CONFERENCE REPORT

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The Society of Craniofacial Genetics and Developmental Biology 35th Annual Meeting

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We review some of the exciting recent discoveries in craniofacial genetics and developmental biology presented at the 35th annual meeting of the Society of Craniofacial Genetics and Developmental Biology (SCGDB). SCGDB, an international organization established in 1975, met in conjunction with the annual meeting of the American Society of Human Genetics in San Francisco in November 2012. The conference was hosted by the Program in Craniofacial and Mesenchymal Biology at the University of California, San Francisco (UCSF), with generous financial support from the UCSF School of Dentistry Dean's office. The conference was also supported by grants from the California Institute for Regenerative Medicine and from New England Biolabs.

The theme of this year's meeting was "Stem Cells in Craniofacial Development and Disease." Experts from diverse fields of science provided insights into the roles of epigenetics, stem cell biology and regenerative medicine in advancing the field of craniofacial genetics and development. Below, we present a brief meeting synopsis followed by the abstracts that were presented as either oral presentations or posters at the meeting.

The objectives of the Society are to promote understanding, research, and interdisciplinary communication in craniofacial genetics and developmental biology, and to apply the results of basic and clinical research to the care and management of individuals with craniofacial problems (http://craniofacialgenetics.org). As part of its mission, the Society strongly encourages the participation of students and young professional scientists. To encourage and reward excellence in science among trainees, cash awards were established for outstanding poster presentations to help defray the registration and travel costs to the meeting. This year's judges for the awards included: Ethylin Jabs (Mt. Sinai Medical Center); Mark Leid (Oregon State University); Eric Liao (Harvard Medical School); Ralph Marcucio (UCSF); and Amy Merrill (University of Southern California). Winners of the Undergraduate awards were Daniel Hunter and Michael Chung (both from Stanford University). Graduate student awards for excellence in science went to Kateryna Kyrylkova (Oregon State University), Gerson Kobayshi (University of Sao Paulo) and Vagan Mushegyan (UCSF). Postdoctoral awards for excellence in science were awarded to Jianxin Hu (UCSF), Pierre LePabic (UC Irvine), and Kerstin Seidel (UCSF).

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The morning began with a session focused on the genetic basis of human syndromes. The session underscored how human genetic studies have come to rely heavily on the integration of sophisticated cell biological and animal studies to dig deep into molecular mechanisms underlying congenital disease pathogenesis.

The first speaker was Dr. Simeon Boyd from UC Davis, who presented the results of a genome-wide association study that identified two loci for non-syndromic sagittal craniosynostosis. One of these is near the *BMP2* gene and was suggested to result in a gain of function of the BMP signaling pathway. The other is in an intronic region of the Bardet-Biedl syndrome (BBS) gene, *BBS9*, which encodes a component of the BBsome in the basal

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body involved with transport in the primary cilia, thereby suggesting involvement of primary cilia in the pathogenesis of craniosynostosis.

Dr. Amy Merrill from the University of Southern California presented the recent discovery of mutations in *FGFR2* in a congenital skeletal disease, bent bone dysplasia. These mutations prevent the proper membrane localization of the receptor, thereby decreasing its ability to respond to extracellular FGF. Interestingly, this mutant receptor retains localization to the nucleus and exhibits enhanced nuclear signaling, revealing a potential role for non-canonical modes of FGFR2 signaling in bone development and disease.

The third presentation was given by Dr. Matthew Warman from Harvard Medical School, who spoke about skeletal malformations caused by somatic mutations that occur early in development. Congenital lipomatous overgrowth with vascular, epidermal, and skeletal anomalies, or CLOVES, is a recently identified non-hereditary syndrome. Dr. Warman reported that CLOVES is caused by somatic mutations in the *PIK3CA* gene encoding the catalytic subunit of Phosphatidylinositol 3-kinase (PI3K). The identified mutations are predicted to result in hyper-activation of the PI3K pathway and are the same mutations that have been previously detected in cancer.

The final speaker of the early morning session was Dr. Ralph Marcucio from UCSF, who discussed mechanisms underlying genotype/phenotype correlation that involve the role of hedgehog signaling in face shape variation. These studies utilize geometric morphometric analysis together with experimental manipulation of the chick embryo. By manipulating Sonic Hedgehog (SHH) pathway activation, the chick embryonic face could be transformed to one that more resembled the mammalian shape. These studies have bearing on the molecular control of craniofacial morphogenesis by SHH signaling in development and congenital disease as well as on the evolution of craniofacial form.

The second morning session featured Dr. Michael Longaker from Stanford University, who brought a clinical perspective with his lecture about the utilization of stem cells in therapeutic applications. He discussed how such stem cells can be practically identified, how they can be isolated from patients, and how their power can be harnessed for repair of craniofacial and other defects. The morning sessions were followed by a luncheon and poster session.

The first afternoon session dealt with dental stem cells. Dr. Tom Diekwisch from the University of Illinois presented his studies on periodontal regeneration and tooth implantation using stem cells isolated from various components of extracted teeth. He showed that these neural crest-derived stem cells could differentiate in vitro into periodontal structures including osteoblasts, cementoblasts, and periodontal ligament cells. Furthermore, the replantation of periodontal stem cell-seeded tooth roots into rat alveolar bone sockets resulted in the complete formation of new periodontal ligament and stable reattachment of teeth. Such in vivo and in vitro studies underscore the potential utilization of stem cell-based therapies in dentistry.

Dr. Frederic Michon from the University of Helsinki demonstrated that *Sox2* marks dental epithelial stem cells that are required for mouse incisor renewal. *Sox2* was shown to be regulated by *Fgf8*, and both genes were regulated by miRNAs. Initial analysis of *Sox2*-deleted mice showed dramatic incisor development defects, and these defects are currently being further characterized. Dr. Michon's studies highlight the mouse incisor as an accessible, informative, and emerging model for the study of epithelial stem cells.

The second afternoon session began with Dr. Joanna Wysocka from Stanford University, who spoke about recent work on the regulation of neural crest cells by unique enhancer elements within genes essential for neural crest induction. Her lab identified these enhancers using neural crest cells differentiated from human embryonic stem cells in combination with a zebrafish model system to determine enhancer activity. Interestingly, not all putative enhancers discovered using the in vitro system were active in the in vivo model. From these studies, Dr. Wysocka proposed that allelic variants in these enhancer regions (SNPs) could affect transcription of genes involved in craniofacial development.

Dr. Tatiana Hochgreb from Caltech presented her work on the roles of *FoxD3* during early cranial neural crest formation and in development of melanophores in zebrafish. Using high-resolution time-lapse imaging, she uncovered three dynamic phases of precursor and migratory neural crest cell movements from the neural keel stage to times of active cell migration. Disruption of the planar cell polarity (PCP) pathway component strabismus alters this dynamic migratory behavior. In addition, Dr. Hochberg assessed early effects of FoxD3 loss-of-function on specification and morphogenesis of dorsal root ganglia, craniofacial skeleton, and melanophores.

Dr. Thomas Schilling from UC Irvine reported new findings on the role of Fat signaling in control of the PCP pathway during zebrafish cartilage development. Loss of Fat, Dachsous or Atrophin-orthologues in zebrafish results in similar skeletal abnormalities, including the shortening of some cartilages, fused joints, and chondrocyte stacking defects. Confocal imaging of Fat- or Dachsous-deficient prechondrocyte condensations revealed loss of stacking and polarity as well as delays in differentiation. In addition, chimeric analysis demonstrated that Fat is both necessary and sufficient to coordinate polarity and differentiation of cartilage in a non-cell autonomous manner. These results provide genetic evidence that skeletal morphogenesis and differentiation are controlled through a conserved Fat signaling pathway, a process that has not previously been associated with defects in skeletal tissue polarity.

Finally, the keynote talk was given by Dr. Gail Martin from UCSF. Dr. Martin provided a fascinating historical view on the winding road leading to the discovery of embryonic stem cells. The talk began with a review of early work done in the middle of the last century on teratocarcinomas and pluripotency. Dr. Martin then covered her seminal studies on the isolation of embryonic stem cells, a term that she coined, from mouse blastocysts. In the latter part of the talk, Dr. Martin discussed the isolation of human embryonic stem cells, which built on her own work and that of others. She concluded with discussions about future directions for the embryonic stem cell field as well as thoughts about potential clinical utilization of such cells in a variety of diseases.

The meeting was followed by a social (happy) hour which was attended by most participants. We are beginning to plan our meeting for 2013, which will take place on October 22 in Boston, MA. Look for details on the SCGDB website: http://craniofacialgenetics.org. We hope to see you there!

