

CONFERENCE REPORT

The Society for Craniofacial Genetics and Developmental Biology 45th Annual Meeting

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Funding information

American Association for Dental, Oral, and Craniofacial Research; American Association for Anatomy; Developmental Dynamics; Illumina

Abstract

The Society for Craniofacial Genetics and Developmental Biology (SCGDB) held its 45th Annual Meeting at the Sanford Consortium for Regenerative Medicine at the University of California, San Diego on October 20th–21st, 2022. The meeting included presentation of the SCGDB Distinguished Scientists in Craniofacial Research Awards to Drs. Ralph Marcucio and Loydie Jerome-Majewska and four scientific sessions that highlighted new discoveries in signaling in craniofacial development, genomics of craniofacial development, human genetics of craniofacial development and translational and regenerative approaches in craniofacial biology. The meeting also included workshops on analysis of single cell RNA sequencing datasets and using human sequencing data from the Gabriella Miller Kids First Pediatric Research Program. There were 110 faculty and trainees in attendance that represent a diverse group of researchers from all career stages in the fields of developmental biology and genetics. The meeting, which also included outdoor poster presentations, provided opportunities for participant interactions and discussions, thus strengthening the SCGDB community.

KEYWORDS

craniofacial, birth differences, development, face, genomics, stem cells

We celebrated the return to an in-person format for the 45th Annual Meeting of the Society for Craniofacial Genetics and Developmental Biology at the Sanford Consortium for Regenerative Medicine in La Jolla, CA. There, we honored leaders in the field with Distinguished Scientist Award presentations, bolstering the scientific program with specialized workshops and lectures from leading investigators and trainees in craniofacial research. Below follows a brief summary of key points and topics discussed at this meeting of the Society.

1 | PROFESSIONAL DEVELOPMENT WORKSHOPS

Chair: Dr. David Clouthier, SCGDB President (University of Colorado Anschutz Medical Campus)

Dr. Nils Lindstrom (University of Southern California) presented a workshop entitled “Single Cell RNA Sequencing Workshop.”

Dr. Lindström, an Assistant Professor at the University of Southern California, started the SCGDB meeting with a workshop focused on basic principles and important technical considerations for creating and analyzing single cell omics datasets. First, Dr. Lindström discussed strategies and techniques to optimize cell dissociation for single cell RNA-seq, which must be adapted to the tissue type and cells of interest. He then reviewed the technology of commercially available fluidic and microwell platforms used to capture single cells and label them with unique barcodes, discussing the benefits and drawbacks of each. Finally, he went stepwise through how to employ the R package *Seurat* to reveal meaningful patterns of expression in groups of cells using a single cell RNA-seq dataset his lab generated from the mouse kidney. The annotated R script and data from Dr. Lindström were made available to the attendees so that they could walk through the analysis independently.

Dr. David Higgins (Children's Hospital of Pennsylvania) presented a workshop entitled "Datasets and Platforms to Support Clinical and Genomic Craniofacial Research."

Dr. Higgins, an Informatics Program Manager at Children's Hospital of Pennsylvania (CHOP), next presented a workshop on accessing and using craniofacial datasets in the Gabriella Miller Kids First Dataset Resource Center at CHOP. The goal of the Dataset Resource Center (DRC) is to work collaboratively with investigators to understand the genetic causes of childhood cancers and structural birth differences. Children with both chromosomal and nonchromosomal birth differences are 11.6x and 2.5x, respectively, more likely to develop pediatric cancers. The DRC works with clinicians to pair high-quality genomic datasets with harmonized clinical data and then make these available to secondary researchers. Numerous data sets are available from both pediatric cancer and structural birth difference studies through the Kids First Dataset Resource Portal (portal.kidsfirstdrc.org). Thus far, the DRC has released 56,212 files from 12,738 participants, which is greater than 1.5 PB of data. Also, an investigator's own data can take advantage of the DRC's bioinformatic pipelines, including alignment and GATK HaplotypeCaller, trio-based joint genotyping and somatic variant calls. This data can then be merged with existing datasets, using the DRC's primary pipeline CAVATICA (cavatica.sbggenomics.com), with individual codes for the data pipeline available on GitHub. An example of querying the Dataset Resource Center was given. Information on accessing KidsFirst data was also provided, as was subsequent analysis of data using R Studio.

2 | SCGDB DISTINGUISHED SCIENTISTS IN CRANIOFACIAL RESEARCH AWARDS

Chair: Dr. David Clouthier, SCGDB President (University of Colorado Anschutz Medical Campus)

The **David Bixler Distinguished Scientist Award** is named in honor of the achievements of the Society's first President, Dr. David Bixler, a pioneer in genotyping craniofacial syndromes. This award recognizes a senior scientist who has provided long-term distinguished leadership to the SCGDB and has made meritorious

contributions to the field of craniofacial sciences. The recipient of this year's award was Dr. Ralph Marcucio, Professor of Orthopedic Surgery at University of California, San Francisco (UCSF). Dr. Marcucio earned his Ph.D. in Animal Science and Reproductive Physiology at Cornell University in the laboratory of Dr. W. Bruce Currie. He then completed a postdoctoral fellowship in the lab of renowned developmental biologist Dr. Drew Noden at Cornell University, followed by a second postdoctoral fellowship in the lab of Dr. Jill Helms at UCSF, where he and his colleagues discovered the Frontal Nasal Ectodermal Zone (FEZ), a signaling center induced by the forebrain that controls patterning and growth of the face. In 2003, Dr. Marcucio joined the faculty at USCF and launched a research program studying the mechanisms that regulate craniofacial shape using innovative approaches in mouse genetics, avian embryology, morphometric analyses, and genomics. His lab has also made important contributions to the understanding of cellular mechanisms that underlie bone fracture repair. Dr. Marcucio has made significant contributions to SCGDB by serving as Vice President, President and Immediate Past President. Dr. Marcucio was instrumental in negotiating the affiliate agreement between SCGDB and the American Association for Anatomy, which had strengthened the SCGDB's financial future, grown our membership, and increased the visibility of its members' research.

