Assessment of Cranial Neural Crest Cell Differentiation Towards an Osteoblastic Fate

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Masters of Science Thesis

Assessment of Cranial Neural Crest Cell Differentiation Towards an Osteoblastic Fate

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**PROPOSAL TITLE**

Assessment of Cranial Neural Crest Cell Differentiation Towards an Osteoblastic Fate

**PROJECT ABSTRACT**

The world health organization estimates that craniofacial defects affect between 2-3% of all live births, with complications ranging from aesthetic issues to severe mental retardation (Marcucio 2015). It is estimated that for a pathology resulting in craniofacial aberrations, each affected individual costs society ~1 million dollars in corrective and palliative care (Singh 2014). Current investigations into genetics underlying these defects have revealed 564 human (HPO 2017) and 3297 murine (MGI 2017) genes thought to be involved in craniofacial pathologies. Facial development requires a population of cells known as Cranial Neural Crest Cells (CNCCs) to properly migrate from the neural plate border and to populate the tissue of the future face (Douarin 2007). CNCCs are multipotent progenitors that respond to extrinsic signaling to make fate choices. Dysregulation of this process can result in craniofacial pathologies, such as clefting. Not only will understanding the genetic factors behind craniofacial development allow for better treatment of craniofacial disease, but can aid in genetic counseling to ultimately save millions of dollars in healthcare costs.

Currently, discovery of genes involved with craniofacial development rely on high throughput genetic manipulations in animal models and/or epidemiological information obtained from human populations. These discoveries mainly employ top-down approaches to understand the genetics involved in craniofacial disorders. We propose that we can employ a bottom-up approach to elucidate novel genetic components involved in
normal craniofacial development to interrogate craniofacial diseases, chiefly, ones affecting craniofacial skeletal development.

Our lab’s focus is to resolve the transcriptic profile of populations of cells, using single-cell RNA sequencing during murine development. Of note is the ability of our lab to identify genes that co-vary with one another in populations of cells and are thought to coordinate genetic programs, a concept known as a metagene (Wilson 2012). Recently, our efforts have been to characterize populations of cells from theiler stage 19 (TS19) murine embryos, a developmental stage critical in craniofacial development (Kawauchi 2005). We propose three specific aims in order to evaluate craniofacial development in a bottom-up manner; (1) identify metagenes in putative CNCCs and osteoblastic progeny in our TS19 data set, (2) create a model system to interrogate CNCC metagene expression patterns and (3) perturb metagenes to broaden our understanding of CNCC fate decisions, particularly towards an osteoblastic fate.

Our proposed research will broadly impact the field by expanding methods to evaluate genetic programs guiding fate decisions. These findings will help to identify novel genes participating in craniofacial development and can be used to understand genetic dysregulation occurring in craniofacial pathologies.
PROJECT NARRATIVE

Investigations into genetic dysregulation of CNCC development have mainly employed a top-down approach. Our proposed studies aim to understand metagenes governing CNCC osteoblastic differentiation to better understand diseases affecting craniofacial skeletal development. This research employs a bottom-up approach to identify potential metagene participants (PMPs) involved in proper craniofacial development to build genetic knowledge of craniofacial pathologies.
SPECIFIC AIMS

Neural crest cells from the cranial region of the developing embryo, called Cranial Neural Crest Cells (CNCCs), support the development of facial tissues by giving rise to melanocytes, odontoblasts, sensory neurons, cartilage and bone, to name a few (Trainor 2013). CNCCs help to construct various bones of the face by producing a population of osteochondral cells. These osteochondral cells help produce both chondrocytes and osteoblasts which give rise to endochondral bone (chondrocytes and osteoblasts) and intramembranous bone (osteoblasts) (Abzhanov 2007). Intramembranous osteoblasts produce many of the rostral bones of the face, including the mandible, maxilla and frontal bone. The rationale of this proposal is to explore elements of genetic programs governing CNCC osteoblastic differentiation. Research into the genetic programs that help to orchestrate CNCC osteoblastic differentiation have helped to reveal many factors involved in craniofacial skeletal diseases (Bengani 2017). Diseases known to affect the ability of CNCCs to undergo osteoblastic differentiation include Frontonasal dysplasia (Msx1) (Nassif 2014), Cleidocranial dysplasia (Runx2) (Otto 2002) and Osteogenesis imperfecta (Osx) (Lapunzina 2010). Many of these craniofacial pathologies were studied in a top-down manner, meaning that the mechanism of the aberration is interrogated after observation of a craniofacial defect (Gordon 2017) (Adhikari 2016) (Suzuki 2015). Genes identified from these top-down studies are necessary for driving proper craniofacial development. By evaluating metagenes (co-varying genes) with these CNCC ‘driver’ genes, we can predict and discover novel genetic components involved in craniofacial development and disease. To evaluate these potential metagene participants (PMPs), we will utilize in vivo data in an in vitro modeling system to understand the role these PMPs play in CNCC osteoblastic differentiation. To elucidate metagenes involved in CNCC functionality, we performed single-cell RNA sequencing of a TS19 murine embryo.

Single-cell RNA sequencing is a technology that interrogates the transcriptional identity of individual cells and can be used to elucidate gene regulatory elements involved in cellular functionality (Aibar 2017). Evaluation of the transcriptional dynamics of single cells offers insight into gene regulatory networks helping to drive fate choices (Wang 2016). Previous studies using single-cell RNA sequencing evaluated the transcriptional profile of cells comprising the population structure of various tissues in early stage murine
embryos (Scialdone 2016). As stated previously, the current focus of our lab is to investigate the transcriptic profile of different populations of cells in a TS19 embryo, a developmental stage where CNCCs are making osteoblastic fate choices (Bhatt 2013). We believe that by using single-cell transcriptional data we can employ a bottom-up approach to identify genes involved in craniofacial pathologies by evaluating metagenes involved in CNCC osteoblastic differentiation. The long-term directive of our lab is to establish a gold standard transcriptional profile for all cell types throughout murine embryonic development, and to understand how metagenes coordinate developmental processes in different cell types. The central hypothesis of this proposal is that information gained from metagenes in our single-cell data set can be used to elucidate novel components involved in craniofacial skeletal development. To test this central hypothesis, we propose three specific aims:

**Aim 1** To identify and characterize putative Cranial Neural Crest Cells (CNCCs), their osteoblastic progeny and associated metagenes, using single-cell mRNA sequencing of murine embryos. The goal of this aim is to test the hypothesis that the transcriptic identity of CNCCs and facial osteoblasts can be broadened by evaluation of murine single-cell RNA sequencing data.

**Aim 2** To develop an in vitro modeling system capable of recapitulating, in vivo, CNCC osteoblastic differentiation. In this aim we design a platform to test the hypothesis that we can model major transcriptional changes occurring in CNCC osteoblastic differentiation, in an in vitro system, to study metagenes.