Abstracts of the 35th Annual Meeting of the Society of Craniofacial Genetics and Developmental Biology in San Francisco, CA on 06 November 2012

A genome-wide association study identifies susceptibility loci for non-syndromic sagittal craniosynostosis near bone morphogenic protein 2 (BMP2) and within Bardet-Biedl syndrome 9 (BBS9)

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Sagittal craniosynostosis is the most common form of craniosynostosis, affecting approximately 1 in 5,000 newborns. We conducted the first genome-wide association study (GWAS) for non-syndromic sagittal craniosynostosis (sNSC) using 130 non-Hispanic white (NHW) case–parent trios. Robust associations were observed in a 120 kb region downstream of BMP2, flanked by rs1884302 ($P = 1.13 \times 10^{-14}$; odds ratio [OR] = 4.58) and rs6140226 ($P = 3.40 \times 10^{-11}$; OR = 0.24) and within a 167 kb region of BBS9 between rs10262453 ($P = 1.61 \times 10^{-10}$; OR = 0.19) and rs17724206 ($P = 1.50 \times 10^{-8}$; OR = 0.22). We replicated the associations to both loci (rs1884302 [$P = 4.39 \times 10^{-31}$]; rs10262453 [$P = 3.50 \times 10^{-14}$]) in an independent NHW population of 172 unrelated sNSC probands and 548 controls. Both BMP2 and BBS9 are genes with a role in skeletal development warranting functional studies to further understand the etiology of sNSC.

Bent bone dysplasia syndrome defines a nuclear role for FGFR2 in skeletal development

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During embryonic development, bone formation requires dynamic regulation of cell proliferation and differentiation. That Fibroblast Growth Factor (FGF) executes such control is made evident by the spectrum of congenital disorders caused by mutations in Fibroblast Growth Factor Receptors (FGFRs). We recently identified *FGFR2* mutations in a birth defect with skeletal abnormalities that are reflective of enhanced proliferation and reduced differentiation of osteoprogenitor cells. This disorder, designated bent bone Dysplasia Syndrome (BBDS), is characterized by deficient ossification of the skull and long bones despite an abundance of pre-osteogenic mesenchyme. Loss-of-function studies in mice have shown that FGFR2 simultaneously promotes osteoprogenitor proliferation and differentiation. Enhanced proliferation and reduced differentiation in BBDS suggests that the causal mutation uncouples the receptor's activities in these processes. The BBDS mutation p.Met391Arg resides in the transmembrane domain, prevents receptor incorporation into the plasma membrane, and consequently reduces receptor responsiveness to extracellular FGFs. Despite decreased canonical FGF signaling, FGFR2M391R retains nuclear localization where it exhibits enhanced responsiveness to intracellular FGF2. Nuclear localization of a receptor principally considered to act at the plasma membrane raises the possibility of noncanonical FGFR2 signaling. We found that FGFR2 acts in the nucleolus where it associates with FGF2 and the RNA polymerase I transcription factor UBF1. This activity is enhanced by the p.Met391Arg mutation, as FGFR2M391R is enriched in the nucleolus with UBF1 and elevates expression of the catalytic 45S pre-ribosomal RNA in the bones of BBDS patients. Together these data suggest that nuclear FGFR2 signaling promotes proliferation by augmenting the capacity of protein synthesis in osteoprogenitors.

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Learning from development: cellular, molecular, and topographical clues for periodontal tissue regeneration and tooth replantation

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The developmental history of tissues provides essential clues for the regeneration and engineering of new tissues. Here we have studied the developmental history of periodontal tissues from neural crest-derived progenitors and examined the natural surface topography environment of tooth root surfaces to successfully develop a strategy for periodontal regeneration and tooth replantation. The tissues that anchor teeth in jaws are derived from neural crest-origin intermediate progenitor cell populations. Intermediate progenitor populations play a crucial role in the regional specification and differentiation of the cranial neural crest. On the basis of global gene expression profiles, gene cohort expression levels, and epigenetic modifications, we have defined key factors involved in the differentiation of dental follicle intermediate progenitors into periodontal lineages, including alveolar bone osteoblasts, cementoblasts, and periodontal ligament cells. These studies indicate that in addition to changes in signature gene expression, unique shifts in gene cohort expression levels, epigenetic modifications, and changes in cell morphology contribute to the individuation of tissue populations from a common neural-crest-derived ancestor. We assessed the suitability of odontogenic progenitor populations for periodontal regeneration when exposed to natural and synthetic apatite surface topographies. We demonstrated that periodontal ligament (PDL) progenitors feature higher levels of periostin and scleraxis expression, increased adipogenic and osteogenic differentiation potential, and pronounced elongated cell shapes on barren root chips when compared with dental pulp and dental follicle cells. When evaluating the effect of surface characteristics on PDL progenitors, natural root surfaces resulted in elongated PDL cell shapes, whereas PDL progenitors on synthetic apatite surfaces were rounded or polygonal. PDL progenitors seeded on natural tooth root surfaces in organ culture formed new periodontal fibers after 3 weeks of culture. Finally, replantation of PDL progenitor-seeded tooth roots into rat alveolar bone sockets resulted in the complete formation of a new PDL and stable reattachment of teeth over a 6-month period. Together, these findings indicate that periodontal

progenitor cell type as well as mineral surface topography and molecular environment play crucial roles in the regeneration of true periodontal anchorage.

Molecular regulation of tooth formation, replacement and renewal

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The embryonic formation and adult homeostasis of organs is of interest to scientists at the molecular, cellular, and evolutionary levels. The continuously growing front tooth (incisor) of the mouse serves as a valuable model to approach these issues. The incisor model system allows reciprocal epithelial/mesenchymal interactions during organogenesis to be elucidated. Moreover, the mechanisms controlling the continuous expansion, migration and differentiation of incisor stem cells can be evaluated in order to develop regenerative strategies based on dental stem cell biology. In order to develop such protocols, we analyzed the genetic network involved in stem cell segregation during tooth development and their maintenance in adult incisor renewal. We revealed the importance of Sox2 during incisor formation and the role of Sox2+ cells in incisor renewal. Moreover, the discovery of fine-tuning of Sox2 and Fgf8 by miRNAs brings further insights into how differentiation of dental epithelial stem cells is controlled. Because "Nothing in biology makes sense except in the light of evolution," we are currently studying the conservation of several genetic networks, such as Wnt, Eda, Sox2, and miRNAs, in species that replace their teeth and but do not renew them.

A novel FoxD3 gene trap line reveals neural crest precursor movement and a role for FoxD3 in their specification

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Neural crest cells migrate extensively and contribute to diverse derivatives, including the craniofacial skeleton, peripheral neurons and glia, and pigment cells. Although several transgenic lines label neural crest subpopulations, few are suited for studying early events in neural crest development. Here, we present a zebrafish gene/protein trap line gt(foxd3-citrine)ct110a that expresses a Citrine fusion protein with FoxD3, a transcription factor expressed in premigratory and migrating neural crest cells. In this novel line, citrine expression exactly parallels endogenous foxd3 expression. High-resolution time-lapse imaging reveals three dynamic phases of precursor and migratory neural crest cell movements from the neural keel stage to times of active cell migration. Disruption of the PCP pathway component strabismus alters this dynamic migratory behavior. In addition, Cre-recombination produces a variant line FoxD3-mCherry-pA; homozygosity of this allele generates a FoxD3 null mutant. Taking advantage of the endogenously regulated expression of FoxD3-mCherry fusion protein, we directly assess early effects of FoxD3 loss-of-function on specification and morphogenesis of dorsal root ganglia, craniofacial skeleton, and melanophores. Whereas there is an initial decrease in number of melanoblasts, later the numbers of melanophores increase at the expense of the DRGs and iridophores. Taken together, our results reveal previously unknown roles for FoxD3 during early cranial neural crest formation and in development of melanophores in zebrafish. These novel lines provide new insights into neural crest specification and migration.