In his award presentation, Dr. Marcucio described many of the key experiments and findings from his career thus far and those trainees and collaborators who made these studies possible. Due to space, most of their names cannot be listed here. Dr. Marcucio first described how the FEZ was identified, and how an anti-Sonic hedgehog (SHH) antibody injected into the neural tube prevented activation of SHH in the telencephalic domain of the brain and the nearby surface ectoderm. The resulting embryos had significant hypoplasia of the upper jaw, due to reduced proliferation of the neural crest cell (NCC)-derived facial mesenchyme. While blocking SHH activity at stage 10 resulted in reduced outgrowth of the upper jaw, blocking at stage 18 did not. Further, outgrowth was rescued by placing a bead soaked in SHH in the neural tube. Knocking out mouse *Smoothed (Smo)* in NCCs resulted in upregulated *Shh* expression in the ectoderm, suggesting that the effect of forebrain SHH was direct and that NCCs might be acting as a sink. Blocking Fgf signaling induced NCC apoptosis and upper jaw hypoplasia, though injecting quail NCCs after blocking Fgf signaling rescued ectodermal *Shh* expression. Further, a gain-of-function experiment for increased SHH levels shifted the shape of the chick midface to resemble a mammalian midface, indicating that SHH levels are intimately involved in determining morphological outcomes.

To better assess morphology from a quantitative approach, Dr. Marcucio started a collaboration with Dr. Benedikt Hallgrímsson at the University of Calgary to employ geometric morphometrics to quantify changes in tissue morphology. Using morphometrics, they showed that the FEZ is highly correlative with the shape of the upper face. The FEZ is shaped differently in different species, so Dr. Diane Hu from the Marcucio lab transplanted the basal forebrain between chick and duck, which showed that the forebrain regulates the shape of the face and FEZ. This also led them to consider the variable

phenotype observed in holoprosencephaly, a common birth difference that ranges from minor hypotelorism and midfacial hypoplasia with a single incisor to cyclopia. Using a dose response experiment, they showed that varying levels of SHH signaling produced a nonlinear variation in facial width. Most phenotypic variation occurred in the steeper part of the curve between wild type embryos and homozygous mutants, explaining in part human variation.

To further examine this variation, his group examined a mouse *Fgf8* allelic series, and found that there was no shape change in heterozygous knockout mice. However, dropping FGF8 levels below 50% induced drastic changes in facial shape. Using RNA sequencing, they found that there were genes in the *Fgf8* allelic series that were anti-correlative with FGF8 levels, suggesting that they normally buffered phenotypic variation. Two of these are *Spry1* and *Fgf17*; their roles in regulating FGF8 levels are currently being examined.

Dr. Marcucio finished by describing a collaboration with Dr. Nathan Young at UCSF. In this work, they created a morphospace map to visualize amniote growth. Looking at principal components, they found that embryo midface principal components are initially quite variable, though come together at a time around with primary palate fusion. This suggests that there are only a few shapes that will allow primary palate fusion. After fusion, the principal components diverge again. The Marcucio lab is utilizing these findings to better understand cleft risk in humans. Further, the Marcucio lab in collaboration with Dr. Licia Selleri at UCSF, is now looking at chromatin changes in depth to understand how specific changes in chromatin accessibility lead to shape changes.

The **Marylou Buyse Distinguished Scientist Award** was created by the SCGDB to honor the memory of the Society's first female President Dr. Marylou Buyse, a prominent clinical geneticist. This award recognizes a mid-career scientist who has made important contributions to the craniofacial sciences. **Dr. Loydie Jerome-Majewska**, who is an Associate Professor (tenured) in the Department of Pediatrics at McGill University in Montreal, Canada was the 2022 recipient of the SCGDB Marylou Buyse Distinguished Scientist Award. Dr. Jerome-Majewska received her B.A. from Wesleyan University and Ph.D. with honors from Columbia University under the guidance of Dr. Virginia Papaioannou. After post-doctoral fellowships with Dr. Papaioannou and later Dr. Elizabeth Lacy, Dr. Jerome-Majewska started her independent career in 2005 as an Assistant Professor in the Department of Pediatrics at McGill University, where she was promoted to Associate Professor with tenure in 2014. Her lab uses mouse genetics to investigate the molecular and cellular bases of human developmental disorders, including those involving core components of the spliceosome that have been linked to craniofacial malformations in human patients. Her work has provided important novel insights into these pathologies. In addition to contributions to the SCGDB since 2017, she is currently co-Editor-in-Chief for *Differentiation*.

Dr. Jerome-Majewska's lab is interested in how disruption in gene splicing leads to a general class of developmental differences referred to as spliceosomopathies. Three of the syndromes her lab studies are cerebro-costo-mandibular syndrome (resulting from variants in *SNRPB*), Nager/Rodriguez syndrome (resulting from variants in

SF3B4), and mandibular facial dysostosis with microcephaly (resulting from variants in *EFTUD2*). These syndromes are characterized by differences that include micrognathia, malar hypoplasia, cleft palate and intellectual disability.

