Transcriptome analysis reveals novel players in the cranial neural crest gene regulatory network

Marcos Simões-Costa,1 Joanne Tan-Cabugao,1 Igor Antoshechkin,1 Tatjana Sauka-Spengler,2 and Marianne E. Bronner1,3

1Division of Biology, California Institute of Technology, Pasadena, California 91125, USA; 2The Weatherall Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DS, United Kingdom

The neural crest is an embryonic stem cell population that gives rise to a multitude of derivatives. In particular, the cranial neural crest (CNC) is unique in its ability to contribute to both facial skeleton and peripheral ganglia. To gain further insight into the molecular underpinnings that distinguish the CNC from other embryonic tissues, we have utilized a CNC-specific enhancer as a tool to isolate a pure, region-specific NC subpopulation for transcriptional profiling. The resulting data set reveals previously unknown transcription factors and signaling pathways that may influence the CNC’s ability to migrate and/or differentiate into unique derivatives. To elaborate on the CNC gene regulatory network, we evaluated the effects of knocking down known neural plate border genes and early neural crest specifier genes on selected neural crest-enriched transcripts. The results suggest that ETS1 and SOX9 may act as pan-neural crest regulators of the migratory CNC.

Taken together, our analysis provides unprecedented characterization of the migratory CNC transcriptome and identifies new links in the gene regulatory network responsible for development of this critical cell population.

[Supplemental material is available for this article.]
and allow identification of new links in the GRN responsible for development of this critical cell population.

Results

Isolation of pure cranial neural crest population

Expression of the specifier gene SOX10 initiates as NC cells commence emigration from the neural tube at all axial levels (Kim et al. 2003; McKeown et al. 2005). Previously, we identified avian NC enhancers that drive reporter expression in a manner that recapitulates endogenous SOX10 expression (Fig. 1A; Betancur et al. 2010b). In particular, Sox10E2 (Fig. 1B,C,C’) mediates expression in the CNC but not in the trunk subpopulations.

To confirm specificity, we compared enhancer activity with endogenous SOX10 expression. Antibody staining in embryos electroporated with Sox10E2:eGFP shows that the enhancer is only active in SOX10+ neural crest (Fig. 1D–F). Although the majority of ectodermal cells are efficiently coelectroporated with Sox10E2 enhancer-driven (eGFP) and ubiquitous promoter-driven (mRFP) constructs, as highlighted by H2B-RFP expression (Fig. 1G), only Sox10+ cells exhibit eGFP expression (Fig. 1H–J). To examine enhancer activity during neural crest migration, we performed time-lapse imaging. Supplemental Movie 1 highlights the remarkable movement of CNC cells as they migrate throughout the embryonic head (Fig. 1K–M). Thus, Sox10E2 enhancer is a useful tool for in vivo labeling and identifying migrating CNCs.

In order to obtain a transcriptional profile of the CNC, Sox10E2:eGFP was electroporated into gastrula-stage chick embryos subsequently incubated to stages of active CNC migration (HH10) (Fig. 2A). After dissection and dissociation of embryos, a pure population of migrating CNC cells was isolated by FACS with 7-AAD exclusion to eliminate unhealthy or dying cells. Approximately 5%–10% of the viable dissociated cranial cells expressed eGFP, resulting in about 500–1000 labeled CNC cells per electroporated embryo. After sorting, cells were tested for enrichment of known neural crest markers such as SOX10 and SNAI2 (Fig. 2C) using qPCR. Isolated GFP+ sorted CNC cells had strong enrichment for both genes relative to GFP− cells (Fig. 3A). SOX10, a key regulator of CNC formation (Thévenot et al. 2007; Betancur et al. 2010b; Simões-Costa et al. 2012), is significantly enriched in the data set although it is strongly expressed in other embryonic tissues such as endothelial cells.

Figure 3B shows genes with high enhancement or depletion in the migratory CNC. In addition to transcription factors, genes known to play important roles in CNC formation are also present. For example, Collagens 2 and 9, crucial for chondrocyte development, are enriched (Fig. 3D), as are nuclear receptors such as RXRG and other transcriptional regulators such as ALXI. Genes visibly down-regulated or absent from the CNC include a number of HOX genes, which is expected since the CNC lacks most HOX genes, GATA factors 4,5,6 as well as TBX5, 20 and 15 (Fig. 3C; Supplemental Table 2). Importantly, ZIC1, important for trunk NC specification and a trunk-specific input in FOXD3 (Simões-Costa et al. 2012), is also absent from the CNC (Fig. 3C).