**Aim 3** To perturb metagenes governing CNCC osteoblastic differentiation, in vitro. We hypothesize that by perturbing metagenes guiding osteoblastic differentiation, in CNC-like cells, we can evaluate craniofacial pathologies involving both driver genes and their PMPs.

**RESEARCH STRATEGY**
A. SIGNIFICANCE

Craniofacial defects, such as cleft palate, affect nearly 1 in 1,000 live births in the United States (NICDR 2017). Efforts to build genetic knowledge concerning craniofacial pathologies mainly rely on information obtained through top-down studies, such as high-throughput animal studies and Genome-Wide Association Studies (GWAS) in humans. Information from animal models are obtained via consortiums such as the KnockOut Mouse Project (KOMP), which sequentially knocks out genes in the murine genome to investigate the phenotypic output. In humans, GWAS help to identify genes involved in a pathology which informs its role in development and disease. Efforts to identify genes involved in specific processes governing CNCC development, such as specification, migration, proliferation and differentiation, have been performed using RNA sequencing (Lumb 2017). Gene regulatory networks governing a number of these CNCC development steps have been characterized using data from both high-throughput and in depth genetic studies (Simoes-Costa 2015). By evaluating metagenes associated with CNCC osteoblastic differentiation, our proposal seeks to expand on the knowledge collected by previous studies.

A1) Our proposal aims to broaden the transcriptional profile of CNCCs and osteogenic progeny

Currently, databases such as Lifemap have a transcriptic profile of over 200 different murine cell types throughout development. Our single-cell analysis of TS19 murine embryo identifies different populations of cells in our data set, based on previously curated transcriptic data and aims to expand their transcriptional information. This will be highly significant as it will be the first in-depth, agnostic approach at evaluation of single-cell sequencing data in a murine embryo at this stage of development. Not only will this data broaden the transcriptic profile of different populations of cells in a TS19 embryo, but this approach can identify metagenes for that cluster of cells. By using the Differential Gene Expression (DGE) lists for a putative CNCC cluster, we can identify genes curated to contribute to a CNCC process (driver genes). By evaluating the highest co-varying genes in that cluster with our driver gene, we can identify PMPs. By evaluating these PMPs we will expand knowledge of different genetic components governing CNCC development, principally, osteoblastic differentiation.
A2) Our proposal employs an in vitro modeling system to recapitulate CNCC osteoblastic differentiation

Using a cell culture system, we will evaluate the transcriptic profile of CNC-like cells, in vitro. We will compare their transcriptic profiles to putative CNCCs and osteoblastic derivatives, found in our TS19 single-cell data set. We will then evaluate transcriptional changes governing CNC-like cells during different stages of differentiation to an osteoblastic fate. This knowledge will be crucial for adjudicating what genetic programs are shared between putative CNCCs in vivo and CNC-like cells in vitro, as to provide a platform of comparison for future studies. To our knowledge, this will be the first time that CNC-like cells will be used to interrogate a hypothesis acquired from a single-cell RNA sequencing data set.

A3) Our proposal will elucidate novel genes involved in CNCC development and disease

Information obtained by our single-cell data set provides a wealth of knowledge which can be used to explore CNCC development. By perturbing both driver genes and PMPs we will be able to provide a high-throughput way to transcriptionally evaluate craniofacial diseases. This knowledge will contribute to a better understanding of how craniofacial diseases manifest and will inform genetic counseling and potential treatment strategies.

B. INNOVATION

B1) Single-cell RNA sequencing data will help discover novel genes participating in CNCC functionality. We propose that our single-cell RNA sequencing data will broaden the transcriptional profile for many different cell types in the developing embryo, including CNCCs and their osteoblastic progeny. By evaluating metagenes in
putative CNCCs and osteoblastic progeny clusters, we will be better informed of genetic components regulating these developmental processes.

**B2)** The modeling system used to interrogate transcription profiles of CNCCs is biologically significant. Our proposed research will be carried out in a well-characterized cell-culture model capable of recapitulating developmentally significant processes in osteoblastic differentiation (Ishii 2012). O9-1 cells are the best for testing our central hypothesis that information gained from CNCC metagenes in our single-cell data set can be used to elucidate novel components involved in craniofacial skeletal development. We believe this for two major reasons. First, O9-1 cells are cultured, ex-vivo CNCCs taken from branchial arch 1 in a murine embryo at TS13. These cells underwent in vivo specification, migration and proliferation before being harvested. Compared to previously used embryonic stem cell cultures, these O9-1 cells have undergone the majority of important developmental steps, in vivo, compared to in vitro. Secondly, O9-1 cells readily possess the ability to differentiate to an osteoblastic fate, both ex vivo and in vitro (Ishii 2012) (Tavares 2017).

**B3)** Our bottom up methodology is technically innovative by employing high-throughput genetic manipulation in an in vitro system, to evaluate novel in vivo findings. We propose that by employing high-throughput genetic manipulation, based on novel in vivo data, we will be able to perturb metagenes that are coordinating cellular processes and learn more about how craniofacial pathologies affect CNCC development. By employing knockdown and overexpression experiments of multiple driver genes and their PMPs, we can systematically uncover novel components in CNCC development. These findings will guide future research to investigate these genes in animal models to help identify novel genes involved in craniofacial disease.

**C. APPROACH**

Our preliminary work has helped to identify clusters of cells in our TS19 single cell data set with putative CNCC identity. DGE lists of these clusters contain a number of genes known to be necessary for CNCC osteoblastic differentiation. These driver genes include Msx1, Runx2 and Osx. By evaluating PMPs
associated with these driver genes, we believe that we can discover novel components guiding CNCC osteoblastic differentiation. We hypothesize that by perturbing CNCC driver genes, we will observe transcriptional dysregulation of their PMPs and by perturbing PMPs, we should see transcriptional dysregulation of driver genes. PMPs found in CNCC metagenes will be investigated in an in vitro system to evaluate participation in CNCC functionality. Our three specific aims address this approach by identification of CNCC metagenes and PMPs (Aim 1), refinement of a modeling system to interrogate transcriptional changes of CNCCs to osteoblasts (Aim 2) and perturbing driver genes and PMPs to monitor transcriptional changes in our modeling system (Aim 3).