To determine how these genes result in facial differences, Dr. Jerome-Majewska's lab first examined the effect of *Eftud2* conditional inactivation in mouse NCCs using the *Wnt1-Cre2* driver strain. Conditional knockout (cko) mutant embryos developed phenotypes that ranged from minor hypoplasia of Meckel's and nasal cartilages to complete absence of these structures. The lab later showed that the phenotypes were due to apoptosis of NCCs after they had migrated away from the neural tube, resulting in fewer cells reaching the pharyngeal arches, which presumably resulted in disrupted craniofacial structures. Subsequent RNA-seq analysis of heads from embryonic day (E) 9.0 conditional knockout mutants found that there were many more genes upregulated than down regulated. Pathway analysis of disrupted genes showed that there was a strong association with the p53 pathway. The Jerome-Majewska lab then found that regulators of p53 were mis-spliced, including *Mdm2*, an inhibitor of p53, though there was not a splicing defect in p53 itself. In fact, p53 protein was stabilized in spliceosome mutants. Inhibition of p53 activity by Pifithrin- α improved craniofacial development in *Eftud2^{cko}* embryos. These changes correlated with reduced accumulation of p53 in *Eftud2^{cko}* embryos and a reduction in neural tube apoptosis. However, as Pifithrin- α treatment caused an increase in first arch apoptosis independent of p53, Dr. Jerome-Majewska used a genetic approach to remove p53 by crossing *Eftud2^{cko}* mutants with the *Tfp53* mutant mouse strain. In *Eftud2^{cko}/Tfp53^{-/-}* embryos, there was reduced cell death in the neural tube without an increase in cell death in the arches. However, this did not improve the observed craniofacial phenotypes in *Eftud2^{cko}* embryos. In contrast, homozygous deletion of p53 in *Snrpb^{cko}* embryos improved craniofacial development. The *Sf3b4^{cko};Tfp53^{-/-}* mutant experiments are ongoing.

One of the common findings in RNA-seq data between the three conditional knockout mouse embryos is an enrichment in genes with skipped introns, with pathway analysis pointing at DNA repair. Even for genes with retained introns, association with the DNA repair pathway is also observed. Overall, there appears to be an evolutionarily-conserved requirement for spliceosome genes in facial development, though changes to craniofacial structures are not solely due to p53 upregulation. The Jerome-Majewska lab is now investigating genes whose expression is disrupted in all three mouse mutants. Of particular interest are genes that are involved in cell-cell communication between NCCs and endodermal cells.

3 | SCIENTIFIC SESSION I: SIGNALING IN CRANIOFACIAL DEVELOPMENT

Chair: Dr. Matthew Harris (Harvard Medical School)

Dr. Thomas Schilling (University of California, Irvine) led off the main scientific program of the meeting with a discussion of his work examining the specification of polarity and growth in the development

of the skeleton. The formation of size and proportion, and thus function of skeletal elements, rely on directional growth of skeletal primordia. Key regulators of this process are part of a conserved feedback network found associated with human craniofacial disorders, such as Robinow syndrome as well as natural variation, suggesting this is a core and important property of normal craniofacial development. Dr. Shilling detailed new data from his lab on zebrafish with defects in maternally and zygotic *ror2* that present with distinct craniofacial and cartilage phenotypes. These phenotypes are similar to those observed in *wnt5b* zebrafish mutants, indicating a genetic pathway affecting cartilage stacking and correct formation of the skeleton. Dr. Schilling then described how polarization signaling regulates growth within chondrogenic bones, focusing on specification of growth zones, both at epiphyses and synchondroses. His lab found that a parathyroid-like hormone paralogue, *pthlha*, is necessary and sufficient to delimitate early growth zones marked by *entp5a* (a diphosphohydrolase). These data lead to a model for understanding how polarity is established in the forming skeletal element regulating size and function. To extend understanding of growth zone and functional implications, Dr. Schilling then outlined experimental approaches his lab has taken to use natural variation to understand changes in postembryonic shaping of the craniofacial skeleton. Genetic association studies between two Lake Malawi Cichlid fish lineages differing in jaw length and shape having distinct ecological feeding specializations showed linkage to retinoid acid receptor *g* (*rarg*). Differences in morphology of the jaw are reflected in the different size or extent of growth zones in the suspensorium of the jaw. They found that *rarg* is expressed in forming cartilage of the jaw, suggesting its potential role in patterning these structures. Interestingly, they found that reduction or elimination of *rarg* in developing zebrafish lead to defects in synchondroses of the adult jaw, including elimination of entire growth zones of the ceratohyal bones. Using these findings to understand the regulation of signaling systems that establishing polarity and growth zones in different areas of the craniofacial complex may reveal modularity and evolvability of the skull. Such modular function may also help explain disease etiology.

Dr. Katherine Fantauzzo (University of Colorado Anschutz Medical Campus) presented her work on signaling from the tyrosine kinase receptor, PDGFR, an essential regulator of palate development and whose disruption leads to clefting in Kosaki and Pentinnen syndromes. Her work has shown that PDGFR α and PDGFR β have major roles in NCC migration and proliferation, respectively. As the two receptors can interact with similar subsets of signaling molecules, are structurally similar and can hetero- or homo-dimerize, it is unclear what establishes the specificity of these different receptor orthologues. Her lab has addressed these questions by generating cell lines stably expressing C-terminal fusions of either PDGFR α or β with bimolecular fluorescence complementation (BiFC) fragments corresponding to the N-terminal or C-terminal regions of the Venus fluorescent protein. They found that PDGFR β receptors homodimerize more quickly than PDGFR α receptors upon PDGF ligand treatment. To assess activation, they measured autophosphorylation and found that PDGFR β receptors have increased levels of autophosphorylation.

Examining cellular localization, they found that PDGFR α homodimers are trafficked more quickly than PDGFR β homodimers and are degraded more quickly. In contrast, PDGFR β homodimers were more likely to be recycled to the cell membrane and led to increased cell proliferation and migration as compared to PDGFR α homodimers. Ongoing work is examining the function of PDGFR α/β heterodimers in cell culture in addition to leveraging new mouse lines encoding BiFC receptors to examine how receptor choice impacts NCC behavior and craniofacial development.