Three-dimensional morphometric analysis of the hypoplastic mandible

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Pierre Robin sequence and Treacher Collins syndrome are both associated with mandibular hypoplasia. However, it has been hypothesized that the mandible may be differentially affected. The purpose of this study was therefore to compare mandibular morphology in children with Pierre Robin sequence to children with Treacher Collins syndrome using three-dimensional analysis of CT scans. A retrospective analysis was performed identifying children with Pierre Robin sequence and Treacher Collins syndrome receiving CT scans. Three-dimensional reconstruction was performed and the following measurements were taken: ramus height, mandibular body length, and gonial angle. These were then compared with the clinical norm corrected for age and sex based on previously published measurements. Three children with Pierre Robin sequence (six hemi-mandibles), four children with Treacher Collins syndrome (eight hemi-mandibles), and two control children (four hemi-mandibles) were identified. The mean age for patients with Pierre Robin sequence was 10.61 years. The mean age in the Treacher Collins syndrome group was 12.07 years. The mean age in the control group was 10.74 years. In our study, mandibular body length was significantly shorted for children with Pierre Robin sequence while ramus height was significantly shorter for children with Treacher Collins syndrome. This resulted in distinctly different ramus height/mandibular body length ratios. In addition, the gonial angle was more obtuse in both the Pierre Robin sequence and Treacher Collins syndrome groups compared with the controls. Three-dimensional morphometric analysis of mandibles in patients with Pierre Robin sequence and Treacher Collins syndrome revealed distinctly different patterns of mandibular hypoplasia relative to normal controls. These findings underscore distinct considerations that must be made in surgical planning for reconstruction.

Regeneration of the dental pulp via amplification of an endogenous Wnt signal

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Adult tissues contain stem cells, which are activated by injury and contribute to the repair of damaged tissues. Dental pulp is no different, but the extent of stem cell activation following injury is unknown. Using Axin2LacZ/+ reporter mice, we identified odontoblasts as being responsive to an endogenous Wnt signal. When the tooth is injured, the dental pulp responds by upregulating Wnt target genes. We tested the consequences of amplifying endogenous Wnt signaling using Axin2LacZ/LacZ mice, where loss of both alleles of the negative Wnt regulator Axin2 leads to an enhanced Wnt responsiveness. In control Axin2LacZ/+ mice the injury site was filled with an inflammatory infiltrate that gradually resolved but resulted in a reduced repair/regenerative response. On post-surgery Day 7 the injury site in Axin2LacZ/+ mice was filled with fibroblastic cells and some blood vessels. By Day 14 the appearance of the injury site had not changed appreciably. Axin2LacZ/LacZ mice, however, exhibited a dramatically different response: on post-surgery Day 7, in addition to fibroblastic cells, the cells lining the injury site had achieved osteoblast-like morphology. By Day 14 the injury site in Axin2LacZ/LacZ mice was filled with a newly formed mineralized tissue. This tissue was localized exclusively at the area of irritation, and had irregular, reduced dentinal tubule architecture. The mineralized tissue stained positive for collagen type I, and thus constituted reparative dentin. The improved regenerative response can be attributed to increased endogenous Wnt signaling and suggests that potential exogenous amplification of the pathway may have therapeutic applications.

Knockdown of *eif4a3* gene results in craniofacial development disorder

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Richieri-Costa-Pereira Syndrome (RCPS) is a rare acrofacial dysostosis characterized by Robin sequence, cleft mandible, short stature, and limb defects and is inherited in an autosomal recessive fashion. Recently, our group identified a homozygous mutation in *EIF4A3* (Eukariotic Initiation Factor 4A3) in RCPS patients as a putative molecular cause underlying this disorder (Favaro et al., ASHG 2012). Our objective was to knockdown *eif4a3* expression in zebrafish embryos in order to phenocopy RCPS and study the malformation of craniofacial structures during embryonic development. We injected zebrafish embryos at the two-cells stage with an *eif4a3* translation-blocking morpholino. Development was observed at 4, 15, 24, and 48 hr post-fertilization (hpf) and 5 days post-fertilization (dpf). For a deeper phenotype characterization, whole-mount RNA in situ hybridization for *myoD*, and *col2a1* expression were performed. Cartilage and bone formation were analyzed by alcian blue and calcein. Acridine orange staining was used to assess apoptosis. At 24 hpf, ~71% of embryos treated with *eif4a3* translation-blocking morpholino presented severely affected as well as dysmorphic craniofacial structures. At 48 hpf, embryos were smaller in size than controls and also presented craniofacial abnormalities. Our results suggest that *eif4a3* embryonic expression is essential for normal craniofacial development. We conclude *eif4a3* knockdown in zebrafish appears to be a good model for future research in Richieri-Costa-Pereira Syndrome.

JNK phosphorylation in periosteum derived fibroblasts harboring the FGFR2 S252W mutation (Apert Syndrome)

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Apert syndrome is characterized by premature fusion of the coronal sutures (craniosynostosis) and symmetric syndactyly of the hands and feet. It is caused by two mutations in the *FGFR2* gene (S252W and P253R). It has been shown that one of the main differentially expressed genes in Apert syndrome fibroblasts is *DUSP2*, whose protein is a MAPK phosphatase. In this study, we aim to evaluate the role of JNK in the augmented osteogenic differentiation potential of fibroblasts from the periosteum. We first assessed the difference in JNK phosphorylation levels between $FGFR2^{S252W}$ fibroblasts and controls, but found no statistically significant differences. However, when we compared the JNK expression in starving and non-starving conditions, starvation increased JNK expression significantly more in S252W cells than in control cells. We then evaluated whether activation of FGFR2 by FGF2 treatment altered JNK phosphorylation. FGF2 treatment increased JNK phosphorylation in S252W fibroblasts by 25% and decreased phosphorylated JNK in controls by 15%. Lastly we assessed the effects of inhibiting JNK activity on osteogenic differentiation. Complete inhibition of JNK activity reduced the osteogenic differentiation levels in $FGFR2^{S252W}$ fibroblasts similar to control levels. In conclusion, we demonstrate that JNK is involved with the greater osteogenic potential in fibroblasts harboring the Apert syndrome mutation.

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Expansion of a repeat motif in the 5′ UTR of *EIF4A3* causes Richieri-Costa-Pereira syndrome (RCPS)

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RCPS (OMIM 268305) is an autosomal recessive acrofacial dysostosis comprising Robin sequence and is characterized by short stature, cleft mandible, and limb defects. Despite previous efforts, no causative genetic mechanism has yet been identified, leading us to try to identify the pathogenic mechanism of RCPS. We analyzed DNA samples of 24 affected individuals and their non-affected relatives, whenever possible (n = 52). We conducted linkage analysis using SNP microarray 50 K in eight affected individuals and in three sibs. This resulted in identification of a homozygous candidate region at 17q25. Segregation analysis with markers mapped at this region in the 72 individuals supported linkage and the most likely location would be between rs2289534 and rs3829612, spanning 128.5 kb. Exome sequencing (ES) did not reveal any obvious pathogenic mutation. Sanger sequencing of 5′ UTR of the *EIF4A3* gene, a CG rich region mapped in this region, revealed 16 repeats of a 20 nucleotide (nt) region in homozygosity among the affected individuals that was absent in the 52 controls. We verified that this motif is transcribed in one of the two *EIF4A3* isoforms and through qRT PCR, whose expression level was very low ($P = 0.006$). Homozygous mutations in *EIF4A3* are very likely the cause of RCPS, which is involved in splicing processes of many types of RNA. This would add RCPS to the list of craniofacial syndromes caused by mutations in genes involved in RNA processing and translation. We propose that the absence of *EIF4A3* interferes in important molecular events during early development. The zebrafish model will be used to confirm a role of this gene in craniofacial development.

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Sinking our teeth into Costello syndrome: RAS signaling regulates enamel deposition in humans and mice

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Receptor tyrosine kinase (RTK) signaling pathways are known to play a central role in tooth development. A group of syndromes, the RASopathies are caused by gain-of-function mutations in the RTK downstream effector Ras/mitogen-activated protein kinase (MAPK) pathway. One of these syndromes, Costello syndrome (CS), is caused by a heterozygous, de novo germline mutation in HRAS that results in a constitutively active Ras protein. CS is characterized by a wide range of cardiac, musculoskeletal, dermatological, and functional abnormalities. In order to further understand the role of Ras signaling in tooth development, we performed craniofacial and dental exams on a total of 46 patients with CS at the CS International Family Conferences in 2009 and 2011. Exams included intra- and extraoral photographs, clinical examinations, X-ray reviews, alginate impressions and exfoliated CS teeth collection. Our dental exams revealed that patients with CS display malocclusion, delayed tooth development, and delayed eruption. Interestingly, 88% of individuals with CS presented with an enamel defect characterized clinically by generalized white spots and striations. Micro computed tomography (microCT) of exfoliated primary teeth from patients with CS showed a significant decrease in enamel thickness compared to control, and scanning electron microscopy (SEM) revealed that the enamel of these individuals contained patchy demineralized zones. Next, we analyzed a CS mouse model expressing HRASG12V and found an enamel defect. Further inspection revealed disorganized enamel structure with demineralized zones. In addition, ameloblast progenitor cells were hyperproliferative in the cervical loop and ameloblasts were disorganized and expressed decreased levels of enamel matrix proteins. Currently, we are analyzing HRASG12V mice treated with inhibitors at multiple points along the Ras effector pathways in order to determine through which pathway Ras is affecting ameloblast proliferation and differentiation and enamel formation.