C1) INTRODUCTION

Overview of Neural Crest Cells. Neural crest cells (NCCs) are a population of cells that arose approximately 450 million years ago when vertebrates began evolving a structure known as the ‘new head’ (Gains 1983). The ‘new head’ replaced the ‘old head’ of primordial vertebrates, with the addition of a hinged jaw, cranial vault, and increased sensory organ perception. These structures allowed jawed vertebrates to engage in a predatory lifestyle (hinged jaw), produce a more robust and highly connected brain (cranial vault) and allowed a more exploratory lifestyle (sensory organs). Neural crest cells are thought to have adopted many genetic regulatory elements from both mesodermal and ectodermal cells in order to create a vast diversity of cell types (Trainor 2015). Neural crest cells possess the ability to give rise to structures in the peripheral nervous system (ganglia and Schwann cells), central nervous system (meninges), enteric nervous system (adrenal-medulla cells), cardiovascular system (heart conotruncus, smooth muscle cells), skeletal muscle (tendons, extraocular muscle), adipose tissue, dermal tissue, and cranial vault (chondrocytes and osteoblasts) (Trainor 2015). NCCs arise in the medial region of the neural plate border (NPB) along the anterodorsal axis of developing vertebrates. NCCs at different positions along this axis will produce different NCC types, based on the expression of Homeobox (Hox) genes (Trainor 2013). Hox genes control the body plan of a developing embryo along the anterodorsal axis and fate restrict NCCs in the developing head region to become Cranial Neural Crest Cells (CNCCs). NCCs more dorsal to CNCCs, become cardiac neural crest cells. NCCs in the most
dorsal regions of the anterodorsal axis, become trunk neural crest cells. CNCCs give rise to skeletal structures in the head, cardiac neural crest cells give rise to smooth muscle and aortic tract cells and trunk neural crest cells give rise to spinal ganglia and enteric nervous system derivatives. Of interest to our proposal are the CNCCs, which are affected in many craniofacial skeletal pathologies.

**CNCC development and disease.** Murine CNCC development begins at embryonic day (E) 7-7.5 (TS10/11), as cells in the medial portion of the NPB receive FGF/ WNT and BMP signaling from the underlying paraxial mesoderm and adjacent lateral ectoderm, respectively (Trainor 2015) (Figure 1A). This signaling acts to fate restrict these cells to a NCC fate by upregulation of NCC specifier genes Pax3+7 and Zic1+2 in mice. In the cranial region of the developing embryo, this NCC specification of Pax and Zic is accompanied by the absence of Hox genes (Elms 2003). The lack of Hox genes demarcates these NCCs from trunk and cardiac NCCs due to the ability for them to produce skeletal tissue. Mutations in Zic2 have been shown to prevent CNCC specification. This results in a pathology known as holoprosencephaly, a disorder which prevents brain lateralization and causes severe craniofacial defects (Weiss 2017) (Maurus 2009). Following NCC specification, CNCCs upregulate Twist, Zeb2, Dlx, and Msx to allow an epithelial to mesenchymal transition, facilitating rostral migration from the NPB to future facial tissue at E8-8.5 (TS13) (Trainor 2015) (Figure 1B). The branchial arches (BA) are segments of embryonic tissue that will eventually produce; facial skeletal structures (BA1), facial expression muscles and stapes (BA2), hyoid bone and thymus (BA3), thyroid and epiglottis cartilage (BA4) and cricoid cartilage (BA6). Diseases such as Mowat-Wilson syndrome result from mutations in Zeb2 by slowing the migration of CNCCs, causing downstream complications in craniofacial development (Wilson 2016) (Rogers 2013). Migrating CNCCs respond to guidance cues, such as ephrins and semaphorins, to find the correct extracellular environment in which to deposit themselves and proliferate (Trainor 2013). CNCCs that enter BA1 produce the maxilla, mandible and other facial bones. Signaling from the proximal and distal ectoderm to CNCCs in BA1 supports proliferation (FGF) and a restricted migration pattern (BMPs) (Trainor 2013). Once CNCCs finish their migration to BA1, they begin proliferation and give rise to the mandibular, maxillary and nasal prominences at E8.5 (TS13) onward (Goodnough 2014) (Figure 1C).
Complications with CNCC proliferation have been observed in Msx1 mutants, causing facial clefting and tooth agenesis in diseases such as Wolf-Hirschhorn syndrome (Nieminen 2003) (Satokata 1994). Once CNCC proliferation has helped to give rise to prominences of the face around E10.5 (TS17), the underlying ectoderm signals using; retinoic acid, FGFs, WNTs, NOTCH, SHH and BMPs to specify CNCCs to a committed lineage (Graf 2016). Although much work needs to be done to elucidate a complete understanding of this signaling, we know that BMP and WNT signaling are necessary, in vivo, for CNCC osteoblastic fate commitment (Abzhanov 2007).

**CNCC osteoblastic development.** As previously mentioned, CNCCs give rise to two types of craniofacial bones: intramembranous bones, such as the mandible and maxilla, and endochondral bones, such as parts of the sphenoid bone and the ethmoid bone. Intramembranous ossification occurs via condensation of intramembranous osteoblasts which allows the production of calcified bone matrix. Endochondral ossification uses chondrocytes to create a scaffold of the developing bone which is then filled in with endochondral osteoblasts (McCaulery 2012). CNCCs differentiate into osteochondral progenitors which give rise to both types of bone by becoming either chondrocytes or osteoblasts (Figure 2A). Issues with osteochondral differentiation are found in patients with mutations in Runx2, resulting in Cleidocranial dysplasia, a condition affecting development of intramembranous bones such as the mandible, maxilla and clavicle (Zeng 2017) (Takarada 2013). Further specification of osteoblasts occurs due to BMP, FGF, HH and WNT signaling which upregulates Osx (Felber 2015). Osx helps to repress the chondrocytic fate of osteochondral progenitor cells (Nakashima 2002). Mutations in Osx can cause Osteogenesis imperfecta, a disease that affects maturing osteoblasts and causes deformation of the skull (Peng 2013) (Lapunzina 2010). In regards to our proposal, we would like to evaluate CNCC development during osteoblastic differentiation. We will examine the role that Msx1, Runx2 and Osx and their PMPs play during CNCC osteoblastic differentiation, using an in vitro model (Figure 2B). Previous studies have shown that Msx1 is necessary for upregulation of Runx2 in the frontonasal process of developing mice (Han 2007) and in cultured human odontoblasts (Goto 2016). Runx2 acts to regulate Osx (Nishio 2006) and in turn, Osx regulates osteoblastic genes such as Satb2, to promote osteoblastic maturation.
Our aim is to evaluate PMPs with these driver genes (Msx1, Runx2 and Osx) during CNCC osteoblastic differentiation.