Dr. Nadege Gougnard (New York University) then presented her work on unexpected roles of a matrix metalloprotease (MMP) in modulating transcriptional regulation of epithelial-to-mesenchymal transition (EMT). MMPs are large family of extracellular proteases that are traditionally associated with the processing and remodeling of the extracellular matrix in cancer and development. Yet, a majority of MMP substrates are nonmatrix molecules and the “moonlighting” activities for MMPs are not well understood. The focus of Dr. Gougnard work has been on MMP28, a relatively understudied secreted MMP family member that regulates EMT and the invasion capacity of cancer cells. MMP28 expression was previously found at the neural plate border, suggesting it may be involved in NCC development. Using *Xenopus* embryos, Dr. Gougnard showed that MMP28 is not expressed in NCC, but rather in cranial placodes. Interestingly, while morpholino knockdown of MMP28 had limited impact on cranial placode development, the expression of EMT genes in neighboring NCCs was reduced. Using NCC explant assays, Dr. Gougnard showed that MMP28 knockdown impaired NCC delamination and migration. To determine how MMP28 expression in the cranial placode influences NCC development, she transplanted wild type donor NCCs into host embryos overexpressing MMP28-GFP and found MMP28-GFP in the nucleus of the donor NCCs. She went on to show that both nuclear localization and proteolytic activity of MMP28 in NCCs is necessary to rescue EMT gene expression following MMP28 knockdown. Some MMPs act as transcription factors or regulate transcription factor complexes. To determine if MMP28 directly regulates transcription of EMT genes in NCCs, Dr. Gougnard performed ChIP-qPCR and found that MMP28 occupies the promoters of *Sox9* and *Twist*. Together, this work demonstrates that MMP28, secreted by the cranial placode cells, is endocytosed by NCCs where it undergoes nuclear translocation and activates EMT gene expression programs to promote NCC delamination and migration.

In the last talk of the signaling session, **Kuo-Chang Tseng** (University of Southern California) reported on his detailed work on the development history of cranial neural crest lineages using transcriptional profiling in zebrafish, focusing on the diversity of NCC-derived connective tissues. Using a specific *sox10* enhancer, NCCs were isolated over the life time of NCC development in the zebrafish head, with their transcriptional and epigenetic signature assessed by single cell multiome technologies. This analysis revealed 23 NCC-derived connective tissue subtypes and allowed the identification of multiple connective tissue-specific enhancers, including hyaline cartilage, gill cartilage and dermal fibroblast enhancers. Instead of validating enhancers one by one in transgenic lines, Kuo-Chang used a “back

mapping technique” to trace connective tissue populations to embryonic stages. Interestingly, this approach indicates that some cells are primed at the epigenetic level to become cartilage as early as 1.5 days post fertilization (dpf). This is also the case for the dermal fibroblast population, which can be traced to the same time point, though in a nonoverlapping pattern suggesting that these cells have a distinct origination from the cartilage population. In contrast, the gill cartilage population, which is distinct from the other two populations at 3 dpf, appears mingled with the cartilage population at 1.5 dpf, indicating that they may have a closer origination. The goal is now to run the same analysis for the remaining 20 connective tissue subtypes identified. This method has the potential to map out multipotency levels and trace the extent of progressive epigenetic maturation of cranial NCCs. To complement this analysis, they are using lineage and spatial tracking methodologies such as scGESTALT and intMEMOIR to look at the generation of lineage-specific differentiation and potency in cranial NCC development.

4 | SCIENTIFIC SESSION II: GENOMICS OF CRANIOFACIAL DEVELOPMENT

Chair: Dr. Jean-Pierre Saint-Jeannet, SCGDB Treasurer (New York University)

The second scientific session of the meeting focused on genomics of craniofacial development. The plenary speaker, **Dr. Yang Chai** (University of Southern California) started the session by presenting work from his laboratory investigating the process underlying cell fate determination of post-migratory NCCs in the first pharyngeal arch. Using scRNA-seq analysis, the Chai laboratory uncovered 13 distinct NCC cluster domains in the embryonic day (E) 10.5 mouse first pharyngeal arch, each contributing over time (E12.5 to E14.5) to specific lineages of the developing mandible, through a process of fate bifurcation. This integrated analysis predicts that cells progressively separate into common progenitors and mesenchymal cells, with common progenitors subsequently undergoing further bifurcation and restriction into osteogenic/odontogenic and chondrogenic/fibroblast lineages. This prediction was confirmed in vivo by cell lineage analyses and perturbation experiments. For example, *Gbx2* is specifically expressed in the proximal domain of the first pharyngeal arch at E10.5. Transgenic animals expressing the *Gbx2CreER;tdTomato* reporter indicate that over time GBX2+ cells split into two domains moving into the oral and aboral directions, and several days later GBX2-derived cells contribute to all the major lineages of the mandible. Disruption of this proximal region of the first pharyngeal arch by knocking out *Alk5* in NCCs results in patterning defects and specific mandible and tooth malformations, validating the binary cell fate restriction process.

In the second part of his presentation, Dr. Chai summarized recent work interrogating cranial NCC-lineage diversification and cellular function during molar morphogenesis. Again, using scRNA-seq analysis from tooth dissected at critical developmental stages, they identified a previously unappreciated heterogeneity within the cranial

NCC-derived dental mesenchyme during molar development and characterized the molecular signature of the dental mesenchyme cellular domains and their contribution to distinct molar mesenchyme-derived tissues over time. Collectively, the single-cell transcriptomic atlases generated by Dr. Chai's lab provide novel insights into the cell fate diversification process of the cranial NCC during craniofacial and tooth morphogenesis.