Biological pathway analysis of cranial neural crest transcriptome

To gain an overview of the molecular processes operational in the CNC transcriptome, we performed a biological pathway analysis of the data set. Confirming its utility, the results reveal enrichment in genes involved in pathways known to be important for neural crest migration, such as integrin (Fig. 4A) and CXCR4 signaling (Fig. 4B). Revealing predictive value, the analysis also suggests molecular processes that have yet to be investigated in depth during neural crest formation. For example, a large number of molecules related to RNA processing are up-regulated in the CNC (Fig. 4C). This suggests that RNA-binding proteins and differential splicing mechanisms may be important in neural crest development. Similarly, a number of molecules involved in post-translational modification, such as the ubiquitination pathway, are present in the data set. This result is consistent with recent findings indicating the importance of molecules involved in post-translational modification in regulating aspects of neural crest development (Lee et al. 2012). The analysis also points to a potential role for pathways that have yet to be investigated in neural crest development, such as the IGFI and JAK/STAT pathways (Fig. 4E).

A number of genes in the CNC transcriptome data set are disease-related. Our analysis revealed association of CNC genes with diseases such as osteochondrodysplasia (Fig. 4D), consistent with the importance of CNC for the formation of the skeletal elements of the face. Furthermore, the data set contains genes linked to malignant diseases, such as neuroblastoma, glioma, and other types of neural crest derived cancers (Fig. 4E). This is expected due to the similar behavior of neural crest cells and metastatic cancers and the fact that several neural crest genes such as MYC and CXCR4 are routinely used as biomarkers for tumor diagnosis.

Novel transcriptional regulators for the cranial neural crest

To validate the CNC transcriptome data set, we performed a secondary in situ hybridization screen (Fig. 5). The results reveal many
interesting candidate genes that may play a role in the process of CNC migration and/or differentiation. Eighty-five genes were cloned and their expression patterns verified by in situ hybridization. Approximately 90% of genes had strong expression in the migrating CNC as expected (Supplemental Table 3). We also tested enrichment of a number of genes by RT-qPCR in GFP+ cells; results are consistent with the RNA-seq data (Fig. 5P).

Our in situ hybridization screen shows specific CNC gene expression for molecules strongly up-regulated in our data set (Fig. 5). Genes such as transcription factor EBF1, adaptor protein

Figure 1. SOX10 cis-regulatory element drives specific reporter expression in the migratory cranial neural crest. (A) Neural crest specifier gene, SOX10, is expressed by all migrating neural crest cells. (B) A chicken embryo electroporated with Sox10E2 enhancer has GFP expression in migratory CNC at HH10. (C, C') Transverse sections of HH10 chicken embryo electroporated with Sox10E2:eGFP plasmid. GFP+ cells are also immunoreactive for the HNK-1 epitope. (D–F) Confocal imaging confirms specificity of Sox10E2 enhancer in the CNC. (G–J) Confocal imaging confirms that of all electroporated cells (red), only the ones with SOX10 positive nuclei (blue) express GFP. (K–M) Time-lapse imaging of a chicken embryo electroporated with Sox10E2:eGFP demonstrates the extensive movements of cranial neural crest in the embryonic head (see Supplemental Movie 1). (NC) Neural crest; (R3) rhombomere 3; (OV) otic vesicle.
Effects of perturbation of neural plate border and neural crest specifier genes on novel targets

To identify novel links in the CNC gene regulatory network, we assayed the effects of knockdown of known neural plate border and neural crest specifier genes on identified targets in the migrating CNC population (Fig. 2B). Four genes were chosen as targets for this analysis: the nuclear receptor RXRG, the tyrosine receptor LTK, the structural protein COL9A3, and the adaptor protein LMO4 (Ochoa et al. 2012; Ferronha et al. 2013), all of which are robustly expressed in the premigratory neural crest. Indeed, both genes have been identified as direct inputs of the Sox10 enhancer (Fig. 1; Betancur et al. 2010b). PAX7 is another important gene in the neural crest GRN, required for neural crest specification (Basch et al. 2006) by directly regulating FOXD3 (Simões-Costa et al. 2012). PAX7 is maintained in the migratory CNC and coexpressed with SOX10 (Supplemental Table 4), and its knockdown causes loss of RXRG, LTK, and COL9A3 (Fig. 6A), but not of LMO4.