**Metagenes and cluster analysis.** As previously mentioned, metagenes are defined as groups of genes with correlated expression involved in cellular functionality (Wilison 2012). Evaluation of PMPs in metagenes guiding CNCC osteoblastic differentiation can reveal novel components involved in these processes (Figure 3). By using single-cell data, we can identify metagenes affecting discreet populations of cells to better resolve specific transcriptional changes that might be lost by evaluating bulk tissue. Bulk sample RNA sequencing is good for understanding the broad transcriptic profile of tissue, but the heterogeneity of transcriptional information within cells comprising that tissue is lost. The ability to perform single-cell RNA sequencing allows us to evaluate a tissue’s transcriptome deeply, while maintaining the heterogeneity of the population structure (Gawad 2016). Our single-cell data analysis employs dimensional reduction techniques to cluster cells of similar transcriptional profiles in order to assign a putative cell identity (Figure 4). We have identified clusters of cells with putative CNCC identity that are composed of, what we believe are, CNCCs in different stages of osteoblastic differentiation (Figure 4B-E). This ability to resolve different populations of cells across pseudo-time in our data set, will allow evaluation of the role that metagenes play during different developmental time points. Of interest is the role that Msx1, Runx2 and Osx metagenes play in CNCC osteoblastic differentiation. We find that Msx1 is in the DGE list for both putative CNCCs and facial osteoblast clusters while Runx2 and Osx are found in the DGE list for putative facial osteoblasts clusters. These findings support current theories on CNCC osteoblastic development by which Msx1 governs expression of Runx2 and Osx (Figure 2). Of particular note, we are interested in how PMPs for Msx1 change between putative CNCC and facial osteoblast clusters.

**C2) PRELIMINARY STUDIES**
Putative identification of cell types in our TS19 data set. Our preliminary results of processing the TS19 single-cell data set have allowed us to assign a number of clusters to a putative cell type (Figure 4C). Clusters that have the highest match to the annotated transcriptic profile for different cell types, as reported by Lifemap, have been identified. The DGE lists for these clusters have been generated and need to be assessed in vivo.

In situ identification. In order to evaluate the patterns of expression for genes in putative CNCC and osteoblastic progeny clusters, in situ, we will use two different levels of resolution using techniques in which our lab has expertise. In order to identify global patterns of expression we will employ whole mount (Figure 5A) and section (Figure 5B) alkaline phosphatase in situ and Laser Capture Microscopy (LCM) with qPCR (Figure 5C). In order to refine expression patterns of different genes at a single cell level we will use branch DNA in situ techniques. We will employ RNA scope on whole embryo and sectioned murine tissue (Figure 5D) and Primeflow on dissociated cells (Figure 5E). These different approaches can help us evaluate both global and single cell expression patterns for driver genes and PMPs in putative CNCCs and their osteoblastic progeny.

In vitro Modeling system. Work done to create a stable line of multipotent neural crest cells, for in vitro use, spans nearly twenty years. Early efforts to produce neural crest cells, in vitro, utilized mouse Embryonic Stem Cells (mESCs) co-cultured with bone marrow derived stromal cells (Kawasaki 2000). These cells were treated with BMP4 to produce PNS neurons, a neural crest derivative. This technique was later applied to human Embryonic Stem Cells (hESCs) with the addition of FACs sorting for NCC cell surface markers P75 and HNK1 (Lee 2007). Recent experiments employ a variety of techniques to produce neural crest cells from embryoid bodies (Yu 2006), neural rosettes (Lee 2012) and monolayer cultures (Mica 2013) (Mimura 2016). These methods utilize mESCs or hESCs in combination with growth factors, such as FGFs, WNTs and BMPs, in an attempt to replicate the extracellular signaling environment experienced by neural crest cells during development. While these methods are able to produce cells that express many of the genes enriched in neural crest cells (Mimura 2016), there has been criticism on the reproducibility and effectiveness of differentiation of ESCs to neural crest cells. Hindering these methods is the correct extracellular environment.
experienced by neural crest cells from their initial conception at the NPB to their final destination. With these considerations, Ishli et al, harvested neural crest cells from a TS13 murine embryo, migrating to BA1. Compared to previous approaches, these cells underwent specification, migration and proliferation, in vivo as compared to in vitro. These cells, designated O9-1 cells, were tested in vitro and differentiated into smooth muscle, glial cells and most importantly, osteoblasts (a hallmark of CNCCs) (Figure 6). These O9-1 cells were also transplanted into frontal bone primordium of the upper half of E13.5 mouse head and gave rise to osteoblasts, ex vivo (Ishii 2012).

Evaluation of in vitro transcriptional changes. Previous research by our group has elucidated important features about cellular transcriptic changes, in vitro, using single-cell approaches (Gibson 2011) (Figure 7). We will employ these same techniques to evaluate transcriptic changes, using qPCR, on bulk and single-cell samples of O9-1 cells undergoing osteoblastic differentiation. Previous assays have been performed to overexpress and knockdown genes in vitro using O9-1 cells and have evaluated transcriptic changes using qPCR analysis (Figure 8) (Tavares 2017) (Sun 2013).

C3) RESEARCH PLAN

Aim 1 To identify and characterize putative Cranial Neural Crest Cells (CNCCs), their osteoblastic progeny and associated metagenes, using single-cell mRNA sequencing of murine embryos. The goal of this aim is to test the hypothesis that the transcriptic identity of CNCCs and their osteoblastic progeny can be broadened by evaluation of murine single-cell RNA sequencing data. To address this hypothesis, we will: (i) determine the transcriptic profile of clusters of cells in our TS19 data set that have a CNC-like identity and (ii) validate co-expression patterns of CNCC driver genes with their PMPs, in situ.

Aim 1.1 Determine the transcriptic profile of clusters of cells in our TS19 data set that have a putative CNCC and facial osteoblast identity
**Rationale:** The information obtained from single-cell assays are informative in understanding genetic programs governing individual cellular processes (Wang 2016). From its humble beginnings of evaluation of a 4-cell murine blastomere (Tang 2009) to the immense evaluation of 1.3 million cells from murine brain tissue (10X 2016), single-cell RNA sequencing has been able to provide a robust transcriptic evaluation of individual cells. These studies have shined light on the importance of heterogeneity of cell types in a tissue such as the pancreas (Baron 2016). Our lab employs single-cell RNA sequencing to better understand transcriptic profiles of populations of cells during murine development (Gibson 2009). The most recent and ambitious endeavor undertaken by our lab is to evaluate single-cell transcriptional changes occurring throughout murine organogenesis. Our goal is to collect all major developmental time points and to be able to map out the developmental trajectory of different populations of cells important for the production of different tissue. We hypothesize that by performing single-cell RNA sequencing from a dissociated murine embryo during theiler stage 19, we will be able to identify clusters of cell with putative CNCC and facial osteoblast identify using DGE lists for those clusters. Since TS19 is a stage where CNCCs are differentiating to different developmental paths, especially osteoblasts, we expect to find CNCCs and their osteoblastic progeny (Bhatt 2013).