The next speaker, **Dr. Licia Selleri** (University of California San Francisco) discussed recent work from her laboratory focusing on the regulatory program driving variation of midfacial outgrowth in evolution and disease. In mammals, the main variable driving species-specific midfacial length is the maxilla, while in avians, it is the premaxilla. To characterize this regulatory program, the Selleri lab performed a comparative RNA-seq profiling of dissected premaxilla, maxilla and posterior palatine/soft palates in embryos from three different species: chick, mouse and pig. In this analysis, pig and mouse maxilla transcriptomes were completely separated from the avian transcriptome. Furthermore, the gene most differentially expressed in the mammalian maxilla was *Zfhx4* (Zinc Finger Homeobox 4), a largely uncharacterized transcription factor. While *Zfhx4* is expressed meridially along the palatal shelf of mouse embryos at E13.5 and the palatal shelf of pig embryos at day 30, it is not expressed in the palatal shelves of avians. Mice with CRISPR/Cas9-mediated loss of *ZFH4* function induces cleft palate and a shorter maxilla, phenocopying the avian midface that presents with a short maxilla and cleft palate in physiological conditions. Strikingly, children with pathogenic variants in *ZFH4* exhibit cleft palate and other craniofacial abnormalities. While the search for a murine *Zfhx4* palatal enhancer was challenging, after testing over 20 regulatory elements, Dr. Selleri's lab was able to identify two enhancers showing palatal expression in transgenic animals. Interestingly, these enhancers are highly conserved between mouse and chick, though the two *Zfhx4* chick enhancer elements failed to show activity in the mouse secondary palate, suggesting that this enhancer is decommissioned in avians. These findings establish the regulatory landscape of midfacial outgrowth variation that has occurred in evolution and identify *ZFH4* as an important regulator of maxillary outgrowth and palate fusion in mammals.

Next, **Dr. Chan Hee Mok** (University of California San Francisco) presented her work on the role of the PBX family of transcription factors in the control of sonic hedgehog (*SHH*) expression in the FEZ in chicken embryos. The FEZ is an important signaling center regulating morphogenesis of the frontonasal process (FNP), and *SHH* is one of the key molecular regulators required for FEZ formation and function. The spatial expression of *SHH* in the chicken FEZ is differentially regulated by two members of the PBX family. While PBX1 enhances *SHH* expression, PBX3 represses this expression, consistent with their complementary expression pattern in the FEZ at Hamburger-Hamilton (HH) stage 22. To understand how PBX1 and PBX3 interacts with the *SHH* locus to differentially regulate its expression, Dr. Mok generated ATAC-seq and ChIP-seq data from the chicken FEZ at HH22 to identify both open chromatin configurations and PBX1/PBX3 binding regions. PBX1- and PBX3-differentially bound sequences at the *SHH* locus spanned ATAC-seq peaks, indicating that they are both associated with open chromatin

regions. One 400 bp PBX1-differentially bound sequence at the *SHH* locus was identified within intron two. The enhancer activity of this region was confirmed by electroporation of a 1.8 kb sequence spanning the 400 bp domain driving *lacZ* in the chick FNP ectoderm at stage HH20. The top two PBX1 motifs analyzed by MEME-ChIP were identified within this PBX1 enriched peak, with GO analysis indicating that these motifs are closely related to DNA binding and transcription regulation. Future work will evaluate poised and active enhancers at the *SHH* locus by analyzing histone mark (acetylation and methylation) to gain further insights into the PBX/SHH-dependent molecular regulatory mechanisms driving FEZ and craniofacial development.

For the last talk of the session, **Mathi Thiruppathy** (University of Southern California) discussed experiments investigating the diversity of cranial NCC-derived cartilage subtypes. Based on location and developmental trajectory, cartilages display a broad range of morphologies and mechanical properties. While hyaline cartilage is abundant throughout the body, elastic cartilage is more craniofacially restricted, found for example in the external ears and larynx of the mammalian head. The regulatory factors driving the divergence of hyaline versus elastic cartilage are largely unknown. Through scRNA-seq analysis of zebrafish cranial NCCs, Mathi identified two separate cell populations enriched for the cartilage-specific genes, *col2a* and *acan*, as well as differentially expressed genes such as the zebrafish orthologs for cartilage associated matrix proteins *ucmaa* and *ucmab*. In situ hybridization confirmed that *ucmab* is enriched in hyaline cartilage, whereas the *ucmaa* is restricted to a specialized elastic cartilage in the flexible gill filaments. Interestingly, zebrafish gill elastic cartilage share important phenotypic features with mammalian “elastic” cartilage of the outer ear, suggesting that may have a conserved regulatory program. To test this possibility, Mathi generated single-cell multiome (ATAC-seq and RNA-seq) datasets from human fetal outer ear (elastic) and nose (hyaline) cartilage and found that human elastic and hyaline cartilages were transcriptomically distinct as observed in zebrafish. Cross-species analyses indicate that zebrafish gill and human ear cartilage are mapping as transcriptomic homologs, with correlation scores comparable to that seen for zebrafish and human hyaline cartilage. Finally, Mathi reported the identification of six ear-specific human enhancers that drive reporter activity in zebrafish gill elastic cartilage, and three nose-specific enhancers active in hyaline cartilage of the zebrafish jaw. Altogether these findings support a conserved cis-regulatory networks driving elastic versus hyaline cartilage identity in human and zebrafish and establish zebrafish gill filament cartilage as a model to study elastic cartilage formation.