Genetic basis of size and shape variation in the neurocranium localized using quantitative trait locus mapping in a model baboon population

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When compared to those of primates and other mammals, the human skull demonstrates a reorganization that has resulted in a large brain case, flat face, and flexed base. These trends have been instrumental in human evolution, are documented in the fossil record, and are likely interdependent, although the nature of their relationships remains unclear. Genetic and developmental work has been conducted on craniofacial dysmorphologies but relatively little is known about the genetic underpinnings of normal variation. This research investigates the relationship between variation in cranial capacity and neurocranial size and shape and identifies regions of the genome (quantitative trait loci, QTL) that are responsible for significant portions of the variation in these traits. A collection of crania (N = 985) was prepared from the colony of

pedigreed baboons housed at the Southwest National Primate Research Center. Cranial capacity was measured from reconstructions of sequential CT scan slices. Linear interlandmark distances were calculated from 3D coordinates captured from the skulls using a Microscribe MX digitizer. The relationship between these datasets was investigated using partial regression analysis to determine which measures of neurocranial form significantly contribute to cranial capacity variation. Quantitative genetic analysis of these data using a variance components approach indicates that cranial capacity is significantly heritable ($h^2 = 0.68 \pm 0.08$ SE) in this population. QTL accounting for cranial capacity variation were identified via whole genome linkage mapping using 331 microsatellite markers. Positional candidate genes within these QTL will be assessed in future studies for their relevance to craniofacial and brain size evolution.

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Mirror the asymmetry: role of BCL11B in epithelial stem cell niche and ameloblast development of the mouse incisor

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The mouse incisor is an asymmetric organ that is characterized by the presence of enamel on the outer (labial), but not the inner (lingual), surface of the tooth. Furthermore, the mouse incisor grows continuously throughout the life of the animal, owing to the presence of epithelial and mesenchymal stem cells that provide a continuous supply of enamel-producing ameloblasts and dentin-producing odontoblasts, respectively. The epithelial stem cells (EpSCs) reside in the structures called cervical loops (CLs) at the posterior end of the incisor. EpSCs in the labial CL give rise to transit-amplifying cells that migrate anteriorly along the epithelium of the incisor while differentiating into mature ameloblasts. In contrast, the lingual CL is presumed to contain a smaller EpSC niche, which does not normally give rise to the ameloblasts. A complex network of fibroblast growth factors (FGFs) and transforming growth factors β (TGFβs) regulate proliferation and differentiation of EpSCs. BCL11B is a transcription factor that plays essential roles in the development and function of the immune, central nervous, and cutaneous systems and is required for perinatal survival. We show that BCL11B is essential for epithelial proliferation, growth, and shape of the mouse incisor. BCL11B controls asymmetric development of the labial and lingual EpSC niches by regulating the expression of FGF and TGFβ signaling members and their antagonists. Subsequently, BCL11B contributes to the proper formation, differentiation, and localization of the ameloblasts.

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A role for dysregulation of DNA damage response in the aetiology of cleft lip/palate

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Non-syndromic cleft lip/palate (NSCL/P) is a multifactorial disease that arises from errors during embryonic development. Since tight regulation of ontogenetic mechanisms is required for appropriate orofacial morphogenesis, our objective was to identify pathways involved in the pathogenesis of NSCL/P. We performed transcriptome profiling of seven dental pulp stem cell cultures from NSCL/P patients and six controls using Affymetrix HuGene 1.0 ST chips. Differentially expressed genes were obtained using SAM and RankProd algorithms. Functional annotation and network analysis were performed using Ingenuity Pathways Analysis, while gene clustering and transcription factor enrichment procedures were carried out using EXPANDER. H_2O_2-induced DNA damage was assessed with flow cytometry for anti-γH2AX in six NSCL/P, two van der Woude syndrome (*IRF6*-haploinsufficient), and seven control stem cell cultures. Finally, RNA in situ hybridization for *Brca1*, *Rad51*, and *E2f1* was carried out on developing CD1 mouse embryos. In NSCL/P cells. We identified a dysregulated network associated with cell cycle progression and response to DNA damage and its co-expression patterns suggested *E2F1* as an upstream regulator. Quantification of γ-H2AX confirmed that the NSCL/P cells exhibited abnormal response to DNA damage, which was also observed in *IRF6*-haploinsufficient cells. Finally, in situ hybridization studies further support our findings, revealing co-localised expression of *Brca1*, *Rad51*, and *E2f1* in the facial primordia and palatal shelves of mouse embryos. Our results suggest that impairment DNA damage response may be involved in the etiology of NSCL/P and indicate that *IRF6* may be functionally relevant in this mechanism.

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RIPK4 mutations explain a portion of IRF6-negative popliteal pterygium cases

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Van der Woude syndrome (VWS) and popliteal pterygium syndrome (PPS) are two allelic, dominant clefting disorders caused by mutations in IRF6. However, 30% of VWS and 3% of PPS patients are mutation-negative. There is substantial clinical overlap between PPS and the recessive pterygia syndrome Bartsocas-Papas syndrome, which is caused by mutations in *RIPK4*. Irf6−/− and Ripk4−/− mice have nearly identical phenotypes and both genes are targets of p63. Given these data, we hypothesized that mutations

in *RIPK4* could explain a portion of mutation-negative PPS or VWS patients. We sequenced *RIPK4* in 27 VWS and 3 PPS patients and identified novel homozygous mutations in 2 PPS patients. The first, A448P, was present in a patient with bilateral cleft lip and palate (CLP), oral synechiae, bilateral popliteal pterygia, and syndactyly. This patient, from consanguineous parents, also had a dorsal-ventral patterning defect of the hands and feet. The second mutation, R618H, was found in a patient with bilateral CLP, lip pits, oral synechiae, and popliteal pterygium from healthy, non-consanguineous parents. R618H was carried by the mother but not by the father and no deletion was detected. SNP genotyping demonstrated maternal isodisomy of chromosome 21, where *RIPK4* is located. Our results show *RIPK4* mutations may explain a significant portion of PPS cases that are *IRF6* mutation-negative and have important implications for genetic counseling given the differing patterns of inheritance for PPS caused by *IRF6* or *RIPK4*. It remains possible that non-coding mutations in *RIPK4* or other members of this pathway could explain the remaining causes of VWS.

Comparative genomics and evolution of dental morphology in mammals

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Mammalian dental morphology is under strong evolutionary pressure because of its impact on mastication and diet. While the mechanisms underlying tooth development have been widely studied in model organisms, the role of genes in patterning of the different elements of the occlusal surface is not fully elucidated. Previous studies showed that *Fibroblast Growth Factor* (*Fgf*) genes are central regulators of tooth development and influence occlusal morphology variation. We hypothesize that the interspecfic variation of murid dental morphology is governed by the variation of the regulatory genetic code, which works through orchestration of the variable spatio-temporal expression of the FGF signaling molecules involved in tooth development. To test this, we used morphometric and functional genomic assays. We characterized the differences in dental morphology among 15 clades of rodents utilizing 55 morphologic character states. Using comparative genomic analyses, we identified six highly conserved non-coding regions (ECRs) in Fgf3, 8, 9, and 10 in *Mus*. Further analysis of ECRs revealed the presence of transcription factor (TF)-binding sites for Runx1, Lef-1, and Pax-9. Preliminary results confirmed TF expression in developing teeth of mouse embryos at E14.5. To identify the relationship between genetic variation in the *Fgf* regulatory genome and dental morphology, we are currently testing the correlation between the variation of ECR haplotypes and individual dental character states using in vivo and in vitro minimal promoter assays.