**Experimental design:** We will process single-cell RNA sequencing data from TS19 embryos, using our lab’s analytical pipeline to cluster cells together based on transcriptic similarity (Figure 3B+C). We will then evaluate DGE lists in those clusters and assign them to a putative cellular identity using transcriptic information obtained by Lifemap database (Figure 3D+E).

**Expected Outcomes:** If our hypothesis is correct, we expect to find putative CNCCs and their osteoblastic progeny in our data set by matching them to curated transcriptic profiles in Lifemap. If we can detect these putative CNCC and facial osteoblast clusters in our data set then we will be able to evaluate their transcriptic profile using DGE lists for that cluster. These DGE lists should contain genes that are involved in CNCC osteoblastic differentiation such as Msx1, Runx2 and Osx.
Potential Problems and Alternatives: Our single-cell RNA sequencing pipeline is still in its infancy, yet we are confident in assigning clusters of cells to a putative cell type using DGE lists and matching them to known transcriptic profiles of cell types, as provided by Lifemap. Our lab has made great progress in trying to set a gold standard for assigning clusters of cells to a cellular identity. Prior methods of assigning clusters to a cellular identity have relied on using a handful of known markers to adjudicate this binning of clusters. This methodology is good for identifying well characterized cell types in a single-cell RNA sequencing data set, but has been criticized as being too subjective to properly identify less well characterized cell types. Our analysis attempts to remove this subjective interpretation and relies on more characterized transcriptic profiles that have been assembled by the Lifemap database. While this methodology might not allow us to perfectly match each of our clusters to a cell type, due to the paucity of transcriptional data for each known cell type, we feel as though it is currently the best method to assign clusters to cell types in an agnostic manner.

Aim 1.2 Evaluation of expression pattern of driver genes and PMPs in putative CNCC and facial osteoblast clusters, in situ

Rationale: Evaluation of DGE lists for clusters with putative CNCC and facial osteoblast identities allows us to identify driver genes for osteoblastic differentiation, such as Msx1, Runx2 and Osx. We can then identify PMPs in the driver gene’s metagene and explore their expression profiles, in situ. It is our hypothesis that CNCC osteoblastic driver genes and their associated PMPs will have similar expression profiles, in situ.

Experimental design: We will employ a number of in situ techniques in which our lab has expertise, to evaluate the expression pattern of driver genes and their PMPs in TS19 murine embryos. We will use alkaline phosphatase in situ hybridization and LCM with qPCR to find global patterns of expressions (Figure 5A-C). By evaluating these global expression patterns, we can become better informed on which PMPs would be best to evaluate in a more refined manner, at single-cell level. To evaluate driver genes and their PMPs at a single-cell
level, techniques employing branched DNA technology will be used to evaluate expression patterns. RNA scope is a branched DNA technology that can be employed on whole mount or sectioned tissue to identify the spatial distribution of these transcripts at a single cell level (Figure 5D). Prime flow is a branched DNA technology that can identify co-localized transcripts in dissociated cells by using FACs (Figure 5E). These techniques assess the expression pattern of driver genes and their PMPs in BA1 of TS19 embryos.

**Expected Outcomes:** If our hypothesis is correct, then we will observe co-localization of CNCC osteoblastic driver genes with their PMPs, in situ. We expect that PMPs that highly co-vary with driver genes in putative CNCCs and facial osteoblasts, will more likely be found to be co-expressed in situ.

**Potential Problems and Alternatives:** Our lab has much experience with many of these in situ techniques to validate novel findings, but alternative methods would rely on employing antibodies to resolve expression patterns. In order to evaluate expression patterns, we would employ immunohistochemistry techniques (IHC) for genes that have well documented antibodies.

**Aim 2** To develop an in vitro modeling system capable of recapitulating developmental steps involved in CNCC differentiation towards an osteoblastic fate. In this aim we design a platform to test the hypothesis that we can generate CNC-like cells and differentiate them to osteochondral-like cells, in vitro. To address this aim we need to (i) culture murine CNC-like cells in vitro, (ii) differentiate murine CNC-like cells to an osteoblastic fate and (iii) evaluate CNC-like cells and osteoblastic derivatives for PMPs found in putative CNCC and facial osteoblast clusters in our TS19 data set.

**Aim 2.1** Culturing of murine CNC-like cells, in vitro

**Rationale:** As previously mentioned, there have been many attempts to generate CNCCs via various cell culture techniques. We believe that O9-1 cells are currently the best option to recapitulate the cellular identity
of CNCCs in an in vitro system. Our hypothesis is that these O9-1 cells have a transcriptic profile similar to putative CNCCs, identified in our single-cell RNA sequencing data set.

**Experimental design:** Utilizing O9-1 cells, we will evaluate the transcriptic identity of these cells and compare them to putative CNCCs. While O9-1 cells have been transcriptically profiled at passage 13, the cells that we will be using are at passage 18 (Ishii 2012). We first need to confirm that the transcriptional profile of these cells have not changed drastically from passage 13 to passage 18. To evaluate this, we will first use RT-PCR to ensure CNCC-specific genes such as Sox9, Pax3, Foxd3, Cdh1, and Nestin are present and that non-CNCC genes such as Sox2 (neuronal), Brachyury (mesodermal), Lrrc10 (cardiac) and Tbx6 (trunk mesenchyme) are not (Figure 9). This RT-PCR analysis will build our confidence that these cultured cells have a CNC-like identity. If these O9-1 cells have many of the same transcripts as reported to be expressed in passage 13, we will use bulk sample qPCR analysis to evaluate levels of reported CNCC genes found in our putative CNCC cluster. These genes include, Tfap2a, Tnfap6, Barx1, Lhx6, Lhx8, Alx1, Alx4, Dlx3, Dlx4, Dlx5, Dlx6, Msx1, Pax7 and Pax3. We will use transcriptional information obtained from qPCR to establish baseline transcript levels for these genes to be used in aim 2.2 and 2.3.

**Expected Outcomes:** If our hypothesis is correct, we predict that many reported CNCC genes will be enriched in passage 18 O9-1 cells. We expect that genes such Msx1, Tfap2a and Pax3 will be detected in passage 18 O9-1 cells, while genes such as Sox2 and Lrrc10 will not.

**Potential Problems and Alternatives:** While we have not personally evaluated the transcriptic profile of passage 18 O9-1 cells, many groups have reported that these cells maintain levels of transcripts profiled by micro array analysis at passage 13 (Ishii 2012) (Tavares 2017) (Sun 2013). Also, the potential for these cells to differentiate into osteoblasts at passage 17 and 22 have been reported (Figure 6B). This information helps us build confidence that the transcriptional information of passage 13 cells is similar to passage 18 O9-1 cells. While these cells should be sufficient for our studies, we are also interested in exploring a monoculture mESC
cell culture system, in parallel. More recent protocols have had success in reliably producing both human and mouse CNC-like cells in a monoculture platform from ESCs (Mimura 2016).