5 | SCIENTIFIC SESSION III: HUMAN GENETICS OF CRANIOFACIAL DEVELOPMENT

Chair: Dr. Rolf Stottmann, SCGDB Secretary (Nationwide Children's Hospital)

Dr. Elizabeth Leslie (Emory University) was the invited plenary speaker for this session devoted to human genetics and craniofacial

development. Dr. Leslie gave a presentation on using genome-wide association studies (GWAS) and genomic sequence to elucidate the genetic architecture of human orofacial clefting. It is quite clear that both genetic and environmental influences such as maternal nutrition play a role in clefting etiology. Mendelian forms of clefting are primarily dominant and nonsyndromic/isolated clefting is thought to be polygenic or multifactorial. Phenotypic subgroups include cleft lip, cleft palate and cleft lip and palate. The search for genetic causes of clefting has been going on for centuries and in the last 10 years, multiple GWAS have been performed and identified 40–50 loci identified with cleft lip and palate and less than five with cleft palate. While these obviously will enrich for single nucleotide polymorphisms in craniofacial regulatory elements, the identification of the associated causal genes is ongoing work. Rare variants (allele frequencies less than 0.1% in the population) of large effect are the next challenge and are currently only accessible through whole genome sequencing. The Leslie lab has been analyzing approximately 2000 trios with genome sequence available through the Gabriella Miller Kids First sequencing project. They have assembled a list of approximately 500 genes from a series of existing gene panels. Interestingly, only 40 of these risk genes are common to all panels. The diagnostic yield in pathogenic variants from the genes on this list was about 9% and varied significantly between cleft lip, cleft palate and cleft lip/palate. Incomplete penetrance was another common finding from this study. These findings together suggest that the genetics of clefting will continue to follow somewhat complex patterns of inheritance with incomplete penetrance. These challenges will require further accompanying validation experiments to truly assign pathogenicity.

Dr. Andre Luiz Pasqua Tavares (George Washington University) discussed *Six1* and *Six1*-associated genes in craniofacial development and disease with a focus on autosomal dominant Branchio-oto-renal Spectrum Disorders (BOR). *SIX1* is a transcription factor required for proper development of several craniofacial developments. 50% of individuals with BOR harbor variants in *SIX1* or the cofactor *EYA1*. To understand the cause of the other 50% of BOR variants, the Tavares lab studied other potential *SIX1* co-factors. First, they studied three factors from those previously discussed in the literature: *Pa2g4*, *Mcrs1* and *Sobp*. All three are expressed in mouse developing craniofacial tissues and biochemical analysis confirmed *MCRS1* and *SOBP* as bona-fide cofactors through co-immunoprecipitation. Co-expression along with *EYA1* and *SIX1* showed these are primarily acting as co-repressors. Expression analysis showed that *SOBP* is always nuclear while *MCRS1* moves from the cytosol to the nucleus upon *Six1* co-expression. Analysis in *Six1* mutant craniofacial tissues shows reduced levels of *Sobp*. Future work will examine genome sequence from BOR patients for variants in these newly verified *SIX1* cofactors. A second line of work is identifying potential novel causative genes to *SIX1*-associated birth differences such as BOR cases that are characterized by structural differences of the outer and inner ear, branchial fistulas or cysts, and hearing loss. Expression and RNA-seq analyses of pharyngeal arches from *Six1* mutants as compared to wild-type embryos identified new potential *SIX1* cofactors and 808 differentially regulated genes. Clustering and gene ontology analyses revealed that many of these genes are associated with translation, NCC cell

differentiation, and osteoblast and cartilage development and therefore could contribute to the structural differences observed in BOR patients.

Dr. Rebekah Charney (University of California, Riverside) gave a talk on the *ZEB2* gene which regulates NCC formation and is affected in Mowat-Wilson syndrome, a neurocristopathy. *ZEB2* is one of the earliest factors expressed when human induced pluripotent stem cells are differentiated into the NCC lineage and continues to be expressed at high levels. The *Zeb2* expression was validated in chicken embryos where it was similarly expressed from early developmental stages and remained expressed through NCC development. Mowat-Wilson syndrome patients with variants in *ZEB2* have a range of phenotypes in the central nervous system and NCC-derived tissues, resulting in developmental delay and craniofacial differences. The better understand the role of *ZEB2* in these phenotypes, a siRNA knock-down of *ZEB2* in NCCs was performed as well as CRISPR-CAS9 mediated-mutation in the *ZEB2* gene. RNA-Seq analysis of *ZEB2* knock-down cells consistently showed downregulation of NCC specifiers, known signaling ligands and genes associated with epithelial-mesenchymal transition and cellular migration. These changes are consistent with *ZEB2* acting as a transcriptional repressor. Parallel experiments were formed in a cell line in which the first 112 amino acids of *ZEB2* were deleted, which resulted in impaired differentiation of osteoblasts and Schwann cells. This truncation prevents proper association with HDAC1 and leads to derepressed chromatin. ATAC-Seq experiments highlighted 69 differentially expressed peaks in common at two stages of NCC development. Motif analysis showed several *ZEB2* binding motifs suggesting at least some of these are directly regulated by *ZEB2*. As *ZEB2* is known to directly regulate BMP signaling, BMP inhibition was tested in this system and indeed showed rescue of some of the gene expression changes in the *ZEB2* mutant cells. Future efforts will include further exploration of this interaction with the BMP pathway and how other *ZEB2* variants or truncations affect the NCC lineage.

The session was concluded by a presentation from **Azeez Alade** (University of Iowa) on a new two-stage analysis of GWAS in African nonsyndromic orofacial clefting cohorts. While a significant proportion of current research is focused on a European population, an analysis of a different cohort may present easily identifiable new variants. GWAS represent an unbiased approach to identify novel association in large cohorts. Some of the associations have been de-emphasized in potentially overly conservative statistical analyses. One approach to overcome this limitation is a two-stage analysis to promote potentially causal single nucleotide polymorphisms (SNPs) which are classified as suggestive. The approach is to study suggestive SNPs in an independent cohort and then combine the analyses in a meta-analysis. Indeed, the replication cohort analysis identified three SNPs that met the statistical significance threshold. Meta-analysis identified the same three loci. One locus was close to the *DACH1* locus. A haplotype analysis of the African population data identified this as inside an enhancer region and is will thus be prioritized for further biological validation. Another significant SNP was near *PTCH1* (again in an enhancer) which has previously been implicated in cleft palate-only etiology. Future

work will focus on characterizing the effects of these SNPs using a human fetal oral epithelial cell line.