Loss of function of SOX10 and TFP2A, key regulators of neural crest development (Rada-Iglesias et al. 2012), similarly affected transcription of LMO4 and RXRG but did not alter LTK or COL9A3 expression. Remarkably, loss of the neural plate border specifier MSX1/2 resulted in up-regulation of three of the genes examined, despite its requirement for neural crest specification (Simões-Costa et al. 2012). The effects of knockdown of neural crest specifiers on transcription factor EBF1 (Fig. 2) yielded completely different results than the other CNC genes analyzed, suggesting that its regulation may be indirect (Supplemental Fig. 1). MYB knockdown had no significant effect in any of the four main CNC transcripts analyzed. Taken together, our results point to ETS1 and SOX9 as key regulators of CNC development and show differential regulation among the CNC genes surveyed.
highlighting the complex nature of the neural crest gene regulatory network.

**Discussion**

Transcriptional regulation of a dynamic cell population like the neural crest is an intricate process. The constantly shifting environment to which the migrating CNC cells are exposed, together with the onset of their differentiation programs, is likely to contribute to a high level of regulatory complexity. By greatly expanding the candidate genes that can be interrogated for their connectivity in the CNC GRN, our data set provides a platform for analysis necessary for completion of the migrating CNC GRN. We have uncovered approximately 100 novel transcriptional regulators, many of which undoubtedly play an important role in determining neural crest identity, potential, and behavior.

Extensive migratory ability is a defining feature of the neural crest. Accordingly, we observe a large number of molecules known to play critical roles in cell migration in our data set such as molecules involved in changes in cell-cell adhesion and membrane receptors. For example, we noted up-regulation of Rho family small GTPases (Fig. 4E), important for planar cell polarity and mediating cytoskeleton dynamics (Clay and Halloran 2011). In fact, nearly half of the up-regulated genes in migrating CNC were
enzymes. This is likely due to the constant structural rearrange-
ments, extensive proliferation, and active signal transduction,
such as the MAPK/ERK pathway, ongoing in migratory CNC cells
(Fig. 4; Newbern et al. 2008; Stuhlmiller and Garcia-Castro 2012).
Furthermore, numerous enzymatic epigenetic modifiers (Hu et al.
2012; Strobl-Mazzulla and Bronner 2012) including helicases and
topoisomerases are active in the CNC.

Consistent with classical experiments revealing differences in
developmental potential between cranial and trunk neural crest,
our data set shows enrichment of major components of the
chondroblast differentiation program in migrating CNCs. These
include SOXD family members, MIA/CD-RAP, TFAP2A transcrip-
tion factors, and cartilage-specific matrix molecules COL2A1,
COL9A3, COL11A1 and ACAN (Supplemental Table 1). The pres-
ence of these transcripts may reflect early specification of a subset
of CNC toward an ectomesenchymal fate, perhaps explaining why
some cranial crest cells differentiate into ectopic cartilage after
grafting to the trunk (Nakamura and Ayer-le Lievre 1982; Le
Douarin et al. 2004). Comparative genomic analysis between dis-
tinct neural crest subpopulations holds the promise of revealing
key circuits that endow the CNC with its unique features.

The complexity of the CNC regulatory program is highlighted
in our functional analysis, which reveals many previously un-
known connections between upstream regulators and the identi-
fied effector genes (Fig. 7). Results reveal important trends in reg-
ulatory control of CNC-specific transcripts. For instance, our data
show that ETS1 and SOX9 are required for the expression of four
of five CNC genes analyzed. This suggests a model in which ETS1
and SOX9 may act as pan-neural crest regulators during CNC
migration (Fig. 7), hinting at a common subcircuit controlling expression of

Figure 4. Pathways, processes, and diseases associated with the CNC transcriptome data set. Biological pathway analysis shows enrichment of mi-
grating cranial crest genes involved in integrin (A) and CXCR4 (B) signaling, as expected since both signaling systems are essential for neural crest
migration. This analysis also reveals potential roles for post-translational regulation in CNC development as a large number of molecules involved in RNA
splicing and processing are up-regulated (C). (D) Genes involved in diseases such as osteochondrodysplasia are present in the data set, which is consistent
with the contribution of the neural crest to the craniofacial skeleton. (E) Enrichment of CNC genes in other pathways, processes, and diseases.
CNC-specific transcripts. This view is supported by cis-regulatory analysis of bona-fide neural crest markers (Betancur et al. 2010b; Simões-Costa et al. 2012; Barembaum and Bronner 2013), identifying SOX9 and ETS1 as direct inputs in neural crest enhancers.