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Additional patients and an association study support role of SOX9 in CD-ACD-PRS phenotypic continuum and in CPO

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SOX9 has an essential role in chondrogenesis. Nonsense mutations and deletions of *SOX9* suggested that haploinsufficiency underlies campomelic dysplasia (CD), a rare autosomal dominant disease characterized by campomelia, skeletal defects and Pierre Robin sequence (PRS). Translocations in the 350 kb region upstream or downstream of SOX9 were reported in less severe CD patients. Some translocations and deletions further upstream have been identified in patients with acampomelic campomelic dysplasia (ACD) and PRS, suggesting that these three syndromes form a continuum of phenotypes. We report the identification of a translocation 600 kb upstream of *SOX9* in a patient with classical features of ACD. In parallel, we identified a deletion in a PRS family that overlaps a deletion reported in a patient with the same phenotype. These data and the literature led us to test whether or not there is a genetic association between the *SOX9* locus and cleft palate (CP) and/or PRS. We used three SNPs in a cohort of case-parent trios analyzed using TDT. While two SNPs, tagging a conserved region upstream of SOX9, were not associated, significant over-transmission of an intragenic SNP was detected in the combined CP + PRS cohort ($P = 0.026$) and PRS cohort ($P = 0.042$), with a relative risk of 0.54 under a dominant model. The association has been replicated in an independent Italian CP-cohort. Our data are in agreement with the hypothesis that removal or disruption of cis-regulatory elements upstream of SOX9 can lead to phenotypes of gradual severity. We show for the first time an association of SOX9 with CP and PRS.

A novel *GNAI3* gain-of-function mutation associated with Auriculocondylar Syndrome in a Brazilian family

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Auriculocondylar syndrome (ACS), mainly characterized by micrognathia, microtia, and mandibular condyle alterations, is an autosomal dominant disorder. We have mapped ACS to a large candidate region at 1p21.1–q23.3 (Masotti et al., 2008). Recently, mutations in two genes, *GNAI3* and *PLCB4* were found in ACS. Therefore, our objective in this present study is to elucidate the mutational mechanism in the Brazilian family linked to 1p21.1–q23.3 (ACS1). We performed exome sequencing in two ACS1 affected individuals (Illumina). Sanger sequencing was used to validate our findings. Given that *DLX5* and *DLX6* expression was decreased in mandibular osteoblasts of ACS patients (Rieder et al., 2012), we verified if expression is altered in mesenchymal stem cell (MSC) of an ACS1 patient. As cartilage is compromised in the ACS1 patients, we also analyzed in vitro MSC chondrogenic differentiation. The N269Y mutation was located in *GNAI3* segregating with the disease in the family and was found to be a conserved amino acid. GNAI3 protein structure modeling of specific amino acid substitution suggests that this N269Y mutation increases the stability of a natural ligand in a novel pocket, decreasing the reliance on the ligand. No significant difference was observed for *DLX5* and *DLX6* in MSCs and neither between patient and control during in vitro chondrogenesis. We conclude that the N269Y mutation in *GNAI3* is different from that previously described. Our findings suggest that it is a gain-of-function mutation and the cause of ACS1 in this Brazilian family. It seems that N269Y mutation does not primarily compromise cartilage differentiation.

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Evaluation of Microfabricated Topographical Cues on Self-renewal and Differentiation of Dental Epithelial Stem Cells

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Dental epithelial stem cells are important for continuous growth in the adult mouse incisor. Although previous studies have combined mesenchymal and epithelial stem cells to artificially recapitulate tooth development, little is known about the basic molecular makeup and minimal extracellular scaffolding necessary to maintain the epithelial stem cell population and induce differentiation. We have successfully isolated dental epithelial stem cells from the cervical loop of the mouse mandibular incisor. Cells were viable in a 2D system atop several different substrates with various results. Our results suggest that E-cadherin and Integrin alpha 6 are potential markers of cervical loop epithelial stem cells. We grew these cells in a 3D microenvironment and obtained spheres with epithelial morphology. Finally, we fabricated scaffolds using polydimethylsiloxane (PDMS) and polyacrylamide (PA) with and without microtopographical features in order to determine the effects of topography on the self-renewal and differentiation of murine dental epithelial stem cells. Insights into the molecular and physical mechanisms responsible for stem cell maintenance could prove beneficial for the successful generation of bioengineered teeth from adult stem cell populations.

Analysis of SPECC1L expression and function in zebrafish model of oblique (Tessier) facial cleft

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The genetic basis of the rare oblique facial cleft was unknown until the identification of SPECC1L from human translocation analysis. We studied SPECC1L homologs (specc1la, specc1lb, specc1) in zebrafish to elucidate the function of these novel genes in craniofacial development. Whole-mount in situ hybridization (WISH) revealed that all homologs were expressed in the craniofacial region during zebrafish development. Morpholino knockdown of specc1lb and specc1, but not specc1la, showed distinct craniofacial phenotypes. Knockdown of specc1lb resulted in bilateral clefts between the median and lateral parts of the palate, while specc1 knockdown led to a fused zebrafish palate. Lineage tracing analysis revealed that in specc1 knockdown embryos, the neural crest cells that normally contribute to the median part of the palate fail to migrate to their proper destination, whereas in specc1lb knockdown embryos, these cells fail to "fuse" with the cells that form the lateral part of the palate. Finally, analysis of neural crest markers revealed that the expression of frzb and bapx1 genes were downregulated in specc1lb morphants, implicating specc1lb to coordinate wnt and endothelin pathways in craniofacial development. Ongoing work is aimed at elucidating the biochemical relationship between specc1lb and its interacting proteins in the context of cranial neural crest morphogenesis.

Phenotypic variation of the cranium of an Apert mouse model expressing the *Fgfr2*^{S252W} mutation exclusively in neural crest

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Lineage mapping has shown that the skull is derived from neural crest (NC) and mesoderm. Holmes and Basilico (2012) used Cre/lox recombination to simultaneously induce expression of *Fgfr2*^{S252W} and *β-galactosidase* in cranial neural crest of the *Fgfr2*^{S252W} Apert mouse model (*NC*$^{S252W/+}$ mice). They reported the absence of coronal suture synostosis—a characteristic feature of Apert syndrome—in the *NC*$^{S252W/+}$ mice. Their findings additionally showed that the *NC*$^{S252W/+}$ mice had short faces at birth (P0) associated with premature fusion of facial sutures and retroflexion of the cranial base. Here, our objective is to identify the overall craniofacial shape variation and associated facial and neurocranial suture fusion patterns between the P0 *NC*$^{S252W/+}$ mice (N = 15) and their non-mutant littermates (N = 19). Images of the skull

were obtained by microCT and 3D coordinates of craniofacial landmarks were analyzed with geometric morphometrics. Our results show that skulls of $NC^{S252W/+}$ mice are distinct from their non-mutant littermates in both shape and size; the mutants being smaller. We focused exclusively on craniofacial shape variation by statistically removing size from analysis. Shape changes show that $NC^{S252W/+}$ mice have a shorter snout and a larger and wider cranium compared to the non-mutants. These shape differences are correlated with the number of prematurely fused cranial sutures and are not restricted to NC-derived bones. The $NC^{S252W/+}$ intra-group shape differences mainly involved cranial base angles with individuals from this group representing the largest and smallest magnitudes for this measure.

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Endothelin signaling regulates neural crest gene expression through a positive feedback mechanism involves MEF2C

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Neural crest cells are a transient, multipotent, migratory cell population that gives rise to multiple cell lineages. Endothelin-mediated cell–cell signaling is essential for the development of the neural crest lineage. Genetic ablation of components of the endothelin signaling system results in defects in craniofacial mesenchyme, melanocyte, and enteric neurons. However, the transcription program downstream of endothelin signaling remains unclear. In this study, we activated endothelin signaling in E9.5 neural crest cells in an embryo explant system with endothelin-1 and analyzed gene expression profile by RNA sequencing. We characterized ~230 novel transcription targets downstream of endothelin signaling in the developing neural crest lineage. Consistent with the proposed role of endothelin signaling, these targets genes are involved in cell adhesion, neuronal differentiation, cell morphology, and cell movement. Interestingly, a consensus MEF2 binding motif is enriched in the putative enhancer regions of these characterized endothelin target genes. Here we show that MEF2C is required for the activation of multiple endothelin target genes. Moreover, endothelin signaling regulates MEF2C by controlling both its expression and activity in the developing neural crest lineage. Our findings established MEF2C as a direct downstream effector of endothelin signaling pathway and suggest that endothelin signal regulates neural crest gene expression through a positive feedback mechanism.