6 | SCIENTIFIC SESSION IV: TRANSLATIONAL AND REGENERATIVE APPROACHES IN CRANIOFACIAL BIOLOGY

Chair: Dr. Amy Merrill, SCGDB Vice-President (University of Southern California)

The final session of the meeting began with a plenary talk by **Dr. Sarah Knox** (University of California, San Francisco), who discussed the key role of nerves during development and regeneration of salivary gland acini. These acini, which produce and secrete saliva, are commonly destroyed during radiation therapy for head and neck cancer, leading to a lifetime of dry mouth and a subsequent increase in the incidence of caries. To develop approaches to regenerate salivary gland acini, the Knox lab is applying lessons learned from development. During salivary gland development, acinar progenitors derived from the oral epithelium undergo branching morphogenesis and become enveloped in the axons of parasympathetic nerves. The Knox lab found that denervation disrupts acinar development, suggesting that nerves play an instructional role. Single cell transcriptomic analysis suggests that nerve-epithelial interactions are mediated by reciprocal expression of the EGF receptor *ErbB3* in acinar progenitors and the EGF ligand *Nrg1* in the nerve. In support of this idea, they demonstrated that acinar cell specification requires *ErbB3* in the oral epithelium and in the absence of nerves, this specification can be rescued by NRG1. Dr. Knox also showed that NRG1-mediated stimulation of *ErbB3* expression in acinar cells activates the mTORC target phospho-Akt to promote acinar formation and lineage progression. Together this work demonstrates that nerves promote acini development through a novel NRG1-ERBB3-mTORC signaling pathway.

In the second half of her talk, Dr. Knox discussed the critical role for parasympathetic nerves in maintenance and regeneration of the adult salivary gland. In humans, denervated salivary glands undergo atrophy. The Knox lab has provided evidence that nerves promote adult salivary gland maintenance by sustaining SOX2+ progenitors that replenishment salivary acinar cells. Dr. Knox hypothesized that SOX2+ cells, which are enriched for muscarinic receptors, rely on nerve-derived acetylcholine. Correspondingly, she demonstrated that synthetic acetylcholine muscarinic receptor agonist cevimeline increases SOX2+ proliferation and lineage contribution to the acini. Next, her group tested the ability of cevimeline to regenerate salivary gland acini following radiation-induced damage in mice. They found that peri-glandular injection of the cevimeline 14 days after radiation promotes acinar cell proliferation and saliva secretion, although the response was not maintained. After honing the frequency and timetable of the treatment, they found that a peri-glandular injection of cevimeline followed by weekly injections into the salivary gland maintained acinar cells and their innervation for 3 months after radiation. Finally, Dr. Knox described that injecting pilocarpine, a broader muscarinic agonist, four times per week results in a profound

functional regeneration of acinar cells 30 days after radiation-induced damage. Currently, the Knox lab is testing the effectiveness of muscarinic stimulation to regenerate acinar cells and maintain their nerve supply after radiation-induced damage in large animal models.

Dr. James Nichols (University of Colorado Anschutz Medical Campus), the next speaker of the session, presented his lab's work on the role of *her* genes in osteoblast differentiation and craniofacial bone patterning. By employing zebrafish models that allow for real time imaging of bone formation, the Nichols lab studies mechanisms that spatially regulate bone formation during craniofacial development. ScRNA-seq analysis of cranial NCCs showed that NCC-derived progenitors that give rise to the dermal bones of the gill chamber, including the opercle and branchiostegal rays, were enriched for the Notch ligand *jag1b* and canonical Notch target *her9*. Interestingly, Jag-Notch signaling regulates craniofacial bone patterning, with the *her9* human ortholog *HES4* associated with craniofacial differences. Dr. Nichols showed that zebrafish harboring a premature stop codon in *her9* completely fail to form mineralized bone in the head despite normal cartilage formation. A closer look revealed “shadow bones,” where osteoblast-derived bone templates were normal in shape but remained unmineralized. He next provided evidence that loss of *her9* does not impact osteoblast maturation and instead functions nonautonomously in cells surrounding the developing bones to confer osteoid mineralized in the local microenvironment. This resembles the Stenciling Hypothesis proposed by McKee and Reznikov, which posits that all tissues contain ample mineral ions for mineralization and the default state is general inhibition of mineralization through pyrophosphate production. In areas where mineralized osteoid is needed, pyrophosphate is selectively degraded by alkaline phosphatase enzymes. Dr. Nichols predicts that *her9* sits atop this stenciling cascade, possibly through local regulation of alkaline phosphatase, to allow for osteoid matrix mineralization. Persistent osteoid seen in the *her9* mutant fish has also been observed naturally in the opercle bone of select sculpin species, suggesting this may also be a mechanism to generate phenotypic diversity in craniofacial bone shape.

Next, **Dr. Xiaolei Zhao** (University of Texas Health Science Center at Houston), presented her work on the role of YAP and TAZ in promoting osteogenesis in NCCs. HIPPO-YAP/TAZ signaling plays an important role in NCC development, with YAP1 variants associated with human craniofacial differences, including orofacial clefts. To test the role of HIPPO-YAP/TAZ signaling on cell fates decisions in NCCs, Dr. Zhao performed a knockdown of *Yap/Taz* in the mouse NCC line O9-1 and found that loss of YAP and TAZ upregulates chondrogenic genes and biased their differentiation into chondrocytes over osteoblasts. Loss of *Yap/Taz* in NCCs had a similar effect in mice, where NCC-derived bones in the calvaria failed to undergo osteogenesis and were coincident with ectopic cartilage formation. This indicates that YAP/TAZ promotes osteoblast over chondrocyte cell fate in NCC-derived skeletal progenitors YAP/TAZ interacts with the transcription factor TEAD to regulate gene expression. To reveal YAP/TAZ-mediated gene regulation during NCC differentiation, Dr. Zhao performed a combination of RNA-seq and Cut&Run-seq. She found that YAP directly regulates genes in osteogenesis, chondrogenesis, and