**Aim 2.2 Differentiation of CNC-like cells to osteoblastic fate, in vitro**

*Rationale:* Previous groups were able to differentiate O9-1 cells to an osteoblastic fate (Ishii 2012) (Sun 2013) using osteogenic differentiation media. These groups identified osteoblastic changes by staining cells with Alizarin Red, a dye which detects calcified tissue, and by evaluating levels of Msx1, Msx2, alkaline phosphate (Alp), Osteocalin (Ocl), and Runx2 (Figure 2B, 6B+C). We *hypothesize* that by differentiating O9-1 cells in vitro, we can recapitulate the transcriptic profile of putative facial osteoblast cells found in our TS19 data set.

*Experimental design:* Employing the same differentiation protocols as previously reported for O9-1 cells (Ishii 2012), we hope to evaluate the transcriptic profile of these cells during different stages of osteoblastic development using qPCR, in a similar manner to previous experiments done by Nelson lab members (Gibson 2009) (Figure 7B+9). Ishii reported that osteoblastic program markers (Msx2, Alp, and Ocl) are not detected in passage 13 undifferentiated O9-1 cells, but are detected after one day of osteoblastic differentiation (Figure 2B). To ensure observation of these markers, we will differentiate O9-1 cells to an osteoblastic fate and use RT-PCR to confirm their expression. Confirming, in house, that we can differentiate these O9-1 cells to an osteoblastic fate, will increase our confidence that these cells share transcriptic profiles resembling cells in putative facial osteoblast cluster. After this RT-PCR analysis, we will perform bulk sample qPCR to check for genes expressed in the DGE list of our putative facial osteoblast cluster that match to the facial intramembranous osteoblast lineage, as reported by Lifemap. These driver genes include Msx1, Msx2, Runx2 and Osx (Figure 9). We will evaluate detection levels of these genes in undifferentiated O9-1cells compared to O9-1 cells undergoing osteoblastic differentiation at different time points to observe how these temporal changes are affecting transcript levels (Figure 9+10A). To evaluate development of facial osteoblast-like cells to more mature osteoblasts-like cells we will use Alizarin Red to test for mineralization.
**Expected Outcomes:** If our hypothesis is correct, we will observe that many of the genes found in the DGE lists of our putative facial osteoblast cluster will also be detected in differentiating O9-1 osteoblastic-like cells. We expect that a number of transcripts will be expressed in both undifferentiated and differentiated O9-1 cells, such as Msx1, due to their multiple roles in CNCC proliferation and osteoblastic differentiation (Figure 2). We expect that other genes such as Osx, Satb2, Alp and Ocl will only be expressed in differentiating O9-1 cells, due to their roles in osteoblastic development.

**Potential Problems and Alternatives:** Due to multiple groups (Ishii 2013) (Sun 2013) having success in osteoblastic differentiation of O9-1 cells and the expertise of current Nelson lab members in cell culture differentiation (Gibson 2009), we feel confident in our ability to generate osteoblastic-like cells from O9-1 cells. If we are unable to utilize these cells in vitro, we will pursue using CNC-like cells from monoculture platform ESCs and differentiate them to an osteoblastic fate using protocols established by previous groups (Mimura 2016).

**Aim 2.3 Evaluation of CNC-like cells and osteoblastic derivatives, for PMPs in metagenes guiding CNCC osteoblastic differentiation**

**Rationale:** By evaluating the transcriptic profile of putative CNCCs and their osteoblastic progeny compared to O9-1 cells during osteoblastic differentiation, we will catalog genes shared in both systems. This data allows us to select metagenes to profile expression levels in O9-1 cells. Using gene analytics, we can interpret the functionality of PMPs in our metagenes. Preliminary results suggest that PMPs in Msx1’s metagene change between clusters, while others do not. Msx1 PMPs in the putative CNCC cluster have annotated molecular functions such as retinoid binding (Crabp1 and Crabp2) and GO terms such as negative regulation of osteoblast differentiation (Id1 and Id2). Msx1 PMPs in the putative facial osteoblast cluster have molecular functions such as BMP signaling (Nbl1 and Smad7) and GO terms such as negative regulation of Notch
signaling pathway (Gata2 and Hey1). Interestingly, alteration of retinoic acid signaling has been shown to cause craniofacial aberrations (Rhinn 2012), while BMP and Notch signaling have been shown to be involved in osteoblastic development (Lin 2011). Msx1 PMPs that are shared between both clusters have molecular functions such as growth factor binding (Igfbp7), GO terms such as mesenchymal cell development (Msx2 and Alx1), and heparin binding (Serpine2). While some of these genes have been reported to co vary with Msx1 in CNCC development and osteoblastic differentiation (Msx2) (Han 2007), many of the genes in our data set have not been annotated in craniofacial development, such as Serpine2. Runx2 PMPs in the putative facial osteoblast cluster include highly co varying PMPs such as Jak2 and Harbi1. Jak2 is a tyrosine kinase that is involved in Stat signaling, a signaling pathway known to be involved with osteoblastic development via upregulation of Runx2 (Dalagiorgou 2017). Harbi1 is a nuclease whose function is not well characterized and is not currently known to be involved in craniofacial development. Osx PMPs include Satb2, a gene known to be involved in osteoblastic maturation (Tang 2011) and Nhlrc2, a poorly studied gene, which is thought to contribute to anti-apoptotic processes (Ruffalo 2004) and whose knockout is embryonic lethal (KOMP) (Figure 3B). Because O9-1 cells undergo many of the same in vivo developmental steps as cells in putative CNCC and facial osteoblast clusters, we believe that these transcriptic profiles will share a high degree of similarity and many of our PMPs will be detected in O9-1 cells. Our hypothesis is that PMPs in metagenes of CNCC osteoblastic driver genes will also be detected in O9-1 cells.

**Experimental design:** We will employ the platform created in aims 2.1 and 2.2 to evaluate the transcriptic profile of O9-1 cells as they undergo osteoblastic differentiation (Figure 10A). To evaluate which PMPs are expressed in O9-1 cells we will employ RT-PCR on bulk sample during different time points during osteoblastic differentiation (Figure 9). Using this prescreening approach will narrow the list of PMPs to interrogate using qPCR. We will employ bulk sample qPCR on our PMPs to see how their levels fluctuate throughout development. We will also employ single-cell qPCR to evaluate how transcript levels of PMPs vary with other genes in the metagene. For example, we will investigate levels of Id2 and Id3 with Msx1 in individual O9-1 cells undergoing osteoblastic differentiation, to evaluate if the expression of these genes directly varies with one
another. We will evaluate if PMPs for metagenes guiding CNCC proliferation and osteoblastic differentiation have a similar detection pattern in vivo versus in vitro (Figure 2+10).