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Systems nanopathology: interpretation of missense variants with all things mappable

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Deep sequencing experiments in search of etiologic mutations underlying craniofacial syndromes commonly yield many non-etiologic variants. For missense variants, protein structure mediates the relationship between genetics and physiology. To address this opportunity, we developed a suite of sequence and structural bioinformatic tools to predict the impact of a missense variant on the maintenance of physiologic function. We present the concept of mapping all known small molecule interactions to the entire human proteome through structural homology of models to crystallographic structures, to sort etiologic from permissive and non-etiologic but function-altering variants. We find that analyzing the physical path from mapped ligands through predictable functionally conserved inter-residue interactions consistently identifies missense variants that affect molecular switches crucial to function, in ways common to other missense variants linked to the same disease. We anticipate that further exploration of this pattern will lead to the first statistical metric of genotype–phenotype linkage involving biomolecular structural interactions, and that this deeper level of understanding will enable steps toward a complete wiring diagram for craniofacial development.

Non-muscle myosin function is required at multiple stages of mouse secondary palate development

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The secondary palate is formed through a multi-step morphogenesis that culminates with the fusion of the palatal shelves. Non-muscle myosins are strong candidate regulators of palate fusion since *myh9*, the gene encoding non-muscle myosin heavy chain type IIA (NMHCIIA) has been identified as associated with human cleft palate Together with essential (ELCs) and regulatory light chains (RLCs), the non-muscle myosin heavy chains constitute non-muscle myosin II (NMII) that can bind actin and regulate a broad array of cellular functions including cell adhesion and migration. The expression of NMHCIIA is elevated in the MEE during palate fusion supporting its potential roles in this process. NMHCIIB shows moderate expression in palate mesenchyme implicating its roles in palate elevation and/or outgrowth. We found that pharmacological inhibition of NMII function in palate explant culture impairs the ability of the palatal shelves to fuse. Small interfering RNA (siRNA) against *myh9* significantly reduced NMHCIIA expression in palate culture and inhibited the fusion process, suggesting that *myh9* specifically has a role in this process. Mice carrying a point mutation (R709C) in the motor domain of NMHCIIB showed defects in the elevation of the palatal shelves resulting in cleft palate. These results strongly implicate NMHCIIA and IIB function in different aspects (fusion and elevation) of palate development. These findings may have particular significance to human orofacial clefting, since four independent human genetics studies have demonstrated linkage of non-syndromic cleft lip with or without cleft palate (CL/P) to the *myh9* gene. This work was supported by grant R03DE022818 from the NIH/NIDCR.

Nitric oxide signaling and histone acetylation coordinate cranial neural crest patterning, differentiation and convergence during craniofacial morphogenesis

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Chemical genetic screening in the zebrafish embryo is a powerful approach to interrogate developmental processes and disease. Here, we report the application of chemical genetics toward the study of cranial neural crest cell (CNCC) and craniofacial morphogenesis. Nitric oxide inhibitor 1-[2-(trifluoromethyl)phenyl] imidazole (TRIM) was identified from a craniofacial chemical screen to induce ectopic pharyngeal skeletal structures. Whole mount RNA in situ hybridization and lineage tracing analysis show that TRIM treatment down-regulated the expression of early CNCC marker *dlx2a* and *sox9a*, abrogated first pharyngeal arch structures, and promoted a progenitor cranial neural crest cell fate in the posterior segments. TRIM treatment also disrupted midline convergence of CNCC. TRIM's effect on CNCC is mediated through inhibition of NO signaling without appreciable effect on global protein S-nitrosylation. Instead, TRIM perturbed *hox* gene patterning and promoted histone hypoacetylation. Rescue of TRIM phenotype was achieved with NO donor, inhibitor of histone deacetylase, and over-expression of histone acetyltransferase *moz* mRNA. These studies demonstrate that NO signaling and histone acetylation are coordinated mechanisms that fine tune CNCC patterning, differentiation and convergence during craniofacial morphogenesis.

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Fat-Dachsous signaling coordinates polarity and differentiation of the craniofacial skeleton in zebrafish

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Little is known about the mechanisms of cell–cell communication necessary to assemble skeletal elements of appropriate size and shape. Skeletal progenitors may behave as coherent units by communicating via the planar cell polarity (PCP) pathway. In Drosophila, two sets of factors control PCP independently: the Fat and the non-canonical Wnt signaling systems. While a requirement for components of the non-canonical Wnt system was recently demonstrated in regulating the oriented divisions and intercalations of chondrocytes in the growth plates of long bones, a role for the Fat system in skeletal development has not been reported. We find that loss of Fat, Dachsous, or Atrophin-orthologues in zebrafish results in similar skeletal abnormalities, including the shortening of some cartilages, fused joints, and chondrocyte stacking defects. Confocal imaging of Fat- or Dachsous-deficient prechondrocyte condensations reveals loss of stacking and polarity—two PCP-regulated behaviors in other contexts such as gastrulation, as well as delays in differentiation. In addition, our chimeric analysis demonstrates that Fat is both necessary and sufficient to coordinate polarity and differentiation of cartilage in a non-cell autonomous manner. These results provide genetic evidence that skeletal morphogenesis and differentiation are controlled through a conserved Fat signaling pathway, a process that has not previously been associated with defects in skeletal tissue polarity.

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Development of the skull in *Fgfr2*$^{+/P253R}$ and *Fgfr2*$^{+/S252W}$ mouse models for Apert syndrome in late embryogenesis

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Mutations in FGFR2 are causative in craniosynostosis and dysmorphology of brain and other cranial soft tissues. Over 98% of all cases of Apert syndrome are caused by one of two gain-of-function mutations in FGFR2 on neighboring amino acids: S252W and P253R. We previously reported quantitative comparisons of skull shape of *Fgfr2*$^{+/P253R}$ and *Fgfr2*$^{+/S252W}$ models of Apert syndrome and unaffected littermates at postnatal Day 0 (P0), and assessed growth of the skull and brain in the *Fgfr2*$^{+/P253R}$ model of Apert syndrome between P0 and postnatal Day 2. Here we examine late embryonic skull and suture development of the *Fgfr2*$^{+/P253R}$ and *Fgfr2*$^{+/S252W}$ Apert syndrome mouse models between embryonic Day 17.5 (E17.5) and post-natal Day 0 (P0), demonstrating specific and localized effects of these mutations at both developmental time points and differences in growth pattern. Precise analysis of micro-computed tomography images revealed differences in the morphology of the facial skeleton, cranial vault, cranial base and palate between mutants and unaffected littermates, with some differences between models. Results indicate a lesser degree of fusion in facial and coronal sutures in mutant mice at E17.5 compared to P0. Our comparative analysis of growth shows that skull growth is different relative to unaffected littermates in mice carrying either Fgfr2 mutation. Differences in growth patterns and suture development provide additional evidence of the critical role that FGF/FGFR signaling plays in skull development during late embryogenesis.

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Neuregulin 1 modulates regeneration and growth of the submandibular gland

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Salivary glands are irreversibly injured after radiation therapy for head and neck cancer, thereby compromising patients' oral health and quality of life. Salivary function, regeneration and the maintenance of epithelial progenitor cells depend on the parasympathetic nervous system, which is reduced after irradiation. Here, we show that the neuronal support cells called Schwann cells (SC) are also depleted after irradiation and that Neuregulin1 type III (Nrg1), a SC survival and proliferation factor binding ErbB2 and 3, is able to regenerate the mouse embryonic submandibular gland (SMG). Radiation treatment of human SMGs decreased innervation and

downregulated cholinergic receptors CHRM1/3 and SC marker SOX10. Irradiation of isolated embryonic mouse SMG ganglia also resulted in a loss of SOX10+ SCs. To determine whether Nrg1 regulated SCs in the SMG, we cultured isolated ganglia with Nrg1 or the ErbB inhibitor PD168393. Nrg1 increased SC number, whereas inhibition of ErbB signaling reduced Sox10+ SC. Blocking of Nrg1 function in the intact SMG resulted in fewer epithelial buds, increased neuronal cell death and aberrant epithelial innervation. To examine whether Nrg1 had a direct effect on the epithelium, epithelial rudiments devoid of SC were treated with recombinant Nrg1. Epithelial morphogenesis and keratin-5+ epithelial progenitor cell proliferation was increased, indicating both SC and epithelial cells are positively regulated by Nrg1/ErbB signaling. Importantly, Nrg1 improved SMG regeneration after irradiation, increasing epithelial morphogenesis fourfold. Collectively, our results demonstrate that irradiation adversely affects Schwann cells and neuronal cell survival, and that Nrg1 is a potential therapeutic candidate for tissue regeneration.