canonical WNT signaling. Motif analysis of open chromatin regions in an ATAC-seq dataset from human NCCs found that 40% of genes regulated by YAP/TAZ are enriched for both TEAD and TCF/LEF motifs, many of which co-localized to shared chromatin regions. For example, *Runx2* and *Sox9* genes contain the TEAD and TCF motif in the same open chromatin region. Dr. Zhao went on to provide evidence that YAP/TAZ works together with canonical WNT signaling to promote osteogenesis and prevent chondrogenesis in NCCs. In O9-1 cells, β -catenin and YAP/TAZ interact and co-localize in the nucleus during osteoblast differentiation. In addition, knockdown of *Yap/Taz* and *Ctnnb1* (encoding β -catenin) increases *Sox9* expression compared to knockdown of *Yap/Taz* alone. Together this work demonstrates that YAP/TAZ and WNT signaling cooperate to promoting osteogenesis in NCCs by preventing chondrogenesis.

The final talk of the session was presented by **Brittany Hufft-Martinez** (University of Kansas Medical Center). Brittany discussed her work on the role of *SPECC1L*, a cytoskeletal protein involved in microtubule stability and actin reorganization, in palate development. Pathogenic variants of *SPECC1L* that cause cleft palate are localized to either the microtubule interacting coil-coil domain (CCD2) or actin interacting calponin homology domain (CHD). While *Specc1l*-null mice do not develop cleft palate, mice homozygous for in-frame deletion of the CCD2 domain (*Specc1l* Δ CCD2) have cleft palate with a penetrance of 50%. Brittany showed that *Specc1l* Δ CCD2 mutant and *Specc1l*-null palate mesenchyme cells have a disorganized actin cytoskeleton and shortened cilia. Since increased actin polymerization inhibits ciliogenesis, Brittany treated *Specc1l* null cells with the actin depolymerizer Latrunculin B and found cilia length was rescued. A closer look at ciliary abnormalities in *Specc1l* Δ CCD2 mutant cells indicated defects in retrograde transport. The Tran group has shown that loss of the intraflagellar transport gene *Thm1* results in shortened cilia and cleft palate. Brittany hypothesized that overlapping phenotypes in *Thm1* and *Specc1l* mutant mice could be explained by genetic interactions between these genes. To test this, she looked at an allelic series from *Thm1*/*Specc1l* mutant mice and found that loss of a single *Thm1* allele in a *Specc1l*-null background increased the penetrance of cleft palate from 0% to 45%. In addition, loss of a single *Thm1* allele in *Specc1l* Δ CCD2 heterozygous mice increased the penetrance of cleft palate from 15% to 35%. As folic acid supplementation has been shown to rescue NCC defects, Brittany treated pregnant mice with folic acid. She found that folic acid ameliorated cleft palate in *Specc1l* Δ CCD2 homozygous mice as well as in *Thm1*; *Specc1l* Δ CCD2 double heterozygous mice. Together, this study demonstrates that genetic interactions between *Specc1l* and *Thm1* regulate cilia length during palatogenesis, and that folic acid has the potential to ameliorate cleft palate associated with cytoskeletal dysfunction.

7 | POSTER SESSION

Organizer: Dr. Amy Merrill, SCGDB Vice-President (University of Southern California)

The first day of the meeting ended with a 2h poster session, in which faculty, post-docs and students presented their poster abstracts in an outdoor venue.

8 | AWARD WINNERS

Poster presentation awards sponsored by the American Association for Anatomy (AAA). Winners of the poster presentation awards, sponsored by AAA, were Drs. Yoon-Gu Jang (1st), Priyanka Kumari (2nd), and Arul Subramanian (3rd) in the post-doctoral fellow category, and Evan Brooks (1st), Brandon Chacon (2nd) and Audrey Nickle (3rd) in the graduate student trainee category.

Platform presentation awards sponsored by American Association of Dental, Oral and Craniofacial Research (AADOCR). Winners of the platform presentation awards, sponsored by AADOCR and which recognized impactful studies in craniofacial research, were Dr. Katherine Fantauzzo in the junior faculty category, Dr. Chan Hee Mok in the post-doctoral fellow category, and Mathi Thiruppathy in the graduate student trainee category.

9 | SUMMARY

It was important to see colleagues after the long pandemic break, with this scientific gathering of the SCGDB members serving as an important platform for disseminating exciting new work and discussion of new advances among colleagues. From the presented talks and posters, it is clear that the field of craniofacial genetics is expanding and entering into new areas of investigation. The excitement for the future of the field was clearly seen in the quality and clarity of talks from young investigators, showing a bright future for the research areas involving craniofacial genetics and development.

AUTHOR CONTRIBUTIONS

Rolf Stottmann, Matthew Harris, Jean-Pierre Saint Jeannet, Amy Merrill, and David Clouthier: Conceptualization, original draft writing, draft review and editing.

ACKNOWLEDGMENTS

This meeting was funded in part by donations from the American Association for Anatomy, The American Association for Dental, Oral and Craniofacial Research, Developmental Dynamics and Illumina. All named authors/presenters have agreed to the publication of the information contained in this manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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How to cite this article: Stottmann, R. W., Harris, M. P., Saint-Jeannet, J.-P., Merrill, A. E., & Clouthier, D. E. (2023). The Society for Craniofacial Genetics and Developmental Biology 45th Annual Meeting. *American Journal of Medical Genetics Part A*, 1–9. <https://doi.org/10.1002/ajmg.a.63206>