancestrais e pode potencialmente surgir em qualquer população contendo alelos com o motivo CGCA-20nt. Além disso, existe uma associação direta entre o número de motivos e a expressão de *EIF4A3*, revelando um potencial mecanismo regulatório de ação cis para esses motivos e sugerindo que a estrutura 5'UTR exerce um papel central na modulação fenotípica. Em seguida, nós revelamos os mecanismos celulares e moleculares responsáveis pela SRCP usando dois modelos complementares, células-tronco pluripotentes induzidas (iPSCs) de pacientes e controles e camundongos haploinsuficientes para *EIF4A3*. As estruturas craniofaciais comprometidas nos pacientes acometidos pela SRCP são sugestivas de distúrbios em células de crista neural (NCCs), uma população de células transiente

que se origina a partir da borda da placa neural (NPB) no embrião em desenvolvimento e dá origem a múltiplos tipos celulares, gerando a maior parte do esqueleto craniano. Então, nós diferenciamos iPSCs de pacientes acometidos pela SRCP e indivíduos controles em NCCs e demonstramos que deficiência de *EIF4A3* prejudica o desenvolvimento de NCCs, levando à migração defeituosa, diferenciação osteogênica prematura e condrogênica desregulada dos derivados mesenquimais de NCCs. Além de apontar que a capacidade de migração prejudicada em NCCs é uma disfunção chave na patogênese de RCPS, nós providenciamos evidência por meio de análise de transcriptoma que o comprometimento da dinâmica de adesão celular está implicado nessa disfunção e envolve componentes de interação das células com a matriz extracelular (ECM). Ademais, nós sugerimos que defeitos nos ribossomos contribuem para a patogênese da SRCP, desde que *splicing* aberrante assim como alteração nos níveis proteicos de componentes ribossomais foram identificados em NCCs derivadas de pacientes. Elucidando os mecanismos patogênicos envolvidos na SRCP também auxilia no entendimento da etiologia de outras síndromes craniofaciais e de como mutações em genes de função básica e ubíqua como o *EIF4A3* leva a fenótipos específicos. Finalmente, nós desenvolvemos um protocolo simples para a rápida geração de NCCs *in vitro* de uma maneira que recapitule os múltiplos estágios do desenvolvimento das NCCs desde a NPB. Esse método representa uma abordagem promissora para o melhor entendimento do desenvolvimento craniofacial humano.