Apart from common inputs from SOX9 and ETS1, each of the target genes analyzed requires a different combination of upstream regulators for transcriptional activation. This is not unexpected since the CNC is complex and its regulation involves the interplay of a large number of transcription factors (Betancur et al. 2010a). Since the four genes analyzed have very similar expression patterns, our results suggest the existence of concurrent genetic programs within the CNC that result in similar transcriptional outcomes. According to this model (Fig. 7), different combinations of transcription factors would result in transcriptional activation of distinct genes in CNCs. SOX9 and ETS1 would thus be a part of a common essential genetic circuit working in conjunction with different regulators (PAX7, SOX10, TFAP2A) to activate transcription.

Interestingly, the same regulator can have distinct effects at different developmental times and in different modules of the CNC GRN. For instance, MSX1 plays a critical early role as an ac-
tivator of neural crest specification (Monsoro-Burq et al. 2005; Simões-Costa et al. 2012), but our data show that in the migrating CNC, it modulates or represses gene expression. Consistent with repressor function, MSX1 has been shown to recruit Polycomb complexes during embryonic development to shut down target genes during myoblast differentiation (Wang et al. 2011). A likely explanation is that the same regulator often can be used re-iteratively but to different effect depending upon available partners. These results highlight the complexity of regulatory interactions in the neural crest GRN and demonstrate that the consecutive programs that drive neural crest gene expression can be quite distinct, even if they utilize some of the same suite of regulators.

The migrating CNC transcriptome data set presented here provides a platform for a thorough exploration of the transcriptional state of CNC cells during their active phase of migration. The data set will facilitate expansion of the terminal modules of the neural crest GRN. This will lead to a better understanding of how the genetic program controlling neural crest identity unfolds and is converted into discrete regulatory modules that drive differentiation into a variety cell types. Our results point to a variety of molecules and pathways that have yet to be explored in the context of neural crest development and offer a panoramic view of the CNC transcriptional landscape.

Methods

Electroporation of enhancers and time-lapse imaging
Chicken embryos were incubated to Hamburger and Hamilton (HH)4 (Sauka-Spengler and Barambaum 2008). Embryos were dis-
sected (Chapman et al. 2001), placed in Ringer’s solution, injected with 2 \( \mu \)g/\( \mu \)L of the pTK-Sox10:e2:eGFP construct (Betancur et al. 2010b), electroporated, and placed in individual culture dishes containing albumen supplemented with Penicillin-Streptomycin (Sigma). For dynamic analysis of the Sox10E2 enhancer, HH4 chick embryos were electroporated as described above. After \( \approx 8 \) h of incubation, embryos were imaged as described (Ezin and Fraser 2008).

**Tissue dissociation and FACS**

After electroporation, embryos were incubated at 37°C until HH10 (\(-14-16 \) h of incubation) and dissected above the level of rhombomere 3 to avoid contamination of otic GFP+ cells. Dissected heads were dissociated in dispase/trypsin. Reaction was stopped by addition of 10 times the volume of Hank’s solution. Cells were washed, passed through a 40 \( \mu \)m strainer (BD) and resuspended in Hank’s solution plus 0.5% BSA, 5 units/mL DNase I (Roche). Cells were sorted using BD FACSAria Cell Sorter (BD Biosciences) with 7-AAD exclusion to eliminate dead/damaged cells. GFP+ cells from dissected heads and GFP/C0 cells from whole embryos (WE) were used for library construction and sequencing.

**RNA Extraction and Amplification**

Sorted cells were pelleted, washed with PBS, and immediately lysed with Lysis Buffer. RNA was extracted with RNAqueous-Micro Kit (Ambion AM1931); genomic DNA was removed by 30 min of TURBO DNase treatment. Quality of the RNA was assayed in an Agilent 2100 Bioanalyzer. All samples had RIN \( \geq 8 \). RNA was subsequently amplified with the Ovation RNA-Seq System V2, yielding double-stranded cDNA that was used for library construction.