**Expected Outcomes:** If our hypothesis is correct, we expect that some, but not all, PMPs for genes necessary for CNCC proliferation and osteoblastic differentiation will be detected in our O9-1 cells during osteoblastic differentiation. We believe that that for Msx1 PMPs, such as Id2 and Id3 (Negative regulators of osteoblastic differentiation), transcript levels will decrease during osteoblastic differentiation and for other Msx1 PMPs, such as Nbl1 and Hey1 (BMP and Notch signaling), transcript levels will increase during osteoblastic differentiation. Because preliminary data suggests that Msx1 levels increase during osteoblastic differentiation (Figure 6C), we will employ single-cell qPCR to evaluate levels of Msx1 and their PMPs during osteoblastic differentiation to see if they co-vary, in vitro.

**Potential Problems and Alternatives:** While we believe that the transcriptic identity of O9-1 cells and their osteoblastic derivatives will be similar to putative CNCCs and their osteoblastic progeny found in our TS19 data set, we expect many transcripts will not be shared between the two. While many of the signaling factors necessary for facial development have not been fully elucidated, we know that FGF signaling alone (signaling factor to maintain O9-1 cells in culture) will not fully recapitulate the transcriptic identity of CNCCs. However, with the plethora of data that our TS19 data set has produced, we are confident that we will find many PMPs that are detected both in vivo and in vitro.

**Aim 3 To perturb CNCC differentiation to an osteoblastic fate.** In this aim we employ the in vitro modeling system, as described in aim 2, to test perturbations of genes thought to contribute to the differentiation of CNCCs to an osteoblastic fate. We hypothesize that we can perturb the ability of these CNC-like cells to differentiate to an osteoblastic fate by altering expression of (i) driver genes previously identified to be necessary for CNCC osteoblastic differentiation and (ii) PMPs of those driver genes.
Aim 3.1 Perturbation of driver genes involved in CNCC osteoblastic differentiation

Rationale: Many in vitro studies have been used to investigate the roles of different genes in CNCC development (Sun 2013) (Shao 2015) (Everson 2017). Previous groups have used human iPSCs from patients with Familial Dysautonomia to understand the role that Ikbkap plays in CNCC differentiation (Lee 2012). Other more recent studies have specifically used O9-1 cells to evaluate the responsiveness of signaling when using overexpression constructs (Everson 2017), and evaluation of the osteoblastic abilities of O9-1 cells using RNAi (Sun 2013) (Figure 8). These studies are very much in line with what we would like to use O9-1 cells for, mainly the evaluation of their osteoblastic potential when using RNAi or overexpression constructs. In vitro knockdown experiments of Msx1 show decreased levels of Runx2 (Goto 2016). Decreased expression of Runx2 prevents osteoblastic differentiation in calvarial osteoblast-like cells (Tu 2008) and overexpression of Runx2 causes rat adipose-derived stem cells to enhance osteoblastic differentiation (Zhang 2006). Runx2 binds to the promoter region of Osx and aids in its expression (Nishio 2006). RNAi of Runx2 and Osx in murine osteochondral cell culture have been shown to prevent osteoblastic differentiation (Shrivatts 2015). These studies guide our efforts to interrogate the involvement of Msx1, Runx2 and Osx in CNCC osteoblastic differentiation. We hypothesize that by perturbing driver genes involved in CNCC osteoblastic differentiation, we will perturb the expression of known osteoblastic markers and associated PMPs.

Experimental design: We will use our modeling platform as described in aim 2 to perturb genes that are reported to be involved in CNCC osteoblastic differentiation (Figure 2+10B). We will perform RNAi knockdown and overexpression experiments to perturb known genes in CNCC osteoblastic differentiation. Previous experiments performed in the Nelson lab, have used lipofection and electroporation to transfect constructs for knockdown experiments in ESCs. For RNAi and over expression experiments we will culture O9-1 cells and transfect them with either pcDNA3-siRNA or pcDNA3-mRNA vectors and select for cells using neomycin, as previously described by Sun 2013. After selecting for cells that are neomycin resistant, we will perform osteoblastic differentiation of O9-1 cells and will interrogate these transcriptional levels using qPCR to
evaluate, over time, if perturbing these driver genes alters canonical expression patterns of osteoblastic genes (Alp, Ocl, Spp1) and PMPs of driver genes. We will also interrogate if these perturbed cells have the ability to give rise to mineralized structures, a hallmark of mature osteoblasts, using Alizarin Red.

**Expected Outcomes:** If our hypothesis is correct, we expect that perturbations in Msx1, Runx2 and Osx would disrupt normal osteoblastic differentiation and alter expression of their PMPs. We expect that knocking down these driver genes will prevent expression of osteoblastic markers as observed in other cell culture systems (Shrivatts 2015) (Goto 2016). We also expect that over expression of Msx1 would increase the proliferation of osteoblastic cells but that these cells would fail to mature to more differentiated mineralized osteoblasts (Nassif 2014). Additionally, it is expected that overexpression of Runx2 and Osx will increase osteoblastic marker expression and will promote earlier osteoblast mineralization (Zhang 2006).

**Potential Problems and Alternatives:** We expect that by perturbing driver genes previously characterized be necessary in CNCC osteoblastic differentiation, we will observe dysregulation of this process, similar to previous assays (Shao 2015) (Goto 2016) (Tu 2008) (Sun 2013). We expect the transcriptic expression pattern for many PMPs to change as we perturb genes necessary for osteoblastic differentiation. However, we believe that many PMPs will not change as a result of perturbing driver genes. Thus, we will evaluate these PMPs with a systematic approach to find candidate PMPs to perturb for aim 3.2.

**Aim 3.2 Perturbation of PMPs associated with driver genes involved in CNCC osteoblastic differentiation**

**Rationale:** By evaluating changes in transcript levels of PMPs in aim 2.3 and 3.1, we can select PMPs to perturb in our cell culture modeling system. Our goal is to validate the biological relevance of PMPs in metagenes for CNCC osteoblastic differentiation. By gaining this insight, not only will we be able to identify novel genetic elements contributing to CNCC osteoblastic differentiation, but also expand the number of genes.
that are currently known to contribute to craniofacial defects. We hypothesize that by perturbing candidate PMPs, we will observe transcriptional dysregulation during CNCC osteoblastic differentiation.

**Experimental design:** We will use our modeling system in the same way that it was employed in aim 3.1 and perform knockdown and overexpression experiments of PMPs for Msx1, Runx2 and Osx (Figure 10B). We will evaluate these perturbed O9-1 cells during osteoblastic differentiation to evaluate if the canonical osteoblastic markers and driver gene expression profiles change. We will also evaluate the ability of these cells to produce mature osteoblasts using Alizarin Red, to interrogate for mineralized structures.