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Characterization of the incisor stem cell niche using gene co-expression network analysis

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The continuously growing mouse incisor provides a unique system for studying the biology of adult stem cells. Its unidirectional growth, with stem cell progeny at progressively increasing stages of maturity arrayed in a linear fashion is of advantage for dissecting stage-specific mechanisms. To date, in-depth analysis has been hindered by our limited understanding of the cellular diversity present in the incisor and by the absence of markers that allow a clear discrimination between distinct cell types. Therefore, we set out to identify novel, cell type-specific markers by analyzing the gene co-expression network organization in the proximal incisor. In this study, expression profiles were generated from 96 individual tissue samples micro-dissected from the proximal incisor region of 96 adult wild-type mice and used for gene co-expression network analysis to identify modules of co-expressed genes. Previous work has shown that co-expression modules identified in heterogeneous tissues frequently relate to distinct biological processes and are often driven by discrete cell types. Therefore, we will investigated whether the identified modules were enriched for the few known markers of specific cell types in the tooth by cross-referencing published gene expression data. Cell type specificity was validated by visualizing gene expression patterns of the highest ranked factors contributing to each module. Analysis of data from a pilot study performed with a smaller dataset yielded promising results, such as the identification of a module that appears to be specific to the population of transient-amplifying cells. The wealth of new information obtained by this study will greatly enhance our understanding of the cellular composition of the incisor system and thereby facilitate our studies of adult stem cell biology.

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Mutations in *HOXB1* cause autosomal recessive congenital facial palsy with sensorineural hearing loss and strabismus

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The HOX genes are a family of highly conserved, homeodomain-containing transcription factors that specify regional differences along the anterior–posterior axis during early development. In this study, we utilized whole exome sequencing to identify a causative gene mutation in *HOXB1* in a family with autosomal recessive congenital facial palsy with hearing loss and strabismus. One hundred seventy-five additional probands with similar clinical features were screened for mutations in *HOXB1* (102 with a diagnosis of Moebius syndrome) and a second family was identified with the same homozygous *HOXB1* c.619C>T mutation. The clinical features of these two families were examined and correlate extensively with the phenotype of the $Hoxb1^{-/-}$ knockout mouse reported previously. This Arg207Cys HOXB1 missense variant identified results in a substitution at the highly conserved arginine 5 residue of the homeodomain, which when mutated in other proteins is known to cause human disease. Modeling of the mutant HOXB1/PBX1 complex shows reduction in affinity for DNA. Additionally, transfection experiments show differential activation of the mutant versus wild-type HOXB1 for its auto-regulatory element. Because both affected families were of conservative German American background, haplotype analysis was performed and both families shared a common haplotype, suggesting a founder mutation. Here is the first demonstration of human developmental disorders that can be classified as HOXB1opathies.

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Craniofacial dysmorphologies caused by an imbalance in Wnt signaling in neural crest cells

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Genetic factors contribute to the high incidence of craniofacial anomalies. Of these, cell-signaling molecules are of particular interest including Wnt growth factors. A loss-of-function mutation in Ror2 can cause imbalances in β-catenin dependent and independent Wnt signaling, leading to the facial dysmorphologies of Robinow syndrome. The proper balance between the two Wnt signaling pathways is crucial for craniofacial development, but how an imbalance in Wnt signaling actually causes the Robinow CFA is still unknown. By comparing Ror2 + /− mouse embryos, which have normal phenotypes, to Ror2−/− embryos, we found that shortening of the maxillary length at embryonic day (e) 12.5 was the first characteristic to show a significant difference. This suggests that defects in the maxillary prominence at e11.5 might later cause severe secondary characteristics and could be caused by intracellular disparities in Wnt signaling. We found that at e11.5, the null mice have increased cell proliferation in the maxillary prominences along with no proliferation and increased cell death in the mandibular prominences. Further, at the same stage, Wnt3a expression domain in the Ror2−/− embryos is restricted to the frontal portion of the frontonasal prominence compared to control embryos, while Wnt5a expression was restricted to the posterior portion of the prominence. We established neural crest (NC) derived cell culture from Ror2+/− and Ror2−/− e11.5 embryos and found that the lack of Ror2 leads to altered sensitivity to different Wnt3a concentration. Altogether, these results indicate how imbalance in Wnt signaling affects both the behavior of a subset of NC cells in the embryonic face.

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Identifying the role of MEF2C and Dlx5 in secondary palate elevation

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Craniofacial defects are among the most common forms of birth defects in the United States. One significant and prevalent craniofacial defect is cleft palate, however, the genetic pathways and cellular mechanisms that regulate palate development remain poorly understood. The MADS box transcription factor MEF2C is required for craniofacial development and functions in a pathway with the homeobox transcription factors Dlx5 and Dlx6. The genes encoding Dlx5 and Dlx6 are closely linked in the genome and are co-regulated by common enhancers. Consistent with their function in a common transcriptional pathway, we identified a genetic interaction between the *Dlx5/6* and *Mef2c* loci that results in lethality of $Dlx5/6^{+/-}$; $Mef2c^{+/-}$ double heterozygous mice at birth due to a failure in secondary palate elevation. We have recently characterized the expression of MEF2C and Dlx5 during palate development and found that both transcription factors are expressed in the craniofacial mesenchyme located just proximal to the palatal shelves at E14.5, the stage when the defect in secondary palate elevation is first detectable. Therefore, to identify genes and pathways important for secondary palate elevation, we performed RNAseq on wt, $Dlx5/6^{+/-}$ single heterozygotes, $Mef2c^{+/-}$ single heterozygotes, and $Dlx5/6^{+/-}$; $Mef2c^{+/-}$ double heterozygotes at E14.5. Multiple co-regulated targets were identified and several of the misregulated genes represent possible candidates for the secondary palate elevation defect.

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Growth accomplishments of children with clefts support neural crest cells in a causual role if they are multivalent stem cells

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Clefts of the lip-and-palate (CLP) are systematic effects, not isolated anomalies of the head-and-face. This is shown by their significantly negative Z (standard deviation) scores for elbow breadth running from −0.40 ($P < 0.05$) in males with unilateral CLP aged 13 years 7 months (13:07) to 18:11, to −1.17 ($P < 0.001$) in those aged 2:00–7:06. Males had significantly low Z for height ages 2:00–18:11, and complete ED growth late, while females (who grow earlier in the life course) had significantly negative Z for height only in the 8:00–18:11 age group. Andrearche (resumption of adrenal secretion of dihydroepiandrosterone) is late in males with clefts. Body Mass Index Z score is negative in some age–sex–cleft groups. This is not due to stress or feeding difficulties because effects are not seen in children with bilateral clefts. The age groups 2:00–7:11, 8:00–13:11, and 14:00–18:11 correspond roughly to the life-cycle stages post weaning dependent, childhood, and adolescence. These differ by the hormones that are active and are separated by inflection points in the growth acceleration curve. Neural crest cells contribute to the formation or control of several of the glands that secrete these hormones as well as to the formation of both membranous and chondrogenic bone. Deficient neural crest cell migration is thought to contribute to clefts directly. The set of alterations in growth in children with CLP could also be accounted for by an early alteration in neural crest cells' contribution to the varied growth cascades the results of which are altered in children with CLP.

Evidence for compensation within the vertebrate skull

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The vertebrate skull functions as a complex structure with close integration with the surrounding soft tissues. Yet, during development, the majority of the bones and cartilages of the skull develop from distinct cell populations compared to the other tissues within the head (e.g., skeletal muscle, the eye, etc.). How this complex integration arises during development is not clear. Similarly, how the skull responds to defects is not understood at the cell and tissue levels. Here, we present examples of compensation as a result of embryonic manipulations within the skull of chicken and fish. We show that in a series of bones, manipulating one element during induction results in compensation by other elements within the series. Furthermore, we show that forcing a reduced eye phenotype or subjecting embryos to an altered gravititational force results in responses in some skeletal elements only while others are

unaffected. Evidence that some bones respond by buckling or bending while others are constrained in their morphology was also determined. Overall, our experiments show that the skull has a remarkable ability to withstand changes to the calvaria, in particular, while enabling other skeletal elements to respond to manipulation. In the long term, this research will help us understand the cell and tissue behaviors underlying complex phenotypes of craniofacial disorders.