**Library building and sequencing**

Sequencing libraries were built according to Illumina Standard Protocols. SR50 sequencing was performed in a HiSeq Illumina machine. Sequence reads were aligned to the *Gallus gallus* genome with Bowtie (version 0.12.7) and TopHat (version 1.3.1.OSX-x86_64). The following reads and percentage of reads mapped were obtained for each of four libraries: CNC1 (21.5 million reads/87% mapped), CNC2 (26.2 million reads/50% mapped), WE1 (21.7 million reads/81% mapped) and WE2 (30.7 million reads/64% mapped). Cufflinks and Cuffdiff were used to calculate gene expression levels and identify differentially expressed transcripts. Two CNC and two whole embryo samples were independently collected, sequenced and mapped, and subsequently analyzed in Cufflinks and Cuffdiff as replicates. The Pearson correlation coefficients obtained between replicates were 0.925 for the CNC samples and 0.957 for the WE samples. A gene was considered to be expressed by the CNC transcriptomic data set if (1) the RPKM values (Reads Per Kilobase of exon per Million fragments mapped) for that gene were above 5 RPKM in the population with higher expression levels; (2) difference in expression levels was significant according to statistical analysis; and (3) the gene had at least twofold up-regulation/depletion in the CNC. The expression levels on the CNC data set (Supplemental Table 1) contain values for samples that were processed as replicates in TopHat. Biological pathway analysis was performed with the aid of Ingenuity iReports, after conversion of the chicken Ensembl gene models to mouse orthologous counterparts.

**cDNA synthesis and qPCR**

RNA from GFP+ and GFP– sorted cells was isolated using the RNAqueous-Micro Kit (Ambion AM1931), according to the manufacturer’s protocol. cDNA was synthesized using SuperScript III First Strand Synthesis kit (Invitrogen) with random primers for RT priming. qPCR was performed using SYBR Green (Bio-Rad) according to the manufacturer’s instructions. For reference genes, we used qPCR primers designed for the *HPRT1* and *TFRC* genes.

**In situ hybridization and antibody labeling**

Whole mount in situ hybridization was performed as described (Acloque et al. 2008). Embryos electroporated with the Sox10E2:
eGFP construct were labeled using HNK-1 and SOX10 antibodies (1:100), and goat anti-mouse IgG Alexa 350 or anti-mouse IgG Alexa 350, respectively (1:1000; Molecular Probes). Whole mount immunostaining was performed as described (Ezin et al. 2009). For in situ hybridization, fragments of candidate genes ranging from 600–800 were PCR cloned using the TOPO TA Cloning Kit from Invitrogen (K4610-20). Clones were sequenced and used as templates for DIG-labeled RNA probes. Expression of approximately 80 candidate genes in the CNC was verified in chicken embryos (Supplemental Table 1).

Loss-of-function studies
For morpholino knockdown, unilateral electroporation was performed in live chick embryos as described (Barembaum et al. 2000), using the following morpholinos: Msx1, Pax7, and Ets1 (Simões-Costa et al. 2012); c-Myc, Sox9, and Sox10 (Betancur et al. 2010b); and Tafp2a (Barembaum and Bronner 2013). Embryos were collected and fixed in 4% paraformaldehyde for 1 h and screened under fluorescence for efficient morpholino incorporation.

Data access
All sequencing data have been deposited in the NCBI BioProject database, under BioProject PRJNA226749 (http://www.ncbi.nlm.nih.gov/bioproject).

Acknowledgments
We thank Diana Perez, Janice Grimm, and Rochelle Diamond for their excellent cell-sorting assistance. The Caltech Flow Cytometry Institute at Caltech. We thank Diana Perez, Janice Grimm, and Rochelle Diamond for technical assistance. This work was supported by NIH grants R01 HD058365, R01 HD058981, and T32 HL007550. We thank Dr. Max Ezin for her help with live time-lapse imaging of enhancer activity, and Mike Stone and Brian Jun for technical assistance. The Caltech Flow Cytometry Institute at Caltech. We thank Dr. Max Ezin for her help with live time-lapse imaging of enhancer activity, and Mike Stone and Brian Jun for technical assistance. This work was supported by NIH HD037105 and DE16459 (to M.E.B.) and the Pew Fellows Program in the Biomedical Sciences (to M.S.-C.), and a Caltech Cell Center fellowship from the Moore Foundation (to M.S.-C.).

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Received May 28, 2013; accepted in revised form November 6, 2013.