**Expected Outcomes:** If our hypothesis is correct, perturbing PMPs of genes known to be necessary for CNCC osteoblastic differentiation will result in transcriptional dysregulation of CNCC osteoblastic differentiation. We expect that by perturbing PMPs of Msx1, Runx2 and Osx we will be able to perturb the levels of these driver genes during O9-1 osteoblastic differentiation. For example, we believe that overexpression of Id2 and Id3, Msx1 PMPs known to be negative regulators of osteoblastic differentiation, will prevent osteoblastic differentiation from occurring. Also, we believe that knockdown of Nhlrc2, a Osx PMP thought to be necessary for embryonic survival, will cause apoptosis of O9-1 cells, a phenomenon we can check using a cell counting assay.

**Potential Problems and Alternatives:** Our data set provides an immense amount of data that we predict will inform our understanding of genetic orchestration of CNCC osteoblastic differentiation. While we believe that by perturbing PMPs we can further elucidate novel genetic components involved in CNCC functionality, we have yet to show that this approach will work experimentally. However, with the wealth of data provided by this bottom up approach, we believe we will be able to produce novel finds and expand our current understanding of genetic elements involved in CNCC osteoblastic differentiation.
C4) OVERALL SUMMARY AND CONCLUSIONS

Our work in elucidating metagenes governing CNCC functional processes will help to better understand craniofacial development and, ultimately, disease. We believe that our bottom-up approach will yield data that can aid in building knowledge of craniofacial diseases by generating candidate genes to further evaluate. If our approaches are successful then we can elucidate novel components disrupting murine CNCC development, in vitro. Our next steps would aim at 1) validation of these findings in vivo and 2) evaluation of these findings in human cell culture. To validate our novel findings in vivo, we would use knockout mice generated by KOMP for genes such as Msx1. We would perform in situ techniques, as described in Aim 1.2, to evaluate populations of CNCCs to check for perturbations of PMPs, specifically using LCM qPCR and RNA scope. Additionally, we would process these KO embryos using our single-cell RNA sequencing pipeline, to observe how metagenes in CNCC and facial osteoblastic clusters vary between KO and WT embryos. These assays would validate our in vitro data, in vivo. To evaluate these findings in human cells we would use a protocol employed by Mimura et al to differentiate hESCs into CNC-like cells (Mimura 2016). We would use these human cells in assays similar to those that were performed in aim 2 + 3 to evaluate if PMPs for driver genes of CNCC osteoblastic differentiation are shared between mice and humans. These future studies could reveal novel genetic components involved in human craniofacial disease. Through the combination of data from our single-cell analysis and in vitro techniques, we believe we can build genetic knowledge about craniofacial disease.

Works Cited


Figure 1-Cranial Neural Crest Cell (CNCC) murine development:
A) Diagram illustrating CNCC specification from the Neural Plate Border (NPB) around TS10/11. B) Illustration of CNCC migration from the NPB around TS10/11, arriving in the branchial arches around TS12/13 and proliferating in the branchial arches starting at ~TS14/15. C) Murine heads with different facial prominences highlighted, each of these prominences contain proliferating CNCCs which are beginning to make fate choices starting around TS17.
Figure 2-Diagram of curated in vivo CNCC osteoblastic differentiation vs in vitro CNC-like cell osteoblastic differentiation. A) Developmental stages of CNCC osteoblastic differentiation, in vivo, illustrating major transcriptional changes as reported by Lifemap, Bhatt 2013 and Tang 2011. B) Stages of CNC-like cell (O9-1 cells) osteoblastic differentiation using osteogenesis differentiation media using data from Ishii 2012 and Sun 2013.

Figure 3-Evaluation of metagenes in CNCCs A) Schematic of metagene discovery in single-cell RNA sequencing assay. Red gene is known to be necessary for CNCC osteoblastic differentiation, both green and blue genes co-vary with red but yellow does not. Red is the driver gene, green and blue are PMPs. B) Evaluation of PMPs for Osx in putative facial osteoblast cluster. Higher co-varying scores indicate that Osx and PMP co-vary greatly in cells of that cluster.
Figure 4-Pipeline for putative cell type identification:
A) Outline of single-cell pipeline. B) Identification of putative clusters of CNCCs. C) Iterative binary splitting of putative CNCC clusters and putative identification. D-E) Evaluation of cluster’s DGE using; Gene analytics to identify top matching cell type and GO terms (D) and Lifemap for possible cell type assessment (E).

Figure 5-Techinques for in vivo assessment:
Figure 6—Previous work performed to validate potential of O9-1 cells: 
A) Procedure to acquire and culture CNCCs from a TS13 murine embryo. B) Differentiation potential of O9-1 cells at passage (P)17 and P22 to an osteoblastic fate by checking for calcified deposits via Alizarin red after 10 days of osteoblastic differentiation. C) RT-PCR analysis of different osteoblastic markers during O9-1 cell differentiation.

Figure 7—Previous work done by Nelson lab members to evaluate expression profiles of single hESCs differentiation: 
A) Relative expression (delta-Ct) of 19 genes in single cells under Activin culture conditions. Rows represent individual cells and columns are gene markers. Markers are grouped as: P = pluripotency, ME = mesendoderm, E = endoderm, ExE = extra-embryonic endoderm. Expression levels range from blue (not detected) to red (highly expressed). 
B) Cell-averaged expression plots of nine genes over the first seven days of differentiation. Lines are splines fit to the average of all cells collected at each time-point and are color-coded to represent different culture conditions (orange = serum, red = Activin, green = BMP4). Relative expression intensity (delta-Ct) is on the X-axis, age of cells (in days) is on the Y-axis.
Figure 8- Previous studies done using O9-1 cells:
A) Transfection of control vs Six1 expression vectors in O9-1 cells and evaluation of different transcript levels using qPCR after 48 hours. B) Transfection of control vs Foxc1 RNAi in O9-1 cells, undergoing osteoblastic differentiation, and evaluation of different transcript levels via qPCR after 48 hours.

Figure 9- Overview of experimental design for Aim 2.
Figure 10-Overview of experimental design for aims 2 +3. A) Quantification of expression patterns of CNCC markers (orange) (Aim 2.2), osteoblastic markers (blue) (Aim 2.2) and osteoblastic driver gene PMPs (Aim 2.3) (grey) using qPCR during O9-1 cell osteoblastic differentiation. B) Quantification of expression patterns of genes from A using qPCR during O9-1 cell osteoblastic differentiation when perturbing osteoblastic driver genes (Aim 3.1) and osteoblastic driver gene PMPs (Aim 3.2).