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Distinct requirements for *wnt9a* and *irf6* in extension and integration mechanisms during zebrafish palate morphogenesis

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Development of the palate in vertebrates involves cranial neural crest migration, convergence of facial prominences and extension of the cartilaginous framework. Dysregulation of palatogenesis results in orofacial clefts, which represent the most common structural birth defects. Detailed analysis of zebrafish palatogenesis is presented to reveal distinct mechanisms of palatal morphogenesis: extension, proliferation and integration. We show that *wnt9a* is required for palatal extension, wherein the chondrocytes form a proliferative front, undergo morphologic change and intercalate to form the ethmoid plate. Meanwhile, *irf6* is required specifically for integration of facial prominences along a V-shaped seam. This work presents a mechanistic analysis of palate morphogenesis in a clinically relevant context.

Wnt and Hedgehog signaling are modulated via the primary cilium on cranial neural crest cells

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Facial deformities are oftentimes harbingers of underlying disease states. For example, decreased Hedgehog activity in the developing craniofacial region causes holoprosencephaly and close-set eyes (hypotelorism). We found that excessive Hedgehog activity, caused by truncating the primary cilia on cranial neural crest cells, causes wide-set eyes (hypertelorism) and frontonasal dysplasias (Brugrnann et al., 2010). Here, using genetic approaches to map Wnt signaling during embryonic development, we demonstrate that the loss of the intraflagellar transport protein Kif3a amplifies Wnt signaling. This amplified Wnt signaling is first evident at neurulation and results in a disproportionally large number of cranial neural crest cells. The neural crest cell populations migrate appropriately but their excessive numbers results in a widened midface. Collectively our data demonstrate that the primary cilia modulates both Wnt and Hedgehog signaling within the cranial neural crest and that the precise balance of both morphogens is essential for normal facial patterning.

Influence of *CYP19A1* single nucleotide polymorphisms in sagittal facial growth: Caucasian and Asian study comparisons

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The enzyme encoded by the *CYP19A1* gene, aromatase, catalyzes the conversion of C19-androgens into C18-esteriol compounds. Aromatase is a vital enzyme in the regulation of estrogen production and it plays a key role in establishing an individual's testosterone-to-estrogen (T:E) ratio. During the pubertal growth period, the development of male and female facial characteristics is strongly influenced by the T:E ratio, and hence it was hypothesized that variations in the DNA code for aromatase may be associated with differences in sagittal facial growth during puberty. In three separate studies, one or more Single Nucleotide Polymorphisms (SNPs) within the *CYP19A1* gene (rs2470144, rs2445761, and/or rs730154) were examined for genotypic association with sagittal jaw growth at puberty in either a Caucasian or Asian population. Using Cephalometrics, annualized sagittal growth was measured with the pre- and post-orthodontic treatment radiographs of male and female subjects demonstrating a significant pubertal growth spurt. A significant growth spurt was defined as a transition from cervical vertebral maturation stage III (CMVS-III) in pre-treatment radiographs to either CMVS-IV or CMVS-V in post-treatment radiographs. All three studies showed that males with a homozygous TT genotype at rs2470144 (and TT at rs2445761, when tested) had significantly enhanced mandibular growth compared to individuals with the homozygous CC genotype at either locus, regardless of ethnicity. By comparison, only one Caucasian study demonstrated that females with the heterozygous CT genotype at rs2470144 or rs2445761 were associated with increased maxillary growth. No association was observed with sagittal pubertal growth in males or females at rs730154.

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Novel molecular pathways elicited by mutant FGFR2 may account for brain abnormalities in Apert syndrome

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Apert syndrome (AS), caused in roughly 70% of the cases by S252W gain-of-function mutation in the FGFR2 gene, is mainly characterized by premature fusion of coronal sutures. Brain dysmorphologies are also present and are not seen in other FGFR2-associated craniosynostosis such as Crouzon syndrome (CS). Here, we tested the hypothesis that S252W mutation leads not only to overstimulation of FGFR2 downstream pathway, as previously proposed, but also induces novel pathological signaling. To address this, we stimulated both wild-type and S252W periosteal fibroblasts with FGF2 to activate FGFR2 in vitro and performed microarray analysis. Results showed that S252W mutation elicits unique molecular pathways. The same was not observed in CS cells. Therefore, FGFR2 downstream activated signaling distinguishes AS from other FGFR2-associated syndromic craniosynostosis, such as CS. Our gene expression profiling suggests that the novel signaling triggered by mutant FGFR2 is associated with development and maintenance of the central nervous system (CNS). Thus, we investigated if any of the differentially expressed genes found in S252W cells were also altered in the CNS of an $Fgfr2^{S252W/+}$ mouse model. We validated Strc (stereocilin) altered expression in newborn Apert mouse brain, and immunostaining experiments suggests a role for endothelial cells and cerebral vasculature in the establishment of the characteristic CNS dysmorphologies in AS. Additional study of the vascularization in the CNS in AS is required for a better understanding of the pathoetiology of AS.

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Growth promotion effect of platelet factors and low oxygen differs in human bone marrow-derived and dental pulp-derived mesenchymal stem cells in vitro

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Activated platelets and low oxygen are two out of many factors involved in wound healing. One of their effects in early stages of wound healing is stimulation of growth of cells involved in repair or regeneration. It has been shown that platelet-derived factors and low-oxygen atmosphere increased proliferation rate of mesenchymal stem cells in vitro. We were interestedin whether effects of platelet factors on human bone marrow-derived (BMSC) and dental pulp-derived (DPSC) mesenchymal stem cells are similar and if they can be reproduced in atmosphere containing normal or low oxygen concentration. Human BMSC were purchased from Lonza. Human DPSC were isolated from healthy third molars (IRB approval 09–99.1). Growth medium consisted of basal medium (Lonza) with 10% human serum, 2 mM L-glutamin, 100 units/ml penicillin G and 100 μg/ml streptomycin. Serum and platelet-derived factors were prepared from human venous blood. Biospherix equipment maintained atmosphere with 5% oxygen and 5% carbon dioxide. Growth stimulatory effects of platelet-derived factors were greater in DPSC than in BMSC. Growth-stimulatory effect of platelet derived factors was more expressed in atmosphere containing 21% oxygen than in 5% oxygen level. It seems that cultured human DPSC are more responsive to growth-promoting influences of platelet-derived factors and low oxygen than cultured human BMSC.

Human fetal dental mesenchyme differentiates human embryonic stem cell-derived epithelial cells to ameloblastic cell lineage

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The developing tooth organ is an interesting model for investigating the role of epithelial-mesenchymal interactions on organogensis. The lack of readily available cell sources for human dental epithelial cells constrains the progress of enamel/tooth bioengineering. Previous animal studies have demonstrated that developing dental mesenchyme can instruct non-dental epithelium to differentiate into enamel forming epithelium. In this study we characterized the capacity of developing (fetal) and fully formed (adult) human dental mesenchyme in promoting the differentiation of human embryonic stem cell-derived epithelial cells to ameloblast lineage cells (ALCs). Differentiation of hESCs into epithelial cells (ES-ECs) was induced by bone morphogenetic protein-4 and retinoic acid. These induced ES-ECs were co-cultured either with human fetal dental mesenchymal cells (FDMCs) or with adult dental mesenchymal cells (ADMCs) in a three-dimensional culture system using Matrigel. After 8 weeks' co-culture, cell/Matrigel complexes were either embedded for histological imaging, or cells were retrieved from Matrigel for gene expression analysis. ES-ECs co-cultured either with FDMCs or ADMCs were also transplanted into the renal capsules of severe combined immunodeficiency (SCID) mice, followed by histological and gene expression analyses. Transcription factors differentially upregulated in FDMCs as compared to ADMCs were characterized. Induced hESCs adopted a typical cobblestone epithelial morphology, with cytokeratin 14 expression levels increased by an average 44-fold as compared to uninduced control. When co-cultured with human FDMCs in vitro, ES-ECs polarized and expressed amelogenin. Enamel-like structures formed at approximately 20% of the frequency of the overall cellular structures. In addition, structures with a morphology resembling bud stage tooth primordia were observed in the tissues derived from ex vivo transplantation of ES-ECs and FDMCs. These results were not found in the co-cultured ES-ECs and ADMCs, where co-cultured cells formed spherical structures that did not resemble either enamel organs or tooth buds. Transcription factors *Msx1*, *Gli1*, *Lhx6*, *Lhx8*, *Lef1*, and *Tbx1* were significantly upregulated in FDMCs as compared to ADMCs.

FDMCs but not ADMCs had the capacity to promote differentiation of recombined ES-ECs into ameloblast lineage cells. Understanding the differences between these two stages of human dental mesenchymal cells could allow us to develop strategies to reprogram ADMCs for tooth organ regeneration.

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